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Enhanced Electrophoretic Separation and Resolution of Myosin Heavy Chains in Mammalian and Avian Skeletal Muscles

Eric R. Blough,* Erik R. Rennie,* Fanjie Zhang,† and Peter J. Reiser*,†,[1]
Department of Exercise Science and †Department of Oral Biology, The Ohio State University, Columbus, Ohio 43210

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We report a sodium dodecyl sulfate–polyacrylamide gel electrophoresis protocol for the reliable separation, with high resolution, of myosin heavy chain isoforms in adult avian (chicken) and mammalian (mouse) skeletal muscles. The sample preparation time can be relatively short, thereby minimizing endogenous proteolytic activity which may otherwise result in dispersed and spurious bands. Inclusion of 2-mercaptoethanol in the upper electrode buffer greatly improves band resolution. Glycerol is commonly included in the reported protocols for myosin heavy chain separation and our results demonstrate that the concentration of glycerol employed can have a marked effect on the relative order of migration among myosin heavy chain isoforms. © 1996 Academic Press, Inc.

Skeletal muscles are composed of a large number of fibers with different physiological and biochemical properties (1). The myosin heavy chain (MHC)[2] molecule is the most abundant myofibrillar protein on a weight basis and plays a crucial role in muscle contraction. For example, maximum velocity of shortening of single muscle fibers (V_{max}) is strongly correlated with MHC isoform composition (2). Skeletal muscle demonstrates a remarkable ability to alter its protein expression during development and in response to a variety of stimuli. The MHC molecule is encoded by a multigene family (3) and MHC expression has been shown to proceed in a tissue-specific and developmentally regulated manner (4). Alterations in MHC expression have been found subsequent to changes in contractile activity, circulating thyroid level, innervation status, and in response to neuromuscular disease (1). Due to these reasons and because of its critical physiological importance, assessment of MHC isoform expression is essential for a further understanding of skeletal muscle contractile properties during development, aging, and disease.

Fast and slow MHC isoforms from avian (5) and mammalian (6) muscle were first successfully separated using SDS–PAGE employing acrylamide percentages between 5 and 7.5%. Danieli-Betto *et al.* (7) were able to resolve both the IIA and IIB MHCs in rat skeletal muscle using 6% SDS–PAGE after increasing the gel glycerol content to 40%. Several laboratories have separated another mammalian MHC isoform, referred to as IID or IIX (8–10), using gradient SDS–PAGE, or after extensive myofibril (11) or myosin isolation (12, 13). The gels utilized in these studies consisted of low percentages of acrylamide and high glycerol concentrations, both of which compromise the mechanical properties of the gel. Alteration of glycerol concentration and pH and the inclusion of 2-mercaptoethanol have been shown to alter the electrophoretic mobility and resolution of large myofibrillar proteins (>500 kDa) (14, 15). To our knowledge, observations concerning MHC separation and these variables have not been adequately described. A simple method of electrophoretic separation of MHC isoforms is reported here. Adjustment of the acrylamide percentage and glycerol concentration in the gels, along with the inclusion of 2-mercaptoethanol in the electrode buffer, allows for consistent separation of four mouse and seven chicken MHCs.

METHODS

Preparation of Muscle Samples

All animals from which muscle samples were obtained for this study were cared for and sacrificed in accordance with an institutionally approved animal care and use protocol. A muscle is quickly removed and placed in cold relaxing solution which has the following

[1] To whom correspondence should be addressed.
[2] Abbreviations used: MHC, myosin heavy chain; DTT, dithiothreitol; bis, *N,N′*-methylene-bis(acrylamide); TEMED, *N,N,N′,N′*-tetramethyethylenediamine.

