Structural Changes and Evolution of Peptides During Chill Storage of Pork

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In this work, we investigated changes in protein structures in vacuum-packed pork during chill storage and its impact on the in vitro protein digestion. Longissimus dorsi muscles were vacuum packed and stored at 4°C for 3 days. Samples were subjected to Raman spectroscopy, in vitro digestion and nano LC-MS/MS. The 3 d samples had lower α-helix content, but higher β-sheet, β-turn, and random coil contents than the 0 d samples (P < 0.05). SDS-PAGE revealed significant protein degradation in the 3 d samples and the differences in digested products across the storage time. Proteome analysis indicated that the 3 d samples had the higher susceptibility to digestion. Increasing protein digestibility was mainly attributed to the degradation of myofibrillar proteins. Thus, exposure of more enzymatic sites in loose protein structure during chill storage could increase protein degradation in meat.

Keywords: pork, chill storage, in vitro digestion, Raman spectroscopy, LC-MS/MS

INTRODUCTION

Postmortem aging and its impact on eating quality of fresh meat has been widely concerned (1–3). Physicochemical and structural changes in meat proteins occurring in postmortem aging and processing may affect the protein digestion and nutritional value of meat (4–6). Postmortem aging for relatively long time may produce bioactive peptides in beef that exhibit DPPH radical scavenging, ACE- and renin-inhibitory activities (7). However, little is known about whether bioactive peptides are produced in a short-term aging of pork.

At early postmortem time, the dephosphorylation of several metabolic enzymes has been shown to affect the rate of glycolysis and pH decline, and subsequently the activation of μ-calpain, the release of lysosomal enzymes and meat tenderization (8). In addition, protein oxidation may occur in fresh meat during chill storage (9), which may cause the formation of disulfide bonds, and a decrease in protein hydrophilicity and water holding capacity. To avoid such changes, vacuum packaging and chill storage have been widely applied (10). However, protein oxidation and structural changes may still occur in vacuum-packed and chilled meat because radical oxygen species widely existing in living muscle tissues could be retained in postmortem muscles (11). Furthermore, protein oxidation also alters the protein structures and digestibility of meat (5). An increase in digestion can be attributed to protein unfolding and increased susceptibility to digestion (4). Previous studies have focused how processing and physicochemical changes affect protein structure and digestion (12, 13). However, few data are available on how structural changes...
of meat proteins enhanced digestibility of fresh meat from a proteomic perspective, in terms of the evolution of peptides from the stomach to the small intestine. In this study, we investigated the structural changes of meat proteins in pork during chill storage and their impacts on protein digestibility and the release of bioactive peptides.

**MATERIALS AND METHODS**

**Reagents**

Ellman’s reagent (4 mg/mL 5,5′-dithiobis-2-nitrobenzoic acid in Tris-Gly) and Tris-Gly (10.4 g Tris, 6.9 g glycine, and 1.2 g EDTA per liter, pH 8.0) were obtained from Sigma Aldrich (St. Louis, MO, USA), as were porcine gastric pepsin (Cat. No. P7125) and porcine pancreatic trypsin (Cat. No. T7409). BCA protein assay kit (No. 23225) and protein marker (No. 26619) were obtained from Thermo Scientific (Rockford, IL, USA). Amicon Ultracel-3 membrane (UFC500396) and Zip Tip C18 pipette tips (ZTC18S096) were obtained from Millipore (Billerica, MA, USA).

**Sample Preparation**

*Longissimus dorsi* muscles (size: 10 × 10 × 5 cm) were obtained from 8 native Suhuai pig carcasses at the same line in a commercial slaughterhouse. Each muscle was cut into four 2.5 × 10 × 5 cm pieces (weight: 50 to 65 g each), vacuum-packed and stored at 4°C for 3 days. Samples were taken on 0, 1, 2, and 3 d for further analyses.

**Raman Spectroscopy**

Raman spectroscopy was performed to evaluate changes in protein secondary structure (LabRAM HR Evolution, Horiba/Jobin, Yvon, Longjumeau, France) as previously described (5). Laser (excitation wavelength: 785 nm, power: 100 mW) was applied and the backscattering Raman signals ranging from 400 to 3,200 cm⁻¹ were collected. Raman spectra were normalized against the band at 1,003 cm⁻¹. The 1,685–1,645 and 1,309–1,229 cm⁻¹ bands correspond to amide I and III vibrational modes of α-helix, random coil, and β-sheet. The 1,341 and 940 cm⁻¹ bands reflect CH-bending and C-C stretching respectively. The range of 1,658–1,650 cm⁻¹ vibrations, C-N stretching, and N-H in-plane bending of peptide groups. The 1,003, 830–850, and 760 cm⁻¹ bands correspond to phenylalanine, tyrosine, and tryptophan, respectively. The α-helix, β-sheet, β-turn, and random coil were quantified (14).

**In vitro Digestion**

The protein digestibility of cooked meat from 0, 1, 2 to 3 d samples was assessed as described by Zou et al. (15). Briefly, meat pieces were packed in plastic bags and cooked in a 72°C water bath until the core temperature reached 70°C. The cooking time was around 20 min. The core temperature was tracked by a portable thermal probe (Pt 100, Testo AG, Mönchaltorf, Schweiz, Germany). After cooking, meat samples were cooled for 2 h to room temperature (22°C). Then, samples (1.00 g) were homogenized (9,600 rpm, 30 s, twice; 13,400 rpm, 30 s, twice) in PBS (10 mmol/L, pH 7.0). The homogenate was digested by gastric pepsin (32 mg/mL in 0.1 mol/L HCl, pH 1.0) at 37°C for 2 h and the digestion was stopped by adjusting the pH to 7.5 with 1 mol/L NaOH. Then the resulting mixture was digested by trypsin (24 mg/mL in 0.1 mol/L PBS, pH 7.0) at 37°C for 2 h and stopped by heating the system at 95°C for 5 min.

