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FAST AND SLOW CHICKEN SKELETAL MUSCLES CONTAIN DIFFERENT α AND β TROPOMYOSINS

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Summary : Avian tropomyosin has been purified from fast skeletal muscles (breast muscle and posterior latissimus dorsi : PLD) and from a slow skeletal muscle (anterior latissimus dorsi : ALD) and the α and β subunits have been further separated using preparative gel electrophoresis. These subunits have been subjected to partial proteolysis using different proteolytic enzymes. In this communication we show that this procedure allows to distinguish not only between fast and slow α tropomyosin but also between fast and slow β tropomyosin. Furthermore we have raised an antiserum against the fast α tropomyosin and we present evidence to show that this antiserum does not cross-react with the slow α tropomyosin. These results are taken to indicate that all these tropomyosin subunits represent different gene products.

Skeletal muscles are classified as fast or slow on the basis of physiological and biochemical properties, which are reflected to a great extent in the specific subset of contractile protein isoenzymes they contain (1-3). Extensive studies have been focused on myosin, the major thick filament constituent ; it has been demonstrated that several myosin isoenzymes exist that can be distinguished on the basis of electrophoretic mobility, immunological properties and partial peptide mapping, at both the haveay-chain and light-chain level (4-7). In the case of tropomyosin, another myofibrillar protein, the situation is less clear. Tropomyosin is a dimer of identical or different polypeptide chains : two major monomer types have been described, α-tropomyosin and β-tropomyosin (8) ; they both possess 284 amino acid residues (9) but their mobility on SDS polyacrylamide gels clearly differs, β-tropomyosin migrating more slowly than α-tropomyosin. The RNAs coding for the two variants appear to exhibit significantly different properties (10). It has been suggested that in higher vertebrates, α-tropomyosin is confined to fast-type muscles and β-tropomyosin to slow-type muscles (11). However, as we have previously shown (12), in the chicken, fast- and slow-type muscles contain different α-tropomyosins. Evidence to the same effect was obtained for canine skeletal muscle (13), and very recently, Billeter and coworkers have suggested that human skeletal muscles contain fast- and slow-muscle specific α- and β-tropomyosins (14).

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In this communication, we present new evidence that not only the α-tropomyosin variants differ between fast- and slow-skeletal muscles of the chicken, but that the β-tropomyosins also display differences when analysed by partial proteolytic cleavage.

Materials and Methods :

Adult chicken tropomyosins were purified from ALD (anterior latissimus dorsi : slow), PLD (posterior latissimus dorsi : fast) and pectoralis major (fast) muscles, as already described (12).

Small scale preparative electrophoresis was employed to further purify and isolate the tropomyosin monomers. Approximately 400 µg of total tropomyosin were applied on 12.5% acrylamide, 0.3% bis-acrylamide SDS gels (15). After electrophoresis, the α- and β- tropomyosins were visualised by KCl "staining" (16). Each band was cut out of the gel, trimmed from both sides and washed for 30 minutes in cold distilled water in the presence of 1 mM DTT ; it was either used immediately or stored at -30°C.

Partial peptide analysis : Gel cuttings, containing approximately 20 µg of protein were placed in Eppendorf tubes, crushed and pushed through a pipette tip several times with the aid of 200 µl of 0.125 M Tris-HCl pH 6.8 and 0.5% SDS (17). Samples were left at room temperature for 1-2 hours. Proteolytic enzymes, i.e. chymotrypsin (Worthingtons), Staphylococcus Aureus protease-V8 (Miles) and papain (Sigma) were added directly onto the mixture. The digestion was left to proceed for 1 hour at 37°C with occasional agitation. Best results were obtained with 10 µg of chymotrypsin, 0.4 µg of V8-protease or 2 ng of papain, per 200 µl of protein eluate. The reaction was stopped with the addition of SDS and β-mercaptoethanol to a final concentration of 2% and 10% respectively, followed by boiling of the samples for 5 minutes.

