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Peculiarities of SDS-PAGE of Titin/Connectin

Ivan M. Vikhlyantsev and Zoya A. Podlubnaya

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Abstract

Titin (also known as connectin) is a giant elastic protein of striated and smooth muscles of vertebrates. The molecular weight of its isoforms is 3.0–3.7 MDa in striated muscles and 0.5–2.0 MDa in smooth muscles. Titin was discovered 40 years ago using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the present time, this method has not lost its relevance but has undergone a number of modifications that improve visualization of giant titin isoforms in the gel. This chapter provides historical insights into the technical aspects of the electrophoresis methods used to identify titin and its isoforms. We focus on the peculiarities of the technique because of which titin molecules remain intact and its high molecular weight isoforms can be visualized. Electrophoretic testing of changes in titin content in muscles can be used in medical practice to diagnose pathological processes and evaluate effective approaches to their correction.

Keywords: striated muscles, titin (connectin), titin isoforms, SDS-PAGE, agarose-strengthened macroporous polyacrylamide gel

1. Introduction

Titin (also known as connectin) is a giant elastic protein of striated [1–6] and smooth [7] muscles of vertebrates. The molecular weight of its isoforms is 3.0–3.7 MDa in striated muscles and 0.5–2.0 MDa in smooth muscles [7]. Titin is the third protein in quantity (after actin and myosin) in the sarcomeres of cardiac and skeletal muscles (**Figure 1**). Titin molecules of about 1 μm in length and 3–4 nm in diameter [8, 9] overlap the half of the sarcomere from the M-line to the Z-line, forming a third filamentous system in myofibrils [10]. In the A-zone of the sarcomere, titin is connected with myosin filaments [11]. In the I-band of the sarcomere, some regions of the titin molecule may interact with actin filaments [12, 13], but most of the titin molecule in this zone passes freely, connecting the ends of myosin filaments with the Z-disc

Figure 1. Sarcomere structure of vertebrate striated muscles. (A) Scheme of sarcomeric structure. Thick filaments consisting mainly of myosin are located in the A-band. Thin filaments consisting mainly of actin are located in the I-band of the sarcomere and in the A-band, where they overlap with thick filaments. The H-zone is the central part of the A-band free from thin filaments. Titin molecules are arranged from the M-line to the Z-disc of the sarcomere. Transversal bands of myosin-binding proteins (H, C, X) in the A-band of the sarcomere are indicated. (B) Microphotograph of sarcomere of demembranized rabbit lumbar muscle. Scale bar: 200 nm.

(Figure 1). Each half of myosin filament in sarcomere includes six titin molecules [14] with N- and C-ends overlapping in the Z- and M-line of the sarcomere, respectively [15].

Titin molecule consists of repeating immunoglobulin-like (IgC2) and fibronectin-like (FnIII) domains. Titin also contains a kinase domain in M-line, unique sequences N2A, N2B, and PEVK in I-zone and phosphorylation sites in Z-disc, M-band, and I-band of sarcomere [15].

The giant size of titin molecule and its location in all zones of sarcomere provide a basis for polyfunctionality of this protein. It has been shown that titin is a framework for the assembly of thick filaments and the sarcomere [16, 17]; is involved in maintenance of the highly ordered sarcomere structure [18, 19]; contributes to the passive tension developed by the muscle during stretching and develops the restoring force during sarcomere shortening [20–22]; is involved in the regulation of actin-myosin interaction [2, 4, 13, 23–31]. The results of recent studies suggest that the elastic protein titin, as a mechanosensor (strain sensor and stress sensor), plays a key role in intracellular signaling processes and in particular, participates in the regulation of muscle gene expression and protein turnover in sarcomere [2, 6, 32–37]. These

conclusions are based on the results of investigations showing the presence in the sarcomere of mechanosensory “hotspots”: complexes of signal proteins joined by titin into a single network, the activity of which varies during muscle stretching or variations in its mechanical loading [32–34, 38]. Titin is supposed to “...sense the mechanical stimuli and transform them into biochemical signals...” [34]. Great attention is now focused on the study of titin role in cardiomyopathies and skeletal muscle diseases [2, 39–47].

This paper provides historical insights into the technical aspects of electrophoresis methods used to identify titin and its isoforms. We focus on the peculiarities of the technique because of which titin molecules remain intact and its high molecular weight isoforms can be visualized.