The undigested proteins were precipitated by adding three volumes of ethanol at 4°C for 12 h. Then the samples were centrifuged at 10,000 × g at 4°C for 20 min. Protein contents in the whole meat samples and the precipitates were quantified by a BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Protein digestibility was calculated as follows:

\[
\text{Protein digestibility} (\%) = \frac{W_0 - W_1}{W_0} \times 100\%
\]

Where \( W_1 \) is the content (g) of undigested proteins precipitated by ethanol. \( W_0 \) is the total protein content (g) in the whole meat before digestion.

The supernatant containing digested products that are ethanol soluble was subjected to nano LC-MS/MS analysis and the precipitate including undigested proteins was separated on the SDS-PAGE gels.

**SDS-PAGE**

The whole proteins were extracted according to the method of Zarkadas and Maloney (16) with some modifications. Briefly, cooked meat samples (0.3 g) were homogenized in 4.5 mL 2% SDS with 3 × 30 s at 10,000 rpm and centrifuged at 4,000 × g for 20 min at 4°C. Two milliliters of the supernatant were dialyzed for 48 h in 1 L distilled water to remove SDS. The dialyzed samples were transferred to new tubes and stored at −80°C for further analyses.

SDS-PAGE was performed to separate proteins or their fragments under reducing conditions (5). Appropriate volumes of samples were mixed with 12.5 μL sample buffer (4 ×) and 5 μL reducing sample agent (10×) and made up to a total volume of 50 μL with ultrapure water. The final protein concentrations were 0.50 μg/μL for all samples. The samples were heated at 70°C for 10 min. Twelve microliters of samples were loaded in triplicate into the wells of 4–12% precast gels (GenScript, Piscataway, NJ, USA). The gels were run in a total 800 mL of SDS running buffer at 150 V till the blue dye front disappeared. The gels were stained with colloidal Coomassie brilliant blue R250 (CBB) for 30 min and then destained for 20 min three times. CBB staining solution contained 0.1% CBB/45% acetic acid/10% ethanol/45% ultrapure water. Destaining buffer contained 10% acetic acid/10% ethanol/80% ultrapure water. The gel images were acquired by an image scanner (GE Healthcare, Little Chalfont, Uppsala, Sweden) and the band intensities were quantified with the Quantity One image analysis software (Bio-Rad, Hercules, CA). The relative intensities of bands were calculated by the actual band intensity divided by that of the 150 kDa band in the calibration marker lane.
under Uniprot (Uniprot-Sus scrofa.fasta, www.uniprot.org); de-isotopic: TRUE; fixed modification: carbamidomethyl (cys); variable modification: oxidation (met), acetyl (protein N-term); label free quantification (LFQ): TRUE; decoy database pattern: reverse; LFQ min ratio count: 1; match between runs: 2 min; peptide false discovery rate (FDR): 0.01; and protein FDR: 0.01. Proteins that could not be annotated were not further used. BIOPEP database was used in the search of similar sequences previously identified showing ACE inhibitory and DPPIV inhibitory activity (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

**Statistical Analysis**

The effects of storage time on measured variables (α-helix, β-sheet, β-turn, random coil, I_760cm^−1/I_1003cm and I_850cm^−1/I_830cm) were evaluated by Student’s t-test using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla). The effects of storage time on other measured variables were evaluated by analysis of variance (ANOVA) and the least significant means were compared by Tukey’s post-hoc t-test. Data were presented as means and standard deviations. The means were considered significantly different if the P-value was smaller than 0.05. Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) were applied to analyze the differences of matched proteins among the four time points.

**RESULTS AND DISCUSSION**

**Changes in Raw Meat Protein Structures Detected by Raman Spectroscopy**

Raman spectroscopy provides powerful information on secondary structural modifications in proteins (18). The α-helix content decreased from 77.41% on 0 d to 67.89% on 3 d (P < 0.05, **Figure 1**), while β-sheet, β-turn, and random coil increased from 3.39, 11.72, and 8.92% on 0 d to 9.75, 13.21, and 9.49% on 3 d, respectively (P < 0.05, **Figure 1**). This indicates that denaturation and unfolding occurred in meat proteins from 0 to 3 d. During aging, meat proteins were degraded by endogenous enzymes into fragments. Degradation of myofibrillar proteins is modulated by protein oxidation and nitrosylation (19).

The side chains of tryptophan and tyrosine residues may change under a polar microenvironment, which results in modifications in the tertiary structure of proteins. The ratios of I_760cm^−1/I_1003cm and I_850cm^−1/I_830cm were related to the exposed or buried status of tryptophan and tyrosine residues, respectively (20). A substantial increase in the ratio of I_760cm^−1/I_1003cm represents changing of tryptophan residues was observed during chill storage (P < 0.05, **Figure 1**), indicating transformation of the tryptophan residues from a buried, hydrophobic state to a polar aqueous state (20). The ratio of I_850cm^−1/I_830cm significantly increased from 0 to 3 d (P < 0.05, **Figure 1**), indicating an increasing number of exposed tyrosine residues and tryptophan residues. In addition, the ratio of I_850cm^−1/I_830cm is also a good indicator for the microenvironment and hydrogen bonds of the ionization of the phenolic hydroxyl group (20). It could be the
FIGURE 2 | Protein digestibility did not differ after pepsin treatment by differ significantly after pepsin and trypsin treatments. (A) D0P, D1P, D2P, and D3P represent 0, 1, 2, and 3 d samples treated by pepsin, respectively. No significant difference was observed among groups \((P > 0.05)\). (B) D0P/T, D1P/T, D2P/T, and D3P/T represent 0, 1, 2, and 3 d samples treated by pepsin and trypsin, respectively. a, b denotes that protein digestibility differ significantly \((P < 0.05, n = 8 each)\).

FIGURE 3 | Typical SDS-PAGE patterns of pork proteins before and after digestion. “Untreated” represents total proteins in cooked pork before digestion. Pepsin represents ethanol-insoluble fraction after pepsin digestion. Pepsin/trypsin represents ethanol-insoluble fraction after pepsin and trypsin treatments. Lanes M, D0, D1, D2, and D3 represent calibration marker, 0, 1, 2, and 3 d samples.

exposure of polar amino acid residues at the surface of the protein molecules. The ordered and steady structure of the protein was broken into disordered and loose fragments during storage, from a buried status of tryptophan and tyrosine residues to an exposed status, which exposed cleavage sites of digestive enzymes. Thus, we postulate that this disordered and hydrophobic status would increase the degree of protein degradation by exposing more cleavage sites to digestive enzymes.

