EXPRESSION AND ANTIBODY PRODUCTION OF CONNECTIN FRAGMENT INCREASED IN SARCOPLASM DURING POSTMORTEM AGING OF CHICKEN MUSCLE

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***Abstract*—A present study aims at clearing relevance of the increase in a connectin fragment in sarcoplasm and meat tenderization caused by aging of meat. In order to prepare antibody against the fragment, we used recombinant connectin fragment as immunogen. The gene coding the connectin fragment was amplified by PCR as template of chicken cDNA library and cloned into the vector pET-22b. Expression of the gene was induced by IPTG in host cells *E.coli* DE3 strain introduced the vector. Expression of recombinant protein was confirmed on the SDS-PAGE gel. After protein purification, the recombinant connectin fragment was immunized to rat and the antiserum colored the 20-kDa band which corresponded to connectin fragment increased in sarcoplasm from aged chicken muscle. These results suggest that antibody against the connectin 20-kDa fragment is useful for monitoring chicken meat tenderization during aging.**

***Index Terms*—connectin fragment, antibody, meat tenderization, aging.**

I. INTRODUCTION

Biochemical and physiochemical changes during post mortem storage of muscles make a dominant contribution to meat quality traits, especially tenderness, water holding capacity and sensory properties. Although those changes and their effects on meat quality are not fully understood, one of the major considerations is postmortem structural protein changes in myofibrils such as fragmentation of myofibrils, the weakening of rigor linkages between actin and myosin, and the splitting of α-connectin into β-connectin fragments (Takahashi, 1992; Koomaraie, 1992). By contrast, sarcoplasmic protein changes during meat aging have been poorly studied except for using the conventional protein separation method of SDS-PAGE and HPLC. Owing to the restricted resolving capacity of SDS-PAGE to separate proteins simply by the molecular weight, only 10-20 protein bands can be resolved simultaneously. With the rapid development of new techniques for understanding protein expression in complicated biological systems, two dimensional gel electrophoresis (2-DE) (O'Farrell, 1975) became a powerful tool for muscle protein separation and analysis (Lametsch, *et al*., 2003; Bouley, *et al*., 2004; Bendixen, 2005). In 2-DE, hundreds of protein spots can be displayed simultaneously in a single gel, thereby much more valuable information about protein changes during meat aging could be disclosed.

When changes in chicken sarcoplasmic protein during aging were analyzed by 2-DE in our laboratory, it was shown for the first time that one protein spot increased in dyeing density during aging and that the spot was 20-kDa fragment of myofibrillar protein connectin from the result of N-terminal amino acid analysis (manuscript in preparation). The connectin 20-kDa fragment existed in the neighborhood of the Z-line of the muscle. It was considered that the increase in the connection fragment in sarcoplasm from aged chicken muscle correlated with changes in myofibtillar structure around Z-line during aging. Therefore, antibody against the connectin 20-kDa fragment was intended to be used as a detection probe to clarify the relevance of the fragment increase in sarcoplasm and meat tenderization during aging.

In the present study, we cloned two length of DNA fragments coding internal parts of the connectin 20-kDa fragment and the DNA fragments were expressed in *E. coli* to purify recombinant proteins and to produce antibodies for detection probe of the fragment during postmortem storage of chicken muscle.

II. MATERIALS AND METHODS

Preparation of Muscle Samples

Chicken breast muscle was taken immediately after slaughter and 10 g of muscle pieces were treated antiseptically by dipping in 1mM NaN_3. The samples, after being respectively wrapped with polyethylene film and aluminum foil, were stored at 4°C for future use. 5 g of muscle at 0, 3, 6, 9, 12, 18, 24, 36, 48, 72 and 96 hours postmortem were weighed respectively and homogenized in 30 ml of a solution containing 10 mM Tris-HCl (pH7.0) and 0.4 ml of protease inhibitor (Nacalai tesque) with a homogenizer (NISSEI DX-8) at 10,000 rpm for 1 min. After centrifugation at 10, 000

rpm for 30 min, supernatant was collected and then stored at -80°C for later analysis by gel electrophoresis.

Determination of Sarcoplasmic Protein Concentration

Protein concentration was determined using biuret method with bovine serum albumin as standards.

