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REVIEW

Nuances of electrophoresis study of titin/connectin

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Abstract Almost 40 years has passed since the discovery of giant elastic protein titin (also known as connectin) of striated and smooth muscles using gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a major technique for studying the isoform composition and content of titin. This review provides historical insights into the technical aspects of the electrophoresis methods used to identify titin and its iso-forms. We particularly focus on the nuances of the technique that improve the preservation of its primary structure so that its high molecular weight isoforms can be visualized.

Keywords Striated muscles · Titin/connectin · Titin isoforms · SDS-Page

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 using gel electrophoresis. They studied chicken striated (breast), cardiac and smooth (gizzard) muscle extracts using 4% polyacrylamide gels (acrylamide/bisacrylamide ratio 50:1,

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Tris glycine pH 8.8; as in Etlinger et al. 1976) in which they identified another very large actin-binding protein, filamin (Wang et al. 1979). Filamin was detected in smooth muscle but not in extracts from cardiac and skeletal muscles of the chicken and rabbit in the presence of sodium dodecyl sulfate (SDS). In another polyacrylamide gel prepared as described by Etlinger et al. (1976), except that 3.2% acrylamide was used instead of 5%, three major bands were observed above the prominent myosin heavy chain. They found a closely spaced doublet and a faster moving singlet band (Wang et al. 1979). Using crosslinked myosin heavy chains (205 kDa) as standards, the authors estimated that each of these doublet bands (1 and 2) has a Mr $\sim 1 \times 10^6$ and that the Mr of band 3 is $\sim 5 \times 10^5$. Proteins 1 and 2 appeared to be immunologi-cally identical and were named titin 1 (T1) and titin 2 (T2). The third protein was thereafter named nebulin (Wang 1982).

Independent of Kuan Wang and his coworkers, another group of investigators headed by Koscak Maruyama discov-ered a protein which they named connectin (Maruyama 1976; Maruyama et al. 1976). The properties of connectin as a pro-tein candidate for the elastic filaments in sarcomeres of striat-ed muscles of vertebrate animals were intensively explored by this group of authors in the late 1970s to early 1980s (Maruyama et al. 1977a, b, c; Matsubara and Maruyama 1977; Toyoda and Maruyama 1978). In 1981, having conduct-ed 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 (Maruyama et al. 1981). Using crosslinked myosin heavy chains as standards, and 1.8–3.0% polyacryl-amide tube gels prepared according to Weber and Osborn (1969), 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 (Maruyama et al. 1984) (Table 1, Reference 2).

Further electrophoretic studies of titin (connectin) using different types of gels (Table 1, References 3, 4, 6, 7, 9, 10, 12) revealed differences in electrophoretic mobility of T1 (α-connectin) in cardiac and skeletal muscles of vertebrates animals (fishes, amphibians, reptiles, birds, mammals). In particular, six skeletal muscles (adductor magnus – AM, psoas – PS, longissimus dorsi – LD, sartorius – SA, soleus – SO, semitendinosus – ST) and cardiac muscle of the rabbit were analyzed on a 2–12% gradient gel (Wang et al. 1991). Plots of molecular mass versus mobility, assuming 2.8 and 2.4 MDa for T1 and T2 of the rabbit PS, respectively, yielded the following set of values for T1: AM and PS, 2.8 MDa; LD and SA, 2.88 MDa; SO and ST, 2.94 MDa (Wang et al. 1991) (Table 1, Reference 6). Cardiac muscle displayed the smallest titin. Based on data obtained the assumption on the existence of isoforms of T1 was made (Wang et al. 1991; Horowits 1992). This assumption is in agreement with the results of our experiments (Vikhlyantsev and Makarenko 2000) in which the differences in electrophoretic mobility of T1 in striated muscles of a ground squirrel were found in a 7% polyacrylamide gel, prepared according to Fritz et al. (1989). The distinguishing feature of an acceptable gel for high-molecular-mass proteins (greater than 500 kDa) is the requirement for a low concentration of bis-acrylamide (bis-acrylamide/acrylamide = 1:200) (Table 1, Reference 5).

In 1995 the complete complementary DNA sequence of human cardiac titin was determined (Labeit and Kolmerer 1995). Further studies showed that the titin gene (TTN) consists of 363 coding exons, which can be differentially spliced and theoretically could generate more than 1 million splice variants in striated and smooth muscles of mammals (Freiburg et al. 2000; Bang et al. 2001; Labeit et al. 2006; Guo et al. 2010; Gerull 2015).