The Degree of Protein Degradation in Cooked Meat

No significant difference was observed in the degree of protein degradation after pepsin digestion among the four time points \((P > 0.05, \text{Figure 2A})\). However, the degree of protein degradation increased after two-step digestion, and the 3 d samples had the highest values \((P < 0.05, \text{Figure 2B})\). In a previous study, postmortem storage did not improve the in vitro digestion parameters of pork \((4)\), in which the structural organization of the muscle cell and the extracellular matrix were not taken into account. To a certain extent, the storage-induced differences in the degree of protein degradation could be attributed to changes in protein secondary or tertiary structures. Such a structural change may further affect protein digestion after meat cooking. Different cooking conditions may affect the structure of meat protein, and the digestibility of meat \((17)\). To avoid it, we cooked meat samples with a similar size for the same time in a water bath. The proteolysis during chill storage may increase protein unfolding and expose more sites to bind to pepsin and trypsin. This is in agreement with the Raman data that reflected structural changes in side chains of proteins, including hydrophobic and electrostatic interactions.

SDS-PAGE of Proteins in Cooked Meat and Their Digested Products

SDS-PAGE was applied to separate soluble meat proteins or their digested products before or after pepsin and trypsin treatments \((\text{Figure 3})\). Before digestion (untreated samples), a reduction in band intensity of several protein bands was observed in cooked pork as the storage time of raw meat increased, indicating fragmentation and breakdown of meat proteins. This result was in agreement with our previous study \((15)\). The appearance of the 30 kDa component \((\text{Troponin-T fragment})\) has been considered
a good indicator of meat tenderization (21). During postmortem storage, high molecular weight proteins (65–200 kDa) may be degraded into smaller fragments (<25 kDa) (22).

In pepsin treated samples, a substantial decrease in band intensity was observed for some large-molecular-weight protein bands (Figure 3), which could be due to degradation of proteins into smaller peptides or free amino acids. In addition, some insoluble proteins could become soluble after enzymatic digestion (23). Intensity loss or even disappearance of bands was observed for pepsin and trypsin treated samples (Figure 3). Most of large molecular weight bands disappeared because proteins or peptides were further degraded into smaller ones after two-step digestion (Figure 3). Similar phenomena have been observed in different pork cuts and pork products (14, 24). However, SDS-PAGE could not separate smaller peptides well that were characterized by nano LC-MS/MS (15).

**Unique Peptides Could Be a Good Indicator for Chill Storage Time**

Pepsin or two-step digestion products were identified by nano LC-MS/MS and matched to proteins. All matched proteins are listed in Supplementary Files 1, 2. Venn diagrams revealed that 97 peptides were matched in all pepsin digested samples and pepsin and trypsin digested samples, respectively (Figures 4A,B). Most of these peptides came from myofibrillar proteins (myosin-1, myosin-7, actin) and sarcoplasmic proteins (phosphoglycerate kinase, creatine kinase M-type). In general, peptide abundances decreased as the storage time of raw meat increased, indicating that these peptides were degraded into smaller ones during digestion. Furthermore, 9 peptides matched to myofibrillar proteins, and 52 peptides matched to sarcoplasmic proteins in pepsin-treated samples (Supplementary File 1). More peptides matched to myofibrillar proteins (48/97) than to sarcoplasmic proteins (22/97) (Supplementary File 2). Taken together, the increased protein degradation could be mainly attributed to the degradation of myofibrillar proteins.

Several common peptides appeared in 1, 2, and/or 3 days samples, which were derived from large molecular weight peptides or proteins. Specifically, 22, 5, 8, and 19 peptides were uniquely matched in pepsin-treated samples on 0, 1, 2, and 3 days respectively, and 18, 3, 33, and 6 peptides were unique for pepsin/trypsin-treated samples at the four-time points (Figures 4A,B). Unique peptides could be a good indicator for chill storage time.

**Digested Products From Myofibrillar Proteins in Pork Evolved With Storage Time**

All peptides matched to myofibrillar proteins were listed in Table 1. Previous studies have shown that actin, myosin-1, myosin-4, myosin-7, and many glycolytic enzymes are highly abundant proteins in pork muscle (15, 25). These proteins are involved in muscle contraction and energy production. In the present study, most of differently abundant peptides are derived from these proteins (Tables 1, 2).

Oxidative modification happened in four peptides (MNVKH WPWMKL, EAFVKHIMSI TVKEDQVFPMNPPKF, MSVK NWPWML) originating from myosin. Long-chain peptides (e.g., EEAEALSHEEGKILRIQIL) were less abundant than short-chain peptides (e.g., EEAEALSHEEGKIL) and some long-chain peptides disappeared in 2nd days samples (Table 1). After gastric digestion, unique peptides were further hydrolyzed by trypsin. Abundances of common peptides (ALIHYAGTV FY, TVKEDQVFPMNPPKF, LEQVDDLEGSLQEK) decreased as the storage time of raw meat increased, indicating that these peptides were digested by pepsin or/ and trypsin (Tables 1, 2). A total of 16 peptides, including...
Table 1: Dynamic evolution of peptides derived from myofibrillar proteins after gastric digestion.

