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Mechanism of Production of Troponin T Fragments during Postmortem Aging of Porcine Muscle

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Troponin T (TnT) is one of the myofibrillar proteins that is easily degraded during postmortem aging of pork. In this study, we determined the N-terminal amino acid sequences of TnT degradation fragments produced during postmortem aging and by m-calpain hydrolysis. The N-terminal amino acid sequences of TnT fragments produced during postmortem aging were EVHEPEEKPRPKLTAP, EKPRPKLTAPKIPEG, and APKIPEGEKVDF. On the other hand, the N-terminal amino acid sequences of TnT fragments produced by the action of m-calpain were APPPPAEV, EVHEPEEK, and APK. These sequences of degradation fragments could be mapped on fast type TnT isoform 2. The peptide bonds of His_{37}−Glu_{38} and Thr_{51}−Ala_{52} in fTnT2 were cleaved during postmortem aging as well as by the calpain hydrolysis; therefore, calpain was concluded to have an important role in TnT degradation during postmortem aging. It was also found that the sourness-suppressing peptide APPPPAEVHEVHEEVH (Okumura et al. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1657−1662) derived from TnT degradation could be produced by the action of calpains on Glu_{21}−Ala_{22} and His_{37}−Glu_{38} sites.

KEYWORDS: Troponin T (TnT); postmortem aging; calpain; porcine; degradation; N-terminal amino acid sequence

INTRODUCTION

It is well-known that muscle is converted to meat as food during postmortem aging. Due to the increase of peptides and free amino acids during postmortem aging, meat taste is improved (*1*−*6*). For example, we have previously reported that during postmortem aging of pork, a peptide APPPPAEVHEVHEEVH was derived from troponin T (TnT) to contribute to improvement of taste (sourness-suppressing) (*1*).

The postmortem degradation of skeletal muscle proteins and its relationship to meat tenderness and quality have been the subject of considerable research. TnT is well-known as one of the myofibrillar proteins to be easily degraded during postmortem aging of meat (*7*). In conditioned meat, a 30 kDa fragment was detected and immunologically identified as a TnT fragment (*8*−*11*). The degradation of TnT progresses simultaneously with the postmortem tenderization of meat, showing a good correlation between the two events (*12*). In addition, we found that the above-mentioned sourness-suppressing peptide was also useful as a conditioning indicator in pork loins (*13*).

However, the details of TnT degradation and the relationship between TnT degradation and meat tenderization remain poorly understood. First, especially in pork, the cleavage sites of TnT are unknown because the amino acid sequence of the degradation products had not been determined. Second, the mechanisms of the fragmentation of TnT proteins during postmortem aging have also not been analyzed. Therefore, although several candidate proteinases, such as calpains and cathepsins, can cleave TnT in vitro (*14*), how much each of them contributes to TnT degradation is not still clarified. Understanding of the mechanism of TnT degradation would lead to control of meat quality during postmortem aging.

Recently, we have determined the amino acid sequence of porcine TnT isoforms. At least eight porcine fast-type TnT (fTnT1/16, fTnT1/17, fTnT2/16, fTnT2/17, fTnT3/16, fTnT3/17, fTnT4/16, and fTnT4/17) and two slow-type TnT (sTnT1 and sTnT2) isoforms have been found so far by our group; these hold the sequential Accession Nos. AB176595, AB176599, AB176596, AB176600, AB176597, AB176601, AB176598, and AB176602 (fTnT) and AB118908 and AB118909 (sTnT) in the DDBJ/EMBL/GenBank nucleotide sequence databases. These sequences suggested that the sourness-suppressing peptide APPPPAEVHEVHEEVH was produced from fTnT isoforms fTnT1, fTnT2, and fTnT3.

