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A Capillary Electrophoresis Method to Study Postmortem Proteolysis in Relation to Pork Tenderness

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Identification of factors that determine meat tenderness is of high priority. The aim of this work was to develop a method that can detect indicators of proteolysis in meat early postmortem. The method was validated on pork samples. A procedure to detect differences of extractable lower molecular weight compounds after a prerigor freeze/thaw cycle of meat was developed using capillary electrophoresis. The procedure was able to separate 39 peaks in the electropherograms. Eight of the peaks were correlated ($P < 0.1$) to Warner−Bratzler shear forces 1 day postmortem (WB1). A multiple linear regression model explained 69% of the variation in WB1 using the areas of four peaks. Several of the peaks used in modeling WB1 were related to the at-slaughter activity of the calpain system. The results presented show that the developed method is able to detect indicators of proteolysis and tenderness at an early time point after slaughter. The method is a new tool intended for studies regarding the mechanisms of postmortem proteolysis and tenderization.

KEYWORDS: Proteolysis; marker; calpain; capillary electrophoresis; Warner-Bratzler; freezing; tenderness

INTRODUCTION

Tenderness of meat is an important aspect of eating quality and identification of the factors that determine final meat tenderness, and the tenderization rate has for long been of high priority. It is well-known that sarcomere length (SL) (*1*), connective tissue (*2*, *3*), intramuscular fat (*4*), and postmortem (PM) proteolysis (*5*) affect meat tenderness. The contributions from connective tissue and intramuscular fat are settled at the time of slaughter, i.e., they do not change PM to an extent that is believed to influence tenderness. The factors that determine the decrease of SL during rigor development are well-understood, and large variation in SL can therefore be controlled and prevented by appropriate handling of animals before and after slaughter (*6*, *7*). PM proteolysis and the mechanism by which meat tenderizes are, however, not well-understood. If the potential for PM proteolysis in a carcass could be determined at an early time point after slaughter, variation in tenderness due to proteolysis could be predicted and it would then be possible to differentiate the time needed for aging of particular carcasses.

Considerable evidence suggests that the activity of several muscle proteolytic enzyme systems is involved in the protein turnover in vivo and in the rate and extent of the tenderness development that occur PM. Several studies have thus observed degradation of muscle proteins during PM storage and the concomitant appearance of protein degradation products (*8*−*11*). The quantity of a fragment often referred to as the 30 kDa fragment, which, at least partly, originates from proteolytic degradation of troponin-T (*12*), correlates to meat tenderness after several days of storage, and this fragment has been suggested as a marker for meat tenderness (*8*, *13*, *14*). The myofibrillar fragmentation index (MFI) is believed to reflect the proteolytic degradation that occurs in meat during storage. Over 50% of the variation in bovine tenderness has thus been explained by MFI values (*15*−*17*), which supports the conclusion that tenderness of meat is highly related to PM proteolysis.

Although the reported correlations between tenderness and indicators of proteolysis are rather high, they are not useful as early PM markers of the proteolytic potential. The quantity of the 30 kDa fragment can first be measured several hours or days after slaughter, and MFI values have to increase to a level that is indicative for tenderness, which also requires several hours or days of storage. The aim of the work presented in this paper was to develop a method that can detect indicators of proteolysis in meat at an early time point after slaughter. The method was validated on pork samples.

MATERIAL AND METHODS

Animals and Sampling. A complete description of the animals and sampling is given in ref *18*. Briefly, samples for capillary electrophoresis (CE), μ-calpain, m-calpain, and calpastatin, were excised from the longissimus dorsi (LD) muscle of 39 pigs 15 min after slaughter, frozen in liquid nitrogen, and afterward stored at −80 °C until analysis. Two

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pieces of the LD, each of 7 cm, were vacuum packed 24 h PM and used for Warner−Bratzler shear force determination. One chop was immediately stored at −20 °C (WB1) and the other chop was aged for an additional 3 days and afterward stored (WB4). The procedures for Warner−Bratzler shear force, calpain, and calpastatin determinations were as described in ref *18*.

