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Identification of Protein Degradation during Post-mortem Storage of Pig Meat

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Eighteen proteins and peptides that were found to change post-mortem in Longissimus dorsi from pig muscle were identified by the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The 18 peptides originate from 9 different proteins including the 3 structural proteins (actin, myosin heavy chain, and troponin T) and the 6 metabolic proteins glycogen phosphorylase, creatine kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase, and dihydrolipoamide succinyltransferase. The molecular weight and estimated sequence length of the identified spots show that these fragments result from proteolytic activity in meat. Identification of the parent proteins and the enhanced post-mortem appearance of the degradation products make these specific peptides good candidates for meat quality markers, and further studies of these specific fragments will lead to a better understanding of the proteolytic activities involved in the post-mortem conversion of muscle to meat.

KEYWORDS: Porcine proteins; skeletal muscle proteins; meat quality; post-mortem changes; protein degradation; proteome analysis; proteolysis; two-dimensional gel electrophoresis; protein identification; matrix-assisted laser desorption−ionization

INTRODUCTION

Conversion of muscle to meat is governed by complex interactions of biochemical processes that take place during post-mortem storage of the carcass (*1*, *2*). Although their influence on the final texture and tenderness of the meat is still not clear, it is well documented that fragmentation of myofibrils takes place during post-mortem storage of meat and that the extent of this fragmentation is related to the tenderness of aged meat (*3*). A number of previous studies indicate that proteolytic degradation of structural proteins including titin, nebulin, troponin T, desmin, filamin, and vinculin plays a major role in the development of meat tenderness (*4*). Especially the degradation of titin and nebulin, both located in the I-band, is of great interest as electron microscopy of post-mortem meat samples has shown that the major fragmentation of the myofibrils occurs in the I-band area (*4*−*6*).

Due to the limited resolving capacity of classical protein separation methods, the post-mortem changes of only a few proteins have been studied so far. The most commonly used method, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) allows the simultaneous analysis of only 10−20 muscle proteins. Two-dimensional gel electrophoresis (2DE) is a technique that separates proteins according to their isoelectric points as well as their molecular weights, providing a much higher resolution than one-dimensional SDS-PAGE. 2DE technology has been greatly improved during the past few years, and it is at present the separation technology that allows the highest resolutions in protein analysis, allowing simultanous quantitative profile analysis of hundreds of proteins. 2DE is a preparative as well as an analytic tool that allows identification of the separated proteins, and it has become a cornerstone of contemporary proteome analysis (*7*). The most commonly used method for identification of proteins from 2DE is peptide-mass fingerprinting by matrix-assisted laser desorption−ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technology is sensitive, allowing identifications of femtomole quantities of proteins and peptides, in a high-throughput mode (*8*, *9*).

We have previously reported the use of comparative 2DE analysis for finding proteins that change during post-mortem storage of porcine meat (*10*). The aim of the present study was to identify these proteins through preparative 2DE and MALDI-TOF MS-based peptide-mass mapping.

MATERIALS AND METHODS

Animals and Tissue Samples. The animals were treated and slaughtered according to Danish regulations on treatment of livestock.

The animals used in this study were seven littermates originating from a cross between a Danish Landrace/Yorkshire sow and a Hampshire boar. The pigs were raised on a commercial farm and

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slaughtered at an average live weight of 100 kg at the experimental slaughter plant at the Danish Institute of Agricultural Sciences (DIAS). One hour post-mortem the carcasses were chilled and thereafter stored at 4 °C.

Muscle samples were taken from the seven carcasses immediately after exsanguination (time 0) and 4, 8, 24, and 48 h post-mortem. The samples were taken from the Longissimus dorsi muscle, at the position of the last rib. Biopsies were removed as described by using the method reported previously (*11*) and were frozen immediately in liquid N_2. The muscle samples were kept at −80 °C until the time of protein extraction.