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As described above, since TnT degradation is thought to be related to meat tenderization and improvement of meat flavor during postmortem aging, examination of its degradation mechanism is a crucial subject in this regard. Thus, the first objective of this study is to determine N-terminal amino acid sequences of TnT degradation fragments during postmortem aging. Since, during postmortem aging of pork, we found sourness-suppressing peptide, APPPPAEVHEVHEEVH, from TnT (*1*), we chose pork as a material in this study. We also aimed to clarify the involvement of calpains as one of the key enzymes acting in the postmortem aging, using m-calpain that digests myofibrillar proteins in a manner closely similar to μ-calpain, although with a different Ca^{2+} dependency (*15−17*). The mechanism of production of APPPPAEVHEVHEEVH is also discussed.

MATERIALS AND METHODS

Meat Sample. Porcine longissimus thoracis muscle (LT) was obtained from the carcasses of porcine crossbreds (Landrace × Large White × Duroc). The LT was stored at 4 °C during the postmortem aging period. Portions of the LT were removed at 1, 3, 5, and 7 days postmortem.

Preparation of Myofibrillar Proteins. In all steps of myofibrillar protein preparation from porcine LT, samples were kept on ice. The meat samples were minced and homogenized with 0.04 M Tris-HCl buffer (pH 7.4) containing 0.16 M KCl. The precipitate obtained was washed and centrifuged three times in 0.16 M KCl at 3000*g* for 15 min. After centrifugation, the pellet was suspended in 0.16 M KCl containing 5 mM NaN_3. Finally, the suspended myofibrillar proteins were filtered through nylon net to remove connective tissue.

Hydrolysis of Myofibrillar Proteins by m-Calpain. m-Calpain from rabbit skeletal muscle was purchased from Sigma (St. Louis, MO). One unit of this enzyme is defined as the production of ΔA_{280} of 0.5 in 30 min at pH 7.5 at 30 °C, measured as trichloroacetic acid- (TCA-) soluble products with *N,N*-dimethylated casein as substrate. Myofibrillar proteins were prepared from LT stored for 1 day after slaughter. Myofibrillar proteins (2.5 mg) were hydrolyzed with m-calpain (125 milliunits) at 30 °C for 180 min in 50 mM acetate buffer (pH 6.2) containing $CaCl_2$ (5 mM) and 2-mercaptoethanol (0.1%). The hydrolysate was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) for analysis of degraded proteins and high-performance liquid chromatography (HPLC) for analysis of fragmental peptides as follows.

SDS−PAGE and Electroblotting. All steps of SDS−PAGE and Western blot analysis were performed at room temperature. After aging or hydrolyzing, the myofibrillar samples were solubilized in 10 mM Tris-HCl buffer (pH 6.8) containing 20% glycerol, 1% SDS, 1% 2-mercaptoethanol, and 0.01% bromophenol blue to prepare SDS samples. SDS samples (300 μg) were loaded onto 55-mm-wide lane of SDS−polyacrylamide gels. The 12.5% gel was run in 25 mM Tris buffer containing 192 mM glycine and 0.1% SDS at a constant current (14 mA) for 2 h 45 min. Prestained Precision Protein Standard (Bio-Rad, Hercules, CA) was used as the molecular mass marker.

Western Blot Analysis. After SDS−PAGE, the gel was soaked in a transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol, and 0.05% SDS). Then the proteins on the gel were transferred to a poly-(vinylidene difluoride) (PVDF) membrane (Immobilon-P_{SQ} Millipore, Bedford, MA) in the same transfer buffer. The current was set at 3 mA/cm^2 of membrane for 90 min. The electroblotted membrane was then blocked by blocking buffer [phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween-20] for 30 min. After blocking, the membrane was incubated for 30 min with anti-fTnT polyclonal goat antibody (primary antibody) raised against a peptide corresponding to the internal region of human fTnT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The primary antibody was used at a 1:100 dilution in an antibody buffer (PBS containing 0.1% BSA and 0.01% Tween-20). After three washes with 0.1% Tween-20/PBS for 5 min each and being blocked with blocking buffer for 30 min, the membrane was incubated with biotin-conjugated anti-goat secondary antibody (Santa Cruz Biotechnology). The secondary antibody was used at a 1:500 dilution in the antibody buffer for 30 min. After three washes, the membrane was incubated with avidin-DH and biotin-conjugated horseradish peroxidase (HRP) (Vectorstain ABC-PO kit, Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. After three washes, the membrane was finally stained with a DAB substrate kit (Vector Laboratories).