2. History of the discovery and study of titin/connectin by SDS gel electrophoresis technique

Titin was discovered in 1979 by Kuan Wang and his coauthors [48] using gel electrophoresis. In macroporous polyacrylamide gel prepared according to Etlinger et al. [49] and containing 3.2% acrylamide, three new bands were found above the myosin heavy chain: a closely spaced doublet and a singlet band with faster mobility [48]. Using crosslinked myosin heavy chains (MHC, 205 kDa) as standards, the authors were able to estimate that each of these doublet bands (1 and 2) has a Mr. $\sim 1 \times 10^6$. Proteins 1 and 2 appeared to be immunologically identical and were named titin 1 (T1) and titin 2 (T2). The third protein had Mr. $\sim 5 \times 10^5$ and was thereafter named nebulin [50].

Another group of investigators headed by Maruyama studied the properties of connectin – the protein they discovered [51]. The properties of connectin as a protein candidate for the elastic filaments in sarcomeres of striated muscles of vertebrate animals were intensively explored by this group of authors in the late 1970s [52–54]. In 1981, having conducted a comparative study of electrophoretic mobility, amino acid composition, and localization in myofibrils of titin and connectin, Maruyama and coauthors showed that the major high molecular weight component of connectin was identical with that of titin [55]. Using 1.8–3.0% polyacrylamide tube gels prepared according to Weber and Osborn [56], and crosslinked MHC as standards, the authors showed that the molecular weights of α -connectin (corresponding to intact molecules of titin-1) and β -connectin (corresponding to proteolytic fragments of T1–T2) of breast muscle of the chicken were 2.8×10^6 and 2.1×10^6 , respectively [57].

Further electrophoretic studies of titin (connectin) using different types of gels (1.8% or 2.3–4% polyacrylamide tube gels, 2–12% gradient polyacrylamide slab gel) [58–63] revealed differences in electrophoretic mobility of T1 (α -connectin) in cardiac and skeletal muscles of vertebrates animals (fishes, amphibians, reptiles, birds, mammals). In particular, plots of molecular mass versus mobility, assuming 2.8 and 2.4 MDa for T1 and T2 of the rabbit psoas, respectively, yielded the following set of values for T1: 2.8 MDa (adductor magnus), 2.88 MDa (longissimus dorsi, sartorius), 2.94 MDa (soleus, semitendinosus) [60]. Cardiac muscle displayed the smallest titin. Similar data were obtained by us using 2.5–9% gradient polyacrylamide slab gel (**Figure 2**). Based on data obtained the assumption on the existence of isoforms of T1 was made [60, 61].

Figure 2. Molecular weights of T1 isoforms from rabbit striated muscles. Electrophoresis was performed in a gradient 2.5–9.0% polyacrylamide vertical gel (8 × 10 × 0.1 cm). (1) myocardium (left ventricle); (2) m. soleus; (3) logarithmic dependence of molecular weight on protein electrophoretic mobility in gel. T1 molecular weight was assessed by the following standards: Cardiac MyBP-C (150 kDa), as well as myosin heavy chains (MHC, 205 kDa), nebulin (770 kDa), and T2-fragment (2400 kDa) of rabbit skeletal muscles [60, 87, 88].

In 1995, the complete complementary DNA sequence of human cardiac titin was determined [64]. Further studies showed that the titin gene (TTN) consists of 363 coding exons, which can be differentially spliced and theoretically could generate more than one million splice variants in striated and smooth muscles of mammals [7, 65–68]. Adult striated muscles express three major titin isoforms: N2A in skeletal muscles (3.35–3.7 MDa), N2B, and N2BA in cardiac muscle (2.97–3.3 MDa, respectively) [65].

2.1. Electrophoretic detection of titin isoforms

To confirm that muscles contain N2A, N2B, and N2BA isoforms of titin, different macroporous gels (2–9.5% gradient polyacrylamide slab gel, 1% agarose slab gel, agarose-strengthened 2% polyacrylamide slab gel, horizontal 1.3% polyacrylamide gel strengthened with 0.5% agarose) were used [69–73]. It was shown that the T1 mobility varied greatly between skeletal and cardiac muscles from different mammals. The major T1 bands were ascribed to the titin isoforms N2B and N2BA in cardiac muscle and the titin isoform N2A in skeletal muscles. According to Western blot data with using antibodies against the N-terminal and the C-terminal ends of titin, it was revealed that the N2A, N2B, and N2BA bands represent full-length titin molecules (titin 1 – T1) [69, 74].

Titin isoform analyses for 37 adult rabbit skeletal muscles showed sizes between 3.3 and 3.7 MDa [75]. N2BA titin isoforms in cardiac muscle of different mammals had sizes between 3.25 and 3.4 MDa [72].

