PE2.03 Influence of early pH drop rates on calpain activity and protein degradation in pork 204.00

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Abstract. **Early post-mortem pH development plays an important role in determining the rate and extent of muscle tenderization. The calpain system was demonstrated to be involved in the proteolytic tenderization of muscle, and is known to be influenced by pH. In the present work we studied the influence of pH drops in porcine *Longissimus dorsi* muscle on the activity of calpain and on myofibril degradation. The activity of calpain was studied with the zymography method. A faster decrease in pH resulted in reduced amount of extractable μ-calpain activity and increased autolysis of the enzyme, and thereby an earlier loss of activity revealing that calpain has a higher activity in muscles with a fast pH drop. The higher calpain activity in the muscles with a fast pH drop also resulted in a higher myofibril fragmentation at 24 h post-mortem, which was no longer evident in the later phase of the tenderization process. In conclusion, the rate of pH decrease early post-mortem influenced the rate, but not the extent of myofibril degradation.**

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***Index Terms*— autolysis, calpain, myofibril fragmentation, pH.**

I. INTRODUCTION

POST-mortem changes in muscle proteins and myofibril fragmentation have been shown to be associated with improved meat tenderness. One of the protease systems involved in the degradation of muscle protein is the calpain system. μ- and m-calpain are calcium dependent and are composed of an 80 kDa and a 28 kDa subunit, and both autolyze in presence of calcium [4]. The 80 kDa subunit, the catalytic part, is reduced to a 76 kDa form by auto proteolysis which reduces its calcium requirement for activity [5]. In addition to calcium concentration, other parameters like temperature and pH influence the activity of calpain [3].

Early post-mortem pH development plays an important role in determining the rate and extent of muscle tenderization [8] and as previously reported, the activity of calpain is also influenced by pH values [3], and consequently the pH drop in muscle after slaughter of animals has effects on the enzyme. In some studies a fast decrease in pH post-mortem was associated with a faster decrease of μ-calpain activity [2; 6; 9; 11] and a low μ-calpain activity was detected in muscles with lower pH post-mortem [10]. Decrease rate of pH also influence muscle protein and myofibril degradation. However, the mechanism is not clear since a faster degradation of proteins has been associated with a fast pH drop [8; 10] as well as a slow pH drop [12].

pH also influence the autolysis of μ-calpain. The role of the autolysis process is not completely understood. It was suggested that the autolysis of the enzyme leads to a reduction in the Ca^{2+} concentration required for the enzyme activity and the autolysis process is also necessary for this molecule to become active towards exogenous substrate [1]. Detection of autolyzed enzyme in muscle and meat is believed to be indicative of proteolytic activity of calpain [5; 7]. pH drop influences also the rate of μ-calpain autolysis and it was shown that a faster pH drop paralleled an earlier appearance of the autolyzed form of the enzyme [7]. In correlation with an earlier appearance of the autolyzed enzyme an earlier degradation of myofibrillar proteins was determined [7].

In the present work we studied the influence of different early pH drop rates in porcine muscles post-mortem. Animals with normal, fast and slow pH decrease rates were selected from a batch handled with a minimum stress before stunning. The activity of calpain and the detection of autolyzed products were analyzed in relation to the three different groups of pH drop using casein zymography.

II. MATERIALS AND METHODS

Eighty Italian Duroc x (Landrace x Large White) crossbred pigs, mainly female, with an average carcass weight of kg 162 ± 12 were pen-fed and finished under standard Parma-ham practice. In *M.longissimus dorsi* (LD), pH was measured (Eutech Instruments pH6) at 1 (pH1) and 3 h post-mortem (pH3) at the 7^{th} rib of the right side of the carcass. Based on pH3 values, 30 pigs were selected and right side loins divided into three groups of 10 based on the rate of the pH drop post-mortem: fast pH drop (F-pH) (pH3 < 6.00), normal pH drop (N-pH) (pH3 in the interval 6.00 – 6.30) and slow