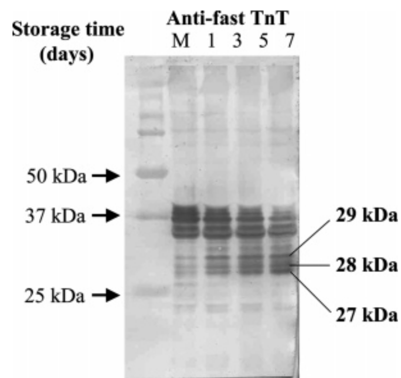

Figure 1. Western blot analysis with anti-fast TnT antibody of TnT fragments produced during postmortem aging. Myofibrillar proteins from porcine longissimus thoracis muscle at 1, 3, 5, and 7 days postmortem were analyzed by Western blotting. The N-terminal amino acid sequences of the 29, 28, and 27 kDa bands (indicated with lines) were EVHEPEEKPRPKLTAP..., EKPRPKLTAPKIPEG..., and APKIPEGEKVDF..., respectively. M, molecular mass markers.

HPLC. Trichloroacetic acid (TCA, 50%) was added to the calpain hydrolysate to a final concentration of 5% TCA, and the resulting mixture was centrifuged at 10000*g* for 10 min. The peptides in the supernatant were analyzed by HPLC on a reversed-phase column (4.6 × 250 mm; Senshu-pak Pegasil-300 ODS-II; Senshu, Tokyo, Japan) with a liner gradient of acetonitorile (0−35%) containing 0.1% trifluoroacetic acid (TFA) at 1.0 mL/min for 70 min. Peaks were detected at 220 nm and collected.

Determination of N-Terminal Amino Acid Sequence of Fragments. The electroblotted proteins on PVDF membrane were stained with Coomassie Brilliant Blue R-250 (CBB; Bio-Rad). The bands and collected HPLC samples of interest were excised, and then the N-terminal amino acid sequences were analyzed by a G1000A protein sequencer (Hewlett-Packard, Palo Alto, CA).

RESULTS AND DISCUSSION

The main objective of this study was to clarify the cleavage sites and degradation pattern of TnT during postmortem aging. For this purpose, we analyzed the time-course changes in TnT fragments in extracts from porcine LT after different times of postmortem aging and m-calpain hydrolysis using Western blot analysis.

The results of Western blot analysis revealed that multiple degradation products of TnT were generated in porcine LT during postmortem aging (**Figure 1**) and by m-calpain hydrolysis (**Figure 2**). Before aging and hydrolysis, four fTnT bands were detected with molecular masses of around 37 kDa, which were thought to be intact TnT isoforms. These bands were decreased with aging and by m-calpain hydrolysis, while their degraded fragments with molecular masses of around 30 kDa increased.

At 7 days postmortem, six anti-fTnT-positive bands were detected (**Figure 1**), and N-terminal sequences were determined for three of six bands with molecular masses of approximately 29, 28, and 27 kDa. The N-terminal amino acid sequences of the 29, 28, and 27 kDa bands were EVHEPEEKPRPKLTAP,

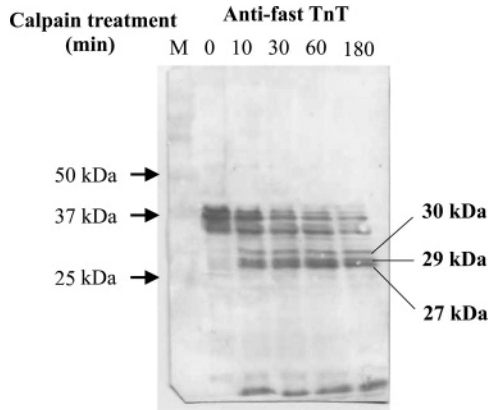

Figure 2. Western blot analysis with anti-fast TnT antibody of TnT fragments produced by m-calpain hydrolysis . Myofibrillar proteins from porcine longissimus thoracis muscle were treated with m-calpain and analyzed by Western blotting. The N-terminal amino acid sequences of the 30, 29, and 27 kDa bands (indicated with lines) were APPPPAEV..., EVHEPEEK..., and APK..., respectively. M, molecular mass markers.