Extraction of Muscle Proteins. Pieces of 100 mg of frozen muscle tissues were cut and weighed at −20 °C to minimize artifactual protein degradation. The frozen muscle tissue was homogenized in 1 mL of 8 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS-O, and 1% carrier ampholytes (Ampholyte 3-10, Pharmacia, Uppsala, Sweden), in a hand-held glass homogenizer. Crude extracts were transferred to an Eppendorf tube and vigorously shaken for 2 h at room temperature, followed by a 30 min centrifugation step at 10000*g* to remove unextracted cellular components, high molecular weight protein complexes, and insoluble proteins.

Two-Dimensional Electrophoresis. The preparation and running of the 2DE analysis were done according to ref *10*. The first dimension of protein separation was made in immobilized pH gradient strips (18 cm IPG strips), which span the pH range of 4−9, and in the second dimension 10% SDS-PAGE (25 × 25 cm) was used. For preparative 2DE, 500 μg of protein was loaded onto every IPG strip, whereas for analytical gels 50 μg of protein was loaded. Preparative gels were silver-stained according to the method of ref *12*, and the analytical gels were stained according to the method of ref *10*. Several preparative gels were made for repeated identification of the spots, and a total of 35 analytical gels were made for the post-mortem study.

Image Analysis. The 2DE gels were scanned using a U-Max office scanner and analyzed using Bioimage 2D analyzer software (Genomic Solutions, Ann Arbor, MI). First, all spot positions were recognized, and relative integrated spot intensities in the individual gels were estimated. Then, the 2DE images were matched by comparing the relative positions and integrated intensities of the individual spots on each gel. For comparative image analysis, the images were grouped, after which the relative expressions of the individual spots were analyzed and compared within and between the image groups. Finally, the spot pattern changes revealed by computer-based image analysis were individually inspected and confirmed.

In-Gel Digestion of Protein Spots. In-gel digestion was performed as described in ref *13*. The excised gel plugs were washed in 50 mM NH_4CO_3/actonitrile (60:40 v/v) and dried by vacuum centrifugation. Trypsin (sequencing grade, Roche), dissolved in 50 mM NH_4CO_3, pH 7.8 (12 ng/μL), was added to the dry gel pieces and incubated for reswelling on ice for 1 h. The supernatant was removed, 30 μL of 50 mM NH_4CO_3 buffer, pH 7.8, was added, and the digest was incubated overnight at 37 °C.

Desalting and Concentration. Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis (*14*). A column consisting of 100−300 nL of Poros reverse phase R2 or Oligo R3 material (20−30 μm bead size, Applied Biosystems, Foster City, CA) was packed in a constricted GeLoader tip (Eppendorf, Hamburg, Germany). A 10 mL syringe was used to force liquid through the column. Ten microliters of the tryptic protein digests was mixed with 40 μL in 0.1% TFA, loaded onto the column, and washed with 20 μL of 0.1% TFA. For analyses by MALDI-TOF MS the peptides were eluted with 0.5 μL of matrix solution (15−20 g/L of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile) directly onto the MALDI target in very small droplets.

Peptide-Mass Mapping by MALDI-TOF MS. A Bruker REFLEX model MALDI-TOF mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with the scout source and variable detector bias gating was used in positive-ion reflector mode for mass analysis of peptide mixtures (peptide-mass mapping). The ion acceleration voltage was 20 kV. Calibration was preformed using the trypsin peaks at *m/z* 842.11 and 2211.1 resulting from autodigestion of trypsin. The average deviation in mass accuracy was 50 ppm (*13*).

