MECHANISM OF THE PRODUCTION OF TROPONIN T FRAGMENTS DURING POSTMORTEM AGING OF PORCINE MUSCLE

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Background

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. During postmortem aging, a peptide APPPPAEVHEVHEEVH derived from troponin T (TnT) was found not only to contribute to improvement of taste (sour-suppressing) but also to be useful as a conditioning indicator in meat (Okumura et al., 2003). However, the details of TnT degradation and the relationship between TnT degradation and muscle tenderization remain poorly understood.

Objectives

The objectives of this study were to determine N-terminal amino acid sequences of TnT degradation fragments during postmortem aging and to clarify the involvement of calpain in the degradation.

Materials and methods

Preparation of myofibrillar proteins

In all steps of myofibrillar proteins preparation from porcine longissimus thoracis muscle (LT), samples were kept on ice. The meat samples were minced and homogenized with 0.04M Tris-HCl buffer (pH 7.4) containing 0.16M KCl. The precipitate obtained was washed and centrifuged three times in 0.16M KCl for 15min at 3,000 x g. After centrifugation, pellet was suspended in 0.16M KCl containing 5mM NaN_3. Finally, the solubilized myofibrillar proteins was filtered by nyron net to remove connective tissue.

Hydrolysis of myofibrillar proteins by m-calpain

m-Calpain from rabbit skeletal muscle was purchased from Sigma (Germany). Myofibrillar proteins were prepared from LT stored for 2 days after slaughter. Myofibrillar proteins (2.5mg) were hydrolyzed with m-calpain (125U) at 30°C for 180 min in 50mM acetate buffer (pH 6.2) containing $CaCl_2$ (5mM) and 2-mercaptoethanol (0.1%). The portion of this mixture (200 μl) was taken after 0, 10, 30, 60, and 180 min and added with 30mM Tris-HCl (pH6.8) buffer (100 μl) containing 60% glycerol, 3% SDS, 3% 2-mercaptoethanol, and 0.03% bromophenol blue to stop the reaction.

SDS-PAGE and electroblotting

All steps of SDS-PAGE and western blot analysis were performed at room temperature. The solubilized myofibrillar proteins (300 μg) were loaded onto 55mm-wide lane of SDS-PAGE gels. The 12.5% gel was run in 25mM Tris buffer containing 192mM glycine and 0.1% SDS at a constant current (14mA) for 2 hr 45 min. Prestained Precision Protein Standard (Bio-Rad, Hercules, CA) was used as the marker.

Western blot analysis

After SDS-PAGE, the gel was soaked in a transfer buffer (25mM Tris, 192mM glycine, 15% methanol, 0.05% SDS). Then, the proteins on the gel were transferred to a polyvinylidenedifluoride (PVDF) membrane (Immobilon-P_{SQ} Millpore, Bedford, MA) in the same transfer buffer. The current was set at 3mA/cm^2-membrane for 90 min. The electroblotted membrane was then blocked by blocking buffer (PBS containing 1% BSA and 0.1% Tween-20) for 30 min. After blocking, the membrane was incubated for 30 min with anti-fTnT polyclonal goat antibodies raised against a peptide corresponding to the internal region of human fTnT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The primary antibodies were 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 antibodies (Santa Cruz Biotechnology). The secondary antibodies were 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 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).

Determination of N-terminal amino acid sequence of proteins

The electroblotted proteins on PVDF membrane were stained with Coomassie Brilliant Blue R-250 (CBB; Bio-Rad). The bands 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 results of Western blot analysis revealed that multiple degradation products of TnT were generated in porcine LT during postmortem aging (Fig. 1) and by calpain hydrolysis (Fig. 2). Before aging and hydrolysis, four fTnT bands were detected molecular masses of around 37 kDa. These bands were thought to be intact TnT isoforms. These bands were decreased with aging and by calpain hydrolysis, and their degraded fragments with molecular masses of around 30 kDa increased. At 7 days postmortem, anti-fTnT-positive six bands were detected, and three of six bands were determined their N-terminal sequences with molecular masses of approximately 29, 28, and 27 kDa. The N-terminal amino acid sequences of 29, 28, and 27 kDa bands were EVHEPEEKPRPKLTAP, EKPRPKLTAPKIPEG, and APKIPEGEKVDF, respectively. After calpain hydrolysis, five anti-fTnT-positive bands were detected and three of five bands were determined their N-terminal amino acid sequences with molecular masses of approximately 30, 29, and 27 kDa. The N-terminal amino acid sequences of 30, 29, and 27 kDa bands were APPPPAEV, EVHEPEEK, and APK, respectively.