The digests were analysed in 12.5 cm long, 1 mm thick SDS-polyacrylamide slab gel, containing 12.5% acrylamide cross-linked with 0.33% bis-acrylamide. Approximately 1-2 µg of total protein were applied per slot. The digestion patterns were visualised using a simplified silver-staining technique (18). In some cases, the digests were also analysed by two-dimensional gel electrophoresis (19), using for the first dimension an Ampholine (Pharmacia Fine Chemicals) range of pH 3.5-10 for the chymotryptic digests and pH 4-6 for the V8 digests.

Immunological studies : α-tropomyosin was prepared from pectoralis major muscle of the adult chicken and purified by preparative SDS/PAGE and electrophoretic elution. Excess SDS was removed by precipitation with 0.3 M KCl in the cold, and 800 µg of protein mixed with Freund's adjuvant were injected in a New Zealand rabbit, in four doses, over a six week period. The immune serum was further purified by adsorption against an α-tropomyosin (purified from pectoralis muscle) -Sepharose-4B affinity column. Serum strength and specificity were tested by an enzyme (peroxidase)-linked immunoassay, on nitrocellulose transfered (after SDS/PAGE) antigens (20) : Each gel was divided in two halves each containing the same series of proteins to be tested. The same amount of protein was loaded per gel slot (1-2 µg). The second half of the gel was used for visualisation of the protein samples by Coomassie Blue staining and destaining in methanol-acetic acid-water (45:10:45). The proteins of the first half of the gel were transfered onto nitrocellulose filters following the "sandwitch" method (20). This results in our hands in more than 70% tropomyosin transfer efficiency after 24 hours. One nitrocellulose copy was stained with amido-black (21) and the other was incubated with the immune serum at appropriate dilutions. The antigen-antibody complexes were visualised by the peroxidase technique (22).

Results and Discussion

As already observed (12), it is possible to distinguish between the α-variants of tropomyosin isolated from fast (PLD, pectoralis) or slow (ALD) muscles, in terms of electrophoretic mobility. On the other hand, the β-variants from the same muscle, are indistinguishable, in the same gel system (12).

One dimensional peptide mapping, using chymotrypsin or V8-protease as proteolytic enzymes, allows for a clear distinction not only between the α- and β-tropomyosins within a single muscle type, but also between the α- or between the β-tropomyosins from different muscle types. One such analysis is presented in Fig.1, where chymotrypsin has been used. Differences in the digestion pattern of the α-tropomyosins versus those of the β-tropomyosins are clearly visible (Fig. 1A, compare lane 1-3 with lane 4-5), as well as between the α-tropomyosin variants (Fig. 1A lane 1-3). In the latter case, α-tropomyosin from ALD muscle presents two peptides which are missing from the "fast" (PLD, pectoralis) α-

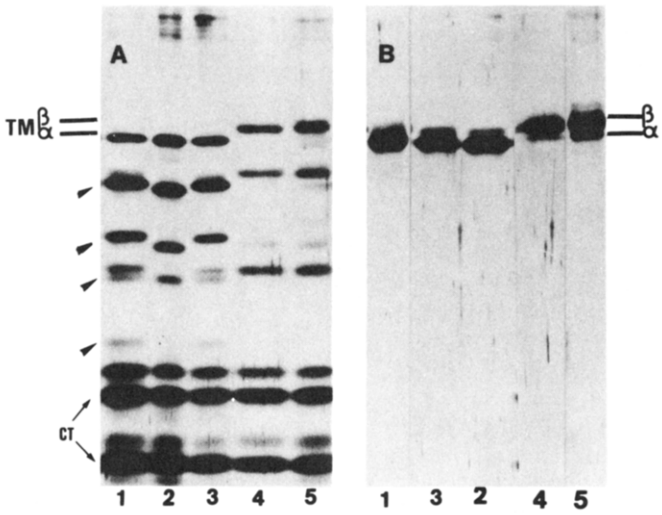

Figure 1 :

(A) Chymotryptic digestion pattern of tropomyosins from fast and slow muscles of the adult chicken.

(B) Tropomyosins from fast and slow muscles of the adult chicken, purified as in Material and Methods section and treated as in (A), without the addition of protease.

Arrowheads indicate the regions of difference between the various α-tropomyosins.

1. α-tropomyosin from pectoralis major muscle
2. α-tropomyosin from ALD muscle
3. α-tropomyosin from PLD muscle
4. β-tropomyosin from PLD muscle
5. β-tropomyosin from ALD muscle

CT indicates the position of chymotrypsin bands.