Incubation and Extraction Procedure. The meat samples frozen 15 min after slaughter, approximately 3 g, were vacuum packed and transferred to a water bath set at 25 °C. This procedure ensured an almost instant thawing of the meat. The samples were incubated at 25 °C for various time periods. An extraction procedure described (*19*) was used with the following modifications. Immediately after incubation, 1.50 g of meat was transferred to a 50 mL centrifuge tube containing 7.50 mL of 3.00% perchloric acid and including 100 μg/mL dityrosin (SIGMA, Sigma Chemicals CO, St. Louis, MO) added as an internal standard (IS). The samples were homogenized using an ULTRA-TURRAX T25 (IKA-WERKE, Staufen, Germany) for 30 s at 20 000 rpm. Undissolved substances were precipitated by centrifugation at 10 000*g* for 20 min, and 5.00 mL of supernatant was stored at −80 °C until CE analysis.

CE. The supernatant was thawed at room temperature and afterward neutralized with 425 μL of 2.00 M K_2CO_3. The pH of the sample was increased to approximately 12 by adding 175 μL of 10.00 M NaOH. Samples were filtrated with 0.2 μm single use syringe filters (Minisart RC 15, Satorius AG, Göttingen, Germany) and diluted once with water before loading onto the capillary. A P/ACE system 5010 CE instrument (Beckmann Instruments, Fullerton, CA) mounted with a diode array detector was used to separate the extracted compounds with the following run parameters: 12 s injection, separation at constant current (250 mA) with 300 mM boric acid (pH 9.00 adjusted with 10 M NaOH) at 20 °C. An uncoated capillary (eCAP capillary tubing from Beckmann Instruments) of 97 cm total length, 90 cm to the detector window, and an inner diameter of 100 μm was used. Peaks were detected at 200 nm.

Peak Identification. Peak areas were corrected for differences in loading volume between samples by dividing with the area of the IS. The velocity-corrected areas were afterward calculated by multiplying with the average velocity (v) of the peak (v = capillary length to the detector/retention time (RT)) (*20*). The electroosmotic flow is the driving force within the capillary, and this flow is highly dependent on buffer strength, pH, applied voltage, diameter of the capillary, and capillary temperature. Variation in the RTs therefore occurs. This variation can be decreased considerably by using RTs relative to two reference peaks. **Table 1** shows the average RTs and the relative RTs for the peaks used in the correlation analysis described later in the paper. Peaks were identified by their relative RT (α) to two of six reference peaks observed in all samples (peaks marked A−F in **Figure 1**) according to the following equation:

$$\alpha = (t_2 - t_1)/(t_x - t_1)$$

where t_x is the RT for the peak of interest and t_1 and t_2 are the RTs for two reference peaks flanking the peak of interest. When describing the peaks, they are referred to by their number of appearance in the electropherograms, i.e., the first peak is named P1, the second peak P2, etc. There were 39 peaks observed; however, not all peaks were observed in all samples. Peaks presented in **Table 1** were observed in all samples and were used in the correlation analysis.

Data Analysis. Correlation coefficients were determined using the CORR procedure of SAS (SAS Inst. Inc., 1999−2000). Multiple linear regression models were obtained by the REG procedure of SAS using stepwise regression. A P value of 0.15 was used to introduce and keep variables in the models.