In our previous study, we have determined the amino acid sequences of porcine fTnT isoforms deduced from the DNA sequences (accession nos. AB176595- AB176602 in DDBJ/EMBL/GenBank nucleotide sequence databases). The number of amino acid residues of the fast and slow TnT isoforms were 270 (fTnT1), 265 (fTnT2), 259 (fTnT3), 249 (fTnT4), 261 (sTnT1), and 250 (sTnT2). The N-terminal sequence EVHEPEEK was found only in fTnT2 and fTnT3 (Fig. 3). As shown in Fig. 4, the cleavage sites His_{37}-Glu_{38} (a1 and b2) and Thr_{51}-Ala_{52} (a3 and b3) were cleaved after postmortem aging as well as by calpain hydrolysis. On the while, Glu_{43}-Glu_{44} (a2) was cleaved only after postmortem aging, and Glu_{21}-Ala_{22} (b1) was cleaved only by calpain hydrolysis. These results suggested that His_{37}-Glu_{38} and Thr_{51}-Ala_{52} were cleaved by calpain hydrolysis in postmortem aging, while Glu_{43}-Glu_{44} was by another proteinases hydrolysis, such as cathepsins. Recently, we found the sour-suppressing peptide APPPPAEVHEVHEEVH from postmortem aged pork (Okumura et al., 2003). From the results in the present study (Fig. 4), the sour-suppressing peptide would be produced by calpain hydrolysis at Glu_{21}-Ala_{22} and His_{37}-Glu_{38} sites. Howevere, APPPPAEVHEVHEEVH sequence was not detected from the N-terminal sequences of TnT fragments in postmortem aged porcine meat. The following thing is a probable reason; in postmortem aged pork, several proteinases, such as calpains and cathepsins, were involved in TnT degradation and, as a result, the peptide was liberated from TnT. On the while, in postmortem aged beef, the sequence APPPPAEVHEVHEEVH was determined from 32.1 kDa fragment (Muroya et al., 2004). These results indicated the ratio of proteinases might be different in porcine and bovine.

In this study, we clarirfied effect of calpain on TnT degradation. Howevere, the involvement of other proteinases such as cathepsins in the TnT degradation needs to be also clarified.

Conclusion

It is highly possible that a peptide APPPPAEVHEVHEEVH is derived from TnT by calpain during postmortem aging.

References

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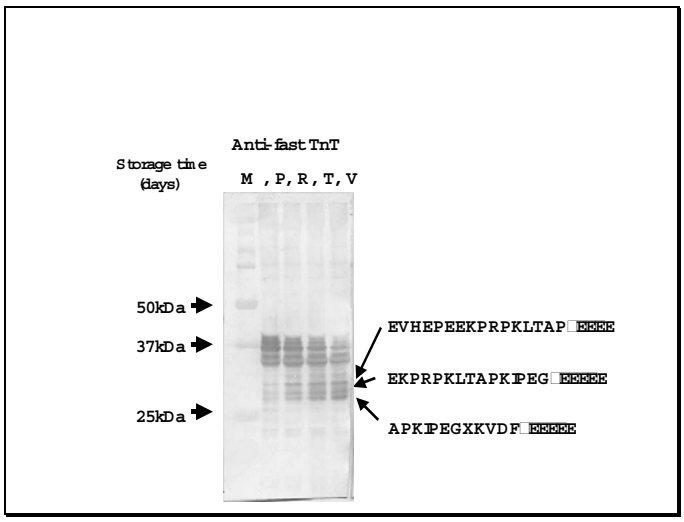

Fig. 1 Western blot analysis of TnT fragments using anti-fast TnT antibody.
Myofibrillar proteins from porcine longissimus thoracis muscle at 1, 3, 5, 7 days postmortem were analyzed.
The N-terminal amino acid sequences of the bands (indicated with arrows) were shown in the figure.
M, molecular mass markers.