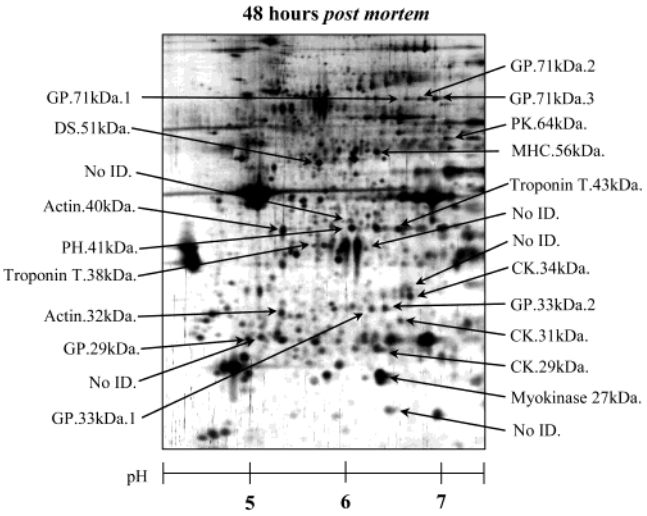

Figure 1. 2DE gel of porcine L. dorsi taken 48 h post-mortem. Arrows show the identified proteins and protein fragments that were found to change post-mortem. GP, glycogen phosphorylase; DS, dihydrolipoamide succinyltransferase; PH, phosphopyruvate hydratase; PK, pyruvate kinase; MHC, myosin heavy chain; CK, creatine kinase.

Protein Identification. Protein identification by peptide-mass mapping was performed using the database search program ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) that searches the NCBI (National Center for Biotechnology Information) protein sequence database. The peptide-mass maps and protein identifications were evaluated as described in ref *13*.

RESULTS

The aim of this work was to identify and characterize proteolytic processes that occur during post-mortem storage of pig meat. We have previously reported the findings of 15 protein spots separated by 2DE, which accumulate during post-mortem storage of pig meat (*10*). These 15 proteins and 8 additional proteins, which by further analysis were found to change post-mortem, were selected for identification. This was basically done by separating and extracting the total of 23 spots from preparative 2DE analysis followed by isolation and MALDI-TOF MS-based peptide-mass mapping.

To perform optimal MALDI-TOF MS analysis of 2DE-separated proteins and peptides, 500 μg of protein was loaded on preparative 2DE gels, and these gels were stained with a MALDI-TOF MS compatible silver stain procedure described in ref *12*.

By comparing the spot pattern of the preparative 2DE gels with the analytic 2DE gels used for comparative image analysis (*10*), the 23 selected spots that change post-mortem were recognized and cut out of the preparative gels. The proteins in the gel plugs were digested with trypsin, which cleaves peptide bonds only on the N-terminal side of lysine and arginine residues. The resulting peptide mixtures were eluted from the gel plugs, and the peptide masses were measured by MALDI-TOF MS. The information of the specific peptide masses was used for searching alignments to proteins found in theoretic digest databases in order to identify the 23 proteins and peptides. Porcine as well as other mammalian sequence databases were used in these searches. This approach allowed the identification of 18 of the 23 selected spots. The 2DE positions of the analyzed proteins and peptides are shown in **Figure 1**.

The identified proteins are listed in **Table 1**, together with the origins of the matched protein sequences, the theoretical molecular weight of the proteins, the estimated sequence length found using the first and last matched peptide of the database

Table 1. Protein Identifications[a]

