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Postmortem Changes in Skeletal Muscle Connectin (titin) and Its Structure

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Abstract

The splitting of the connectin (titin) molecule during meat conditioning and its primary structure were analyzed. Using immunofluorescence microscopy and immunoelectron microscopy, it was showed that the connectin molecule split into β-connectin and 1,200 kDa-subfragment at a point 0.34 μm apart from the Z-disc during meat conditioning. Differences in molecular weight and partial amino acid sequences of connectin were determined for cattle, pig and chicken skeletal muscles. Results of peptide mapping analysis differed according to animal species. Amino acid sequences deduced from partial nucleotide sequences of connectin also differed according to animal species at immunoglobulin-like (Ig) and fibronectin type 3(FN3) domains. In chicken, the molecular weight of connectin from leg muscles differed from the value recorded in pectoral muscles. It is suggested that meat texture and conditioning may be related to the splitting of the connectin molecule and its structure.

Discipline: Animal industry
Additional key words: splitting of connectin, localization of 1,200 kDa-subfragment, primary structure, meat conditioning, meat texture

Introduction

Connectin is an elastic protein of striated muscle initially reported by Maruyama et al.[16], and also called titin[30]. The apparent molecular mass of connectin is 3,000 kDa[14,28]. Each connectin molecule extends as a long (~1.2 μm) filament from the Z disc to the M line in a sarcomere and comprises an elastic segment at the level of the I band and an inelastic segment at the level of the A band. Connectin filaments keep the thick filaments centered within the sarcomere during force generation[6].

During meat conditioning, the connectin molecule splits into 2 polypeptides. The molecular mass of one peptide is 1,200 kDa (1,200 kDa-subfragment); the other peptide is called β-connectin. This splitting occurred nonenzymatically when myofibrils were treated with a solution containing 0.1 mM $CaCl_2$ and 30 μg of leupeptin/ml[22]. This splitting is very important for meat texture, because it reduces the elasticity of meat during conditioning[23]. Connectin filaments split more rapidly in more tender meat[1,8]. However, Fritz et al.[3] concluded that the connectin content did not enable to distinguish "tough" from "tender" beef. The time required for conditioning varies according to animal species. Within meat conditioned quickly, splitting is also rapid[1,8,18]. Differences in molecular structures of connectin may thus be a determinant of meat texture.

Labeit and Kolmerer[12] determined the complete cDNA sequence of human cardiac connectin and showed that its structure consisted primarily of 2 main types of repeating domains (an immunoglobulin-like (Ig), a fibronectin type 3 (FN3)) and a PEVK domain. They suggested that the PEVK domain primarily accounted for the elasticity of the connectin molecule. They indicated that after the extensibility of the PEVK domain had been exhausted, the stable fold of the Ig domains would resist further extension. These domains may thus determine the meat texture. The molecular structures of connectin in humans, rabbit and experimental animals have been studied extensively[4,9,10,12,15,17,21]. However, there are few reports on the molecular structure of connectin in domestic animals.

In this report, it was revealed that the splitting of the connectin molecule occurs at a point 0.34 μm apart from the Z-disc during meat conditioning[25,26]. The differences in the molecular weight of connectin

and amino acid sequences of Ig, FN3 and PEVK domains in cattle, pig, chicken and muscle types were described[24].

Materials and methods

1) Preparation and Ca treatment of myofibrils

Myofibrils were prepared according to the method of Perry and Grey[19] from cattle, pig and chicken. To induce the splitting of connectin into a 1,200 kDa-subfragment and β-connectin *in vitro*, freshly prepared myofibrils were treated with 0.1 mM $CaCl_2$ as described by Takahashi et al.[22].

2) Electrophoresis

Myofibrillar proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Tatsumi and Hattori[27].