| Origin          | Accession | Sequence                  | Modifications | MH+ [Da] | Area^c |
|-----------------|-----------|---------------------------|---------------|----------|--------|
| Myosin          | Myosin-1  | F1SS64 MNVKHPWMKL          | M9(Oxidation) | 1469.75  |        |
|                 |           | MNVKHPWMKL                |               | 1485.74  |        |
|                 |           | TVKEDQFPMPNPKF             |               | 1776.90  |        |
|                 |           | TVKEDQFPMPNPKF             | M10(Oxidation)| 1792.90  |        |
|                 |           | EEEAESLHEESEGKIL           |               | 1683.81  |        |
|                 |           | EEEAESLHEESEGKILRIQKL     |               | 2194.13  |        |
|                 |           | ETLKRENKLQEOESDL           |               | 2058.08  |        |
|                 |           | IEKHFITGTLKL               |               | 1242.73  |        |
|                 |           | EHEESEGKILRIQKL           |               | 1484.82  |        |
|                 |           | SLHAYGTVDY                |               | 1238.60  |        |
|                 |           | EDDOISANPLL               |               | 1212.65  |        |
|                 |           | MLDFRENOQIL               |               | 1319.66  |        |
|                 |           | ITGSQAKQKTNVRKIQYF        |               | 2169.16  |        |
|                 |           | WMVTRINQQL                |               | 1288.68  |        |
|                 | Myosin-1 (Fragment) | F1SS62 SELKTKEEQQRULNDL |               | 2073.08  |        |
|                 |           | KTKEEEQQRULNDLTACQRARL    |               | 2540.39  |        |
|                 | Myosin-7  | F1S9D6 MSVKHPWMKL          | M9(Oxidation) | 1419.73  |        |
|                 |           | MSVKHPWMKL                |               | 1435.72  |        |
|                 |           | ALIHYGTVDY                |               | 1222.61  |        |
|                 |           | IDSRKGAEKLLGS           |               | 1468.86  |        |
|                 |           | EEEAESLHEESEGKILRAQGL    |               | 2152.09  |        |
|                 |           | RQYRILIPTPAPEGQF          |               | 2029.11  |        |
| MLC2v (Fragment) | A1XOV9   | NAFFYDPEGKOWL             |               | 1520.81  |        |
|                 |           | DYNKLNIVHTHGEEKD          |               | 1910.96  |        |
|                 |           | AAFPPOTVNL                |               | 1101.56  |        |
| Myosin light chain | Q29069  | RALGTNPTEANKKVLGNPSNEEMNAGKGE | M10(Oxidation) | 3399.77  |        |
| Tropomyosin     | Troponin T fast skeletal muscle type | Q7SN6 GYLEDIFK |               | 1055.54  |        |
| Tropomyosin     | Tropomyosin TM30-ipl (Fragment) | P79309 LEEELKNVTNLNL | EAFVKHMSI | 1415.74  |        |
|                 | Beta-tropomyosin (Fragment) | Q8MKF3 LEEKLEGAEATRF | EAFVKHMSI | 1692.88  |        |

| Protein database ID (Uniprot-Sus scrotus). | Peptides obtained by MS/MS analysis. | Different color indicates different relative abundance of peptides. Red color represents the highest abundance, and the green color represents the lowest abundance. | DOP | D1P | D2P | D3P |
|-------------------------------------------|------------------------------------|--------------------------------------------------------------------------------|-----|-----|-----|-----|
| KPPAAAAPAPAPAPAPAPAPAPPA PAPAPAPKEEK, TKLEQQVDDLEGSEQEK, and TKLE QQVDDLEGSEQEKK were cleaved into 1 to 4 amino acids peptides by trypsin (Table 2). From the digested products, 117 peptides were selected to match the BIOPEP database showing ACE-inhibitory (angiotensin-converting enzyme inhibitory) and dipeptidyl peptidase IV (DPPIV) inhibitory activities (Table 2). Eighteen peptides were estimated to have ACE-inhibitory and DPPIV inhibitory activities, which have been identified in the database (26). ACE inhibitors are known to beneficial to treat arterial hypertension (27), diabetes mellitus, ischemic heart disease, chronic heart failure, angioedema, chronic kidney disease, and Parkinson's disease (28, 29). DPPIV inhibitor may be beneficial for NASH subjects (30). Four peptides (TKLEQQVDDLEGSEQEKK, LEQQVDDLEGSEQEKK, VKLEQHVDDLEGSEQ EKK, LEQHVDDLEGSEQEKK) were identified, which contain sequences VDDELEGSEQEKK and DDLEGSEQEKK that were previously reported to have antioxidant peptides (31). It is of note that some identified peptides maintain a long sequence after the two-step digestion. However, almost all the peptides would be hydrolyzed by peptidases into smaller fragments (dipeptides and tripeptides) located in the outer layer of intestinal epithelium before reaching the blood stream (32). Thus, moderate chill storage may enhance the production of antioxidant, ACE inhibitor and DPP-IV inhibitory peptides. Table 2 illustrates dynamic evolution of 117 peptides derived from cooked meat samples. The potential bioactive peptides were predominantly originated from myosin, actin, tropomyosin and troponin. Peptides EDQVFPMNPPK (Oxidation) and IEDQALALQLOQK appeared in 0 day samples, whereas peptides EFEMSNKQISEDEQALQLOQK, IEDQALAMQLO, and SKQLEDLVSQLOK appeared only in 2 day samples. Abundances of LAQESIMDIENEK, LAQESTMDIENDKQQQLDEK, and their |
### TABLE 2 | Dynamic evolution of peptides derived from myofibrillar proteins after two-step digestion.