Cloning and Expression

Both of DNA fragments coding connectin 12-kDa and 16-kDa fragments were amplified by PCR with chicken cDNA library as template (Fig. 1). Four kinds of primers, two sense primers and two anti-sense primers were designed based on well-known chicken connectin database. The PCR fragments of connectin 12-kDa and 16-kDa fragments after digestion of *Nde*I and *Bam*HI were electrophoresed on agarose gel, and then each fragment was dissected out to ligate to pET-22b vector (Novagen). The constructs were transformed into *E.coli* strain DE3 (TAKARA BIO). Expression of both fragments were induced with IPTG. Expression of recombinant 12-kDa and 16-kDa fragments were confirmed by SDS-PAGE.

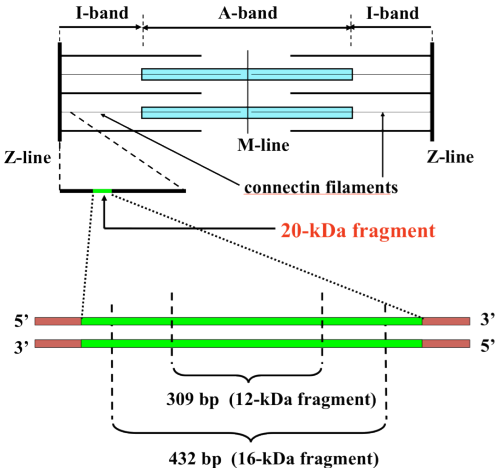

Fig. 1. Topological positions of connectin 20-kDa fragment and DNA fragments in a sarcomere .

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples for SDS-PAGE were prepared as below: Sarcoplasmic protein concentration was diluted to 0.2 mg/ml with sample buffer (0.1 M Tris-HCl, pH 6.8, 0.02% Bromophenol Blue, 10% Glycerol, 4% SDS, 2% 2-mercaptoethanol and 0.02% NaN_3), followed by placing them into boiling water for 3 min, and cooled at room temperature. SDS-PAGE was carried out according to the method of Laemmli with running buffer (0.025 M Tris-HCl, 0.192 M glycerol and 0.1% SDS). The gels were run at a constant current of 10 mA during the initial stacking period and 30mA during the separation period, and then stained with CBB.

Protein Purification

Separated recombinant 12-kDa and 16-kDa fragments on the SDS-PAGE gel were dissected and electrically eluted from the gel with a Centrilutor micro-electroelutor (Millipore).

Antibody Production

Antibodies against both recombinant 12-kDa and 16-kDa fragment were raised in Wister rats according to 'The Guidelines of Animal Experimentation' of Kobe University. Rats were injected with the antigen 4 times for 5 weeks, as a mixture with complete adjuvant for the first time and with incomplete adjuvant afterwards. Antiserums were collected at weekly intervals for subsequent immunizations and checked the production of antibodies by ELISA method. After production of antibodies, whole blood were collected and placed at 4°C overnight, then centrifuged to separate antiserums.

ELISA

ELISA was conducted to detect production of antibody against recombinant 12-kDa and 16-kDa fragments according to the direct assay method of Tsitsilonis *et al.* (2002). ELISA 96-well microtitration plates were coated with recombinant connectin fragment and blocked with PBS containing 1% bovine serum albumin (BSA). Rat antiserums were incubated at various dilutions for 1 h at room temperature. After washing with PBS-T, the plates were incubated with goat anti-rat IgG secondary antibody conjugated with peroxidase (Sigma) and 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

Separated proteins were electrotransferred from polyacrylamide gel to PVDF membranes with a method of Towbin *et al*. (1979). Membranes were blocked with 7.5 % non-fat dry milk in TBS-T solution, and then incubated with antiserums. The antiserums 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.

III. RESULTS AND DISCUSSION

Figure 2 shows agarose gel electrophoresis patterns of second PCR products. Several bands appeared which consist of specific and non-specific products in first PCR with cDNA library as template, but after the second PCR with first PCR products as template, both of 432 bp (Fig. 2A) and 309 bp (Fig. 2B) of PCR products were clearly appeared, as

expected. Each product band was dissected from gel and ligated to expression vector pET-22b after the sequence was confirmed. When the constructs were transformed into *E.coli* and expression of both fragments were induced with IPTG, recombinant 12-kDa and 16-kDa fragments were appeared on SDS-PAGE gel of IPTG induction *E.coli* lysates. Figure 3 shows the bands of recombinant connectin fragments. After the lysates were centrifuged, both fragments were included in precipitation probably because of formation of inclusion bodies in expressing recombinant connectin fragments (Fig.3A, b and d). In order to purify recombinant connectin fragments from precipitation fractions, we adopted electro-elution methods and succeeded in purification of both fragments without any protein breakdown in appearance (Fig. 3B). Purified recombinant 12-kDa and 16-kDa fragments immunized to Wister rats and antiserums were collected at weekly intervals for subsequent immunizations.