3) Preparation of antiserum and immunohistochemical observation

Antiserum against the 1,200-kDa subfragment of the connectin molecule was prepared as previously described[26]. Indirect immunofluorescence microscopy and immunoelectron microscopy observation using that antiserum was carried out as previously described[26].

4) Peptide mapping

Connectin was digested by V8 protease as described previously[24]. The peptides produced by V8 protease digestion were separated by SDS-PAGE.

5) PCR amplification and sequencing of segments from the connectin gene

Sequences of the oligonucleotide primers were based on the sequences of pig connectin reported by Fritz et al.[4]. The connectin molecule has Ig

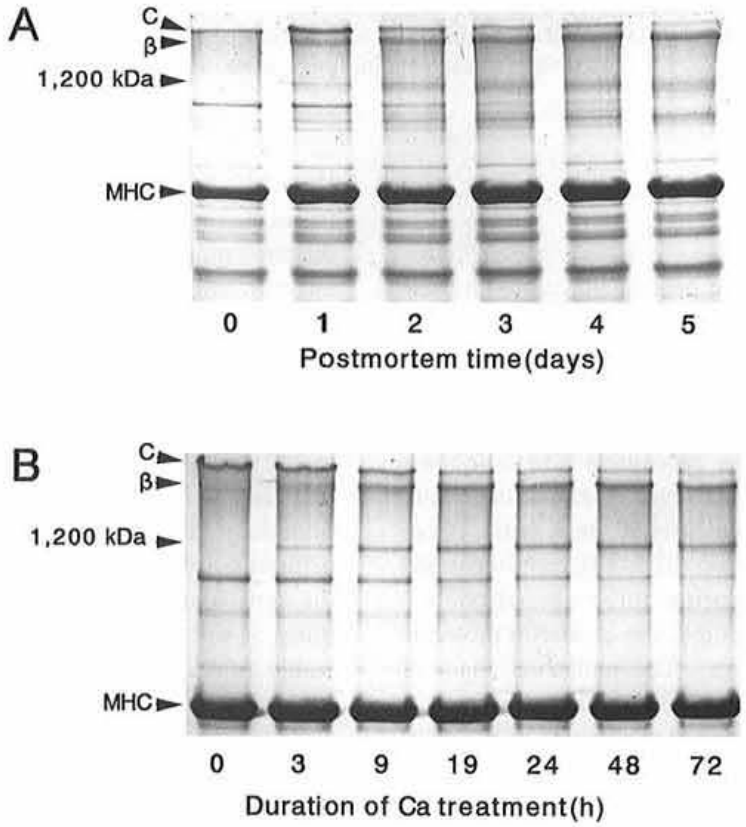

Fig. 1-A. Postmortem changes of connectin in chicken *M. tensor fasciae latae* stored at 4°C

-B. Changes of connectin during Ca treatment of chicken leg myofibrils

C: connectin, β: β-connectin, 1,200 kDa: 1,200 kDa-subfragment, MHC: myosin heavy chain.

and FN3 domains. The sense primer for amplification of the Ig domain was 5'-CAGGTGGCTCCTTAAGGTTATTTGTTCC-3' and the antisense primer for the Ig domain was 5'-CAAAGGCAGACTTTGTTCCACTGCTGTT-3'. The sense primer for amplification of the FN3 domain was 5'-CCAGGACCTCCAAACAATCCC AAAGT-3' and the antisense primer was 5'-AGCACAGATACGGAAGTTGTATTCATG-3'. The positions of these primers on the sequence determined by Fritz et al.[4] were 509–536, 691–718, 154–179 and 361–387, respectively. Genomic DNA was extracted from cattle, pig and chicken. PCR amplification was conducted according to the method of Chikuni et al.[2]. Both strands of amplified DNA were sequenced. From the nucleotide sequence, the amino acid sequence was deduced.