EKPRPKLTAPKIPEG, and APKIPEGEKVDF, respectively. On the other hand, five anti-fTnT-positive bands were detected by the action of m-calpain (**Figure 2**). The N-terminal amino acid sequences of the three bands migrating at 30, 29, and 27 kDa were APPPPAEV, EVHEPEEK, and APK, respectively. The N-terminal amino acid sequence EVHEPEEK was found only in fTnT2 and fTnT3 (**Figure 3**).

As shown in **Figure 4**, the peptide bonds $His_{37}-Glu_{38}$ (a1 and b2) and $Thr_{51}-Ala_{52}$ (a3 and b3) were cleaved during postmortem aging as well as by the action of m-calpain. $Glu_{43}-Glu_{44}$ (a2) was cleaved only during postmortem aging, and $Glu_{21}-Ala_{22}$ (b1) was cleaved only by m-calpain hydrolysis. These results suggested that $His_{37}-Glu_{38}$ and $Thr_{51}-Ala_{52}$ were cleaved by calpains during postmortem aging, while $Glu_{43}-Glu_{44}$ was due to hydrolysis by another proteinase, such as cathepsin. These results also revealed that the sourness-suppressing peptide would be produced by the action of calpains on the peptide bonds $Glu_{21}-Ala_{22}$ and $His_{37}-Glu_{38}$.

The results of this study showed that TnT was degraded by m-calpain and these cleavage sites were present in the N-terminal region. On the other hand, Hughes et al. (*18*) reported that the C-terminal region of TnT, which was purified from rabbit skeletal muscle, was cleaved by μ-calpain. It was probable that the purified TnT might not be associated with other proteins and, thus, its C-terminal region might be easily degradated by μ-calpain. On the other hand, TnT in the myofibrillar structure is associated with other proteins such as tropomyosin, and this association may protect the C-terminal region of TnT from proteolytic degradation.

Figure 5 shows the HPLC profiles on an ODS column of extracts prepared from myofibrillar proteins treated with m-calpain for 1 and 180 min. Although there were few peaks at 1 min, many peaks gradually increased as the m-calpain treatment proceeded. Most peptides in the extract increased during hydrolysis; among them, peptide peaks P1, P2, and P3 increased noticeably. It was found that the N-terminal amino acid sequences of these peptide peaks began with APP. We previously found the sourness-suppressing peptide APPPPAEVHEVHEEVH from postmortem aged porcine meat by almost the same HPLC analysis (*1*, *13*). Thus, it was highly probable that at least one of these three peptide peaks would be the sourness-suppressing peptide and would be liberated from TnT by calpain hydrolysis.

In this study, we used m-calpain for the following two reasons: m-calpain digests myofibrillar proteins in a manner closely similar to μ-calpain, although with a different Ca^{2+} dependency (*15*−*17*); and Goll et al. (*19*) reported that the free calcium concentration increases gradually with increasing time of postmortem storage and may reach millimolar levels after 24 h postmortem. Nevertheless, it is necessary to evaluate the effects of μ-calpain on TnT degradation, since it is generally more active than m-calpain in postmortem aging (*20*). Moreover, since it was reported that TnT was degraded also by cathepsins L (*21*) and D (*22*), the involvement of cathepsins or other proteinases in the TnT degradation needs to be clarified.

