Detection of paratropomyosin binding site on connectin filaments at the A-1 junction region of skeletal muscle myofibrils

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Background

During postmortem ageing of muscle, a myofibrillar protein paratropomyosin weakens the rigor linkages between actin and myosin and contributes to the increased meat tenderness (Takahashi,1996). In living and immediately postmortem muscles, paratropomyosin was found at the junction of A- and I-bands of sarcomeres (Hattori and Takahashi, 1988). The increase of calcium ion concentration to 1 x 10^{-4} M in postmortem muscle resulted in the translocation of paratropomyosin from its original position to thin filaments (Hattori and Takahashi, 1988). We have shown that paratropomyosin bound to β-connectin (titin 2) in examining binding of paratropomyosin at the A-I junction region and that the interaction of paratropomyosin with β-connectin was weakened by above 10^{-4} M calcium ions which corresponded to the calcium ion concentration in postmortem skeletal muscle (Fei, *et al.*, 1999). Thus, we have suggested that paratropomyosin bound to connectin filaments at the A-I junction region of sarcomeres.

Objective

The aim of this study was to clarify the position where paratropomyosin bound to connectin filaments at the A-I junction region of sarcomeres.

Materials and Methods

Chicken breast muscle was used. Paratropomyosin was purified with a hydroxyapatite column according to the procedure described previously (Yamanoue *et al.*, 1998). β-Connectin was separated by the method of Kimura and Maruyama (1983), and purified by the method of Itoh *et al.* (1986). SDS-PAGE was done according to the procedure of Laemmli (1977) using a 2-17.5% gel. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out using the Multiphor II with Immobiline DryStrip gels (Amersham Pharmacia Biotech UK Ltd. England). β-Connection fragments produced by protease digestion were electrotransferred on to a PVDF membrane by the method of Towbin *et al.* (1979).

Results and Discussion

In order to clarify the binding position of paratropomyosin on connectin at the A-I junction region of sarcomeres, β-connectin was digested by various proteases for peptide mapping. After β-connectin fragments were separated by SDS-PAGE method and then transferred on to a PVDF membrane, the fragments that paratropomyosin bound were detected by avidin-biotin complex (ABC) method using biotinylated paratropomyosin modified with sulfo-NHS-biotin (Pierce Chemical Co., Illinois, U.S.A.) for a probe. And the fragments that corresponded to the A-I junction region were detected by immunoblotting method using anti-connectin monoclonal antibody (T11) (Sigma, Missouri, U.S.A.) for the antigenic probe of the region.

When β-connectin was digested by *staphylococcus aureus* V8 protease (Pierce Chemical Co., Illinois, U.S.A.) under the conditions containing 4 M urea, biotinylated paratropomyosin mainly bound to the bands of 150 kDa, 100 kDa, 70 kDa, 50 kDa, 43 kDa and 20 kDa, and in the detection with T11 antibody there was remarkable coloring of 150 kDa, 100 kDa, 70 kDa, 50 kDa, 43 kDa and 40 kDa bands (data not shown).

Since β-connectin is an enormous molecule of about 2,000 kDa of chain weight (Maruyama, 1994) and is a repetitive structure of the modules of immunoglobulin-like domains and fibronectin-3-type domains (Labeit and Kolmerer, 1995), it was considered that many fragments of the same peptide chain weight were produced by protease digestion. So 2D-PAGE method was adopted in order to analyze

the fragments that were not separated by SDS-PAGE. The two dimensionally separated fragments were transferred on to PVDF membranes and similarly detected using both probes. Many fragments above 35 kDa of chain weight were mainly colored in the area of pH 5-7 in detecting with biotinylated paratropomyosin (Fig.1). When T11 monoclonal antibody was overlayed on the membrane, the fragments above 35 kDa were strongly colored in the area of pH 5-6, but little fragments were colored in high chain weight area at pH 6-7 (Fig. 2). On the other hand, there was no fragment of coloring when the same experiments were carried out without both probes.