RESULTS AND DISCUSSION

An approach to discover markers for proteolysis is to search for protein/peptide degradation products. One problem in using this approach is that the products might only be detectable several hours or days after slaughter. However, it is possible to accelerate the proteolytic processes by including a prerigor freezing of the samples. A freeze/thaw cycle of meat prerigor is known to have a major effect on meat quality caused by a severe thaw contraction (*21*). Thaw contraction is mainly caused by a rapid increase of the free calcium concentration in the sarcoplasma upon thawing. Besides driving the contraction process, calcium also activates the calpain system, which is one of the proteolytic enzyme systems within muscles (*22*). Dransfield (*23*) froze prerigor beef samples at −70 °C and thawed the samples quickly at 30 °C. Twenty minutes after thawing, the activity of μ-calpain was declined to 14% of the initial activity and m-calpain to 70% of the initial activity. The rapid decline in calpain activity was suggested to be due to activation, caused by the increased calcium concentration, followed by autolysis and loss of enzyme activity. In unfrozen meat that

Table 1. RT, Relative RT, Mean Peak Areas, and SDs for Peaks Used in Correlation Analysis[a]

peak[b]	RT (min)	relative[c] RT	mean[d]	SD
P1	10.8	A-0.000	696 347	57 468
P2	12.9	A-0.482	31 418	6464
P3	15.0	A-0.930	18 745	3594
P4	15.3	B-0.000	624 752	74 158
P5	16.1	B-0.222	13 536	1783
P6	16.3	B-0.271	786	243
P10 (IS)	19.0	C-0.000	100 000	0
P12	20.2	C-0.163	14 027	9180
P21	26.6	D-0.000	26 967	3261
P22	29.4	D-0.166	1082	377
P26	33.0	D-0.384	5181	3075
P31	43.2	E-0.000	60 637	4960
P32	51.1	E-0.386	19 890	4799
P35	56.2	E-0.636	3208	831
P36	63.7	F-0.000	25 591	1857
WB1 (N)			63.9	13.7
WB4 (N)			47.4	8.7
μ-calpain (U/g)[e]			5.35	1.10
m-calpain (U/g)			7.69	1.10
calpastatin (U/g)			15.0	4.12

[a] Mean and SD for at-slaughter activities of μ-calpain, m-calpain, and calpastatin and Warner–Bratzler shear force 1 and 4 days PM (WB1, WB4). [b] Only peaks observed in all animals are presented. [c] Relative RT to two flanking peaks. See text for detail. [d] Corrected peak area. See text for detail. [e] Units per gram of meat.

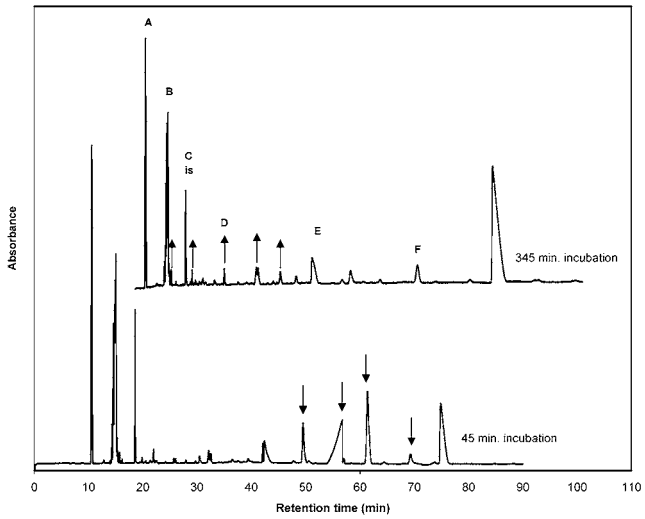

Figure 1. Electropherograms obtained from extract of meat 45 and 345 min after a prerigor freeze/thaw cycle. The upper electropherogram has been transposed 10 min for clarity. The peaks marked with letters A–F are used for identification of single peaks. See text for details; IS equal to C. Arrows indicate examples of either increasing or decreasing peaks as a function of incubation time.

has a normal rigor development, the calcium concentration also increases; however, the increase will take place later PM when the pH has dropped to approximately 6. A freeze/thaw cycle applied to a prerigor muscle is therefore likely to accelerate the proteolytic processes caused by the calpain system. The lysosomal cathepsins have also been linked to PM proteolysis and tenderization of meat (*24*, *25*), and it is most likely that the proteolytic activity of these proteases also will be accelerated by a prerigor freeze/thaw cycle due to membrane disruption.