ID	database alignment[b]	estimated seq length[c]	min seq coverage[d] (%)	matched peptides[e]
GP.71kDa.1	glycogen phosphorylase (humam, 96 kDa)	162–609 (842)	19	14
GP.71kDa.2	glycogen phosphorylase (human, 96 kDa)	67–601 (842)	12	10
GP.71kDa.3	glycogen phosphorylase (human, 96 kDa)	162–609 (842)	18	12
GP.33kDa.1	glycogen phosphorylase (human, 96 kDa)	49–258 (842)	7	6
GP.33kDa.2	glycogen phosphorylase (human, 96 kDa)	24–219 (842)	5	5
GP.29kDa	glycogen phosphorylase (human, 96 kDa)	628–828 (842)	35	12
PK.64kDa	pyruvate kinase (human, 59kDa)	33–455 (531)	33	17
MHC.56kDa	myosin heavy chain (pig, 223 kDa)	27–618 (1935)	15	10
DS.51kDa	dihydrolipoamide succinyltransferase (pig, 49 kDa)	69–440 (455)	25	14
CK.34kDa	creatine kinase (human, 43 kDa)	31–156 (381)	19	5
CK.31kDa	creatine kinase (human, 43 kDa)	12–242 (381)	21	6
CK.29kDa	creatine kinase (human, 43 kDa)	87–177 (381)	24	7
troponin T 43kDa	troponin T (rat, 32 kDa)	49–272 (272)	33	10
troponin T 38kDa	troponin T (rat, 32 kDa)	55–241 (272)	21	5
PH.41kDa	phosphopyruvate hydratase (human, 47 kDa)	6–228 (433)	37	12
actin 40kDa	α-actin (pig, 42 kDa)	31–374 (377)	45	15
actin 32kDa	α-actin (pig, 42 kDa)	216–328 (377)	10	4
myokinase 27kDa	myokinase (pig, 22 kDa)	10–166 (194)	31	7

[a] Identified protein fragments that were found to change post-mortem in pig L. dorsi are listed. The list of best database alignments shows the identities of the proteins that were used to identify the post-mortem markers. [b] Species origins and calculated MW of the full-length proteins are listed in parentheses. [c] Estimated sequence lengths of the post-mortem fragments based on first and last matched peptides in the database alignment. Number of amino acids of the full-length protein is listed in the parentheses. [d] Minimum coverage of the matched peptides in relation to the full-length sequence. [e] Number of matched peptides is listed.

search, which illustrates from which part of the protein sequence the peptides used in the database searches align to, the sequence coverage of the matched peptides in relation to the full-length sequence, and the number of matched peptides in the database search.

The 18 identified spots originate from 9 different proteins; 3 structural proteins, namely, actin, myosin heavy chain, and troponin T, and the 6 metabolic proteins glycogen phosphorylase, creatine kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase, and dihydrolipoamide succinyltransferase. Myosin heavy chain, dihydrolipoamide succinyltransferase, actin, and myokinase were identified by alignments to porcine proteins, whereas the identitites of glycogen phosphorylase, pyruvate kinase, creatine kinase, troponin T, and phosphopyruvate hydratase could be established by homologous alignments to human and rat proteins.

Two fragments derived from actin (40 and 32 kDa) and one fragment derived from myosin heavy chain (56 kDa) were identified as spots with increasing intensities post-mortem, suggesting that both actin and myosin are degraded post-mortem (**Figure 2**). The sequence of the myosin heavy chain fragment is estimated to include the amino acids 27−618, indicating that full-length myosin is cleaved to form a globular myosin head domain fragment (*15*). This is in agreement with previous studies that show degradation of myosin heavy chain into a globular myosin head fragment during post-mortem storage of bovine muscle (*16*). Two spots were identified as troponin T as seen in **Figure 2**: the full-length troponin T (43 kDa) decreased in intensity, whereas the 38 kDa troponin T fragment accumulated during post-mortem storage. Post-mortem degradation of troponin T is well-known, and it is believed to be related to tenderness (*17*, *18*).

Fourteen of the 18 identified spots (listed in **Table 1**) were fragments of 6 different metabolic proteins. Five of these are sarcoplamic proteins, namely, glycogen phosphorylase, creatine kinase, pyruvate kinase, phosphopyruvate hydratase, and myokinase, whereas dihydrolipoamide succinyltransferase are located in the mitochondria. Six spots are considered to be fragments of glycogen phosphorylase. **Figure 3** shows how the fragment spots accumulate during post-mortem storage. We found three fragments of 71 kDa, two of 33 kDa, and a single fragment of 29 kDa. The estimated sequence length in **Table 1** indicates

