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A non-destructive method to monitor changes in a troponin T peptide in beef drip with a monoclonal antibody

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ABSTRACT

To simplify the monitoring of postmortem beef aging, we established a system to detect a troponin T (TnT) peptide fragment in bovine muscle drip (natural exudates) with an original monoclonal antibody. The antibody was raised against a synthetic peptide APPPPAEVPEVHEEVH corresponding to the N-terminal region of bovine fast-type TnT. In a competitive enzyme-linked immunosorbent assay (ELISA), our antibody detected the standard peptide dose-dependently. According to the monitoring examination with a competitive ELISA during 22 days postmortem, the concentration of the peptide in both the drip and trichloroacetic acid extracts from the longissimus muscle ($n = 4$) significantly increased in parallel, up to 10 nmol/ml and 16.4 nmol/g at day 14 postmortem, respectively. These events were accompanied by an increase in the conventional 30 kDa fragment in western blot analysis and a decrease in the Warner–Bratzler shear force value of the beef from 5.0 to 2.4 N/cm^2. The peptide detection system using drips with the antibody has advantages applicable to a non-destructive, simple, quick, and on-site monitoring method, such as immunochromatography.

1. Introduction

Meat aging improves the taste, tenderness, and juiciness of meat, particularly pork and beef. These quality traits have a direct impact on consumer preferences, and controlling the quality is therefore the most important factor that raises the value of meat in the market. Retailers also potentially require a simple, quick, and precise method of monitoring meat aging to control and check the quality on site in terms of consumer preference. Providing consumers with the monitoring results would enable them to make an informed choice about which meats to buy.

To predict and monitor meat quality traits, suitable indicators have been explored in muscle proteins related to molecular events during meat aging, especially in recent cases by the proteomic method (Hwang, Park, Kim, Cho, & Lee, 2005; Lametsch et al., 2003; Morzel et al., 2004). One of the most important events is protein degradation, which is thought to be mostly caused by enzymes. The proteolysis of some myofibrillar proteins easily occurs during meat aging and contributes to muscle structure weakening, which contributes to meat tenderization.

One of the easily degraded proteins during meat aging is troponin T (TnT), a myofibril contractile component (MacBride & Parrish, 1977; Olson & Parrish, 1977). In aged beef, a degradation product of TnT, the conventional 30 kDa polypeptide, was detected and immunologically identified (Ho, Stromer, & Robson, 1994; Huff-Lonergan et al., 1996; Negishi, Yamamoto, & Kuwata, 1996). The degradation of TnT progresses simultaneously with the postmortem tenderization of beef, showing a good correlation between the two events (Penny & Dransfield, 1979). At least ten isoforms of TnT are expressed in bovine and porcine skeletal muscles (Kitamura, Muroya, Nakajima, Chikuni, & Nishimura, 2006; Muroya, Nakajima, & Chikuni, 2003), and thereby various types of fragments, including polypeptides of around 30 kDa, are generated during meat aging (Muroya, Nakajima, Oe, & Chikuni, 2006; Muroya et al., 2004). In addition, peptide fragments with less than 20 amino acid residues are thought to be released from the N-terminal region of TnT that is specifically cleaved at several hydrophilic sites in the N-terminal region (Kitamura et al., 2005; Muroya, Ohnishi-Kameyama, Oe, Nakajima, & Chikuni, 2007; Muroya et al., 2004).

One of the peptides detected in aged beef and pork has been determined to be APPPPAEVPEVHEEVH (Nakai, Nishimura, Shimizu, & Arai, 1995; Stoeva, Byrne, Mullen, Troy, & Voelter, 2000; Voelter et al., 2000). The peptide was found to be useful as an aging indicator in beef or pork loins (Okumura, Yamada, &

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Nishimura, 2003; Voelter et al., 2000), as well as a sourness-suppressing peptide that is thought to improve beef taste (Okumura, Yamada, & Nishimura, 2004). In addition, the peptide was identified as a fragment of the fast-type TnT isoform based on nucleotide sequencing of bovine TnT cDNA (Muroya et al., 2004). Here we refer to this peptide as the APn peptide after the well-conserved alanine and following clustered proline residues in bovine, porcine, rabbit, rat, mouse, chicken, and quail TnT amino acid sequences (Kitamura et al., 2006; Muroya et al., 2003). The APn peptide is actually generated when fast-type TnT is proteolyzed by m-calpain (Kitamura et al., 2005).

The 30 kDa TnT fragment persistently remains in myofibrils during homogenization and subsequent centrifugal separation of muscle tissue proteins in low salt aqueous conditions (Kitamura et al., 2005; Muroya et al., 2004). Myofibrils are an assembly of muscle structural and contractile proteins that are thought to interact tightly together. Compared to polypeptides such as the 30 kDa fragment, the APn peptide is expected to be useful as a meat aging indicator because it is expected to be easily released from muscle tissue and thereby able to be detected in a muscle drip. In our hypothesis, it is possible that there is an increase in bovine APn peptide in beef drip accompanying postmortem aging and that the increase can be monitored using an immunological detection system.