Feidt et al. (*19*) found, using an extraction procedure similar to the procedure used in this study, that an extract from kid muscle contained compounds with a range of molecular sizes up to 13 kDa, the majority being less than 4 kDa. Using size exclusion chromatography for fractionation of the extract, aging of kid muscle changed the profile of the extract to a higher content of low molecular weight compounds, which indicates proteolytic activity during storage of the meat (*19*). Numerous papers have linked proteolytic enzyme activity to the increase in meat tenderness, which occurs after slaughter (for review, see ref *26*).

In the present paper, we have used the superior separation capability of CE to quantify compounds in an extract from pork obtained early PM after a prerigor freeze/thaw cycle of a small sample of meat. A standard capillary zone electrophoresis procedure was optimized using boric acid as electrolyte buffer. The optimization was aimed at a maximum number of peaks and involved the buffer strength, buffer pH, injection time, applied voltage, length of capillary, diameter of the capillary, and capillary temperature. After testing several small peptides as ISs, dityrosin was chosen as it was not interfering with other peaks and also showed high storage stability. The optimization resulted in the CE procedure described in the Materials and Methods section.

To study the development of extractable compounds in pork after a prerigor freeze/thaw cycle, meat from one animal was incubated for 345 min and samples were removed after time periods of 45, 105, 225, and 345 min. **Figure 1** shows the complete electropherograms obtained after 45 and 345 min of incubation. Several peaks either decreased or increased during the incubation, indicating that the quantity of the extracted compounds is changing (arrows in **Figure 1**). Whether all of these changes are due to proteolytic activity is questionable. A part of the extracted compounds is probably unrelated to proteolysis; however, as shown later in this paper, a number of the peaks are very likely to be associated to proteolysis.

The method was tested on 39 pork samples to validate whether some of the observed changes could be related to meat tenderness and calpain-mediated proteolysis. Samples from all animals were analyzed using 225 min of incubation following a prerigor freeze/thaw cycle. In total, 39 peaks were observed; however, not all peaks were observed in all samples, an effect possibly due to the detection limit of the instrument or due to large variations between samples in their relative RTs. Even though the variation in RTs was minimized considerably by using relative RTs to two reference peaks, there were still several peaks that we were unable to identify in all samples. Therefore, only the 15 peaks observed in all samples were used to validate the obtained method. The RTs, relative RTs, mean peak areas, and standard deviations (SD) are presented in **Table 1**, which also includes data on WB1, WB4, μ-calpain, m-calpain, and calpastatin. The first peak (A, **Figure 1**) was observed with a RT of 10.8 min, the IS (C) with a RT of 19.0 min, and the last peak with a RT of 72.7 min. **Table 2** presents the peaks that were found to correlate with WB1 and/or WB4. These eight peaks are marked on the electropherograms in **Figures 2** and **3**.

Table 2. Pearson Correlation Coefficients (*r*) between Peaks Presented in **Table 1** and Warner–Bratzler Shear Force 1 and 4 days PM (WB1, WB4)[a]

peak	WB1	WB4	peak change	peak	WB1	WB4	peak change
P3	0.43[d]	ns	↓	P22	0.28[b]	ns	÷
P4	0.34[c]	0.27[b]	*f*	P26	0.30[b]	ns	↑
P6	0.67[e]	0.35[c]	↑	P35	0.30[b]	ns	↓
P12	0.37[c]	0.33[c]	↑	P36	0.55[e]	0.34[c]	↓

[a] Only peaks with *P* values less than 0.1 are presented. Changes in peak areas from 45 to 334 min of incubation are indicated by arrows; ns, $P > 0.1$. [b] $P < 0.1$. [c] $P < 0.5$. [d] $P < 0.01$. [e] $P < 0.001$. [f] Could not be determined due to multiple, partly separated peaks in P4.