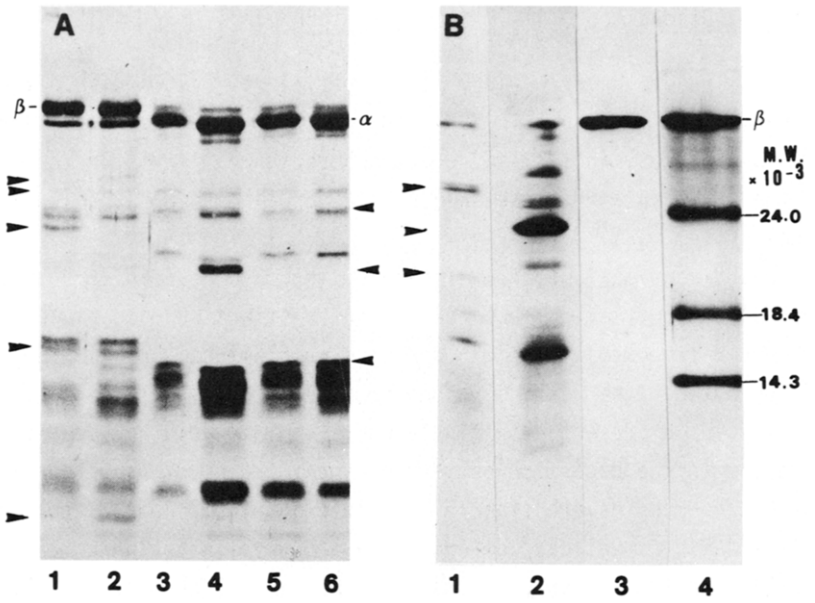

Figure 2 :

(A) Staphylococcus Aureus - V8-protease digestion pattern of tropomyosins from fast and slow muscles of the chicken. TM indicates the position of intact tropomyosin.

Arrowheads indicate the regions of difference between α-tropomyosins and between β-tropomyosins.

1. β-tropomyosin from ALD muscle
2. β-tropomyosin from PLD muscle

3&6 α-tropomyosin from pectoralis major muscle

4. α-tropomyosin from ALD muscle
5. α-tropomyosin from PLD muscle

(B) Papain digestion pattern of β-tropomyosins from fast and slow muscles of the chicken (15 % acrylamide, Coomassie Blue stained gel).

Arrowheads indicate regions of difference.

1. β-tropomyosin from PLD muscle
2. β-tropomyosin from ALD muscle
3. Intact β-tropomyosin from PLD muscle
4. Intact β-tropomyosin from ALD muscle plus molecular weight markers.

tropomyosins, and is in turn lacking four peptides which are present in the other α-variants (arrowheads indicate the regions of difference in Fig. 1A) ; no difference can be detected in the chymotryptic digestion pattern of the β-tropomyosins.

Under the digestion conditions we employed, Staphylococcus Aureus protease-V8 recognises mainly glutamate residues (23) and can therefore be used to probe the differences in regions of similar molecules that are unaffected by chymotrypsin (which recognises aromatic and hydrophobic residues). Again, characteristic differences can be observed between the α- and β-tropomyosins and between the fast (PLD, pectoralis) and slow (ALD) α-tropomyosins (Fig.2A). It can also be