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composition: 2.0 mM EGTA, 4.4 mM MgATP, 10.0 mM imidazole, 1.0 mM Mg^{2+}, sufficient KCl to achieve a final ionic strength of 180 mM and pH 7.00 (2). The muscle is pinned to a silicone elastomer lining (Sylgard 184, Dow Corning; Midland, MI) in a petri dish which is filled with relaxing solution and placed on a chilled stage of a dissecting microscope. Bundles (1–2 mm thick) of fibers are cut parallel to fiber longitudinal axis, tied to glass capillary tubes, and placed in glycerinating solution (relaxing solution prepared with 50% (v/v) glycerol and leupeptin at a final concentration of 5 μg/ml). Bundles are stored in this solution for several days at −22°C. Small pieces are cut from the bundles (3–4 mg total, typically from four bundles prepared from a given muscle) in cold relaxing solution and macerated utilizing forceps. Large pieces of connective tissue are removed. The pieces are then blotted to remove excess relaxing solution, weighed and placed in 30 μl of sample buffer per milligram of sample in a 1.5-ml microcentrifuge tube. The sample is then vortexed, placed in a 100°C heating block for 2 min, and immediately placed on ice for 5 min. The sample is then centrifuged at 12,000*g* for 1–2 min. The supernatant is transferred to another microcentrifuge tube and the pellet is discarded. All of the gels shown in this report were run with samples prepared as described above. The advantage of preparing samples in this manner is that most of the soluble, nonsarcomeric proteins diffuse out of the muscle fibers and the samples are, therefore, reduced to essentially myofibril preparations. This greatly facilitates the identification of proteins on stained gels, especially in the low-molecular-weight region (<45 kD) in other studies. However, identical MHC separation is obtained (not shown) when small pieces of fresh muscle are processed in the same manner as described above for small pieces which are cut from a glycerinated bundle.

Composition and Preparation of Sample and Electrode Buffers

Several sample buffers have been tested and found to be variable in terms of the amount of myosin heavy chain extracted from the sample and flatness of the heavy chain bands on stained gels. The sample buffer that consistently yielded the best results consists of 8 M urea, 2 M thiourea, 0.05 M Trizma base, 0.075 M dithiothreitol (DTT), 3% (w/v) SDS, pH 6.8, and 0.004% (w/v) bromophenol blue. The urea and thiourea are initially dissolved with stirring and mild heating. This solution is stirred for 15 min with 25 g of a mixed bed resin (AG 501-X8, Bio-Rad Laboratories) and filtered and the resin is discarded. The other ingredients are added and the pH is adjusted. This solution is filtered and stored in 1-ml aliquots at −22°C and completely thawed and mixed before use. This buffer is identical to one described by Fritz *et al.* (14) except the Tris concentration in our buffer is greater and DTT is substituted for 2-mercaptoethanol.

The lower electrode buffer in the electrophoresis unit consists of 25 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3. The upper electrode buffer is identical except 800 μl of 2-mercaptoethanol is added per liter.

Composition and Preparation of Gels

All of the gels in this study were run in a Hoefer SE 600 gel unit at 18°C with a Hoefer PS 500 XT power supply. The gels are 14 cm wide, 16 cm long, and either 0.5 or 0.75 mm thick. The stacking gels consist of either 2.9 or 4.0% (w/v) acrylamide (with no apparent difference in MHC isoform separation between these two percentages) with an acrylamide:*N,N′*-methylene-bis(acrylamide) (bis) ratio of 20:1, 0.125 M Tris, pH 6.8, and 0.1% (w/v) SDS. Five milliliters of the gel solution is degassed before adding 30 μl of fresh 10% (w/v) ammonium persulfate and 6 μl of TEMED for polymerization.

Our standard separating gels consist of 9% (w/v) acrylamide with an acrylamide:bis ratio of 200:1, 0.75 M Tris, pH 8.8, and 0.1% (w/v) SDS. Fifteen milliliters of the gel solution is degassed before adding 120 μl of fresh 10% (w/v) ammonium persulfate and 12 μl of TEMED. The separating gel is poured and allowed to polymerize for 1 h before pouring the stacking gel which, in turn, is allowed to polymerize for 30 min before loading samples.