Thus, the fragments of 150 kDa, 100 kDa, 70 kDa and 43 kDa chain weights were simultaneously detected by both biotinylated paratropomyosin and T11 antibody (Fig. 3). They seemed to be the fragments including the binding site of paratropomyosin at the A-I junction region of connectin. The fragments above 70 kDa chain weight probably include 43 kDa fragment in their structures. It is important to determine the amino acid sequence of 43 kDa fragment for clarifying more precise binding site of paratropomyosin on connectin filaments.

Conclusion

When β-connectin fragments digested by V8 protease were detected by both biotinylated paratropomyosin and T11 antibody, 43 kDa fragment and the other fragments with higher chain weight were colored simultaneously by both probes. Therefore, it is suggested that paratropomyosin binds to the 43 kDa region of connectin filaments at the A-I junction in living and immediately postmortem muscles.

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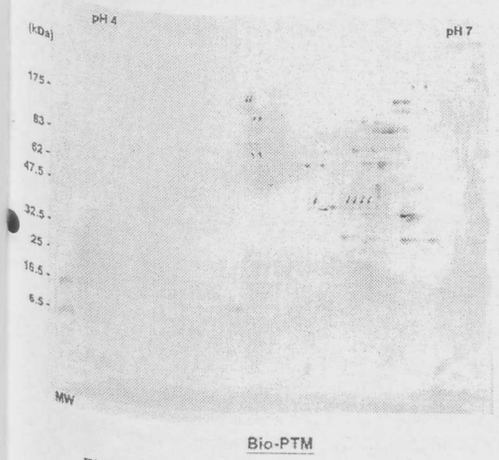
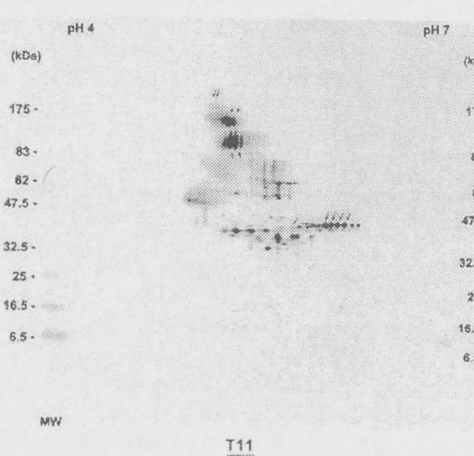


Bio-PTM

Fig. 1. Detection of β-connectin fragments digested by *staphylococcus aureus* V8 protease with biotinylated paratropomyosin

β-Connectin in a solution containing 0.15 M NaCl, 4 M urea and 0.125 M K-phosphate buffer (pH 7.0) was digested by V8 protease at 1 : 10 weight ratio to β-connectin for 24 hours at 25°C. The β-connectin fragments were separated by 2D-PAGE method. Arrows show the fragments detected by T11 antibody.



T11

Fig. 2. Detection of β-connectin fragments digested by *staphylococcus aureus* V8 protease with T11 antibody

β-Connectin fragments were separated as shown in the legend of Fig. 1. Arrows show the fragments detected by biotinylated paratropomyosin.

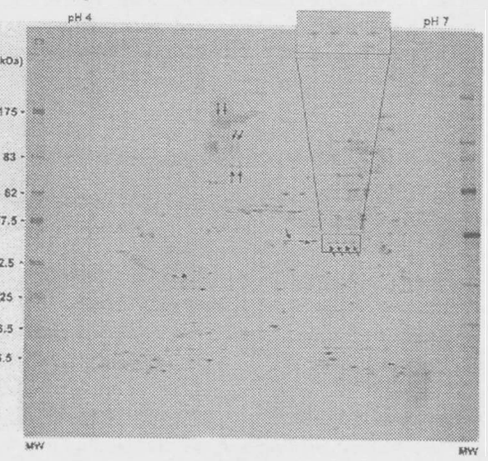


Fig. 3. Two dimensional-PAGE pattern of β-connectin fragments digested by *staphylococcus aureus* V8 protease

β-Connectin fragments were separated as shown in the legend of Fig. 1. The gel was stained by silver stain method. Arrows show the fragments detected simultaneously by both probes. Four spots in the square show 43 kDa fragments.