Results and discussion

Fig. 1-A shows the postmortem changes of myofibrillar proteins in chicken *M. tensor fasciae latae* stored at 4°C. With increasing postmortem time, the band of connectin became weaker, while the band of β-connectin became stronger. Furthermore, the 1,200 kDa-subfragment appeared during storage. The

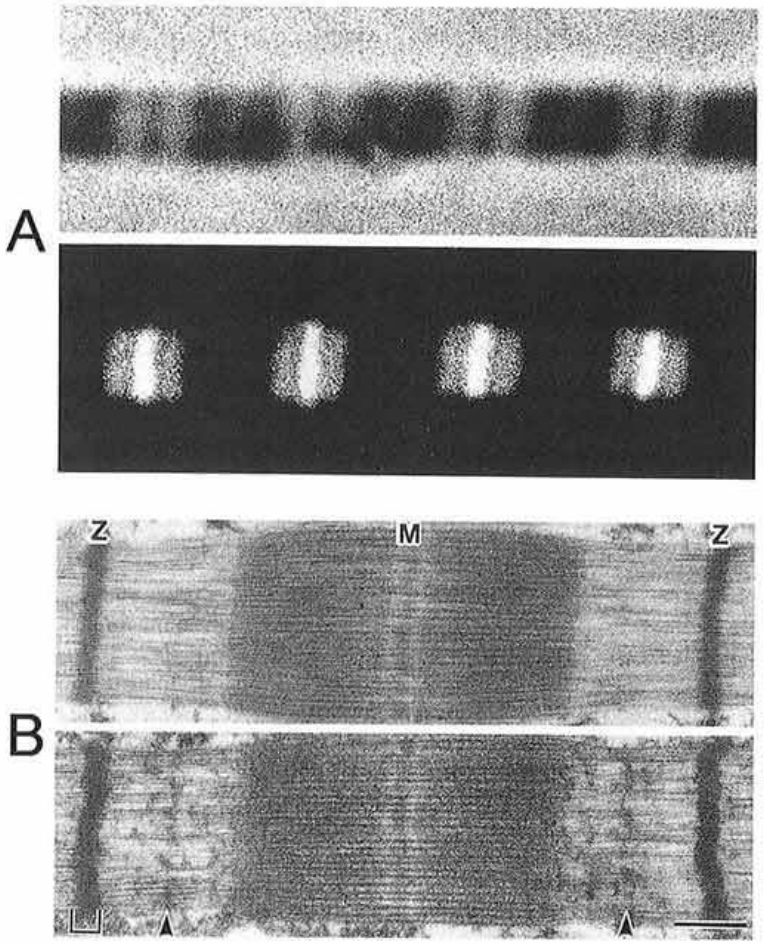

Fig. 2-A. Immunofluorescence staining of myofibrils with antiserum against the 1,200 kDa-subfragment
Upper panel: phase-contrast image, lower panel: immunofluorescence image.
-B. Immunoelectron micrographs showing the localization of the 1,200 kDa-subfragment
Antibodies were heavily deposited on the Z-disk indicated in brackets, and formed a stripe in each I-band indicated by an arrowhead.
Upper panel: control, lower panel: treatment with antiserum. Z: Z-disk, M: M-line. Scale bar: 0.3 μm.

densitometric analysis suggested that these changes continued until about 4 days postmortem, and β-connectin and the 1,200 kDa peptide were produced stoichiometrically from the entire connectin molecule (data not shown). The same changes were observed in chicken *M. semimembranosus* and *M. adductor*[25]. These data suggest that the connectin molecule is degraded to β-connectin and the 1,200 kDa-subfragment until about 4 days postmortem in chicken leg muscles. Fig. 1-B shows the changes of myofibrillar proteins of chicken leg muscles during the Ca treatment. Basically, the changes of connectin during the Ca treatment were the same as those occurring in stored muscles. However, the band of the 1,200 kDa-subfragment was stronger and sharper than in the stored muscles. It was confirmed immunologically that the 1,200 kDa-subfragment was derived from the connectin molecule and not from β-connectin by immunoblot analysis using the antiserum against the 1,200 kDa-subfragment[26]. Takahashi et al.[22] demonstrated that connectin is not degraded in a Ca treatment solution containing 5 mM EDTA. Therefore, they concluded that these changes are nonenzymatically induced reactions caused by 0.1 mM Ca ion.