| Origin | Accessiona | Sequence | Modifications | MH+ [Da] | Area | BIOSEP sequences | Activity |
|---|---|---|---|---|---|---|---|
| Myosin | F1SS64 | EDQVFPMNPPK | M7(Oxidation) | 1301.62 | MNPPK | ACE-inhibitory |
| Myosin-1 | | EDQVFPMNPPK | MNPPK | 1317.61 | | | |
| Myosin-1 | | LAQESMDIENKE | M7(Oxidation) | 1519.73 | | | |
| Myosin-1 | | LAQESMDIENKE | M7(Oxidation) | 1535.72 | | | |
| Myosin-1 | | TVKEDQVFPMNPPKF | | 1776.90 | | | |
| Myosin-1 | | EDQISANPLL | | 1212.65 | | | |
| Myosin-1 | | IEDEQALALQOLQK | | 1498.81 | QK | | ACE-inhibitory |
| Myosin-1 | | NDLOQOVAEAEGLADAER | | 2199.06 | | | |
| Myosin-1 (Fragment) | F1SS62 | WESMQMSLDAEIR | M5(Oxidation) | 1623.77 | | | |
| Myosin-1 | | WESMQMSLDAEIR | VVESMQSMLDAEIR | 1607.77 | | | |
| Myosin-1 | | LAQESMDIENKEQOLQKE | M7(Oxidation) | 2251.04 | | | |
| Myosin-1 | | LAQESMDIENKEQOLQKE | 2235.05 | | | | ACE-inhibitory |
| Myosin-1 | | NLTEEAEQIDETIK | M6(Oxidation) | 1650.79 | | | |
| Myosin-1 | | NLTEEAEQIDETIK | 1634.79 | | | | ACE-inhibitory |
| Myosin-1 | | AGLGILLEEER | | 1201.66 | | | |
| Myosin-1 | | AGLGILLEEER | 1217.66 | | | | ACE-inhibitory |
| Myosin-1 | | LQQEDVLMDOVER | M9(Oxidation) | 1718.83 | | | |
| Myosin-1 | | LQQEDVLMDOVER | 1702.84 | | | | ACE-inhibitory |
| Myosin-1 | | LQQEDVLMDOVER | 1718.83 | | | | ACE-inhibitory |
| Myosin-1 | | LQQEDVLMDOVER | 1702.83 | | | | ACE-inhibitory |
| Myosin-1 | | KLETIDOIQQGEDEIQK | M13(Oxidation) | 2462.20 | | | |
| Myosin-1 | | KLETIDOIQQGEDEIQK | 2446.21 | | | | ACE-inhibitory |
| Myosin-1 | | NAYEESLDQLETLK | | 1652.79 | | | |
| Myosin-1 | | NAYEESLDQLETLK | 1808.90 | | | | ACE-inhibitory |
| Myosin-1 | | TKEQQDDLLEGEK | | 2089.03 | | | |
| Myosin-1 | | TKEQQDDLLEGEK | 2217.13 | | | | ACE-inhibitory |
| Myosin-1 | | DIDQDLTLK | | 1245.66 | | | |
| Myosin-1 | | DIDQDLTLK | 1601.86 | | | | ACE-inhibitory |
| Myosin-1 | | LNQQEDVLQOEGAQR | | 1972.99 | | | |
| Myosin-1 | | LNQQEDVLQOEGAQR | 2129.08 | | | | ACE-inhibitory |
| Myosin-1 | | LEQVDLLEGEK | | 1859.89 | | | |
| Myosin-1 | | LEQVDLLEGEK | 1878.98 | | | | ACE-inhibitory |
| Myosin-1 | | AITDAAMMADELK | | 1303.67 | | | |
| Myosin-1 | | AITDAAMMADELK | 1521.77 | | | | ACE-inhibitory |
| Myosin-1 | | EFESNLSNOSKEDEGAL | | 2710.31 | QK | | ACE-inhibitory |
| Myosin-1 | | EFESNLSNOSKEDEGAL | 2838.40 | | | | ACE-inhibitory |
| Myosin-1 | | AEDEEEINAELAK | | 1561.72 | | | |
| Myosin-1 | | ALQAEQHTLDDOAEK | | 2838.41 | | | |
| Myosin-1 | | DKVNTLTK | | 1398.76 | | | |
| Myosin-1 | | IOLEENOK | | 1084.63 | | | |
| Myosin-1 | | KIDQDLTLK | | 1373.75 | | | |
| Myosin-1 | | NDLOQOVAEAEGLADAER | | 2215.05 | | | |
### TABLE 2 | Continued

| Origin | Accessiona | Sequenceb | Modifications | MH+ [Da] | Area[c] | BIOSEP sequences | Activity |
|--------|------------|-----------|---------------|---------|---------|-----------------|----------|
|        |            |           |               | D0P/T   | D1P/T   | D2P/T           | D3P/T   |
|        |            |           |               |         |         |                 |          |
|        |            |           |               | SEIQAALEEASELEHEEGK | 2170.02 |         |                 |          |
|        |            |           |               | SQEDKEQALMVER | 1675.83 |         |                 |          |
|        |            |           |               | VAEOELLDAER | 1359.68 |         |                 |          |
|        |            |           |               | SLHYAGTVDY | 1238.60 |         |                 |          |
|        |            |           |               | DTLHLDALR | 1296.66 |         |                 |          |
|        |            |           |               | DTLHLDALR | 1296.65 |         |                 |          |
|        |            |           |               | IEELEELAEER | 1488.71 |         |                 |          |
|        |            |           |               | IEELEELAEER | 1488.71 |         |                 |          |
|        |            |           |               | MEGDLNMEIQNLHANR | 2013.91 |         |                 |          |
|        |            |           |               | RANLQABEELR | 1554.86 |         |                 |          |
|        |            |           |               | TLEDQSEIK | 1175.62 |         |                 |          |
|        |            |           |               | MNVKHPWMKL | 1469.76 |         |                 |          |
|        |            |           |               | IEKPMGIF | 934.51  |         |                 |          |
|        |            |           |               | NTOQILDKQHLDALR | 2051.08 |         |                 |          |
|        |            |           |               | TNEKIQQFF | 1154.58 |         |                 |          |
|        |            |           |               | ENKIDQCQLDTQIEAGGKR | 2500.27 |         |                 |          |
|        |            |           |               | IEDEQALAMOLQK | 1516.77 |         |                 |          |
|        |            |           |               | KAITDAAMMAEEIKK | 1665.85 |         |                 |          |
|        |            |           |               | KALQEAHQTDLQAAE | 2966.51 |         |                 |          |
|        |            |           |               | DKVNTLTQK | 2808.40 |         |                 |          |
|        |            |           |               | KMEGDLNMEIQNLHANR | 2142.01 |         |                 |          |
|        |            |           |               | Myosin-7 F1S9D6 |  |         |                 |          |
|        |            |           |               | NLTLEMAQLDEIAK | 1646.83 |         |                 |          |
|        |            |           |               | NLTLEMAQLDEIAK | 1662.83 |         |                 |          |
|        |            |           |               | LEDEEMMAELTAK | 1621.72 |         |                 |          |
|        |            |           |               | LEDEEMMAELTAK | 1637.72 |         |                 |          |
|        |            |           |               | VKLEQHVDLGLSEQEK | 2096.06 |         |                 |          |
|        |            |           |               | VKLEQHVDLGLSEQEKK | 2224.15 |         |                 |          |
|        |            |           |               | LEQHVDLGLSEQEK | 1868.89 |         |                 |          |
|        |            |           |               | LEQHVDLGLSEQEKK | 1996.98 |         |                 |          |
|        |            |           |               | ALQEAHQLDLQAAE |  |         |                 |          |
|        |            |           |               | DKVNTLTQK | 2808.40 |         |                 |          |
|        |            |           |               | LEQHVDLGLSEQEK |  |         |                 |          |
|        |            |           |               | LEQHVDLGLSEQEKK |  |         |                 |          |
|        |            |           |               | Myosin light chain 1/3 A1XQT6 |  |         |                 |          |
|        |            |           |               | KPAAAAPAPAPAPAPA | 2098.18 |         | PK             | DPPPIV inhibitory |
|        |            |           |               | KPAAAAPAPAPAPAPAPAPAPAPK | 2484.36 |         | EK             | ACE-inhibitory |
|        |            |           |               | KPAAAAPAPAPAPAPAPAPAPAPAPAPK |  |         |                 |          |
|        |            |           |               | ITLSQVGDVLK | 1200.71 |         |                 |          |
|        |            |           |               | DOGYEFVLEGRL | 1514.68 |         |                 |          |
|        |            |           |               | Myosin heavy chain Q95249 | 2432.20 |         |                 |          |
|        |            |           |               | KLETDISQIQGEMEDIVGEAR | 2212.06 |         |                 |          |