From our present data and previous results, there exist similarities and differences between postmortem aged porcine and bovine meat. By HPLC analysis, APPPPAEVHEVHEEVH was detected from aged porcine meat (*1*, *13*), and APPPPAEVPEVHEEV was detected from aged bovine meat (*23*). Thus, such TnT fragment peptides may be generally produced without regard to animal species. On the other hand, as for degraded TnT bands, the N-terminal amino acid sequence APPPPAEV was determined from a 32.1 kDa fragment of TnT in aged bovine meat (*8*), while a protein band containing N-terminal amino acid sequence of APPPPAEV was not detected in postmortem aged porcine meat in the present study. This difference might be caused by a difference in the ratio of proteinases (for example, calpains vs cathepsins) between porcine and bovine meat.

In conclusion, the N-terminal amino acid sequences of TnT degradation fragments in postmortem aged pork were determined. The possible involvement of m-calpain in this degradation was clearly shown. It is highly possible that a sourness-suppressing peptide APPPPAEVHEVHEEVH is derived from TnT by calpains during postmortem aging. These results greatly

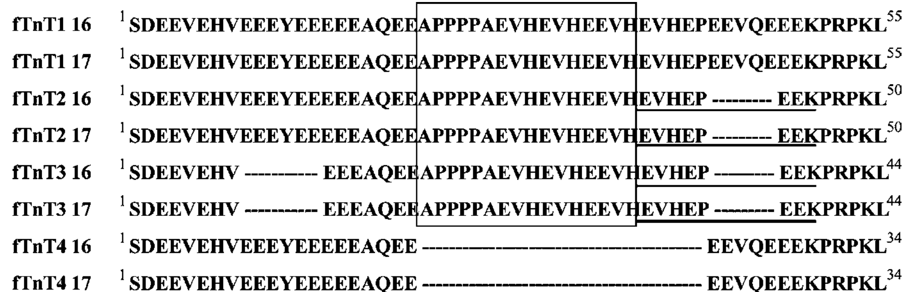

Figure 3. Amino acid sequences of N-terminal region of eight porcine fTnT isoforms. The sourness-suppressing peptide is boxed (Accession Nos. AB176595, AB176599, AB176596, AB176600, AB176597, AB176601, AB176598, and AB176602 in the DDBJ/EMBL/GenBank nucleotide sequence databases). The numbers of amino acid residues of the fTnT isoforms are 270, 265, 259, and 249 for fTnT1, fTnT2, fTnT3, and fTnT4, respectively. Underlining indicate the N-terminal amino acid sequence of 29 kDa fragment (**Figures 1** and **2**). ---, exon regions of alternative splicing.

Figure 4. Amino acid sequence of N-terminal region of porcine fast-TnT2 (fTnT2) and its cleavage sites. The sourness-suppressing peptide is underlined. a1–a3, cleavage sites detected after postmortem aging; b1–b3, cleavage sites detected by calpain hydrolysis.

Figure 5. Changes in HPLC profile of extracts of pork loins hydrolyzed with m-calpain. P1, P2, and P3 indicate the peptides whose N-terminal amino acid sequences were analyzed. Trace A, 1 min treatment; trace B, 180 min treatment.

contribute to the understanding of TnT degradation during postmortem aging of meat at the molecular level.

LITERATURE CITED

(1) Okumura, T.; Yamada, R.; Nishimura, T. Sourness-suppressing peptides in cooked pork loins. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1657−1662.

(2) Nishimura, T. Palatability on texture, aroma, and taste of meat. *Jpn. J. Taste Smell Res.* **2001**, *8*, 161−168.

(3) Nishimura, T. Mechanism Involved in the Improvement of Meat Taste during Postmortem Aging. *Food Sci. Technol. Int.* **1998**, *4*, 241−249.

(4) Nishimura, T.; Rhue, M. R.; Okitani, A.; Kato, H. Components contributing to the improvement of meat taste during storage. *Agric. Biol. Chem.* **1988**, *52*, 2323−2330.

(5) Caul, F. Study on the development of beef flavor in U.S. choice and U.S. commercial cuts of sirloin, In Survey of the Progress of Military Subsistence Problem Ser. I 9; *Quarter Master Food Container Inst.* **1957**, No. 9, p 152.

(6) Smith, G. C.; Culp, G. R.; Carpenter, Z. L. Postmortem aging of beef carcass. *J. Food Sci.* **1978**, *43*, 823−826.