Adult striated muscles express three major titin isoforms named N2A (skeletal muscles), N2B, and N2BA (cardiac muscle). Multiple splicing pathways result in titin isoform diversity (Freiburg et al. 2000). In particular, in soleus and psoas skeletal muscles, different exon-skipping pathways produce titin transcripts that code for 3.7 and 3.35 MDa isoforms, respectively (Freiburg et al. 2000). In the heart, ventricular muscle exons separated by 101 kb lead to the exclusion of 155 exons and the expression of a 2.97-MDa cardiac titin N2B isoform. The atria express exclude 90–100 exons resulting in a 3.3-MDa N2BA titin isoform (Freiburg et al. 2000).

Electrophoretic detection of titin isoforms

To confirm that muscles contain N2A, N2B and N2BA isoforms of titin, different macroporous gels have been reported by different authors (Table 1, References 13–15). These gels showed that the T1 mobility varied greatly between skeletal and cardiac muscles from different mammals (Cazorla et al. 2000; Trombitás et al. 2000; Neagoe et al. 2003; Warren et al. 2003a, b; Lahmers et al. 2004; Makarenko et al. 2004; Udaka et al. 2008; Guo et al. 2013). The major T1 bands were ascribed to the titin isoforms N2B and N2BA in cardiac muscle and the titin isoform N2A in skeletal muscles. Western blots using antibodies against the N-terminal and the C-terminal ends of titin revealed that the N2A, N2B and N2BA bands represent full-length titin molecules (T1) (Cazorla et al. 2000; Wu et al. 2000; Lahmers et al. 2004).

Using agarose-strengthened 2.0% polyacrylamide gel, isoform diversity of skeletal N2A-titin in rabbit muscles was detected (Prado et al. 2005). In particular, titin isoform analyses for 37 adult rabbit skeletal muscles showed sizes between 3.3 MDa and 3.7 MDa (Prado et al. 2005, human soleus N2A (3.7 MDa) and human cardiac N2B (3.0 MDa) and N2BA (3.3 MDa) were as markers for molecular weight detection). N2BA titin isoforms in cardiac muscle of mammals (mouse, hamster, rat, rabbit, cat, and cow) had sizes of approximately 3.25–3.4 MDa (Neagoe et al. 2003; Table 1).

With the help of the electrophoresis system using 1% vertical agarose gel (Warren et al. 2003a), at least four classes of cardiac N2BA titin isoforms were observed. In particular, two rat embryonic/neonatal forms (N2BA-N1, N2BA-N2) with apparent sizes of approximately 3710 and 3590 kDa, respectively, were found during late embryonic and immediately post-natal (Warren et al. 2004). These were gradually replaced by adult forms (N2BA-A1, N2BA-A2) with sizes of 3390 kDa and 3220 kDa, respectively (Warren et al. 2004). Similar titin isoform transformations were observed in embryonic/neonatal hearts of rat and other mammals and reported by the researchers (Lahmers et al. 2004; Opitz et al. 2004; Greaser et al. 2005; Krüger et al. 2006).

Giant titin isoforms expressed in rat striated muscles with an RBM20 autosomal dominant mutation were recently reported (Greaser et al. 2008; Li et al. 2012; Guo et al. 2012). The molecular masses of these isoforms were estimated from their electrophoretic mobility in 1% vertical agarose gel to be 3750 kDa and 3830 kDa (Li et al. 2012).

More than 10 years ago, our group headed by Zoya Podlubnaya compared the isoform composition of titin in mammalian striated muscles under conditions of hibernation, microgravity, and during the development of pathological processes. Vertical agarose-strengthened 2.1–2.3% polyacrylamide gels ($8 \times 10 \times 0.1$ cm in the Laemmli buffer system, pH 9.0) prepared according to Tatsumi and Hattori (1995; Table 1, Reference 11) were used to separate titin isoforms and their fragments. Our first experiments showed that, in addition to N2A, N2BA, N2B and T2 bands, there exist one or two more high Mr bands (named NT) (Vikhlyantsev et al. 2004a, b). Staining the gels with ethidium bromide revealed