pH drop (S-pH) (pH3 > 6.30) (table 1). The pH and temperature (digital thermometer TFA DT 90) of the LD were monitored at 1, 3, 6, 24 and 72 h post-mortem. Samples for calpain activity were taken at 1 and 6 h post-mortem at the 8th rib and immediately frozen in liquid nitrogen. Additional samples for calpain activity were taken at 24 and 48 h, and samples for myofibril fragmentation at 24 h, 3 d and 6 d post-mortem, and then frozen and stored at – 80 ºC. Frozen meat samples (- 80°C) were finely chopped. One g of each sample was homogenized (13.500 rpm) in 6 ml of an extraction buffer at pH 8.0 and centrifuged for 30 minutes at 4°C at a speed of 15000 x *g*. To determine the activity of μ-calpain the zymography method was used. After addition of sample buffer, 15 μL supernatant was loaded in each well of casein minigels with 26 wells. Gels were run at 80 V for 3 hours at 4 ºC before incubation with calcium for one hour. After staining and destaining, the density of each band was measured and quantified relative to reference standards within each gel. The myofibril fragmentation was measured using a Malvern Mastersizer Micro Plus (Malvern Instruments Ltd, Worcestershire, UK). Samples of 2.5 g were homogenized in 30 mL cold buffer (100 mM KCl, 20 mM Potassium Phosphate, 1mM EGTA, pH 7.00) at 20,500 rpm and analyzed in duplicates. The data were analyzed using Proc Mixed (SAS, ver. 9.1). The model included pH drop class and time post-mortem as fixed effects and animal as random effect.

III. RESULTS AND DISCUSSION

Results

The calpain activity was analyzed with casein zymography. At 1 h post-mortem μ-calpain activity and autolyzed μ-calpain activity did not differ between groups. At 6 h post-mortem the activity of both native and autolyzed μ-calpain appeared stable in the groups with slow and normal pH drop, while in the group with F-pH drop the μ-calpain activity was lower and the autolyzed form higher ($P < 0.01$) compared to the other two pH groups and to 1 h post-mortem (table 1). At 24 h post-mortem the extractable activity of the native isoform had decreased in all groups ($P < 0.01$), and with the fast pH drop group showing a lower activity compared to the slow pH drop group ($P = 0.01$). For the autolyzed form a significant increase was determined in all groups at 24 h compared to 6 h post-mortem, with the F-pH group showing the higher amount of extractable autolyzed μ-calpain activity. Also at 72 h post-mortem a decrease ($P < 0.01$) of μ-calpain activity compared to 24 h post-mortem was detected in all groups, and the S-pH group showed a higher activity compared to the N-pH drop group ($P < 0.05$) and to the F-pH group ($P < 0.01$). Concerning the autolyzed form of μ-calpain at 72 h post-mortem a decrease in the activity was detected in the N-pH and S-pH but only the second group showed a significant decrease ($P < 0.01$); in the F-pH group the activity of the autolyzed form did not change compared to 24 h post-mortem and it was higher than both the N-pH ($P < 0.01$) and the S-pH ($P < 0.01$) groups (table 1).

The myofibrillar fragmentation was measured by determine the size-distribution of the myofibrillar fragments (surface mean diameter D (3,2)). At 24 h post-mortem the F-pH group had a higher level of myofibrillar fragmentation compared to the other two groups ($P < 0.01$), suggesting a higher proteolysis in relation to the faster drop of pH. Later, at 72 and 144 h post-mortem, no significant differences were found between groups (fig. 1).

Discussion

The analysis of calpain activity with the zymography method revealed a decrease of μ-calpain activity parallel to the pH decrease: faster the pH drop faster the loss of enzyme activity. While at 1 h post-mortem no differences in the three groups were detected, at 6 h post-mortem the group with fast pH drop showed a reduction of the activity of the enzyme and at the same time an increase in the autolysis, both in relation to the other two groups and in relation to the 1 h samples. It has previously been suggested, that the detection of autolyzed enzyme in muscle and meat can be considered an indication of proteolytic activity [5; 7]. The detection in our samples of increased autolysis and less native μ-calpain activity suggests a faster activation of the enzyme at lower pH values. Previous studies have also shown faster decline of μ-calpain activity in samples with faster pH drop [2; 6; 9; 11] and at the same time an increase in the rate of autolysis of the enzyme [7]. The activity of μ-calpain decreased significantly in all group at 24 h post-mortem. The group with faster pH decline showed also at this time point a lower activity of μ-calpain in accordance with previous reports [10; 11] and a higher detection of activity of autolyzed μ-calpain. At 24 h post-mortem also the N-pH and S-pH showed significantly higher activity of the autolyzed form compared to the earlier time points. As expected the activity of the enzyme continued decreasing at 72 h post-mortem in all groups, and the S-pH group showed a slower decrease of enzyme activity compared to the other two groups. The activity of the autolyzed form instead was constant for the F-pH and N-pH groups, and decreased in the S-pH group. Looking at the overall picture of the decrease of μ-calpain activity within 3 days post-mortem and the autolysis process of the enzyme it could be concluded that faster pH drop in the muscle after slaughter