The objectives of the present study were (1) to develop a monoclonal antibody that recognizes the APn peptide, (2) to use the antibody to determine if the APn peptide is present in beef drip, and (3) to determine if the relative amount of APn peptide in beef drips changes with postmortem aging and tenderness. To this end, we developed a monoclonal antibody raised against the APn peptide and examined the usefulness in a competitive enzyme-linked immunosorbent assay (ELISA) for a beef drip that naturally exudates for monitoring beef aging.

2. Materials and methods

2.1. Animals

The animals were cared for as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee of National Institute of Livestock and Grassland Science, National Institute of Livestock and Grassland Science, Japan). Four Japanese black cattle (ages 53–134 months) were slaughtered after captive bolt gun stunning. The muscle samples from the cattle were used for both measurement of beef tenderness and detection of APn peptide in beef and the drip as described below.

For antibody production, commercial Balb/c female mice (ages 6 weeks and 18 months) were used for preparation of monoclonal antibody and collection of ascites, respectively. The mice were fed with commercial pellets until slaughter.

2.2. Meat sample preparation

After overnight hanging of the carcasses at 4 °C, 7 cm-long muscle sections were excised from the thoracic and central regions of the right side longissimus muscle of each carcass. The serial muscle sections from the 6th rib toward the caudal end were assigned for days 1, 3, 7, 10, 14, and 22 postmortem (PM) samples in the order of sampling day. After sampling of a section for day 1, each 7 cm section was bagged (not vacuum packed) and stored at 4 °C for subsequent ELISA and shear force examination. On the specified sample day most of each section was frozen for measurement of the Warner–Bratzler shear force value (WB-SFV). For western blot analysis, approximately 1 cm^3 piece of the muscle was sampled from the dorsal side of each section at the designated sampling days and frozen at −20 °C until use. Only the samples at day 0 PM for western blot analysis, taken within 1 h of slaughter at the 7–9th lumbar vertebrae, were prepared from the longissimus muscle of the left side of the carcass, in order to avoid possible effects of abnormal rigor mortis caused by the excision of the muscle.

For ELISA examination, drip (exudates) and muscle extract samples were prepared. The exudate in the bags was briefly stirred and collected on days 3, 7, 10, 14, and 22 after slaughter, and frozen at −20 °C until use for ELISA. In addition, approximately 500 mg pieces of the beef aged 1, 3, 7, 10, 14, and 22 days were homogenized with 10-times volume of 20 mM potassium-phosphate buffer (pH 6.8) containing 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1% protease inhibitor cocktail specific to serine, cysteine, aspartate proteases and metalloproteinases (Sigma). The homogenate was then centrifuged at 2060*g* for 15 min at 4 °C to remove the myofibril fraction. The supernatant of the homogenate samples was treated with 5% trichloroacetic acid (TCA) for more than 30 min on ice, followed by centrifugation at 2060*g* for 15 min. ELISA samples were then prepared by neutralizing the TCA extracts with 1 N NaOH to pH 7.7–7.8.

2.3. Preparation of monoclonal antibody

2.3.1. Establishment of monoclonal cell lines

The bovine APn peptide (APPPPAEVPEVHEEVH) conjugated to keyhole limpet hemocyanin (KLH) was synthesized by Sigma–Genosys (Hokkaido, Japan) as an antigen. The mice were injected with the antigen every two week for 5 months, as a mixture with complete adjuvant for the first time and with incomplete adjuvant afterwards. Every week after the injection, the sera were collected from the mice to check the production of antibodies by the non-competitive direct ELISA method. The specificity and reactivity were tested to screen the positive monoclonal hybridoma, according to the non-competitive ELISA assay method of Tsitsilonis et al. (2002). The procedure after coating with peptide is described below (see Section 2.4).

After being fully immunized, the mice were slaughtered to prepare the spleen cells for establishment of monoclonal cells. In brief, the spleen tissue was dispersed into the cells on a 75 μm mesh and washed by centrifugation. The spleen cells were mixed and fused to mouse myeloma SP2 cells in the presence of 50% of polyethylene glycol (Sigma, St. Louis, MO, USA) at a ratio of 6:1 to produce immortal hybridoma cell lines. Then each of the cells was placed into a well of 96-well plates (Nunc, Roskilde, Denmark) and was cultured with RPMI 1640 medium containing 10% fetal bovine serum (FBS; Sigma). After screening of the hybridoma cells by the addition of 1/50 volume of HAT (hypoxanthine, aminopterin, and thymidine) medium (Sigma) to the culture, the hybridoma was rescued with the culture medium containing 1/100 volume of HT (hypoxanthine and thymidine; Invitrogen Japan K.K., Tokyo, Japan). After growth of the cell colonies, the antibody production was examined by ELISA. According to the reactivity of the antibody, ten monoclonal hybridoma cell lines were finally selected to be stored for use thereafter.

The antibodies produced by the monoclonal cell lines were typed using a mouse monoclonal antibody isotyping kit (HyCult Biotechnology b.v., Uden, Netherlands) according to the manufacturer's protocol.

2.3.2. Ascites preparation and purification of antibody

Among the ten hybridoma cell lines, a monoclonal cell line 10H2, one of the most reactive, was used to obtain a large amount of the monoclonal antibody. The cells were maintained with RPMI 1640 medium containing 10% FBS which was changed every third day. Prior to the injection, the mice were injected with pristine (Sigma) at the tail to raise the immunization. Then 2×10^6 cells