Using 1% vertical agarose gel [70] at least four classes of cardiac N2BA titin isoforms were observed, of which two rat embryonic/neonatal forms (N2BA-N1, N2BA-N2) had sizes of 3710 and 3590 kDa. These isoforms were found during late embryonic and immediately post-natal period [76]. These were gradually replaced by adult forms (N2BA-A1, N2BA-A2) with sizes of 3390 and 3220 kDa, respectively [76]. Similar titin isoform transformations were observed in embryonic/neonatal hearts of rat and other mammals and reported by the researchers [74, 77–80].

Giant titin isoforms expressed in rat striated muscles with an RBM20 autosomal dominant mutation were reported [81–83]. The molecular masses of these isoforms were estimated from their electrophoretic mobility in 1% vertical agarose gel to be 3750 and 3830 kDa [83].

2.2. New high molecular weight forms of titin in striated muscles of mammals: aggregates or intact isoforms?

Our group headed by Zoya Podlubnaya conducts a comparative study of titin isoform composition in mammalian striated muscles under conditions of hibernation, microgravity, and during the development of pathological processes [84]. Vertical agarose-strengthened 2.2% polyacrylamide gel prepared according to Tatsumi and Hattori [85] was used to separate titin isoforms and their fragments.

Our first experiments, conducted more than 10 years ago, showed that, in addition to N2A, N2BA, N2B and T2 bands, there exist one or two more high Mr. bands (named NT) [84, 86]. Staining the gels with ethidium bromide revealed no nucleic acids in the bands, although western blots with 9D10 antibodies revealed titin bands. The bands were visualized in the electropherograms of striated muscles of mammals, but in the electropherograms of striated muscles in other groups of vertebrates (amphibians and birds) revealed no NT bands [84].

The content of NT titins in muscles of animals and humans was as follows: Mongolian gerbil (8–14%), mouse (13–18%), rat (9–26%), rabbit (13–30%), ground squirrel (24–33%), and human (29–41%) [73, 84]. Using human and animal skeletal muscle myosin heavy chain (205 kDa) and nebulin (770–890 kDa), as well as the N2A titin isoform (~3600 and 3700 kDa) of rabbit and human soleus as standards [60, 75, 87, 88], we estimated that the NT has a Mr. of $\sim 3.8\text{--}3.9 \times 10^6$ [73]. Expression of titin isoforms with these molecular weights is not excluded [66, 67, 82, 83], but titin aggregates in gels could not be excluded either [69, 88]. Data published in 2003 demonstrated in electropherograms of the dog heart left ventricle, together with the known N2BA and N2B isoforms and T2-fragments of titin, the presence of higher molecular weight double protein bands that were named titin aggregates [70].

We were also not absolutely sure that titin NT bands were not aggregates of its lower molecular weight isoforms and their fragments. If this were so, then the proteolytic cleavage of titin accompanied by an increase in the content of its fragments must result in the higher content of aggregates. Experiments on proteolytic cleavage of titin in muscle tissue under the influence of endogenous proteases were performed to test this assumption [84] (**Figure 3**).

Figure 3. Proteolytic changes in titin in ground squirrel muscles. Electrophoresis was performed in vertical agarose-strengthened 2.1% polyacrylamide gel ($8 \times 10 \times 0.1$ cm). (1) m. soleus (control); (2) m. soleus (proteolysis, 1 h); (3) left ventricle of heart (control); (4) left ventricle of heart (proteolysis, 30 min). Proteolytic cleavage of titin was performed under the influence of endogenous muscular proteases. To this end, small pieces of muscle tissue (20–30 mg) were held for 30–60 min at 25–30°C. Then, 2–3 mg pieces were taken from the muscle sample and placed into solubilizing solution (10 mM Tris-HCl, 1.2% SDS, 10% glycerol, 2% β -mercaptoethanol or 75 mM DTT, 8–10 μ g/ml of leupeptin or E64, pH 7.0) for the extraction and further electrophoretic testing of the proteins. T3300 is probably the proteolytic fragment of NT titin with molecular weight of ~3300 kDa.

It was found that proteolysis of titin in m. soleus for 30–60 min resulted in a reduction (six-fold to sevenfold) of the content of NT titin and twofold reduction of the content of N2A titin (**Figure 3**, lanes 1 and 2). At the same time, a considerable increase in the content of T2 and appearance of a band with a molecular weight of ~3200–3300 kDa (T3300) were detected, which is probably a product of NT titin degradation.

