TWO-DIMENSIONAL GEL ANALYSIS OF TROPONIN T ISOFORMS AND FRAGMENTS DURING POSTMORTEM AGING

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Introduction

Troponin T (TnT) is degraded mainly into the 30-kDa peptide, during postmortem tenderisation of beef. A peptide generated by TnT degradation was found to suppress sourness of lactic acid and is useful as a conditioning indicator in pork. Despite the significance of elucidating postmortem TnT degradation, the details of TnT degradation are still not understood to date. In bovine muscles, at least eight fast-type TnT (fTnT) (fTnT1/16 and 17, fTnT2/16 and 17, fTnT3/16 and 17, and fTnT4/16 and 17) and two slow-type TnT (sTnT) (sTnT1 and sTnT2) isoforms have been found to be expressed so far. These fTnT and sTnT isoforms are generated by the alternative splicing of the mRNA originated from fTnT and sTnT genes, respectively. The molecular weight (MW) and isoelectric point (pI) of the isoforms are estimated to range widely from 32.1 to 29.8 kDa, and from 5.61 to 9.23, respectively (Muroya *et al.*, 2003). To clarify degradation of multiple TnT isoforms, the analysis is required to be highly resolutive, by which the isoform proteins can separate from one another. Taking advantage of high resolution, 2DE analysis provides the benefits of being able to analyze complex mixtures of myofibrils and sarcoplasm. In the present study, in order to comprehend the changes in TnT isoforms accompaning beef aging, whole muscle proteins including all TnT-related polypeptides were applied to 2DE analysis. To identify TnT spots, western blotting, using isoform-specific anti-TnT antibodies on 2DE gels were performed.

Materials and Methods

The longissimus thoracis (LT) for 0 d postmortem samples were excised from a Holstein and a Japanese Black steer within 1 hr after slaughter. After overnight hanging of the carcasses at 2°C, the muscle blocks were excised from the carcass. At 1 and 14 d postmortem, the muscle samples were prepared from the central part of the block, and bagged during aging at 2°C. The sample for whole muscle proteins was prepared by homogenizing the muscle sample in lysis buffer containing 8 M urea, 50 mM dithiothreitol (DTT), 4% CHAPS, and 0.2% Bio-Lyte 3/10 Ampholyte (Bio-Rad, CA, USA.), to prepare 2DE samples. For myofibril preparation, muscle samples were homogenized and extracted with homogenizing buffer, 20 mM potassium-phosphate buffer (pH 6.8) containing 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1mM 2-mercaptoethanol, and 0.05% sodium azide. Then, the samples were solubilized in lysis buffer. After determining protein concentration, 100-200 µg of protein was loaded onto a Bio-Rad ReadyStrip, 11 cm, pH 3-10 non-linear and immobilized pH gradient (IPG) strips. Isoelectric focusing (IEF) was performed in a Protean IEF cell (Bio-Rad). Strips were rehydrated overnight. For the subsequent IEF, voltage was increased gradually up to 8,000 V for 2.5 h, and then was maintained at a total voltage of 60,000 or 80,000 Vh. The second dimensional SDS-PAGE was performed in a Criterion cell (Bio-Rad) on 12.5% polyacrylamide gel at 100 V for 1 h. Overall proteins in preparative gel were stained in SYPRO Ruby (Bio-Rad). Experimental pI was determined using a 2DE protein standard (Bio-Rad). After 2DE, the proteins on the gel for detecting phosphorylation were fixed and stained with Pro-Q® Diamond phosphoprotein gel stain (Invitrogen, Eugene, OR, USA) according to manufacture's protocol. After destaining, the fluorescence of the gel was scanned at excitation 540 nm and emission 595 nm with Ettan DIGE Primo (GE Healthcare, Fairfield, CT, USA). As for western blot analysis, the proteins on the gel were transferred to a polyvinylidenefluoride membrane. The electroblotted membrane was incubated with commercial anti-fTnT or -sTnT polyclonal goat antibodies (Santa Cruz Biotechnology, Inc., CA, USA). The positive spots were detected with avidin-DH and biotin-conjugated HRP, and DAB substrate kit (VECTORSTAIN® ABC kit, Vector Laboratories, CA, USA.) as performed previously (Muroya *et al.*, 2006).

Results and Discussion

The 2DE western blot image showed that at least nine and three anti-fTnT antibody-positive spots were distributed on the 0 and 14 d gel, respectively. The MW of the fTnT spots were 36.5, 35.4, 34.8, and 32.8 kDa on the 0 d gel, and were 32.1, 28.3, and 26 kDa on the 14 d gel. The spots of four different MW isoforms on the 0 d gel were of fTnT1, fTnT2, fTnT3, and fTnT4 isoforms, respectively (Muroya *et al.*, 2004, 2006). In the overview of 2DE map of intact fTnT isoforms, the highest MW isoform was acidic, and in contrast, the lowest MW isoforms tended to be basic, with intermediate MW isoforms having broad pI range. As for the N-terminal variants, fTnT spots consisted of fTnT2 and fTnT3 isoforms in most part, plus fTnT1 and fTnT4 isoforms in minor part, in the case of bovine LT muscle. On the other hand, 32.1, 28.3, and 26 kDa polypeptides on the 14 d gel were degradation products generated during postmortem aging, since they appeared with aging, showing lower MW than that of intact TnT isoforms. The fTnT isoform spots on the 0 d gel were distributed within a range of pI 5.7-10, while on the 14 d gel, spots of fTnT fragments

were exclusively located at pI of more than 9.6. These results clearly demonstrated that, in spite of the broad pI range, all of the fTnT isoforms were degraded into basic polypeptides during postmortem aging of beef. Similarly, two and one anti-sTnT antibody-positive spots were detected on the 0 and 14 d gel, respectively. The sTnT-related spots on the 0 d gel were of pI 5.6 and 5.7, while the degraded sTnT-spot was at pI of more than 9.6 on the 14 d gel. The MW of the sTnT peptides were 34.5 kDa on the 0 d gel, and 31.0 kDa on the 14 d gel. This result indicates that sTnT isoforms were also degraded exclusively into basic polypeptides. Moreover, we detected spots of phosphorylated myofibril protein by gel staining specific to phosphorylated protein. Since no difference in the TnT spot pattern between beef whole muscle and myofibril samples was observed by western blot analysis, the myofibril fraction was used to improve the content of TnT proteins. Consequently, three of the fTnT-related spots on the 0 d gel were shown to be phosphorylated, but none of the phosphorylated spots matching anti-TnT antibody-positive spots was found on the 14 d gel. It is likely that TnT proteins are phosphorylated in an isoform-specific manner in live bovine skeletal muscle, and that changes in phosphorylated spots were caused by a cut-off of the N-terminal region of TnT during beef aging.

Conclusion

Whole beef longissimus muscle proteins were developed on 2DE gel and multiple TnT-related spots were identified by western blotting. The results showed that more than ten fTnT isoform spots (pI 5.7-9.6<) and two sTnT spots (pI 5.6-5.7) present at slaughter were degraded exclusively into basic spots (pI 9.6<) at day 14 postmortem, indicating that TnT is cleaved primarily in the glutamic acid-rich amino-terminal region.

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