Gel Fixation and Staining

Fixation and silver-staining of gels is performed according to Guilian *et al.* (16) with modifications. The gels are initially fixed in a solution consisting of 50% (v/v) ethanol and 10% (v/v) acetic acid for approximately 1 h and are then directly transferred to a solution of 5% (v/v) glutaraldehyde for 12–24 h. The gels are washed in distilled water for approximately 5 h with six water changes during this time. The staining step is a 6-min soak in the silver stain solution consisting of 46 mM silver nitrate, 207 mM ammonium hydroxide, and 18.9 mM NaOH. The gels are washed three times for 5 min each in distilled water. Color development is performed in a solution consisting of 0.24 mM citric acid and 1.9% (w/v) formaldehyde. The gels are transferred from one development tray to a second tray, containing the identical development solution, as soon as the myosin heavy chain bands are first observed. The gels are kept in the second development tray until color development of the protein bands is complete and are then transferred to 5% (v/v) acetic acid for approximately 1 min to prevent background staining. Gentle agitation of the gels is employed during the entire process. The gels are stored in distilled

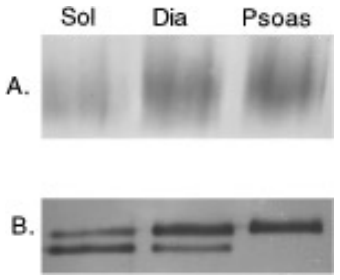

FIG. 1. Effect of 10 mM 2-mercaptoethanol on myosin heavy chain isoform resolution during electrophoresis. The same samples from rabbit soleus (Sol), diaphragm (Dia), and psoas muscles were electrophoresed on identically prepared gels (9% acylamide, 200:1 acrylamide:bis, 12% glycerol) which were run under the same conditions (constant current throughout at 12 mA/gel initially and 25 mA/gel when the dye front reached the interface between the stacking and separating gels) without (A) or with (B) 2-mercaptoethanol in the upper electrode buffer.

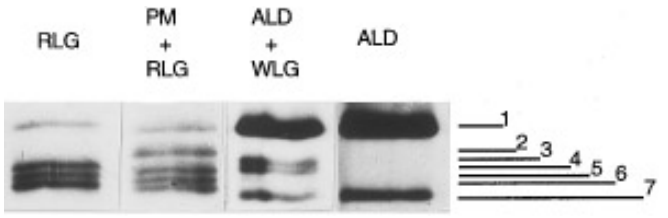

FIG. 2. Resolution of seven myosin heavy chain (MHC) isoforms in samples from adult chicken anterior latissimus dorsi (ALD), red portion of the lateral gastrocnemius (RLG), white portion of the lateral gastrocnemius (WLG), and pectoralis major (PM) muscles. The two slow-type MHC isoforms, SM2 and SM1, are labeled as bands 1 and 7, respectively. Adult fast MHC, found in the PM, is labeled as band 2. Bands 3–6 are other unidentified embryonic or neonatal MHC isoforms that others have shown to be expressed in adult lateral gastrocnemius muscle (see Discussion).

water until prepared for drying which is performed as in Guilian *et al.* (16).

Chemicals

Acrylamide, bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate, TEMED, DTT, 10× electrode buffer, and ion exchange resin (AG 501-X8) were purchased from Bio-Rad Laboratories (Hercules, CA). Urea (enzyme grade) was obtained from Life Technologies, Inc, (Gaithersburg, MD). All other chemicals utilized in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Fritz *et al.* (14) demonstrated that inclusion of 2-mercaptoethanol in the upper electrode buffer, to a final concentration of 10 mM, resulted in much sharper bands of very high molecular weight proteins. We observed the same, very striking effect on the MHC isoform bands which are electrophoresed to near the bottom of our gels, as shown in Fig. 1. All of the other gels shown in this report were run with the same concentration (i.e., 10 mM) of 2-mercaptoethanol in the upper electrode buffer.

The separation of seven MHC isoforms that are expressed in chicken skeletal muscles is shown in Fig. 2. The two slow-type MHC isoforms that are expressed virtually exclusive of other MHC isoforms in posthatch anterior latissimus dorsi muscle, i.e., SM1 and SM2, migrate faster and slower, respectively, of all the fast-type MHC isoforms on what is currently our standard gel for separating these isoforms (9% acrylamide, 12% glycerol, acrylamide:bis = 200:1). Separation of four MHC isoforms, which are expressed in adult mouse diaphragm, is also consistently achieved with the identical gel and running conditions, as shown in Fig. 3. The greatest separation is achieved when these gels are run for 21 h at constant current throughout with current initially set so that the voltage is 50 V and increased so that the voltage is initially 200 V when the tracking dye migrates to the interface between the stacking and separating gels. The final voltage for these gels varies between approximately 375 and 425 V with different gel runs. The MHC bands migrate to within about 4 cm from the bottom of the separating gel under these conditions. Identification of the mouse MHC isoforms in this study was based on the histochemically determined fiber-type compositions of adult mouse muscle as reported by Parry and Zardini (17).