(Continued)
TABLE 2 | Continued

| Origin                  | Accession   | Sequence                    | Modifications | MH+ [Da] | Area | BIOSEP sequences | Activity |
|-------------------------|-------------|-----------------------------|---------------|----------|------|------------------|----------|
|                         |             |                             |               | D0P/T    | D1P/T | D2P/T | D3P/T     |          |
| Myosin, heavy chain 7   | H6SHX6      | KLETDISQGEMEDIVQEAR          | M13(Oxidation)| 2448.19  |       |                  |          |
|                         |             | LGSLDIDHNOY                 |               | 1274.60  |       |                  |          |
| Actin (Fragment)        | B2ZF7N7     | IGMESAGIHETTY               | M3(Oxidation) | 1424.64  |       |                  |          |
|                         |             | IGMESAGIHETTY               |               | 1408.64  |       |                  |          |
|                         |             | MKLTERGYSF                  |               | 1344.70  |       |                  |          |
|                         |             | MKLTERGYSF                  |               | 1360.69  |       |                  |          |
|                         |             | FQPSFIMGESAGIHETTY          |               | 2014.92  |       |                  |          |
|                         |             | QPSFIMGESAGIHETTY           |               | 1867.85  |       |                  |          |
|                         |             | SYELPDGQVITIGNER            |               | 1790.89  |       |                  |          |
| Tropomyosin             |             |                             |               |          |      |                  |          |
| Tropomyosin 4           | D0G7F7      | MELGEMQLK                   | M6(Oxidation) | 1165.56  |       |                  |          |
|                         |             | MELGEMQLK                   |               | 1149.56  |       |                  |          |
|                         |             | LVILEGEGLER                 |               | 1170.67  |       |                  |          |
|                         |             | KLVLVGEGLER                 |               | 1298.77  |       |                  |          |
| Tropomyosin alpha-1 chain | F2Z5B6    | AISEELDHALNDMTSI            | M13(Oxidation)| 1774.82  |       |                  |          |
|                         |             | AISEELDHALNDMTSI            |               | 1758.82  |       |                  |          |
|                         |             | MELQEIQLK                   | M1(Oxidation) | 1147.60  |       |                  |          |
|                         |             | MELQEIQLK                   |               | 1131.61  |       |                  |          |
|                         |             | KLVIESDLER                  |               | 1314.76  |       |                  |          |
|                         |             | LVIESDLER                   |               | 1186.67  |       |                  |          |
|                         |             | IQLVEEELDR                  |               | 1243.65  |       |                  |          |
|                         |             | IQLVEEELDRAGSER             |               | 1272.89  |       |                  |          |
|                         |             | QLEDDELVSLOK                |               | 1301.70  |       | QK                | ACE-inhibitory |
|                         |             | SKQLEDDELVSLOK              |               | 1516.82  |       | QK                | ACE-inhibitory |
|                         |             | KAISEELDHALNDMTSI           |               | 1886.91  |       |                  |          |
| Tropomyosin 3           | Q2XQY5      | LVIEGDLER                   |               | 1156.66  |       |                  |          |
| Troponin                |             |                             |               |          |      |                  |          |
| Troponin I              | B3V70       | SMLQIAATELEK                |               | 1432.77  |       |                  | EK       |
|                         |             | SMLQIAATELEK                |               | 1415.76  |       |                  | EK       |
| troponin C              | A1XQV5      | AAFDMDADAGGDDSVK            |               | 1715.76  |       |                  |          |
|                         |             | SYLSEEMIAEFK                |               | 1446.68  |       |                  |          |
| Troponin T              | Q75NG6      | YOLEIDKF                    |               | 1055.54  |       |                  |          |

aProtein database ID (Uniprot-Sus scrofa). bPeptides obtained by MS/MS analysis. cD0PT, D1PT, D2PT, and D3PT, 0, 1, 2, and 3 d samples treated by pepsin and trypsin. Different color indicates different relative abundance of peptides. Red color represents the highest abundance, and the green color represents the lowest abundance.

oxidative peptides and KPAAAAAPAPAPAPAPAPAPKEK were the lowest in 2 day samples. Abundances of SVMLQIAA TELEK, DIDDLETLAKVEK, and TKEQQVDLEDLEGSLQEIK were the highest in 2 day samples. This could be mainly attributed to the degradation of proteins and accumulation of peptides in the first stage, which would be further degraded into smaller peptides that have ACE and DPPIV inhibitory bioactivities. Thus, 2 days could be a key time point for chill storage of pork in terms of meat nutrition.