Table 1 Types of gels for electrophoretic study of titin

Reference	Type and size of gel, percentage of acrylamide (agarose)	Ratio of acrylamide and bis-acrylamide	Gel buffer, pH	Sample buffer, pH	Heating of samples	Tissue/muscle source	Molecular mass of titin (connectin)	Nuances
1. Wang et al. 1979	3.2% polyacrylamide tube gel, 0.5 × 10 cm	50/1	0.1 M Tris-glycine, pH 8.8, 0.4% SDS	100 mM Tris-HCl, 10 mM EDTA, 40 mM DTT, 10% SDS, 20% glycerol, pH 8.0	Samples were boiled for 2 min	Purified striated muscle myofibrils (chicken breast muscle, rabbit back muscle) prepared according to Ettinger et al. (1976)	Doublet bands (T1 and T2) has Mr ~1 × 10^6.	This procedure was a modification of the Ettinger et al. method (1976)
2. Maruyama et al. 1984	1.8–3.0% polyacrylamide tube gel, 0.6 × 10 cm	37/1	7.8 g NaH$_2$PO$_4$ × H$_2$O and 38.6 g Na$_2$HPO$_4$ × 7H$_2$0, pH 7.5	0.01 M sodium phosphate buffer, pH 7.0, 1% SDS, and 1% β-mercaptoethanol, 8 M urea	37 °C for 2 h	Chicken breast muscle	α-connectin – 2.8 MDa; ß-connectin – 2.1 MDa. A faint band above α-connectin is visible in gel (see fig. 2 in Maruyama et al. 1984)	Connectin was extracted from exhaustively washed myofibrils of chicken breast muscle in 0.1 M phosphate buffer, pH 6.6.
3. Hu et al. 1986	1.8% polyacrylamide tube gel, 0.6 × 10 cm	20/1	7.8 g NaH$_2$PO$_4$ × H$_2$O and 38.6 g Na$_2$HPO$_4$ × 7H$_2$0, pH 7.5	10% SDS, 40 mM DTT, 10 mM EDTA, 0.1 M Tris-HCl, pH 8.0	2–3 min at 100 °C	Striated muscles of carp, goldfish, bull frog, newt, turtle, snake, chicken, rabbit	Decrease in mobility of α-connectin was in the following order: fish muscles > frog, newt, chicken, snake, rabbit muscles > turtle muscles. As standard, chicken breast muscle was used (MWs of α- and ß-connectins were estimated to be 2.8 and 2.1 × 10^6, respectively).	This procedure was a modification of the Weber–Osborn method (1969)
4. Wang and Wright 1988	2–12% gradient polyacrylamide slab gel, 8 × 10 cm	50/1	40 mM Tris-acetate, 20 mM Na-acetate, 2 mM EDTA, 0.1% SDS, pH 7.6	1% SDS, 5–10% sucrose, 1 mM EDTA (pH 8.0), 40 mM DTT, 10 pg/ml of pyronin Y (tracking dye), 10 mM Tris-HCl, pH 8.0	90 s at 95 °C	Rabbit striated muscles (longissimus dorsi, psoas, semitendinosus, soleus, diaphragm, adductor magnus, sartorius, heart)	Decrease in mobility of T1 was in the following order: heart > psoas > adductor magnus > longissimus dorsi > semitendinosus, soleus, diaphragm, sartorius.	Muscle tissues were snap-frozen in liquid nitrogen and pulverized to fine powders. Snap-frozen tissue powders were treated with SDS sample buffer preheated to 95 °C. This procedure was a modification of the Somerville and Wang method (1981)
5. Fritz et al. 1989	10% polyacrylamide slab gel (small resolving gel, 6 × 8.2 × 0.075 cm) and 4–10–15% polyacrylamide slab gel (layer resolving gel (12 × 14 × 0.15 cm, each layer 4 cm high).	200/1	0.75 M Tris, 10% glycerol, 0.1% SDS, pH 8.6, 8.8, 9.3	8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT, 25 mM Tris-HCl, pH 6.8.	Samples were boiled for 2–8 min	Myofibrils from rabbit psoas and bovine rectus abdominis muscle	–	Myofibrils were prepared in a buffer containing 50 mM KCl, 20 mM Tris, pH 7.0, 2 mM EDTA, 4 mM MgCl$_2$, 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 1% Triton X-100. Myofibrils were stored at −20 °C in rigor buffer containing 75 mM KCl, 10 mM KH$_2$PO$_4$, 2 mM MgCl$_2$, 2 mM EGTA, 50% (v/v) glycerol, pH 7.0.

Table 1 (continued)

