Characterization of Antibodies against Connectin/Titin 20-kDa Fragment Increased in Chicken Sarcoplasm during Postmortem Aging

Yamanoue M.*, Ueda S., Matsunaga K., Onishi K. and Sioyama N.

Laboratory of Chemistry and Utilization of Animal Products, Graduate School of Agricultural Science, Kobe University, Kobe, Hyogo 657-8501, Japan

Abstract—**In this study, polyclonal and monoclonal antibodies produced against connectin/titin 20-kDa fragment, which increased in chicken sarcoplasm with postmortem aging, were characterized in order to utilize for estimating meat aging. Recombinant 12- and 16-kDa fragments of internal sequence of the 20-kDa fragment were prepared as immunogens. The result of ELISA with rat anti-12-kDa fragment antiserum indicated that the absorbance was higher with sarcoplasm of aged chicken muscle than that of immediately postmortem muscle, and an indirect immunofluorescence microscopy showed that the antiserum bound to the Z-line of sarcomeres. Two stable hybridoma cell lines (16-4B4 and 1-3C) were produced with mouse spleen cell immunized by recombinant 12-kDa fragment and myeloma cell (P3U1). Isotypes of monoclonal antibodies (mAbs) 16-4B4 and 1-3C secreted from the fused hybridoma cells were IgG3 and IgG2b with κ-light chain, respectively and both mAbs were speclfic for the 20-kDa fragment increased in chicken sarcoplasm with postmortem aging time. While, the results of western blotting proved that the antibodies cross-react to the bands in sarcoplasm from aged pork and beef muscles similarly with chicken sarcoplasm. Therfore, it is concluded that the antibodies against connectin/titin 20-kDa fragment are capable of use for estimating meat aging.**

Keywords—**Connectin/Titin, Antibody, Postmortem aging.**

I. INTRODUCTION

Connectin/titin is a huge molecule of 3000-kDa to connect with the M-line from the Z-line of sarcomere and contributes to maintenance of sarcomere structure [1]. So far, there are many reports on the degradation of connectin/titin as a factor of meat tenderization during postmortem aging. Structural protein changes in myofibrils such as fragmentation of myofibrils and the splitting of α-connectin into β-connectin fragments were observed in aged muscles [2, 3]. We have shown that connectin/titin 20-kDa fragment increased in chicken sarcoplasm with postmortem aging [4]. Increase of the fragment in sarcoplasm from aged chicken muscle seemed to correlate with changes in myofibrillar structure around Z-line during postmortem aging. Therefore, it was considered that antibody against connectin/titin 20-kDa fragment was capable of a candidate as a detection probe to clarify the relevance of the fragment increase in sarcoplasm and meat tenderization. In this study, recombinant 12- and 16-kDa fragments were prepared as the immunogen of internal sequence of the 20-kDa fragment. Polyclonal and monoclonal antibodies were developed and characterized by binding to the fragments in order to utilize for estimating meat aging.

II. MATERIALS AND METHODS

Preparation of Recombinant Connectin/Titin Fragment: Recombinant connectin/titin 12- and 16-kDa fragments were prepared by the method described previously [4]. Briefly both DNA fragments coding 12- and 16-kDa fragments were amplified by PCR. The PCR fragments after digestion of *Nde*I and *Bam*HI were electrophoresed on agarose gel, and then DNA bands were dissected out to ligate to pET-22b vector (Novagen). The constructs were transformed into *E.coli* strain DE3 (TAKARA BIO). Expression of DNA fragments was induced with IPTG and was confirmed by SDS-PAGE.

Antibody Production: Antibodies against both recombinant 12- and 16-kDa fragments were raised in Wister rats according to previously described method [4]. Polyclonal antibodies (pAbs) were purified by affinity column with HiTrap NHS-activated HP (GE Helthcare).

Monoclonal antibodies (mAbs), namely, 1-3C and 16-4B4 were developed in our laboratory according to

the conventional method [5]. Mouse (Balb/c) was immunized with recombinant 12-kDa fragment and produced antibodies reactive in ELISA. The spleen was removed and spleen cells were fused with myeloma cells (P3U1) in polyethylene glycol (mol. wt 1500, Sigma). The cells were then cultured in HAT medium in 96-well sterile cell plates. Medium from hybridoma colonies were tested by indirect ELISA against recombinant 12-kDa fragment. Two cell lines producing antibodies that were specific for the fragment were cloned by limiting dilution in 96-well plates and expanded before freezing in liquid nitrogen.

Sandwich ELISA: Sandwich ELISA was conducted to detect connectin/titin 20-kDa fragment in chicken sarcoplasm during postmortem aging. ELISA 96-well plates were coated with rat anti-12-kDa fragment antiserum and blocked with PBS containing 1% bovine serum albumin (BSA). Chicken sarcoplasm prepared at various postmortem time were incubated for 1 hr at room temperature. After washing with PBS-T, excess amounts of rabbit anti-rat IgG (Sigma) were incubated to mask free anti-12-kDa fragment antibody for 1 hr. After washing with PBS-T, the plates were incubated with rat anti-16-kDa fragment antiserum and then goat anti-rat IgG secondary antibody conjugated with peroxidase (Sigma). Detection was completed by the addition of the enzyme substrate, o-phenylenediamine (OPD) and H_2O_2 followed by adding 2N H_2SO_4 to stop the development. The antibody reactivity was quantified by measurement of spectrophotometric absorption at 450 nm.