Two peptides were observed to have the same sequence but their molecular weights were different, which is attributed to protein oxidation based on the database. Eighteen peptides from two-step digestion were observed to have oxidation modification. It is known that protein oxidation occurs during storage and the extent of protein oxidation may be enhanced as meat storage time increases. In the present study, pork samples were vacuum packed and stored at low temperature (4°C) for 3 days. In such a condition, protein oxidation could be alleviated. And thus more cleavage sites could be exposed in loose protein structure and protein degradation increased. The number of bioactive peptides may increase after the two-step digestion.

The above results could be associated with rigor mortis and subsequent aging of muscle (33). It is well-known that glycolysis occurs in skeletal muscles after slaughter (34, 35). This process is accompanied by the dephosphorylation of energy metabolic enzymes, the formation of actomyosin,
and the shortening of sarcomeres (6, 36). Such activities may make some energy metabolic enzymes and myofibrillar proteins less susceptible to pepsin and trypsin digestion under in vitro condition on day 1. However, prolonged storage can tenderize meat, and make the myofibrillar proteins and bound energy metabolic enzymes more digestible. This is because the endogenous enzymes, e.g., μ-calpain and cathepsins, catalyze the degradation of myofibrillar proteins into smaller fragments (33), which will expose the cleavage sites for digestive enzymes. It is notable that, too long-time storage of fresh meat may have the problems of food spoilage, discoloration, and the decreased digestibility of meat proteins due to oxidation (37).

CONCLUSIONS

In this study, postmortem aging was shown to have significant impacts on the structural characteristics and in vitro digestion of pork proteins. It is observed that the 3 d samples had lower α-helix content and the peak intensity at 760 cm⁻¹ (tryptophan residues), but higher β-sheet, β-turn, and random coil contents than the 0 d samples. This is accompanied by significant protein degradation and increased susceptibility to digestion. The structural changes of proteins altered the accessibility of proteolytic enzymes to the cleavage sites, and consequently the protein digestion. Further work is necessary to evaluate the effect of postmortem aging on in vivo digestion of pork protein.

REFERENCES

1. Li CB, Wu JQ, Zhang N, Zhang S, Liu J, Li JP, et al. Effects of boning method and postmortem aging on meat quality characteristics of pork loin. Anim Sci J. (2009) 80:591–6. doi: 10.1111/j.1740-0929.2009.00677.x
2. Channon HA, Kerr MG, Walker PJ. Effect of Duroc sex and aging period on meat and eating quality attributes of pork loin. Meat Sci. (2004) 66:881–8. doi: 10.1016/j.meatsci.2003.08.010
3. Lepper-Bilani AN, Berg EP, Buchanan DS, Berg PT. Effects of post-mortem aging time and type of aging on palatability of low marbled beef loins. Meat Sci. (2016) 112:63–8. doi: 10.1016/j.meatsci.2015.10.017
4. Bax ML, Sayd T, Aubry L, Sante-Lhotellier V. Muscle composition slightly affects in vitro digestion of aged and cooked meat: identification of associated proteomic markers. Food Chem. (2013) 136:1249–62. doi: 10.1016/j.foodchem.2012.09.049
5. He J, Zhou GH, Bai Y, Wang C, Zhu SR, Xu XL, et al. The effect of meat processing methods on changes in disulfide bonding and alteration of protein structures: impact on protein digestion products. RSC Adv. (2018) 8:17595. doi: 10.1039/C8RA02310G
6. Sante-Lhotellier V, Aubry L, Gatellier P. Effect of oxidation on in vitro digestibility of skeletal muscle myofibrillar proteins. J Agric Food Chem. (2007) 55:3343–8. doi: 10.1021/jf070252k
7. Fu Y, Young JF, Therkildsen M. Bioactive peptides in beef: endogenous generation through postmortem aging. Meat Sci. (2017) 123:134–42. doi: 10.1016/j.meatsci.2016.09.015
8. Li CB, Li J, Zhou GH, Lametsch R, Erbregg P, Brüggemann DA, et al. Electrical stimulation affects metabolic enzyme phosphorylation, protease activation, and meat tenderization in beef. J Anim Sci. (2012) 90:1638–49. doi: 10.2527/jas.2011-4514
9. Rowe LJ, Maddock KR, Lonergan SM, Huff-Lonergan E. Influence of early postmortem protein oxidation on beef quality. J Anim Sci. (2014) 82:785–93. doi: 10.1093/ansci/82.3.785
10. Zhou GH, Xu XL, Liu Y. Preservation technologies for fresh meat - A review. Meat Sci. (2010) 86:119–28. doi: 10.1016/j.meatsci.2010.04.033
11. Warner RD, Dunsea EP, Ponnampalam EM, Cottrell JJ. Effects of nitric oxide and oxidation in vivo and postmortem on meat tenderness. Meat Sci. (2005) 71:205–17. doi: 10.1016/j.meatsci.2005.04.008
12. Rysman T, Hecke TV, Poucke CV, Smet SD, Roeyen GV. Protein oxidation and proteolysis during storage and in vitro digestion of pork and beef patties. Food Chem. (2016) 209:177–84. doi: 10.1016/j.foodchem.2016.04.027
13. Du XJ, Sun YY, Pan DD, Wang Y, Ou CR, Cao JX. The effect of structural change on the digestibility of sarcomplasmic proteins in Nanjing dry-cured duck during processing. Poultry Sci. (2018) 1–8. doi: 10.1002/ps.8815
14. Alik AP, Pedanou G, Berjot M. Fast determination of the quantitative secondary structure of proteins by using some parameters of the Raman Amide I band. J Mol Struct. (1988) 174:159–64. doi: 10.1016/0022-2860(88)80151-0
15. Zou XY, Zhou GH, Yu XR, Bai Y, Wang C, Xu XL, et al. In vitro protein digestion of pork cuts differ with muscle type. Food Res Int. (2018) 106:344–53. doi: 10.1016/j.foodres.2017.12.070
16. Zarkadas CG, Maloney SA. Assessment of the protein quality of the smooth muscle myofibrillar and connective tissue proteins of chicken gizzard. Poultry Sci. (1998) 77:770–9. doi: 10.1093/ps/77.5.770
17. Wen SY, Zhou GH, Li L, Xu XL, Yu XB, Bai Y, et al. Effect of Cooking on in vitro digestion of pork proteins: a peptidomic perspective. J Agric Food Chem. (2015) 63:250–61. doi: 10.1021/jf505323g
18. Herrero AM. Raman spectroscopy for monitoring protein structure in muscle food systems. Crit Rev Food Sci. (2008) 48:512–23. doi: 10.1080/10408390701537385
19. Liu R, Li Y, Wang M, Zhou GH, Zhang WG. Effect of protein S-nitrosylation on autolysis and catalytic ability of μ-calpain. Food Chem. (2016) 213:470–7. doi: 10.1016/j.foodchem.2016.06.104
20. Li-Chan ECY. The applications of Raman spectroscopy in food science. Trends Food Sci Tech. (1996) 7:361–70. doi: 10.1016/0924-2244(96)10037-6