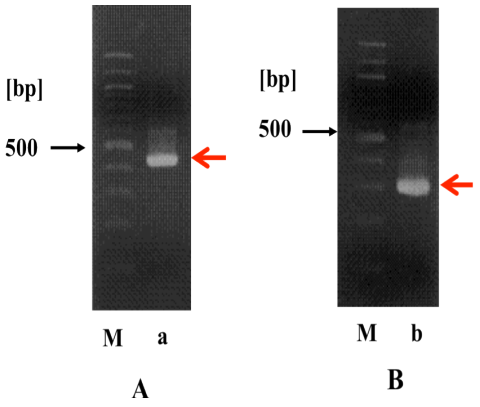

Fig. 2. Agarose gel electrophoretic patterns of DNA fragments amplified by PCR. (A) 432 bp. (B) 309 bp.

Figure 4 shows antibody production against both recombinant 12-kDa and 16-kDa fragments by ELISA method. Before immunization of the antigens, both absorbances (anti-12-kDa, pre and anti-16-kDa, pre) showed no changes in spite of antiserum dilution, as quantified by spectrophotometric absorption at 450 nm. Antibodies increasingly produced in rats from the second to the fourth booster immunisation. After the fourth immunisation, antibody reactivities were about 0.8 and about 0.6 in 1:1000 dilution of of anti-12-kDa and anti-16-kDa fragment antiserums, respectively. So, sufficient antibodies were produced in rats and then whole bloods were collected to prepare antiserums.

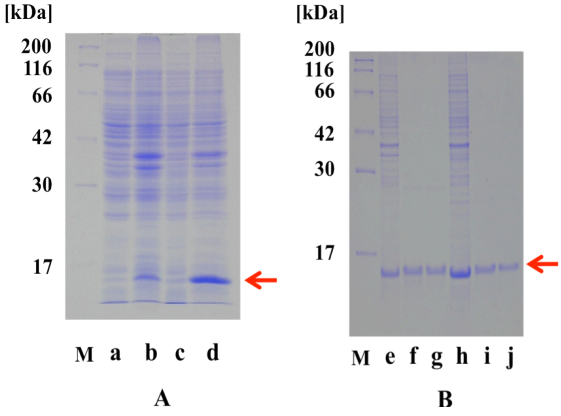

Fig. 3. SDS-PAGE patterns of (A) the E. coli lysates after IPTG induction and (B) purified recombinant connectin fragments. a and b, sup. and ppt. of 16-kDa fragments, c and d, sup. and ppt. of 12-kDa fragments; e,f and g, 16-kDa fragment; h, i and j, 12-kDa fragment.

Binding with the antiserums and purified recombinant connectin fragments was checked by immunoblotting method (Fig. 5). As the results, anti-16-kDa fragment antibody was bound to recombinant 12-kDa and 16-kDa fragments specifically, and both protein bands colored on PVDF membrane (Fig. 5A). Also, both bands colored specifically again as to incubation with anti-12-kDa fragment antiserum (Fig. 5B). Therefore, it was considered that the antibodies against recombinant 12-kDa and 16-kDa fragments could be useful for detection of the connectin 20-kDa fragment in sarcoplasm during aging of chicken meat, because both fragments were constituent parts of the connectin 20-kDa fragment.

When sarcoplasmic proteins of chicken breast muscle from 0 to 96 hours postmortem storage were separated by 2-DE, it was shown that the connectin 20-kDa fragment appeared and increased in sarcoplasm with aging time. In order to check the usefulness of antibodies against recombinant 12-kDa and 16-kDa fragments for detection of the connectin 20-kDa fragment, sarcoplasmic proteins from aged chicken muscles were analyzed by immunoblotting method. Sarcoplasmic proteins at 0 and 96 hours postmortem were separated by SDS-PAGE method and electrotransferred to PVDF membranes, and then anti-12-kDa and anti-16-kDa fragment antibodies were overlaid on the membranes (Fig. 6).