Reference	Type and size of gel, percentage of acrylamide (agarose)	Ratio of acrylamide and bis-acrylamide	Gel buffer, pH	Sample buffer, pH	Heating of samples	Tissue/muscle source	Molecular mass of titin (connectin)	Nuances
								Migration rates of titin and nebulin in gel are especially affected by 2-mercaptoethanol inclusion in the upper buffer.
6. Wang et al. 1991	2–12% gradient polyacrylamide slab gel, 8 × 10 cm	50/1	40 mM Tris-acetate, 20 mM Na-acetate, 2 mM EDTA, 0.1% SDS, pH 7.6	1% SDS, 5–10% sucrose, 1 mM EDTA (pH 8), 40 mM DTT, 10 pg/ml of pyronin Y (tracking dye), 10 mM Tris-HCl, pH 8	90 s at 95 °C	Rabbit striated muscles (adductor magnus, psoas, longissimus dorsi, sartorius, soleus, semitendinosus, cardiac muscle)	Molecular mass of T1: AM and PS, 2.8 MDa; LD and SA, 2.88 MDa; SO and ST, 2.94 MDa. T2: 2.4 MDa. Cardiac muscle displayed the smallest titin.	Muscle tissues were snap frozen, pulverized in liquid nitrogen, solubilized in hot SDS
7. Horowits 1992	2–12% gradient polyacrylamide slab gel	50/1	40 mM Tris-acetate, 20 mM Na-acetate, 2 mM EDTA, 0.1% SDS, pH 7.6	1% SDS, 10% glycerol, 1 mM EDTA (pH 8), 70 mM β-mercaptoethanol, 0.05% bromophenol blue, 10 mM Tris-HCl, pH 8	Samples were boiled for 1–2 min	Chemically skinned strips of rabbit psoas and soleus muscles	Soleus titin migrated in gel slightly slower than psoas titin.	Strips of rabbit psoas and soleus muscle were chemically skinned (see the article for more details). After measuring its mechanical properties and dimensions, each single fiber was dissolved in sample buffer.
8. Granzier and Wang 1993	3.3–12% gradient polyacrylamide slab gel, 8 × 10 × 0.075 cm	50/1	40 mM Tris-acetate, 20 mM Na-acetate, 2 mM EDTA, 0.1% SDS, pH 7.6	Laemmli (1970) sample buffer (75 mM Tris-Cl, 3% w/v SDS, 120 mM DTT, 55 μg/mL Pyronin Y, 15% v/v glycerol, pH 6.8) was used. Sample buffer of Fritz et al. (1989) (8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT, 25 mM Tris-Cl, pH 6.8) was also used.	23–100 °C for 1–8 min. Optimal solubilization temperatures for fibers were in the range of 55–75 °C.	Skinned rabbit psoas fibers.	T1–2.8 MDa; T2–2.4 MDa; A faint band above T1 was detected.	Skinned rabbit psoas fibers were used (see the article for more details). Presoaking in a low ionic strength alkaline buffer was identified as an important step in obtaining complete solubilization of titin from purified myofibrils.
9. Kawamura et al. 1994	2.3–4% polyacrylamide tube gel, 0.6 × 10 cm	36.5/1	0.375 M Tris-HCl, 0.1% SDS, pH 8.8	10% (w/v) SDS, 40 mM DTT, 10 mM EDTA, 0.1 M Tris-HCl buffer, pH 8.0	Samples were boiled for 2 min	Skeletal muscles of lamprey, electric ray, horse mackerel and chicken.	The mobility of ray α-connectin in gel was slower than that of chicken and other animals α-connectins.	SDS gel electrophoresis was carried out according to Laemmli (1970). The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine, 0.1% SDS
10. Granzier and Irving 1995	Slab gel	–	–	50 mM Tris-Cl, 2% SDS, 10% glycerol,	90 s at 90–95 °C		2.49 ± 0.03 MDa for rat cardiac T1	SDS gel electrophoresis was carried out according to

Table 1 (continued)

Reference	Type and size of gel, percentage of acrylamide (agarose)	Ratio of acrylamide and bis-acrylamide	Gel buffer, pH	Sample buffer, pH	Heating of samples	Tissue/muscle source	Molecular mass of titin (connectin)	Nuances
				80 mM DTT, 30 µg/ml Pyronin Y, pH 6.8		Rat heart and rabbit skeletal muscles (semitendinosus and psoas)		Granzier and Wang (1993). Muscle tissues were quick-froze in liquid nitrogen and pulverized to a fine powder.
11. Tatsumi and Hattori 1995,	2% polyacrylamide slab gel strengthened with agarose (0.5%), 8.3 × 7.2 × 0.15 cm	20/1 (Fairbanks et al. 1971) 37.5/1 (Laemmli 1970)	40 mM Tris-acetate, 20 mM Na-acetate, 2 mM EDTA, 0.1% SDS, pH 7.4 (Fairbanks buffer system) (1971). 0.375 M Tris-HCl, 0.1% SDS, pH 8.6 (Laemmli's buffer system)	1% SDS, 1% β-mercaptoethanol, 5 mM EDTA, 5 mM Tris-HCl, pH 8.0, 10% glycerol, 30 µg/ml leupeptin	Samples were boiled for 2 min	Myofibrils of chicken breast muscle (M. pectoralis superficialis) and rabbit skeletal muscles (M. longissimus thoracis, m. soleus)	α-connectin of rabbit muscles had a lower mobility in gel than chicken muscle α-connectin.	Protein samples were frozen at −80 °C until use. Glass cell were cooled for 5 min before pouring of gel. Laemmli's system provided higher resolution to titin and nebulin.
12. Spierts et al. 1997	2.4–12% gradient polyacrylamide slab gel	–	–	50 mM Tris-Cl, 2% SDS, 10% glycerol, 80 mM DTT, 30 µg/ml Pyronin Y, pH 6.8	90 s at 80 °C	Red and white axial muscles of carp (Cyprinus carpio L.)	Psoas and semitendinosus muscles from rabbit were used as high-molecular-mass standards, and titin of these muscles had a lower mobility than carp muscle T1.	Fiber bundles were quick frozen in liquid nitrogen and pulverized to a fine powder.
13. Cazorla et al. 2000	2–9.5% gradient polyacrylamide slab gel	–	–	–	–	Human soleus, mouse, rat, rabbit, dog, bovine, pig, cow and human myocardium.	Two T1 bands in cardiac muscle and one T1 band in m. soleus were detected. Using the mobility of human soleus T1 (3.7 MDa) and rat cardiac titin T1 (2.97 MDa, bottom band) as standards the authors showed that molecular mass of top cardiac T1 band is ~3.3 MDa. In addition to T1 and T2 some samples also contained a band that barely entered the gel. Western blotting with titin-specific antibodies indicated that this band is titin. The authors suggested that this is titin aggregates.	Muscle samples were quick-frozen in liquid nitrogen, pulverized, and then rapidly solubilized. SDS gel electrophoresis was carried out according to Granzier and Irving (1995).
14. Warren et al. 2003a, b	1% agarose slab gel, 16 × 18 × 0.15 cm	–	30% v/v glycerol, 50 mM Tris-base,	8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT,	100 °C for 3 min, 60 °C for 10–20 min	Rabbit tibialis anterior, semitendinosus, soleus, psoas, left ventricle, right	Decrease in mobility of titin was in the following order: cardiac N2B > cardiac N2BA	Skeletal and cardiac tissue was dissected from New Zealand rabbits (2–3 kg), mongrel