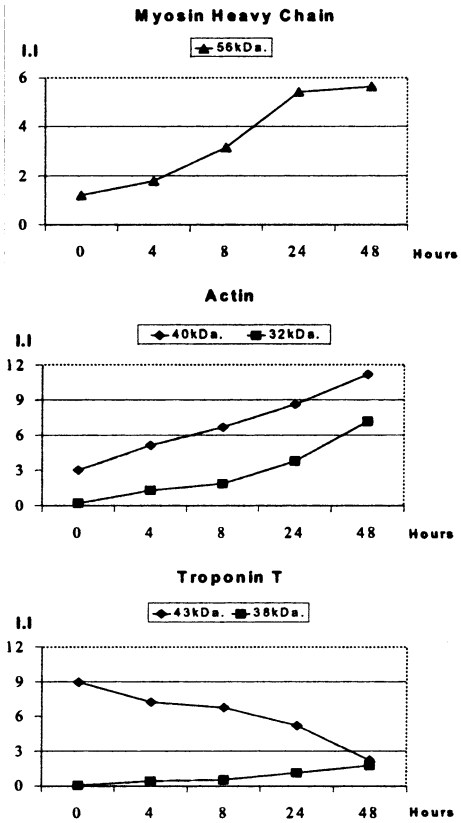

Figure 2. Post-mortem profiles of protein fragments from structural proteins. Curves represent the relative integrated intensities of the identified protein spots observed in 2DE analysis of L. dorsi samples taken at 0, 4, 8, 24, and 48 h post-mortem. Curves show the average intensities observed in seven animals.

that the multiple fragments at 71 and 33 kDa are modified isoforms of two fragments. The three glycogen phosphorylase fragments of 71 kDa have identical estimated sequence lengths, and this is also the case for the two fragments at 33 kDa. The estimated sequence length data of the six glycogen phosphorylase fragments also show that the 71 and 29 kDa fragments are a result of a specific cleavage of the full-length protein, whereas the 33 kDa fragments result from subdegradation of

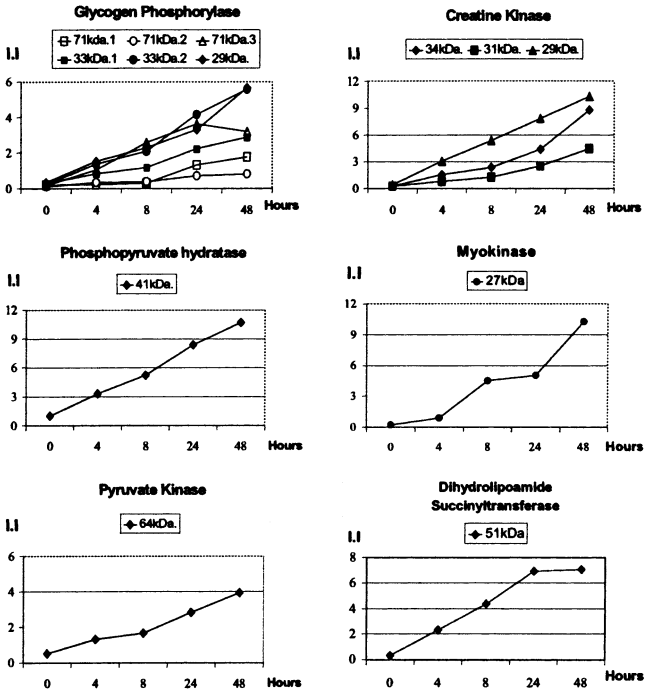

Figure 3. Post-mortem profiles of protein fragments from metabolic proteins. Curves represent the integrated intensities of the identified spots from L. dorsi samples taken at 0, 4, 8, 24, and 48 h post-mortem. Curves are based on the average intensities observed in seven animals.