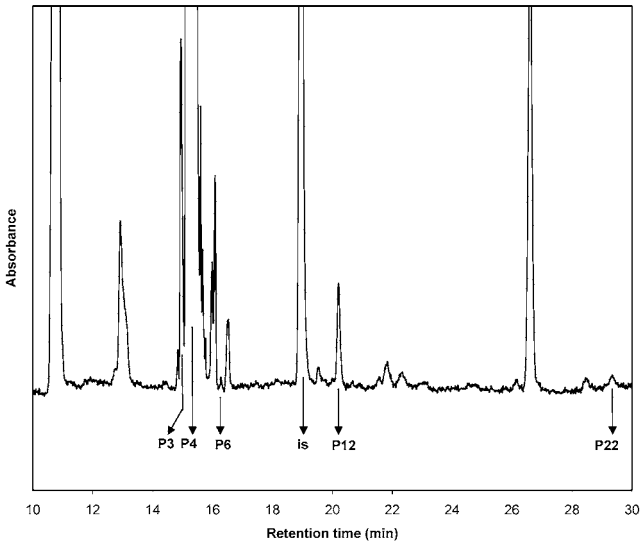

Figure 2. Electropherograms obtained from extract of meat 225 min after a prerigor freeze/thaw cycle. RTs are from 10 to 30 min. The absorbance scale is 10-fold lower than in **Figure 1**. Numbered peaks are presented in **Table 2**.

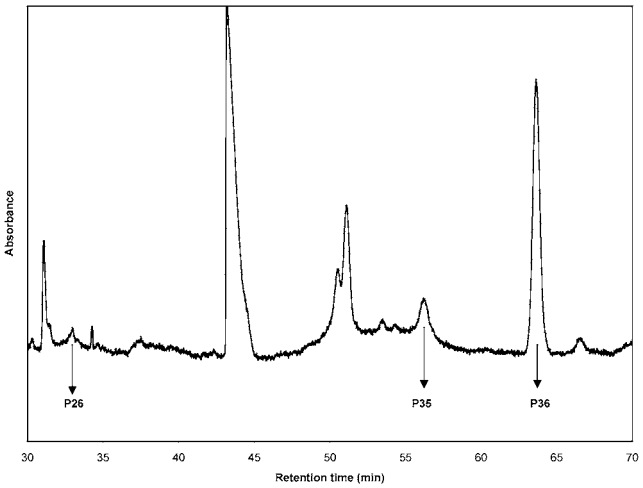

Figure 3. Electropherograms obtained from extract of meat 225 min after a prerigor freeze/thaw cycle. RTs are from 30 to 70 min. The absorbance scale is 10-fold lower than in **Figure 1**. Numbered peaks are presented in **Table 2**.

Overall, the obtained correlation coefficients were higher for WB1 than for WB4 (**Table 2**), which may be due to the longer

Table 3. Multiple Linear Regression Models on Warner–Bratzler Shear Force 1 and 4 Days PM (WB1, WB4) Using Peaks Presented in **Table 2**[a]

dependent variable	independent variable	partial R^2	P value	model R^2	model P value
WB1	P6	0.45	<0.001		
	P36	0.13	0.002		
	P22	0.05	0.038		
	P3	0.05	0.025	0.69	<0.001
WB4	P6	0.12	0.034		
	P4	0.07	0.100	0.19	0.028

[a] A P value of 0.15 was used to introduce and keep variables in the models.