Table 1 (continued)

Reference	Type and size of gel, percentage of acrylamide (agarose)	Ratio of acrylamide and bis-acrylamide	Gel buffer, pH	Sample buffer, pH	Heating of samples	Tissue/muscle source	Molecular mass of titin (connectin)	Nuances
			0.384 M glycine, and 0.1% w/v SDS, pH 8.5 (no pH adjustment necessary).	0.03% bromophenol blue, and 0.05 M Tris-Cl, pH 6.8.		ventricle, dog triceps, left ventricle, rat soleus. The cardiac samples contain N2BA and N2B isoforms whereas the skeletal samples contain only N2A type of titin.	> N2A (rabbit psoas) > N2A (rabbit tibialis anterior, semitendinosus, soleus, rat soleus) > dog triceps. The rabbit psoas muscle has two differently sized N2A bands. The rabbit left and right ventricles have two N2BA bands. Two faint bands above N2B and N2BA were detected. The authors suggested that this is titin aggregates.	dogs, or Sprague Dawley rats and flash frozen in liquid nitrogen. The frozen tissue was pulverized and placed in preweighed Dounce homogenizers. The sample heating comparisons showed that the optimal temperature for solubilization of cardiac titin with minimal breakdown was 60 °C for 10 or 20 min.
15. Neagoe et al. 2003	Agarose-strengthened 2.0% polyacrylamide slab gels with a Laemmli buffer system (Tatsumi and Hattori 1995)	37.5/1	0.375 M Tris-HCl, 0.1% SDS, 0.5% agarose, pH 8.6	1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 8 μg/ml leupeptin, 6 μM bromphenol blue, 4.3 mM Tris-HCl, pH 8.8, 4.3 mM EDTA	Samples were boiled for 3 min	Frog, mouse, hamster, rat, rabbit, cat, cow and human heart, rabbit soleus (freshly excised and frozen muscle tissue).	N2B titin isoform – 3000 MDa; N2BA isoforms – 3.25–3.4 MDa. Rabbit soleus N2A-titin (3.7 MDa) and skeletal nebulin (700–800 kDa) were used as markers for molecular weight detection.	Samples were solubilized by quickly homogenizing 30–60 mg of frozen tissue in 100 μl ice-cold relaxing buffer supplemented with 40 μg/ml leupeptin. Samples were centrifuged briefly and the pellet fraction was used for further analysis.
16. Vikhlyantsev and Podlubnaya 2006	Horizontal 1.3% polyacrylamide gel strengthened with agarose (0.5%), 8.5 × 12.5 × 0.2 cm	37.5/1	0.5 M Tris-HCl, 0.1% SDS, pH 9.0	10 mM Tris-HCl, 1.5% SDS, 1% β-mercaptoethanol, 10% glycerol, 2.5 mM EGTA, 8 μg/ml leupeptin or E64, pH 7.0	Fresh or frozen muscle tissues were incubated in sample buffer for 30–40 min at 20–25 °C (reference samples), after which a small amount of the protein extract was taken for subsequent heating at: 35–60 °C for 10 min, 65–75 °C for 5 min, 80–90 °C for 2–4 min, 95–100 °C for 1–4 min.	Mouse, rat, rabbit, ground squirrel and human striated muscles (freshly excised and frozen muscle tissue).	One or two titin bands (called NT) above N2A, N2BA, N2B and T2 bands were detected. Using the mobility of human and animal nebulin (770–890 kDa) and myosin heavy chain (205 kDa) as standards the authors showed that molecular weights are: 2.08–2.30 MDa (T2), 2.42–2.45 MDa (N2B), 2.56–2.8 MDa (N2A), 3.23–3.3 MDa (cardiac NT), 3.38–3.73 MDa (skeletal NT).	Fresh muscle tissues and frozen in liquid nitrogen were used. Frozen muscles were stored at –80 °C. Recommended temperature for solubilization of titin without breakdown is 35–40 °C.