Immunoblotting: Sarcoplasmic proteins prepared from 0 to 96 hours postmortem were separated by SDS-PAGE and separated proteins were electrotransferred from polyacrylamide gel to PVDF membranes. Membranes were blocked with 7.5 % non-fat dry milk in TBS-T solution, and then incubated with rat anti-12-kDa antiserum. The antiserum were washed out and the membranes were overlaid with goat anti-rat IgG antibody conjugated with alkaline phosphatase (Sigma), then incubated with substrate of NBT/BCIP for coloring.

Indirect immunofluorescence microscopy: Antiserum against recombinant 12-kDa fragment was used for an indirect immunofluorescence staining of myofibrils. Myofibrillar suspension was mounted on a slide glass and treated with anti-12-kDa fragment rat antiserum and fluorescein isothiocyanate (FITC)-labeled anti-rat IgG. Free IgG were washed out thoroughly with PBS solution. The specimen was observed under a fluorescence microscopy (Olympus).

III. RESULTS AND DISCUSSION

Figure 1 shows the results of sandwich ELISA conducted to detect connectin/titin 20-kDa fragment in chicken sarcoplasm during postmortem aging. When the 20-kDa fragment was measured in chicken sarcoplasm prepared from 0 to 96 hours postmortem, absorbance at 450 nm was increased with post-mortem aging in measuring sarcoplasm at higher protein concentration. These results show that connectin/titin 20-kDa fragment was released from myofibrils to

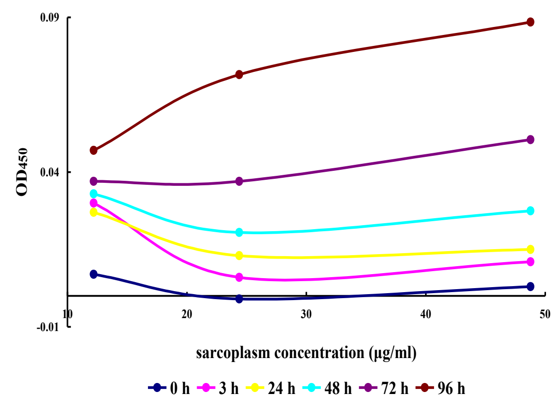

Fig. 1 Sandwich ELISA of chicken sarcoplasm with rat antiserums during postmortem aging.

sarcoplasm and the amount increased with postmortem time.

Binding of anti-12-kDa antibody to the 20-kDa fragment appeared in chicken sarcoplasm was checked by immunoblotting method. Figure 2 shows that the 20-kDa band was specifically colored in incubating with rat anti-12-kDa antiserum. The colored band appeared slightly at 24 hours postmortem and then became thicker with postmortem time. As the result, anti-12-kDa fragment antibody bound specifically to connectin/titin 20-kDa fragment increased in sarcoplasm during storage of chicken muscle.

Indirect immunofluorescence microscopy was carried out to determine where recombinant 12-kDa

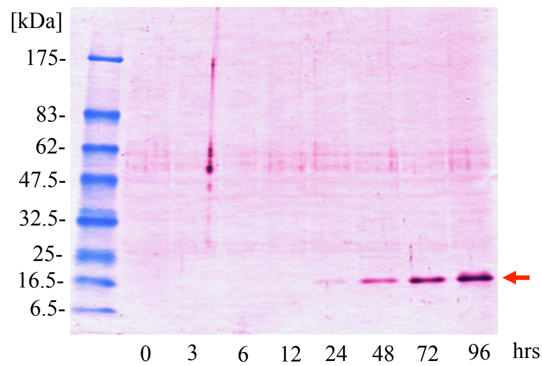

Fig. 2 Immunoblotting of chicken sarcoplasmic protein with rat anti-12-kDa fragment antiserum during postmortem aging.

fragment of internal sequence of connectin/titin 20-kDa fragment was localized in sarcomeres. Clearly detectable fluorecences were observed at Z-lines of sarcomeres, as arrowheads pointed out in Figure 3. Rat anti-12-kDa antibody bound to some structure around the Z-lines, suggesting that connectin/titin 20-kDa fragment is a part of connectin/titin filaments near the Z-line. Therefore, it was considered that the antibody against recombinant 12-kDa fragment could be useful for detection of connectin/titin 20-kDa fragment in sarcoplasm during postmortem aging of chicken muscle.