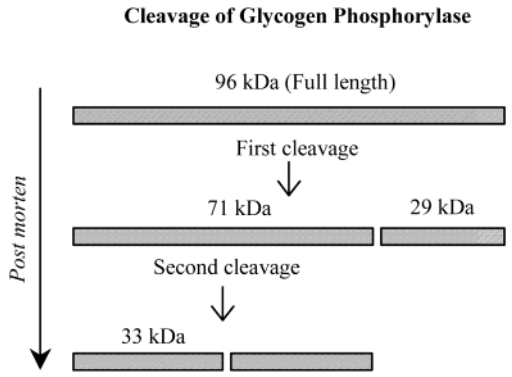

Figure 4. Illustration of the suggested cleavage pattern of glycogen phosphorylase, as observed at times 0–48 h post-mortem. The full-length 96 kDa protein is initially cleaved to form 71 and 29 kDa fragments. The 71 kDa fragment is then further cleaved into a 33 kDa fragment.

the 71 kDa fragments as illustrated in **Figure 4**. Three spots were identified as fragments of creatine kinase at the positions of 34, 31, and 29 kDa. These spots were observed to accumulate during post-mortem storage as seen in **Figure 3**. The 2DE position of the full-length creatine kinase is known from previous studies (*10*). Creatine kinase is highly abundant in muscle tissues, as also observed in **Figure 1**, and the decrease of the full-length protein during post-mortem storage could therefore not be observed. Fragments of pyruvate kinase, phosphopyruvate hydratase, and myokinase were detected in spots at 64, 41, and 27 kDa, respectively.

DISCUSSION

The above results show that structural as well as metabolic proteins are degraded during post-mortem storage of porcine muscle. The MALDI-TOF MS-based peptide-mass mapping used to identify the post-mortem-altered proteins proved to be a powerful method, resulting in an identification rate of 78%. This rate is much higher than could have been expected, taking into account the rather limited number of porcine protein sequences available from the protein sequence databases. Of the protein sequences in the nonredundant SWISS-PROT database, only a total of 784 proteins are of porcine origin, and in the NCBI protein database 5141 proteins are of porcine origin. However, our results show that the extensive sequence homologies between human and porcine proteins, as presented in **Table 1**, make MALDI-TOF MS a useful method for the characterization of porcine proteins.

Of the nine identified proteins, four were based on homology alignments to human proteins, and one protein was matched to a rat protein. Close examination of the informative peptide matches confirmed that they all belong to protein regions that are highly conserved between species.

Post-mortem fragmentation of the structural protein troponin T was confirmed by coherent spot pattern changes of concomitantly decreasing full-length protein spots and increasing fragment spots. No decrease of the full-length actin and myosin heavy chain was detected in connection with the increasing spot intensity of the identified fragments, and by comparing the spot intensities of the actin and myosin heavy chain fragments with the full-length proteins, we estimate that only a minor part of actin and myosin is degraded post-mortem. The estimated sequence length of myosin heavy chain (**Table 1**) shows that degradation occurs in the globular myosin head domain. It has previously been shown that a fragment containing the globular myosin domains is formed during post-mortem storage of bovine muscle (*16*, *19*). Our present study is in agreement with this observation. Post-mortem degradation of actin has not previously been reported, most likely due to the restricted resolution of one-dimensional SDS-PAGE and the fact that only a minor part of the actin is degraded. A relationship between post-mortem degradation of myosin heavy chain and actin and meat texture has not been shown, and further studies have to be made to clarify this.