no nucleic acids in the bands, although western blots with 9D10 antibodies revealed titin bands (Vikhlyantsev et al. 2004a). Our subsequent studies showed that these titin bands appear in the electrophoregrams of striated muscles of humans (Vikhlyantsev and Podlubnaya 2008) and other mammals, including mouse, rat, rabbit (Vikhlyantsev and Podlubnaya 2006; Ulanova et al. 2015), ground squirrel (Vikhlyantsev et al. 2008,), Mongolian gerbil (Vikhlyantsev et al. 2011; Okuneva et al. 2012), and bear (Salmov et al. 2015). Titin isoform composition in striated muscles in other groups of vertebrates (amphibians and birds) revealed no NT bands (Vikhlyantsev and Podlubnaya 2012).

The content of NT titins in the muscles of small rodents was 10–20% compared to 30–40% for ground squirrel, bear and humans. Using human and animal skeletal muscle myosin heavy chain (205 kDa) and, nebulin (770–890 kDa), as well as the N2A titin isoform (~3600 kDa and 3700 kDa) of rabbit and human soleus as standards (Wang et al. 1991; Krüger et al. 1991; Granzier and Wang 1993; Prado et al. 2005), we estimated that the NT have a Mr of ~3.8–3.9 × 10^6 (Vikhlyantsev and Podlubnaya 2006). Expression of titin isoforms with these molecular weights is not excluded (Bang et al. 2001; Guo et al. 2010, 2012; Li et al. 2012), but titin aggregates in gels could not be excluded either (Granzier and Wang 1993; Cazorla et al. 2000; Warren et al. 2003a). 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.

Accordingly, we tried to increase the time of electrophoresis in the vertical agarose-strengthened 2.0–2.3% polyacrylamide gels (8 × 10 × 0.1 cm) from 2 to 4–5 h. However, this led to a strong diffusion of titin bands. Then, we tried using 1.7–1.8% instead of 2.0–2.3% polyacrylamide gels, but the attempt failed because the gels slid down between the glass plates or broke during electrophoresis. We then developed a horizontal agarose-strengthened 8.5 × 12.5 cm gel system using 1.3% polyacrylamide and 0.5% agarose (Table 1, Reference 16). The gels showed that mobility of the NT bands varied greatly between muscle sources.

Using human and animal skeletal muscle myosin heavy chain (205 kDa) and nebulin (770–890 kDa) as standards, we estimated the molecular masses of N2A, N2BA, N2B, T2 and NT titin bands. The results obtained were impressive. The NT bands were 3230–3730 kDa, compared to the N2A, N2BA, N2B and T2 bands (Mr ~2.1–2.8 × 10^6) (Table 1, Reference 16), that corresponded to a set of values for T1 (α-connectin) and T2 (β-connectin) (Maruyama et al. 1984; Wang et al. 1991; Granzier and Wang 1993). Similar data were obtained for vertical agarose-strengthened 1.9% polyacrylamide gels (14.5 × 16.0 × 0.15 cm). In Fig. 1, the gels resolved a doublet band at 3300–3400 kDa for cardiac muscle and a singlet band of 3600–3700 kDa for skeletal muscles of mammals. We therefore hypothesized that the NT bands are
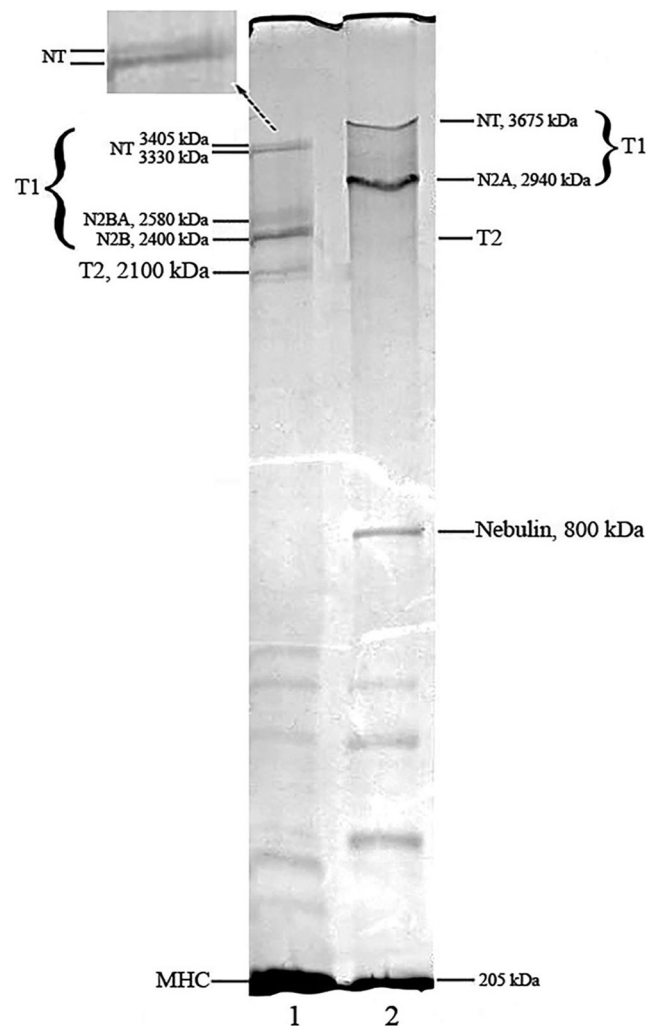