induced faster decrease in calpain activity and faster autolysis. An early pH drop is accompanied by an early increase in intracellular calcium concentration [9]. Consequently μ-calpain activation and autolysis occurs earlier and eventually results in an earlier loss of proteolytic activity and subsequently lower activity at later time points.

In concomitance with faster decrease of μ-calpain activity and higher autolysis in the F-pH group, myofibrillar fragmentation was higher at 24 h post-mortem. The detection of faster myofibrillar degradation in muscles with a faster pH drop was previously reported [8], as well as a faster degradation of those myofibrillar proteins like desmin and talin that are thought to be mainly degraded by μ-calpain in post-mortem muscles [2; 7]. In the later time points, at 72 and 144 h post-mortem, these differences were no longer observed. In agreement with our results Uytterhaegen et al. [10] reported faster protein degradation within the first 24 h post-mortem in muscles with faster pH decrease, but the rate of the degradation during ageing, in the fast and slow pH drop, was the same and at the latest time point no differences were detected. The same pattern was reported by White et al. [12], they suggested that temperature and pH drop can influence the rate but not the extent of proteolysis.

μ-Calpain was demonstrated to be responsible of the proteolysis of those muscle proteins that are degraded during post-mortem ageing. From the results presented in this and in previous studies we can suggest that faster decrease in muscle pH post-mortem leads to a faster activation of the protease and consequently to higher protein degradation in longissimus dorsi muscle. At the same time we should consider that at 72 and 144 h post-mortem, the differences in size of the myofibrillar particles did not present any differences within the three groups. The earlier activation of the protease has an influence in the early time points, but this effect is lost in the later time points where, in agreement with previous works [10; 12], no differences in myofibril degradation and meat tenderness was reported. Probably the earlier effect of proteolysis on meat tenderness is lost during ageing because of an earlier exhaustion of the enzyme activity in the group with faster pH decrease [6] suggesting that the rate of pH decrease early post-mortem can influence the rate but not the extent of myofibrillar degradation.

IV. CONCLUSION

The present results show that a fast pH fall leads to earlier μ-calpain activation and autolysis and eventually results in an earlier loss of proteolytic activity. Likewise, myofibril fragmentation occurs earlier following a fast pH-fall. In conclusion, the rate of pH decrease early post-mortem affected the rate but not the extent of myofibrillar degradation.

REFERENCES

[1] Baki, A., P. Tompa, A. Alexa, O. Molnar, and P. Friedrich. 1996. Autolysis parallels activation of mu-calpain. Biochemical Journal 318:897-901.

[2] Bee, G., A. L. Anderson, S. M. Lonergan, and E. Huff-Lonergan. 2007. Rate and extent of pH decline affect proteolysis of cytoskeletal proteins and water-holding capacity in pork. Meat Science 76:359-365.

[3] Camou, J. P., J. A. Marchello, S. Mares, R. Vazquez, and D. E. Goll. 2006. Effect of pH and temperature on activity of μ- and m-calpain. FASEB J. 20:A50-A5b.

[4] Cong, J. Y., D. E. Goll, A. M. Peterson, and H. P. Kapprell. 1989. The role of autolysis in activity of the Ca^{2+}-dependent proteinases (μ-Calpain and m-Calpain). Journal of Biological Chemistry 264:10096-10103.

[5] Goll, D. E., V. F. Thompson, H. Q. Li, W. Wei, and J. Y. Cong. 2003. The calpain system. Physiological Reviews 83:731-801.

[6] Hwang, I. H. and J. M. Thompson. 2001. The interaction between pH and temperature decline early postmortem on the calpain system and objective tenderness in electrically stimulated beef longissimus dorsi muscle. Meat Science 58:167-174.