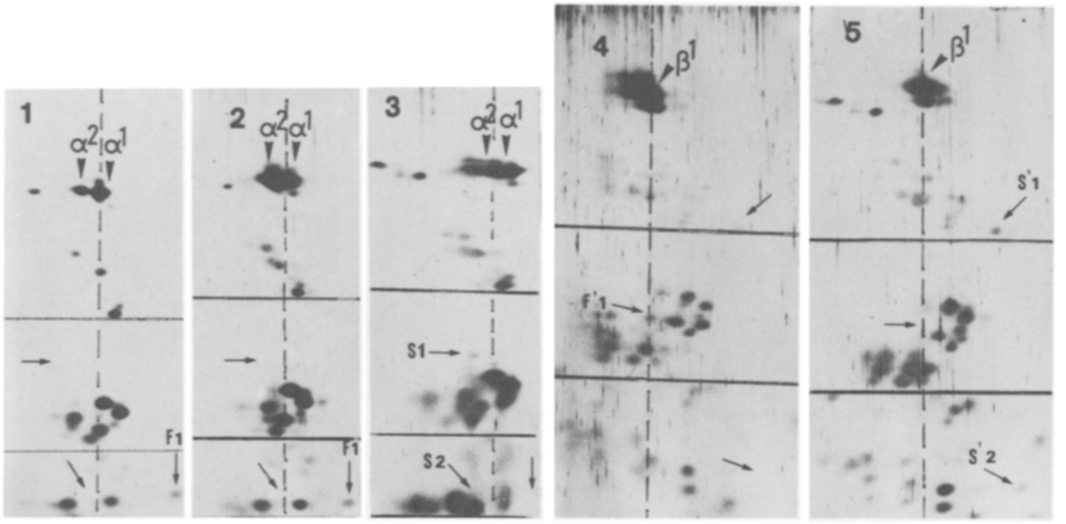

Figure 3 :

Two-dimensional peptide mapping of tropomyosins from fast and slow chicken muscles, after digestion with Staphylococcus V8-protease.

Arrows indicate differences between the α-tropomyosins (1-3) and between the β-tropomyosins (4-5). α1 indicates the position of the unphosphorylated undigested α-tropomyosin. α2 indicates the position of the phosphorylated undigested α-tropomyosin. β1 indicates the position of the unphosphorylated undigested β-tropomyosin.

1. α-tropomyosin from pectoralis major muscle
2. α-tropomyosin from PLD muscle
3. α-tropomyosin from ALD muscle
4. β-tropomyosin from PLD muscle
5. β-tropomyosin from ALD muscle

F1 : peptide uniquely found in α-tropomyosin from fast muscle
S1 and S2 : peptides uniquely found in α-tropomyosin from slow muscle
F'1 : peptide uniquely found in β-tropomyosin from fast muscle
S'1 and S'2 : peptides uniquely found in β-tropomyosin from slow muscle.

clearly seen that with V8-protease differences become evident in the digestion pattern of fast (PLD) β-tropomyosin versus that of the slow (ALD) β-tropomyosin (Fig. 2A, lane 1,2). In agreement to the V8-protease difestion evidence, the use of papain as the proteolytic agent, again indicates differences in the difestion pattern between PLD- and ALD- derived β-tropomyosins (Fig. 2B).

When the V8-protease digests are analysed by two dimensional electrophoresis, even when a narrow pH range (pH 4-6) is employed for the first dimension, it can immediately be observed that all peptides are distributed over an even narrower pH zone, around pH 5 (Fig.3). This can be taken to reflect the periodicity of charge distribution along the entire length of tropomyosin molecule ; amino acid sequence analysis (24, 25) of tropomyosin shows a more or less regular alternation of polar and non-polar amino acid residues.

Glutamate residues are to be found in the borders of those regions, from one end of the molecule to the other (24,25). The lower horizontal third of the peptide map exhibits a higher scatter of peptide distribution in the case of β-tropomyosins (Fig.3, 4-5), from either fast or slow muscle, in consistence with a somewhat reduced regularity in β-tropomyosin within the -COOH half of the molecule (9) ; this also implies that the lowest molecular weight peptides in the V8 digestion pattern are largely derived from the carboxylic half of the molecule, which also contains most of the amino acid substitutions between α- and β-tropomyosins (9). The section in the middle of the peptide map exhibits a very characteristic peptide distribution, depending on whether the digests originate from an α- or from β-tropomyosin (Fig. 3, 1-5). The particular peptide differences between fast and slow muscle α-tropomyosins and between fast and slow muscle β-tropomyosins are indicated by arrows in Fig.3 (1-5). It can be seen that there is a number of peptides characteristic for each type of tropomyosin, like, for instance, peptide F1 for α-tropomyosin from PLD and pectoralis muscle, peptides S1 and S2 for α-tropomyosin from ALD muscle, peptide F'1 for β-tropomyosin from PLD muscle and peptides S'1 and S'2 for β-tropomyosin from ALD muscle (Fig.3, 1-5).