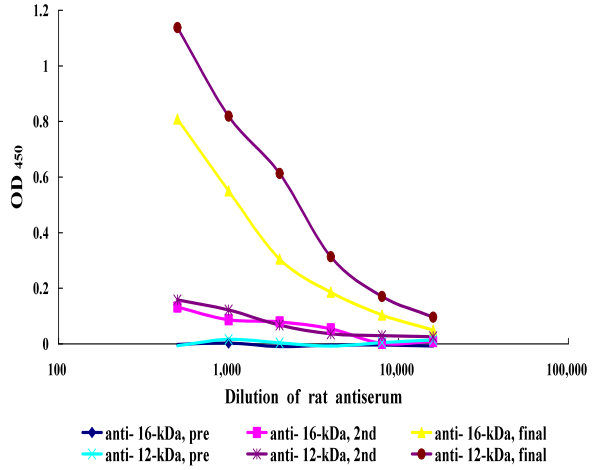

Fig. 4. Estimation of rat anti- 16-kDa and 12-kDa fragment antiserums by ELISA.

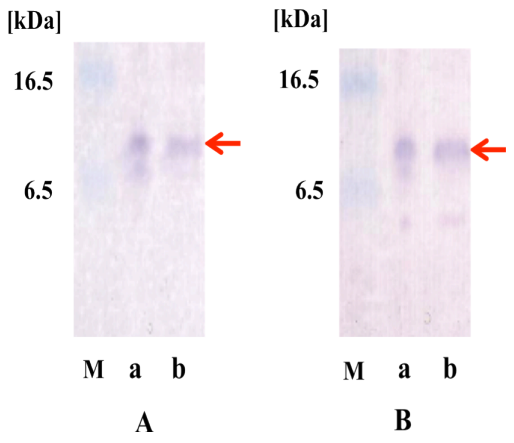

Fig. 5. Binding of (A) anti- 16-kDa and (B) 12-kDa fragment antiserums to recombinant (a) 16-kDa and (b) 12-kDa connectin fragments by immunoblotting.

There appeared no coloring band when anti-16-kDa fragment antiserum was incubated with sarcoplasmic proteins at 0 hours postmortem (Fig. 6A-a). On the other hand, a colored band to which the connectin 20-kDa fragment corresponded occurred in sarcoplasm at 96 hours postmortem (Fig. 6A-b). Similarly, the same colored band occurred in sarcoplasm at 96 hours postmortem with anti-12-kDa fragment antiserum (Fig. 6B-b). Thus both antibodies succeeded in detecting the connectin 20-kDa fragment increased in chicken sarcoplasm with aging time.

Binding of the antibodies to the connectin 20-kDa fragment in sarcoplasm during postmortem storage of chicken muscle could be quantitatively recognized by the method such as ELISA.

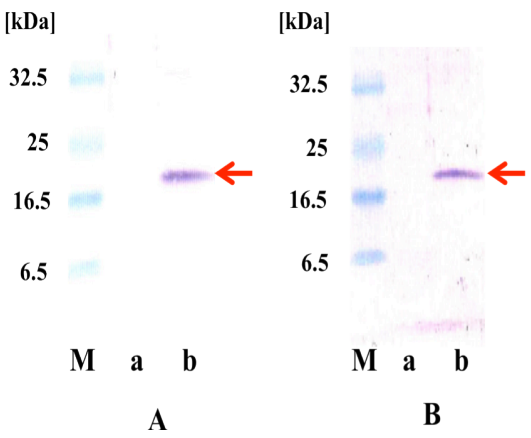

Fig. 6. Immunoblotting of chicken sarcoplasmic protein with rat (A) anti- 16-kDa and (B) anti- 12-kDa fragment antiserums. a, sarcoplasm at o hr; b, sarcoplasm at 96 hr.

IV. CONCLUSION

In this study, we represent antibody production against the connectin 20-kDa fragment and binding of the antibodies to the fragment in sarcoplasm from aged chicken muscle. These results show the possibility that the antibodies against the connectin 20-kDa fragment are useful for monitoring increase of the fragment and applicable as a aging index of chicken muscle during postmortem storage.

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