Localization of the 1,200 kDa-subfragment in a sarcomere was revealed by indirect immunofluorescence microscopy (Fig. 2-A). When myofibrils were treated with antiserum against the 1,200 kDa-subfragment, Z-disks were strongly fluorescent, the regions of both sides of the Z-disks were fairly fluorescent, and the A-band was absent. Immunoelectron microscopy revealed a heavy deposition of antibodies on the Z-disk and the formation of a stripe in each I-band, which was about 0.34 μm apart from the Z-disk for a sarcomere length of 2.6 μm (Fig. 2-B). The other deposits were scattered and not common to both I-bands. These results indicate that the 1,200-kDa subfragment is a portion of connectin filaments that may become attached to the Z-disk. A single connectin molecule forms an elastic filament connecting a thick filament with a Z-disk, extending its distal end nearly to the M-line. The distance between the Z-disk and the stripe in the I-band, 0.34 μm, was in good agreement with the difference between the length of a connectin molecule (approximately 1.30 μm, which is half the length of the sarcomeres we observed) and β-connectin (approximately 0.90 μm). Therefore, we estimated that the length of the 1,200 kDa-subfragment was about 0.34 μm for a sarcomere length of 2.6 μm.

These results indicate that the splitting of connectin filaments occurs at a point 0.34 μm apart from the Z-disc in a sarcomere during meat conditioning. Fig. 3 gives a schematic diagram of that splitting.

Locker and Wild[13] reported that the molecular weight of connectin was essentially the same in vertebrate skeletal muscle. However, Hu et al.[7] observed differences in the molecular weight depending on animal species and muscle types after analyzing many sources of vertebrate striated connectin and non-vertebrate connectin-like protein. Tatsumi and Hattori[27] noted that the molecular weight differed between chicken leg, rabbit leg and rabbit back muscles using the same SDS-PAGE conditions as those applied in this study. Molecular weight was found to differ according to the muscle type in chicken (Fig. 4). The molecular weights in *M.*

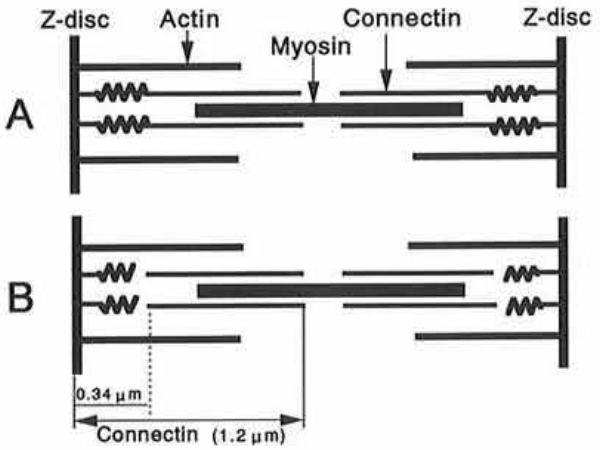

Fig. 3. Splitting of connectin molecule in a sarcomere during meat conditioning
A: just after slaughtering, B: after conditioning