Several studies have shown troponin T to be degraded post-mortem, and this degradation is believed to be closely related to meat tenderness (*1*, *17*). Our results show that degradation of troponin T results in a decrease of a 43 kDa fragment, which may be identical to the decreasing fragments of 39 kDa previously reported in ref *20*. The accumulating fragment of 38 kDa may be identical to an accumulating fragment of 32 kDa reported by the same authors as above. Fragments from metabolic proteins were the most abundant post-mortem changes identified in this study. Previous studies of post-mortem protein degradation in bovine muscle using peptide profiling and amino acid sequence analysis of protein fragments extracted during meat aging showed that creatine kinase and glycogen phosphorylase are degraded post-mortem (*18*, *21*, *22*). With the use of yeast two hybrid analysis, as well as by in vitro incubations with calpain, Purintrapiban et al. (*22*) found that both creatine kinase and glycogen phosphorylase are calpain substrates, making calpain a good candidate for being the enzyme that degrades the two proteins post-mortem. Further studies will show if degradation of glycogen phosphorylase with calpain results in the same fragments as observed post-mortem in the present study and as illustrated in **Figure 4**.

Several in vitro studies have shown that many structural proteins are substrates for calpain, and it has been found that there is a relationship between calpain activity and tenderization of meat (*3*, *23*, *24*). Proteolysis of metabolic proteins has probably no direct influence on meat texture, but peptides resulting from degradation of metabolic proteins may be useful as markers of proteolytic activity and may therefore be useful as indicators for meat quality.

The present study failed to show/confirm some of the previously reported post-mortem degradations, including the structural proteins titin, nebulin, desmin, filamin, and vinculin (*4*, *25*). The main reason for this might be that these proteins are degraded by unspecific cleavage or by multiple enzymes, which results in low yields of a large number of different peptides. Studies of titin show two specific degradation products at 2400 and 1200 kDa, which with time post-mortem are degraded to smaller peptides; however, these peptides cannot be observed on SDS-PAGE (*25*).

Five of the post-mortem changes could not be reliably identified by the MALDI-TOF MS-based methods we have used. We will attempt to identify these proteins in future work by using tandem mass spectrometry approaches that result in partial de novo sequence data (*9*), hence allowing more specific database matching than what is possible with information obtained by MALDI-TOF analysis.

CONCLUSIONS

From the above work, it can be concluded that MALDI-TOF MS-based peptide-mass mapping is a highly efficient method for the identification of 2DE-separated porcine proteins. By this method we have identified 18 peptides, which were a result of post-mortem proteolysis. Further analysis may reveal which proteases are involved in these fragmentations.

Our finding have confirmed previously reported protein degradations, as well as demonstrated for the first time that also actin is degraded post-mortem. We have also observed that metabolic enzymes are degraded post-mortem. The role of these enzymes in the post-mortem metabolism of meat has not yet gained much attention, and our findings may help future meat science projects to focus on novel post-mortem processes that may be relevant to meat quality.

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LITERATURE CITED

(1) Ouali, A. Proteolytic and physicochemical mechanisms involved in meat texture development. *Biochimie* **1992**, *74*, 251−265.

(2) Koohmaraie, M. Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Sci.* **1996**, *43*, 193−201.

(3) Koohmaraie, M. The role of Ca^{2+}-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. *Biochimie* **1992**, *73*, 239−245.

(4) Taylor, R. G.; Geesink, G. H.; Thompson, V. F.; Koohmaraie, M.; Goll, D. E. Is Z-disk degradation responsible for postmortem tenderization? *J. Anim. Sci.* **1995**, *73*, 1351−1367.

(5) Boyer-Berri, C.; Greaser, M. L. Effect of postmortem storage on the Z-line region of titin in bovine muscle. *J. Anim. Sci.* **1998**, *76*, 1034−1044.

(6) Huff-Lonergan, E.; Parrish, F. C.; Robson, R. M. Effect of post mortem aging time, animal age, and sex on degradation of titin and nebulin in bovine longissimus muscle. *J. Anim. Sci.* **1995**, *73*, 1064−1073.

(7) Pandey, A.; Mann, M. Proteomics to study genes and genomes. *Nature* **2000**, *405*, 837−846.

(8) Gevaert, K.; Vandekerckhove, J. Protein identification methods in proteomics. *Electrophoresis* **2000**, *21*, 1145−1154.

(9) Roepstorff, P. Mass spectrometry in protein studies from genome to function. *Curr. Opin. Biotechnol.* **1997**, *8*, 6−13.