[7] Melody J.L., Lonergan S.M., Rowe L.J, Huiatt T.W, Mayes M.S., and Huff-Lonergan E. 2004. Early post-mortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. J. Animal Sci. 82:1195-1205.

[8] O'Halloran, G. R., D. J. Troy, and D. J. Buckley. 1997. The relationship between early post-mortem pH and the tenderization of beef muscles. Meat Science 45:239-251.

[9] Rhee, M. S., Y. C. Ryu, and B. C. Kim. 2006. Postmortem metabolic rate and calpain system activities on beef Longissimus tenderness classifications. Bioscience Biotechnology and Biochemistry 70:1166-1172.

[10] Uytterhaegen, L., E. Claeys, and D. Demeyer. 1992. The Effect of Electrical-Stimulation on Beef Tenderness, Protease Activity and Myofibrillar Protein Fragmentation. Biochimie 74:275-281.

[11] Whipple, G., M. Koohmaraie, M. E. Dikeman, and J. D. Crouse. 1990. Effects of High-Temperature Conditioning on Enzymatic-Activity and Tenderness of Bos-Indicus Longissimus Muscle. J. Anim Sci. 68:3654-3662.

[12] White, A., A. O'Sullivan, E. E. O'Neill, and D. J. Troy. 2006. Manipulation of the pre-rigor phase to investigate the significance of proteolysis and sarcomere length in determining the tenderness of bovine M-longissimus dorsi. Meat Science 73:204-208

Table 1. Native and autolyzed μ-calpain activity analyzed with the zymography method.

Time Post-mortem, hours	Slow pH drop (pH3 < 6.00)	Normal pH drop (6.00 < pH3 > 6.30)	Fast pH drop (pH3 > 6.30)
μ-Calpain activity			
1 h	1.07 ^{a,x} ± 0.034	1.09 ^{a,x} ± 0.033	1.04 ^{a,x} ± 0.034
6 h	1.01 ^{a,x} ± 0.033	1.12 ^{b,x} ± 0.033	0.87 ^{c,y} ± 0.033
24 h	0.67 ^{a,y} ± 0. 033	0.59 ^{a,b,y} ± 0.033	0.51 ^{b,z} ± 0.033
72 h	0.41 ^{a,z} ± 0. 033	0.30 ^{b,z} ± 0.033	0.22 ^{b,w} ± 0.033
Autolyzed μ-Calpain activity			
1 h	0.03 ^{a,x} ± 0.081	0.02 ^{a,x} ± 0.079	0.02 ^{a,x} ± 0.082
6 h	0.01 ^{a,x} ± 0.080	0.00 ^{a,x} ± 0.079	0.10 ^{b,y} ± 0.080
24 h	0.16 ^{a,b,y} ± 0.080	0.12 ^{a,y} ± 0.080	0.19 ^{b,z} ± 0.079
72 h	0.08 ^{a,z} ± 0.079	0.09 ^{a,y} ± 0.080	0.18 ^{b,z} ± 0.079

^{a,b,c} Within rows, mean values without a common superscript differ ($P < 0.05$).

^{x,y,z,w} Within columns, mean values without a common superscript differ ($P < 0.05$).

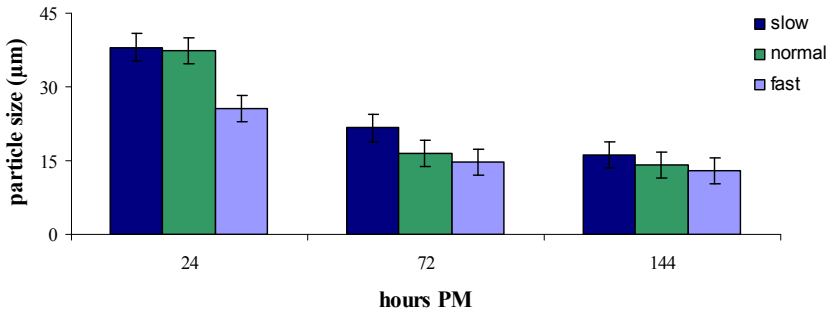

Figure 1. Myofibrillar fragmentation.