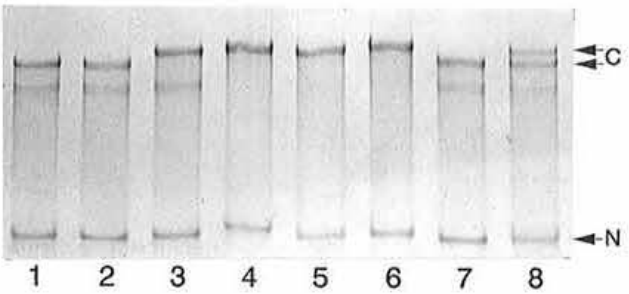

Fig. 4. SDS-PAGE analysis of connectin from various muscle types in chicken
1: *M. pectoralis profundus*, 2: *M. pectoralis superficialis*, 3: *M. semimembranosus*, 4: *M. semitendinosus*, 5: *M. sartorius*, 6: *M. biceps femoris*, 7: mixture of 1 and 2, 8: mixture of 1, 3, 4, 5 and 6. C: connectin, N: nebulin (700 kDa).

pectoralis profundus and *M. pectoralis superficialis* were lower than in *M. semitendinosus*, *M. semimembranosus*, *M. sartorius* or *M. biceps femoris* (Fig. 4, lane 1–6). The sample, which consisted of a mixture of 2 types of pectoral muscles, produced a single band of connectin (Fig. 4, lane 7). On the other hand, the sample which consisted of a mixture of a pectoral muscle and leg muscles produced 2 bands of connectin (Fig. 2, lane 8). Thus, it was confirmed that the molecular weight of connectin differed according to the muscle types in chicken. Many muscle proteins are present in different isoforms[20]. Connectin in rabbit fast muscles showed a higher mobility on SDS-PAGE than connectin in slow muscles and this difference was correlated with the resting tension of muscle fibers[5,29]. Differences in the molecular weight of connectin in chicken depending on skeletal muscles would thus appear to be derived from physiological differences between fast and slow muscles.

By peptide mapping analysis using V8 protease, SDS-PAGE patterns of cattle, pig and chicken were clarified. They indicated that the primary structure of the entire connectin molecule differed according to animal species (Fig. 5). V8 protease specifically cleaved peptide bonds on the carboxyl side of aspartic and glutamic acid residues. Due to the large molecular mass and the existence of repeating domains in a connectin molecule, V8 protease digestion produced many bands with varying intensities. The patterns of peptide mapping were reproducible. Arrowheads indicate typical bands, which differed from those in other animal species (a, b: cattle; c: pig; d: chicken). Although the 'b' and 'c' bands were close in mobility, the 'c' band was distinctly lower than the 'b' band.

To reveal differences in the primary structure of connectin according to animal species more clearly, partial nucleotide sequences encoding connectin were determined. The connectin molecule contains the Ig and FN3 domains[10,12]. Most of the connectin molecule consists of repeats of these domains in succession. Partial nucleotide sequences encoding single Ig and FN3 domains of pig, cattle and chicken muscle connectin were determined, on the basis of which, the amino acid sequences were identified (Fig. 6). Amino acid sequences of pig domains were the same as those indicated by Fritz et al.[4]. The amino acid sequences of the Ig domain in cattle and

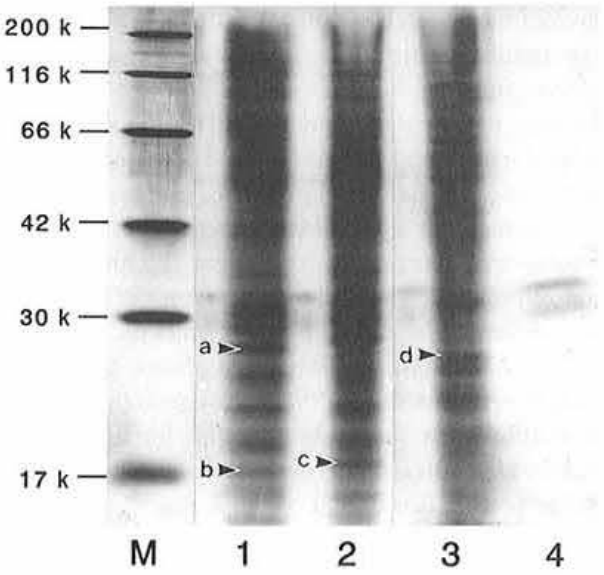

Fig. 5. Digestion patterns of connectin in *M. semimembranosus* by *S. aureus* V8 protase
Arrowheads indicate typical bands, which differ from those in other animal species. M: marker, 1: cattle, 2: pig, 3: chicken, 4: protease alone, a, b: typical bands of cattle, c: typical band of pig, d: typical band of chicken.