Fig. 1 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis of titin isoforms in striated muscles of ground squirrel (*Spermophilus undulatus*); a modified view from (Vikhlyantsev and Podlubnaya 2008). *1* Myocardium (left ventricle), *2* m. soleus. Protein bands are indicated: heavy chains of myosin (MHC); nebulin–cytoskeletal protein of thin filaments; proteolytic fragments of titin (*T2*); intact titin (*T1*); isoforms of T1 (N2B, N2BA, N2A, NT). Vertical agarose-strengthened 1.9% polyacrylamide gel (14.5 × 16.0 × 0.15 cm) was used to separate the titin isoforms. Enlarged fragment of the gel with NT-titin bands is in the *inset*. As standards for estimation of molecular weights, the MHC (205 kDa), nebulin (770–890 kDa), titin-2 (2100–2400 kDa) of rabbit and human striated muscles were used (Wang et al. 1991; Krüger et al. 1991; Granzier and Wang 1993)

intact N2A, N2BA, N2B titin isoforms (Vikhlyantsev and Podlubnaya 2008).

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 (Vikhlyantsev and Podlubnaya 2008), thereby agreeing with our hypothesis. Although this requires further research, we cannot exclude the possibility that the NT bands are the other protein immunologically identical to titin. Agarose-strengthened 2.1% polyacrylamide gels revealed no NT bands

in rat heart in day 19 embryos or 2–14 days neonatal hearts (Fig. 2; unpublished data) but NT titin was detected in rats 30 days old (data not shown).

Peculiarities of sample preparation for electrophoresis study of titin

Granzier and Wang (1993) paid particular attention to sample preparation of titin. They found that titin was extraordinarily sensitive to proteolysis in situ by endogenous proteases and by proteases in buffers contaminated with bacteria and fungi. Even in SDS-solubilized myofibril samples, appreciable degradation of titin by residual protease activity can occur in a few days at room temperature (Wang 1982, 1985; Granzier and Wang 1993). To limit proteolysis, the authors suggested the inclusion of protease inhibitors in SDS samples prior to electrophoresis. Leupeptin, E-64, and a protease inhibitor cocktail

Fig. 2 Perinatal titin isoform switching in rat heart. *1* Adult rat heart expresses N2B, N2BA and NT-titins. *2* Fetal hearts 3 day before birth (e19) expresses more long (embryonic) N2BA-isoform of titin and N2B-titin. *3, 4* Two days after birth (*3*) and 14 days after birth (*4*), N2B and N2BA-titins are expressed in rat heart. *MHC* Heavy chains of myosin; *T1* intact titin; *T2* proteolytic fragments of titin. Vertical agarose-strengthened 2.1% polyacrylamide gel (8 × 10 × 0.1 cm) was used to separate the titin isoforms

(Sigma) have been used to inhibit proteolytic degradation of titin (Granzier and Irving 1995; Tatsumi and Hattori 1995; Linke et al. 1997; Neagoe et al. 2003; Warren et al. 2003a; Vikhlyantsev and Podlubnaya 2006). In order to attain better solubilization of titin, it has been proposed to use urea–thiourea SDS DTT sample buffer (Fritz et al. 1989; Warren et al. 2003a). It is conceivable that urea–thiourea may facilitate the access of SDS to titin by rapidly solubilizing other myofibrillar proteins in the sarcomere lattice.