Proteolysis of titin in cardiac muscle for 30–60 min resulted in a 2–3-fold decrease in the content of NT and N2BA (**Figure 3**, lanes 3 and 4). At the same time, the increase in the content of not only T2-fragments but also N2B isoform of titin was observed, which could be explained by the presence of fragments of NT and N2BA titins in this protein band. Densitometry data showed that the total titin content (relative to MHC content) in muscles as a result of 30–60 min proteolysis has not changed.

Thus, the results did not confirm our assumption that NT bands are aggregates of lower molecular weight titin isoforms and their fragments. However, these data did not exclude the aggregative origin of NT bands. Assuming that molecular masses of titin aggregates should considerably exceed 3800–3900 kDa, we decided to find out more about the differences in electrophoretic mobility of the observed bands. We developed a horizontal agarose-strengthened gel system using 1.3% polyacrylamide and 0.5% agarose [73]. The gels showed that mobility of the NT bands, as well as other titin bands, varied greatly in different muscles.

Using human and animal skeletal muscle nebulin (770–890 kDa) as well as MHC (205 kDa) as standards the molecular masses of N2A, N2BA, N2B, T2, and NT titin bands were estimated. The results obtained were unexpected for us. The NT bands had sizes between 3230 and 3730 kDa,

whereas the N2A, N2BA, N2B, and T2 bands had sizes between 2100 and 2800 kDa [73, 84]. The last values corresponded to a set of values for T1 (α -connectin) and T2 (β -connectin) [57, 60, 88]. Similar data were obtained for vertical agarose-strengthened 1.9% polyacrylamide gels (**Figure 4**). The gel resolved a doublet NT band at 3300–3400 kDa for cardiac muscle and a singlet band of 3600–3700 kDa for skeletal muscles of mammals.

Results from western blots with Z1/Z2 antibodies against the N-terminal end and AB5 antibodies against the C-terminal end of titin revealed the NT bands were full-length titin molecules [89] (**Figure 5**). We, therefore, hypothesized that the NT bands are intact N2A, N2BA, N2B titin isoforms [89]. Although this requires further research, we cannot exclude the possibility that the NT bands are the other protein immunologically identical to titin, for example, a protein whose long thin filaments were revealed in the “shades” of rabbit psoas myofibrils remaining after sequential removal of myosin, actin, tropomyosin, troponins, and the minor M-band proteins [90] (**Figure 6**).

Figure 4. SDS-PAGE analysis of titin isoforms in striated muscles of ground squirrel (*Spermophilus undulatus*); a modified view from [89]. Vertical agarose-strengthened 1.9% polyacrylamide gel (14.5 × 16.0 × 0.15 cm) was used to separate the titin isoforms. (1) Myocardium (left ventricle); (2) m. soleus. T1 molecular weight was assessed by the following standards: MHC (205 kDa), nebulin (770–890 kDa), titin-2 (2100–2400 kDa) of rabbit and human striated muscles [60, 87, 88].

Figure 5. Western blotting of titin in striated muscles of ground squirrel; a modified view from [89]. As primary antibodies, the following were used: Z1/Z2 to N-end of titin molecule located in Z-disc of sarcomere (1, 2); AB5 to the part of titin molecule in A-disk located near the M-line of sarcomere (3,4); (1,4) m. soleus; (2,3) myocardium (left ventricle).

2.3. Nuances of sample preparation of titin

In the paper of Granzier and Wang [88], particular attention was paid to sample preparation of titin. The authors pointed to the fact that titin is extraordinarily sensitive to proteolysis *in situ* by endogenous proteases and by exogenous proteases such as those found in buffers that are contaminated with bacteria and fungi. According to these and other authors, even in SDS-solubilized myofibril samples, appreciable degradation of titin by residual protease activity can occur in a few days at room temperature [50, 88, 91].

To limit proteolysis, a number of authors suggested the inclusion of protease inhibitors in SDS samples prior to electrophoresis. Leupeptin, E-64, and a protease inhibitor cocktail have been used to inhibit proteolytic degradation of titin [21, 70, 72, 73, 85, 92]. In order to attain better solubilization of titin, it has been proposed to use urea-thiourea SDS DTT sample buffer [70, 93].