Our results clearly show that it is possible to distinguish between the β-tropomyosins from PLD and ALD muscle. These results are in very good agreement with those obtained by Billeter and coworkers working with human skeletal muscle (14). Despite the difference in origin (human versus avian) tropomyosins display striking similarities. For example, chymotrypsin leads to identical patterns for the β-tropomyosins from fast and slow muscle in both species, while V8 protease allows the detection of differences.

It seems probable that the differences in the partial-cleavage peptide maps of various tropomyosins reflects differences in the primary sequence of these proteins. No operational modification of the proteins could be detected when screened for purity by two-dimensional electrophoresis. Phosphorylation, a post translational modification found to occur in tropomyosin, although mainly in embryonic muscle (12), does not affect the V8-protease or chymostryptic digestion pattern of tropomyosin (E. Kardami, unpublished observations).

In the case of the α-variants, there exists additional support that they constitute different gene products :

1) A serum that has been raised against α-tropomyosin from pectoralis muscle cross-reacts very weakly with either β-tropomyosin (Fig. 4B, lane 3) or α-tropomyosin from ALD muscle (Fig. 4B

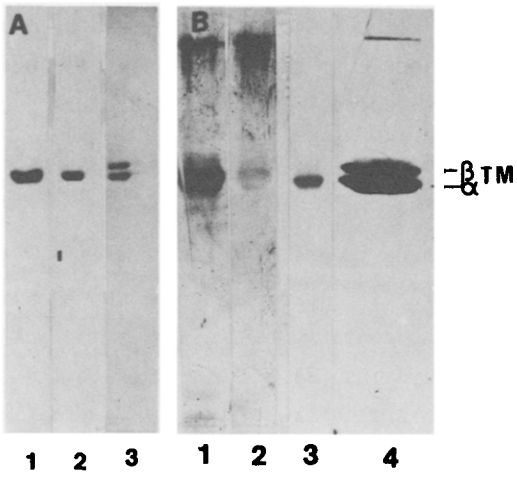

Figure 4 :

(A) 12.5 % acrylamide gel of tropomyosins (identical to the gel used for the protein transfer in (B)).
1. α-tropomyosin from pectoralis major muscle
2. α-tropomyosin from ALD muscle
3. α-) and β-tropomyosin from PLD muscle

(B) Enzyme-linked Immunoassay : Peroxidase visualisation of the immobilised antigen-antibody complexes on nitrocellulose filter.

1 & 2 : Non purified anti α-tropomyosin (pectoralis) serum, at a 1/200 dilution, with 1. α-tropomyosin from pectoralis major muscle,
2. α-tropomyosin from ALD muscle.

3 & 4 : Purified immune serum, reaction with α-) and β-tropomyosin from PLD muscle
3. at a serum dilution of 1/200
4. at a serum dilution of 1/20.

lane 2) as judged by the degree of "peroxidase-staining" of the immobilized antigen-antivody complexes. Several immune serum dilutions were assayed (1 :10 to 1 : 1000) and care was taken to insure that the same amount of each protein to be tested is transfered on the nitrocellulose filter (never in excess of 1 µg), by densitometric scanning of the identical to the transfered gel, as well as of the "blotted" gel after transfer, and inspection of the transfered proteins by amido-black staining of one nitrocellulose "copy". This assay is important since as shown in Fig. 4B the same serum may or may not recognize a protein (compare lane 3 and 4, Fig. 4B) depending on the dilution of serum.

2) Molecular cloning of cDNA prepared against tropomyosin mRNA (26) has shown that this RNA recognises two mRNA molecules which code for two proteins having electrophoretic mobilities similar to those we have described for α-tropomyosin from fast and slow muscle (A.R. McLeod, to be published).

Although no evidence of that sort exists as yet for the β-tropomyosins it is worth mentioning that in rabbit skeletal muscle, consisting

of a mixed fiber population, a minor and a major β-tropomyosin form were found, in a ratio similar to that of slow to fast fiber within the muscle (9). This, in conjunction with our results and those of Billeter and coworkers (14) implies that the β-tropomyosins from fast and slow type fibers may be different gene products.

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