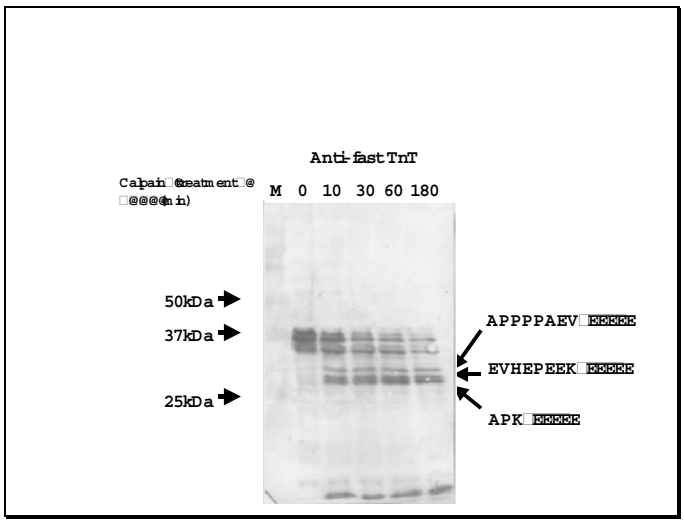

Fig. 2 Western blot analysis of TnT fragments using anti-fast TnT antibody.
Calpain hydrolysate of myofibrillar proteins from porcine longissimus thoracis muscle were analyzed.
The N-terminal amino acid sequences of the bands (indicated with arrows) were shown in the figure.
M, molecular mass markers.

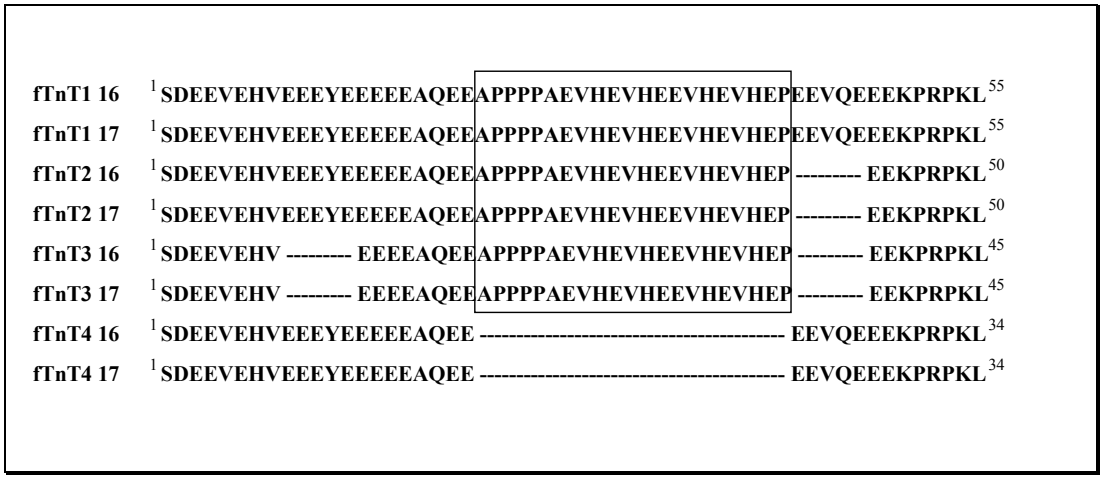

Fig. 3 The amino acid sequences of N-terminal region of eight porcine fTnT isoforms.
Sour-suppressing peptide is boxed.
--- indicates exon regions of alternative splicing.

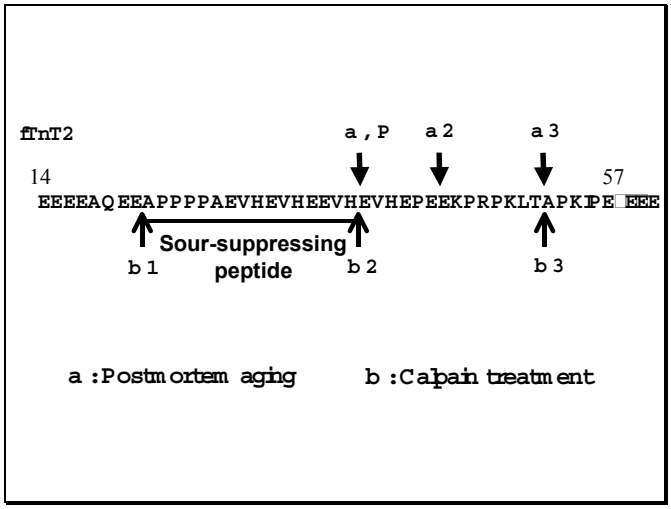

Fig. 4 The amino acid sequence of N-terminal region of porcine fast-TnT2 (fTnT2) and its cleavage sites.
a, indicates cleavage sites observed after postmortem aging.
b, indicates cleavage sites observed by calpain hydrolysis.
Sour-suppressing peptide is underlined.