Another crucial methodical nuance that should be taken into account during sample's preparation of titin is heat treatment of the samples. SDS samples are usually prepared by heating them in boiling water for several minutes. This process promotes denaturation of proteins and facilitates disulfide reduction. However, different authors have shown that boiling degrades titin [70, 88, 94, 95]. Samples heated at 100°C had less intact titin and more breakdown products

Figure 6. Microphotograph of isolated negatively stained myofibril of rabbit lumbar muscle after the extraction of myosin and actin filaments. The remaining "Z-discs" are kept in the register by longitudinal filaments continually passing through the entire myofibril (indicated by the arrow). Scale bar: 100 nm.

(smears migrating near the bottom of the gel) than those at 60°C [70]. A temperature of 50–60°C for 10–20 min has been considered optimal for the extraction and preservation of intact titin [70, 88, 95]. Results of our studies demonstrated that heating of SDS sample at the said temperature may lead to artifacts in the content of titin. SDS samples of mammalian cardiac muscle heated at 60–65°C for 20 min had another N2BA/N2B ratio than those at 30–40°C [96]. We recommended heating titin in SDS at 35–40°C for 30–40 min [73, 84].

2.4. Other details of the electrophoretic study of titin

It is suggested that titin has a tendency to aggregate during electrophoresis, especially in gel systems that use a stacking gel or discontinuous buffers [88]. Fritz et al. [93], as well as Greaser and Warren [97, 98], recommended the inclusion of β -mercaptoethanol in the top anodic buffer to prevent disulfide crosslinking.

There is some peculiarity that should be noted with regard to the preparation of agarose-strengthened 2% polyacrylamide gel. Tatsumi and Hattori [85] to prevent polyacrylamide polymerizing before agarose is polymerized, cooled the glass cell with agarose solution (40°C) for 5 min in ice water. Similarly, we added the agarose solution to glass cells that were pre-cooled to 8–10°C and left the gel for 10 min in the refrigerator at 5°C. Then, we kept the gel for 30 min at 20°C and then for 2–2.5 h at 27°C.

It is recommended to perform electrophoresis using macroporous, agarose-strengthened polyacrylamide gels at low currents. Neagoe (of Wolfgang Linke's group) noted that the best separation of the high molecular weight proteins was obtained by running the electrophoresis overnight at 2 mA per 8.6×7.7 cm gel [99]. In our studies with the use of similar gels ($8.0 \times 10.0 \times 0.10$ cm), we perform electrophoresis at 3 mA for 40 min, then increasing the current strength up to 7–8 mA [96].

Granzier and Wang recommended to refresh the tank buffer once at 2.5 h to limit pH changes caused by electrolysis during electrophoresis [88].

3. Conclusion

In summary, it should be noted that SDS-PAGE of titin is quite difficult and not a routine procedure. Giant molecular mass and the susceptibility of titin to degrade during preparation significantly complicate the study of this protein by electrophoresis. It is necessary to know the three main rules for a successful study of titin by electrophoresis: (1) use protease inhibitors (leupeptin, E-64, protease inhibitor cocktail); (2) do not heat SDS samples higher than 40–60°C; and (3) judiciously select the type of the gel. Currently, the most suitable gels for analyzing titin are the following: (1) vertical agarose-strengthened 2% polyacrylamide gel [85]; (2) vertical 1% agarose gel [70]; and (3) horizontal agarose-strengthened 1.3% polyacrylamide gel [73].

It should be noted that we have obtained experimental evidence of existence in mammalian striated muscles of higher molecular weight isoforms of titin, named NT. According to our data, the development of pathological processes leads to the destruction of NT titins (**Figure 7**),

Figure 7. Increased proteolysis of titin and reduction of T1 content in m. Soleus of post-apoplectic patient. Vertical agarose-strengthened 2.3% polyacrylamide gel ($8.0 \times 10.0 \times 0.10$ cm) was used to separate titin isoforms. (1) Human m. soleus (control); (2) m. soleus of post-apoplectic patient.

which is accompanied by disorders of sarcomeric structure and impairment of the contractile ability of muscles [84]. In addition to fundamental value, these findings are of great practical value, because the testing of changes in titin content in muscles can be used in medical practice to diagnose pathological processes and evaluate effective approaches to their correction.

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Conflict of interest

Ivan M. Vikhlyantsev and Zoya A. Podlubnaya declare that there is no conflicts of interest.

Author details

Ivan M. Vikhlyantsev^{1,2*} and Zoya A. Podlubnaya^{1†}

*Address all correspondence to: ivanvikhlyantsev@gmail.com

1 Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Russia

2 Pushchino State Institute of Natural Science, Pushchino, Russia

† Zoya Podlubnaya died on March 26, 2018

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