Table 4. Pearson Correlation Coefficients (r) between Peaks Included in Modeling WB1 (**Table 3**) and the At-Slaughter Activities of μ-Calpain, m-Calpain, and Calpastatin

peak	μ-calpain	m-calpain	calpastatin
P3	ns	–0.41[b]	ns
P6	ns	ns	0.71[c]
P22	ns	ns	ns
P36	ns	–0.31[a]	0.52[c]

[a] $P < 0.1$. [b] $P < 0.5$. [c] $P < 0.001$; ns, $P > 0.1$.

period of tenderization that has taken place in WB4 samples. It is well-known that tenderness of meat increases during aging to a point described as "background toughness" whereafter only minor changes in tenderness occur (*27*, *28*). The background toughness is mainly determined by SL, intramuscular fat content, water content, and the content and quality of connective tissue. As discussed in ref *18*, the variations in the degree of proteolysis PM are to a large extent reflected in the variation of WB1 and to a lesser extent in the variation of WB4, whereas the background toughness is much more related to WB4. If the peaks quantified (**Table 2**) represent compounds that are changing in quantity due to proteolysis, this may explain why the correlations are higher to WB1 than to WB4.

On the basis of the peaks that correlated to WB1, multiple linear regression models were calculated (**Table 3**). Peak number 6 had the highest partial correlation coefficient followed by P36, P22, and P3. Overall, the model explained 69% of the variation in WB1. Only two peaks were included in the model calculated for WB4, and the model explained 19% of the variation in WB4 (**Table 3**). Peak number 6 had the highest partial correlation coefficient followed by P4. The relatively better model obtained on WB1 as compared to WB4 can be explained using the same arguments as used in connection to **Table 2**.

The calpain system has been suggested as a catalyst of the PM tenderization process in beef (*10*, *29*) and has also been linked to proteolysis in pork (*18*, *30*). **Table 4** shows the correlation coefficients between the at-slaughter activity of μ- and m-calpain and their inhibitor calpastatin and the peaks that were included in the model obtained on WB1. None of the peaks correlated to the at-slaughter activity of μ-calpain. Peak numbers 3 and 36 slightly correlated to m-calpain activity, whereas peak numbers 6 and 22 were uncorrelated. The activity of m-calpain has been linked to PM proteolysis and tenderization of meat (*31*, *32*), although this linkage has been questioned (*33*). Peak number 36 and especially P6 were strongly correlated to the calpastatin activity. The significant correlations between peak numbers 6 and 36 and the calpastatin activity can be explained by at least two mechanisms: (i) the peaks represent degradation products of calpastatin. Alternatively, (ii) the quantity of the compounds represented by the peaks is determined by calpastatin, i.e., the compounds are proteins/peptides that are either degraded or produced by proteolytic enzymes of which the activity is controlled by calpastatin (the only known enzymes that are inhibited by calpastatin are the calpains). That the latter mechanism is plausible is partly verified by the negative correlation between m-calpain activity and P36. The negative correlation implies that the quantity of the compounds represented by P36 is reduced after thawing due to proteolysis, which is supported by the results presented in **Table 2** where the peak is shown to decrease with time. Peak number 6 was positively correlated to calpastatin activity implying that the peak would decrease with time; however, the peak increased with time (**Table 2**). During PM proteolysis, several proteins are cleaved into smaller fragments (primary fragments). These primary fragments will be further degraded into even smaller secondary fragments. This statement is supported by Feidt et al. (*19*) who observed a decrease in high molecular weight compounds and an increase in low molecular weight compounds during storage of meat. If P6 is a primary fragment originating from a protein degraded very fast PM and the degradation of the primary fragment is controlled by calpastatin, then the correlation between P6 and calpastatin will be positive and the peak will increase with time as observed in **Table 2**. Another explanation of P6 is that it is a degradation product of calpastatin.

The results presented in **Tables 2**–**4** suggest that the method described in this paper is able to detect indicators of proteolysis and tenderness at an early time point after slaughter. The method is a new powerful tool for studies regarding the mechanisms of PM proteolysis and tenderization. A logical follow-up on the present work is to identify importance peaks in order to further understand the mechanisms of PM proteolysis and tenderization and, in the long-term, to identify possible markers of meat tenderization.

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