Heat treatment of the samples is also important. SDS samples are usually prepared by heating them in boiling water for 2–5 min, a process thought to promote denaturation of proteins and facilitate disulfide reduction. Other authors have shown that boiling degrades titin (King and Kurth 1980; Granzier and Wang 1993; Mitsuhashi et al. 2002; Warren et al. 2003a). Samples heated at 100 °C had less intact titin and more breakdown products (smears migrating near the bottom of the gel) than those at 60 °C (Warren et al. 2003a). A temperature of 50–60 °C for 10–20 min has been considered optimal for the extraction and preservation of intact titin (Granzier and Wang 1993; Mitsuhashi et al. 2002; Warren et al. 2003a). We recommended heating titin in SDS at 35–40 °C for 30–40- min (Vikhlyantsev and Makarenko 2000; Vikhlyantsev and Podlubnaya 2012).
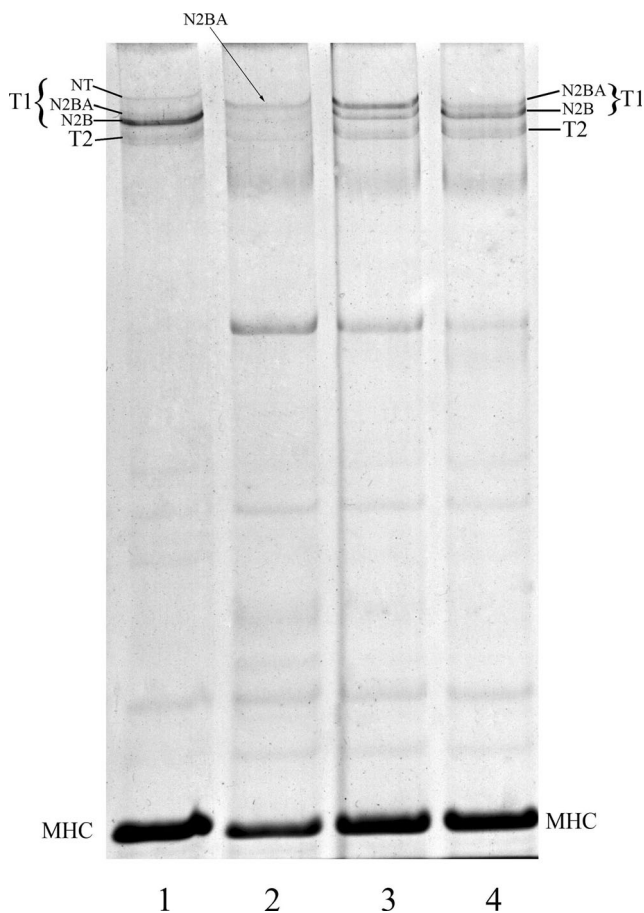
Other details of the electrophoretic study of titin

Titin has a tendency to aggregate during electrophoresis, especially in gel systems that use a stacking gel or discontinuous buffers (Granzier and Wang 1993). To prevent disulfide cross-linking, Fritz et al. (1989), as well as Greaser and Warren (2009, 2012), recommended the inclusion of β-mercaptoethanol in the top anodic buffer. Granzier and Wang (1993), using 3.3–12% linear gradient gels, noted the importance of polymerizing the gel for at least 12 h prior to sample application. Titin bands are often diffuse in non-aged gels, presumably because the low percentage acrylamide near the top took much longer to properly polymerize (Granzier and Wang 1993).

Agarose-strengthened 2% polyacrylamide gels exhibit some peculiar characteristics. To prevent polyacrylamide polymerizing before agarose is polymerized, Tatsumi and Hattori (1995) cooled the 40 °C agarose solution 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.

Our recommendation is to perform electrophoresis using macroporous, agarose-strengthened polyacrylamide gels at low currents. For instance, Neagoe (of Wolfgang Linke's group) noted that the best separation for the high molecular weight proteins was obtained by running the electrophoresis

overnight at 2 mA per 8.6 × 7.7 cm gel (Neagoe 2008). Similarly, we performed the initial electrophoresis of 8.0 × 10.0 × 0.10 cm gels at 3 mA for 40 min, then increasing the current strength up to 7–8 mA. It is recommended to refresh the tank buffer once at 2.5 h to limit pH changes caused by electrolysis during electrophoresis (Granzier and Wang 1993).

Conclusion

From the above discussion, it is clear that SDS-PAGE electrophoresis of titin is a complex procedure that is certainly not routine. The combination of giant molecular mass and the susceptibility of titin to degrade during preparation compound the problem. Thus, for a successful study of titin by electrophoresis, one must follow three main rules: (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 formats for analyzing titin are: (1) vertical agarose-strengthened 2% polyacrylamide gel (Tatsumi and Hattori 1995); (2) vertical 1% agarose gel (Warren et al. 2003a); and (3) horizontal agarose-strengthened 1.3% polyacrylamide gel (Vikhlyantsev and Podlubnaya 2006).

Compliance with ethical standards

Conflict of interest Ivan M. Vikhlyantsev declares that he has no conflicts of interest. Zoya A. Podlubnaya declares that she has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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