(10) Lametsch, R.; Bendixen, E. Proteome analysis applied to meat science: characterizing postmortem changes in porcine muscle. *J. Agic. Food Chem.* **2001**, *49*, 4531−4537.

(11) Bergström, J. Muscle electrolytes in man determined by neutron activation analysis on needle biopsy specimens. *Scand. J. Clin. Lab. Invest.* **1962**, *14* (Suppl. 68), 11−12.

(12) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850−858.

(13) Jensen, O. N.; Larsen, M. R.; Roepstorff, P. Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. *Proteins* **1998**, *Suppl. 2*, 74−89.

(14) Gobom, J.; Nordhoff, E.; Mirgorodskaya, E.; Ekman, R.; Roepstorff, P. Sample purification and preparation technique based on nanoscale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **1999**, *34*, 105−116.

(15) Rayment, I.; Rypniewski, W. R.; Schmidt-Base, K.; Smith, R.; Tomchick, D. R.; Benning, M. M.; Winkelmann, D. A.; Wesenberg, G.; Holden, H. M. Three-dimensional structure of myosin subfragment−1: a molecular motor. *Science* **1993**, *261*, 50−58.

(16) Greaser, M. L. Conversion of muscle to meat. *Muscle as Food*; Peter, J., Ed.; Academic Press: London, U.K., 1986; pp 37−102.

(17) Ho, C. Y.; Stromer, M. H.; Robson, R. M. Identification of the 30 kDa polypeptide in post mortem skeletal muscle as a degradation product of troponin-T. *Biochimie* **1994**, *76*, 369−375.

(18) Troy, D. J.; Patyaryas, T.; Tsitsilonis, O. E.; Yialouris, P. P.; Vazeou, S.; Healy, A.; Stoeva, A.; Voelter, W. Sequence analysis of protein extracted from bovine myofibrillar extracts during the aging period. *Proceedings of the 43rd International Congress of Meat Science and Technology*; Rice Printers: Hamilton, New Zealand, 1997; Vol. 92, pp 698−699.

(19) Bandman. An immunological method to assess protein degradation in post-mortem muscle. *Meat Sci.* **1988**, *22*, 1−19.

(20) Ertbjerg, P.; Henckel, P.; Karlsson, A.; Larsen, L. M.; Moller, A. J. Combined effect of epinephrine and exercise on calpain/calpastatin and cathepsin B and L activity in porcine longissimus muscle. *J. Anim. Sci.* **1999**, *77*, 2428−2436.

(21) Stoeva, S.; Byrne, C. E.; Mullen, A. M.; Troy, D. J.; Voelter, W. Isolation and identification of proteolytic fragments from TCA soluble extracts of bovine M. longissimus dorsi. *Food Chem.* **2000**, *69*, 365−370.

(22) Purintrapiban, J.; Wang, M.; Forsberg, N. E. Identification of glycogen phosphorylase and creatinee kinase as calpain substrates in skeletal muscle. *Int. J. Biochem. Cell Biol.* **2001**, *33*, 531−540.

(23) Uytterhaegen, L.; Claeys, E.; Demeyer, D. Effect of exogenous protease effectors on beeftenderness development and myofibrillar degradation and solubility. *J. Anim. Sci.* **1994**, *72*, 1209−1223.

(24) Delgado, E. F.; Geesink, G. H.; Marchello, J. A.; Goll, D. E.; Koohmaraie, M. The calpain system in three muscles of normal and callipyge sheep. *J. Anim. Sci.* **2001**, *79*, 398−412.

(25) Huff-Lonergan, E.; Mitsuhashi, T.; Parrish, F. C.; Robson, R. M. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting comparisons of purified myofibrils and whole muscle preparation for evaluating titin and nebulin in post mortem bovine muscle. *J. Anim. Sci.* **1996**, *74*, 779−785.

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