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                    10         20         30         40         50
A   CATTLE   IRGRPTPEVK WGKVDGEIRD AAIIDSTSSF TSLVLDNVNR YDSGKYTLTL E
    PIG      •K•••••••• •••••••••• •••••••••• •••••••••• •••••••••• •
    CHICKEN  •••••••••• •••••••••E •••••T•••• ••••••S••• F•T••••••• •

                    10         20         30         40         50         60
B   CATTLE   TDITRSSVFL SWGKPIYDGG CEIQGYIVEK CDTSVGEWTM CTPPTGINKT NIEVEKLLEK
    PIG      I••••••••• •••••••••• •••••••••• ••V••••••• •••••••••• ••••••••••
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Fig. 6. Comparison of the corresponding amino acid sequences of connectin
Identity of amino acids is indicated by a single dot.
A: partial sequence of immunoglobulin-like (Ig) domain, B: partial sequence of the fibronectin type 3 (FN3) domain.
The nucleotide sequence data revealed in this report appeared in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers D85838–D85842.

chicken differed from those of the pig at 1 and 6 points, respectively. Amino acid sequence of the FN3 domain in cattle was different from that of the pig at 2 points. The Ig and FN3 domains of connectin were thus found to display amino acid sequences that differed according to animal species. These results indicate that the primary structure of connectin is completely different in cattle, pig and chicken.

Labeit and Kolmerer[12] determined the complete cDNA sequence of human cardiac muscle connectin. The partial sequences of skeletal muscle connectin of several animal species such as humans, rabbit and chicken have been elucidated[11,12,15]. It was suggested that tissue-specific connectin was expressed by alternative splicing. Those studies showed that the primary structure of connectin differed according to animal species and muscle types. These findings were confirmed by our data for cattle, pig and chicken.

Connectin is an elastic filament whose splitting results in changes in the meat texture during conditioning[23]. The primary structure of connectin was different in cattle, pig and chicken. And the Ig and FN3 domains have been shown to interact with F-actin and myosin[9,11]. The connectin molecule is anchored to the Z-disc by the binding of the 1,200 kDa-subfragment to α-actinin, which is the major protein constituent of the Z-disc[25]. Although the sequence changes were mostly conservative (T for S; D for E; Y for F), the interactions between connectin and myofibrillar proteins such as actin, myosin and α-actinin may not be equally strong in different animal species. In many studies, conditioning changes in meat are considered to result from endogenous protease concentration or levels of activation. However, conditioning of meat may be related to the structure of myofibrillar proteins such as connectin.

In this paper, it was revealed that the connectin molecule split at a point 0.34 μm apart from the Z-disc during the conditioning of meat, and that this site corresponded to the N2-line of a sarcomere. The region of connectin molecules, which is situated near the N2-line, is called the PEVK region and is comprised predominantly of 4 amino acid residues. The sequence of this region differs from those of the Ig and FN3 domains[12]. The PEVK region may also account for the capacity of connectin filament extension[12], and thus may also be a factor of meat texture and rate of conditioning. Primers for the amplification of the PEVK region of the connectin gene were synthesized, based on sequences that would not include introns and would be conserved in human skeletal and cardiac muscle connectin. These primers, however, failed to amplify the sequence of the PEVK region in cattle, pig and chicken (data not shown). The primary structure of the PEVK region thus appears to differ in those species. It was suggested that the PEVK domain primarily accounted for the elasticity of the connectin molecule[3]. Therefore, meat texture may be related to differences in the PEVK domain of connectin.

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