(7) MacBride, M. A.; Parrish, F. C., Jr. The 30,000-dalton component of tender bovine longissimus muscle. *J. Food Sci.* **1997**, *42*, 1627−1629.

(8) 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.

(9) Huff-Lonergan, E.; Mitsuhashi, T.; Beekman, D. D.; Parrish, F. C., Jr.; Olson, D. G.; Robson, R. M. Proteolysis of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *J. Anim. Sci.* **1996**, *74*, 993−1008.

(10) Negishi, H.; Yamamoto, E.; Kuwata, T. The origin of the 30 kDa component appearing during post-mortem aging of bovine muscle. *Meat Sci.* **1996**, *42*, 289−303.

(11) Muroya, S.; Kitamura, S.; Tanabe, S.; Nishimura, T.; Nakajima, I.; Chikuni, K. N-terminal amino acid sequences of troponin T fragments, including 30 kDa one, produced during postmortem aging of bovine longissimus muscle. *Meat Sci.* **2004**, *67*, 19−24.

(12) Penny, I. F.; Dransfield, E. Relationship between toughness and troponin T in conditioned beef. *Meat Sci.* **1979**, *3*, 135−141.

(13) Okumura, T.; Yamada, R.; Nishimura, T. Survey of conditioning indicator for pork loins: changes in myofibrils, proteins and peptides during postmortem conditioning of vacuum-packed pork loins for 30 days. *Meat Sci.* **2003**, *64*, 467−473.

(14) Hopkins, D. L.; Thompson, J. M. Factors contributing to proteolysis and disruption of myofibrillar proteins and the impact on tenderisation in beef and sheep meat. *Aust. J. Agric. Res.* **2002**, *53*, 149−166.

(15) Sasaki, T.; Kikuchi, T.; Yumoto, N.; Yoshimura, N.; Murachi, T. Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic. *J. Biol. Chem.* **1984**, *259*, 12498−12494.

(16) McDermott, J. R.; Mantle, D.; Biggins, J. A.; Kidd, A. M.; Davison, K.; Lauffart, B.; Pennington, R. J. T. Specificity of neuropeptide degradation by two calcium-activated neutral proteases from human skeletal muscle. *Life Sci.* **1985**, *37*, 725−730.

(17) Goll, D. E.; Thompson , V. F.; Li, H.; Wei, W.; Cong, J. The calpain system. *Physiol. Rev.* **2003**, *83*, 731−801.

(18) Hughes, M. C.; Geary, S.; Dransfield, E.; McSweeney, P. L. H.; O'Neill, E. E. Characterization of peptides released from rabbit skeletal muscle troponin-T by μ-calpain under conditions of low temperature and high ionic strength. *Meat Sci.* **2001**, *59*, 61−69.

(19) Goll, D. E.; Otsuka, Y.; Nagainis, P. A.; Shannon, J. D.; Sathe, S. K.; Muguruma, M. Role of muscle proteinases in maintenance of muscle integrity and mass. *J. Food Biochem.* **1983**, *7*, 137−177.

(20) Koohmaraie, M.; Schollmeyer, J. E.; Dutson, T. R. Effect of low-calcium-requiring activated factor on myofibrils under varying pH and temperature conditions. *J. Food Sci.* **1986**, *51*, 28−32.

(21) Mikami, M.; Alan, H. W.; Mark, A. J. T.; Rose, A. M.; David, J. E. Degradation of myofibrils from rabbit, chicken and beef by cathepsin L and lysosomal lysates. *Meat Sci.* **1987**, *21*, 81−97.

(22) Okitani, A.; Matsumoto, T.; Kitamura, Y.; Kato, H. Purification of cathepsin D from rabbit skeletal muscle and its action towards myofibrils. *Biochim. Biophys. Acta* **1981**, *662*, 202−209.

(23) Nakai, Y.; Nishimura, T.; Shimizu, M.; Arai, S. Effects of freezing on the proteolysis of beef during storage at 4 °C. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 2255−2258.

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