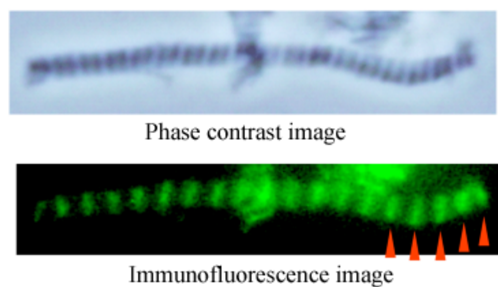

Fig. 3 Indirect immunofluorescence and phase contrast images of chicken myofibrils prepared at 0 hours post-mortem and incubated with rat anti-12-kDa antiserum.

Monoclonal 16-4B4 and 1-3C antibodies were developed with recombinant 12-kDa fragment as immunogen. Both of mAbs 16-4B4 and1-3C secreted from the hybridoma cells were speclfic for connectin/titin 20-kDa fragment increased in chicken sarcoplasm. The 20-kDa bands were detected from 48 hours postmortem and became thicker with postmortem time (Fig. 4). Delay in post-mortem time to detect the 20-kDa bands by mAbs probably dues to the difference in antibody titers between polyclonal and monoclonal antibodies.

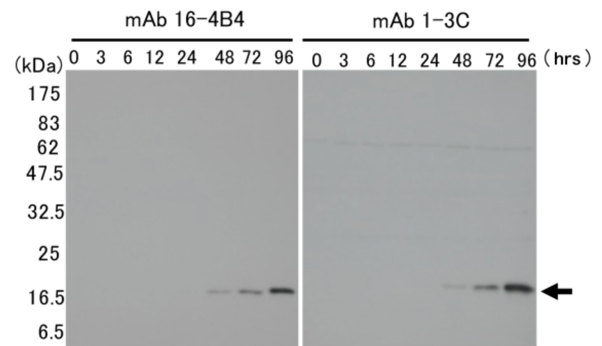

Fig. 4 Immunoblotting of chicken sarcoplasmic protein with monoclonal 1-3C and 16-4B4 antibodies during postmortem aging.

When isotypes of monoclonal antibodies were determined with Isostrip Mouse Monoclonal Antibody Isotyping kit (Roch), mAbs 16-4B4 and 1-3C were IgG3 and IgG2b with κ-light chain, respectively (Fig. 5, arrows).

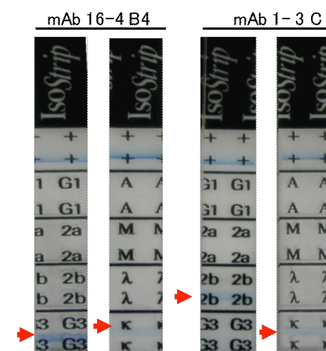

Fig. 5 Determination of isotypes of monoclonal 16-4B4 and 1-3C antibodies with Isostrip Kit.

In order to examine usefulness of monoclonal antibodies among meat species, chicken, pork and beef were sufficiently aged and each sarcoplasm was prepared to separate 20-kDa fragments by SDS-PAGE.

After electrotransferred onto PVDF membranes, the membranes were incubated with mAbs, and then secondery antibody conjugated with peroxidase followed by the substrate for detection. Both mAbs exhibited cross-reactions with any of meat species tested (Fig. 6). Thus it was shown that monoclonal 16-4B4 and 1-3C antibodies could be useful for estimating appearance of connectin/titin 20-kDa fragment in sarcoplasm from aged pork and beef muscles similarly with chicken muscle.

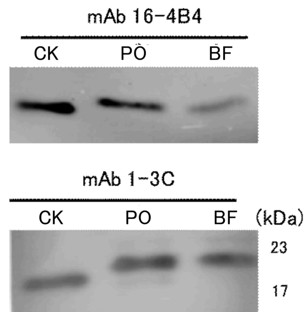

Fig. 6 Immunoblotting of sarcoplasmic protein from aged chicken, pork and beef muscles with monoclonal 16-4B4 and 1-3C antibodies.
CK, chicken; PO, pork; BF, beef.

IV. CONCLUSIONS

It is concluded that polyclonal and monoclonal antibodies against connectin/titin 20-kDa fragment are capable of use for estimating meat aging.

ACKNOWLEDGMENT

A part of this study was supported by grants-in-aid for scientific research of Japan Society for the Promotion of Science (No. 21580330).

REFERENCES

1. Maruyama K (1994) Connectin, an elastic protein of striated muscle. Biophys. Chem. 50: 73-85
2. Boyer-Berri C, Greaser ML (1998) Effect of postmortem storage on the z-line region of titin in bovine muscle. J. Anim. Sci. 76: 1034-1044
3. Takahashi, K (1992) Non-enzymatic weakening of myofibrillar structures during conditioning of meat: Calcium ions at 0.1 mM and their effect on meat tenderization. Biochimie. 74: 247-250.
4. Yamanoue M, Kubo T, Matsunaga K et al (2010) Expression and antibody production of connectin fragment increased in sarcoplasm during postmortem aging of chicken muscle. Proc. 56th ICoMST in USB memory, Jeju, Korea, C0025
5. Galfre G, Milstein C (1981) Preparation of monoclonal antibodies: Strategies and procedures. Methods Enzymol 73: 3-46