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in Proteome Xchange, Accession No. PXD020595.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of Experimental Animal Center of Nanjing Agricultural University.

AUTHOR CONTRIBUTIONS

CL designed the experiments. XZ, JH, DZ, MZ, YX, and CW conducted the experiments. XZ, JH, and CL wrote the paper. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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21. Macbride MA, Parrish FC. The 30,000 dalton component of tender bovine longissimus muscle. J Food Sci. (1977) 42:1627–9. doi: 10.1111/j.1365-2621.1977.tb08442.x

22. Naveena BM, Kiran M, Sudhakar RK, Ramakrishna C, Vaithiyananathan S, Devatkal SK. Effect of ammonium hydroxide on ultrastructure and tenderness of buffalo meat. Meat Sci. (2011) 88:727–32. doi: 10.1016/j.meatsci.2011.03.005

23. Diaz M, Vattem D, Mahoney RR. Production of dialysable and reduced iron by in vitro digestion of chicken muscle protein fractions. J Sci Food Agric. (2002) 82:1551–5. doi: 10.1002/jsfa.1219

24. Li L, Liu Y, Zou XY, He J, Xu XL, Zhou GH, et al. In vitro protein digestibility of pork products is affected by the method of processing. Food Res Int. (2017) 92:88–94. doi: 10.1016/j.foodres.2016.12.024

25. Kim GD, Yang HS, Jeong JY. Intramuscular variations of proteome and muscle fiber type distribution in semimembranosus and semitendinosus muscles associated with pork quality. Food Chem. (2018) 244:143–52. doi: 10.1016/j.foodchem.2017.10.046

26. Minkiewicz P, Dziuba J, Iwaniak A, Dziuba M, Darewicz M. BIOPEP database and other programs for processing bioactive peptide sequences. J AOAC Int. (2008) 91:965–80. doi: 10.1093/jaocas/91.4.965

27. Zhou M, Daubresse M, Stafford RS, Alexander GC. National trends in the ambulatory treatment of hypertension in the United States, 1997-2012. PLoS ONE. (2015) 10:e0119292. doi: 10.1371/journal.pone.0119292

28. Weir MRMD. Effects of renin-angiotensin system inhibition on end-organ protection: can we do better? Clin Ther. (2007) 29:1803–24. doi: 10.1016/j.clinthera.2007.09.019

29. Montinaro V, Cicardi M. ACE inhibitor-mediated angioedema. Int Immunopharmacol. (2020) 78:106081. doi: 10.1016/j.intimp.2019.106081

30. Balaban YH, Korkusuz P, Simsek H, Gokcan H, Tatar G. Dipeptidyl peptidase IV (DDP IV) in NASH patients. Ann Hepatol. (2007) 6:242–50. doi: 10.1016/S1665-2681(19)31905-2

31. Je JY, Qian ZJ, Kim SK. Antioxidant peptide isolated from muscle protein of bullfrog, Rana catesbeiana Shaw. J Med Food. (2007) 10:401–7. doi: 10.1089/jmf.2006.169

32. Babusiak M, Man P, Petrik J, Vyoral D. Native proteomic analysis of protein complexes in murine intestinal brush border membranes. Proteomics. (2007) 7:121–9. doi: 10.1002/pmic.200600382

33. Geesink GH, Kuchay S, Chihihi AH, Koohmaraie M. µ-Calpain is essential for postmortem proteinolysis of muscle proteins. J Anim Sci. (2006) 84:2834–40. doi: 10.2527/jas.2006-122

34. Hollung K, Veiseh E, Froystein T, Aas L, Langsrud O, Hålårum KL. Variation in the response to manipulation of post-mortem glycolysis in beef muscles by low-voltage electrical stimulation and conditioning temperature. Meat Sci. (2007) 77:372–83. doi: 10.1016/j.meatsci.2007.03.029

35. Wang SD, Li CB, Xu XL, Zhou GH. Effect of fasting on energy metabolism and tenderizing enzymes in chicken breast muscle early postmortem. Meat Sci. (2013) 93:865–72. doi: 10.1016/j.meatsci.2013.11.053

36. Hjalund K, Brown BP, Hwang H, Flynn CR, Madreaddy L, Geetha T, et al. In vivo phosphoproteome of human skeletal muscle revealed by phosphopeptide enrichment and HPLC-ESI-MS/MS. J Proteome Res. (2009) 8:4954–65. doi: 10.1021/pr9007267

37. Lund MN, Heinonen M, Baron CP, Estévez M. Protein oxidation in muscle foods: a review. Mol Nutr Food Res. (2011) 55:83–95. doi: 10.1002/mnfr.201000453

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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