Increasing the glycerol concentration in the separating gel to 40% has a marked effect on the migration order of chicken MHC isoforms (Fig. 4), similar to the effect of glycerol on fish MHC isoform migration order, as reported by Huriaux *et al.* (15). Both slow-type MHC isoforms, i.e., SM1 and SM2, migrate faster than embryonic, neonatal, and adult pectoralis muscle isoforms in 40% glycerol gels but SM1 still migrates faster than SM2. Also, the migration of neonatal MHC relative to adult MHC is reversed between 12 and 40% glycerol such that neonatal MHC migrates slower than adult MHC at the higher glycerol

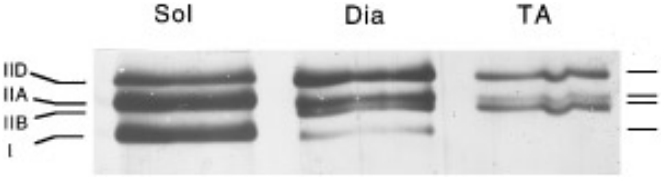

FIG. 3. Resolution of four myosin heavy chain (MHC) isoforms in samples from adult mouse soleus (Sol), diaphragm (Dia), and deep portion of the tibialis anterior (TA) muscles. The identification of the MHC isoforms is based on the correspondence between relative band intensities for each sample and the reported histochemically determined fiber-type proportions (17).

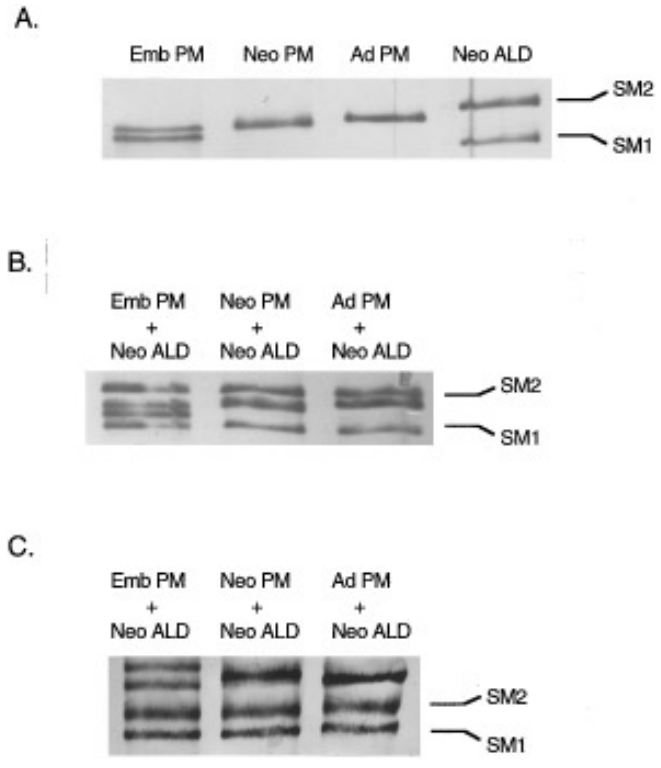

FIG. 4. The effect of different concentrations of glycerol in the separating gel on the relative electrophoretic migration order of chicken myosin heavy chain isoforms. Samples of embryonic (Emb), neonatal (Neo), and adult (Ad) pectoralis major (PM) and anterior latissimus dorsi (ALD) were electrophoresed in different lanes in the separating gel shown in A which contained 12% (v/v) glycerol. Embryonic, neonatal, and adult PM samples were each coelectrophoresed with neonatal ALD in different lanes in the separating gel shown in B which also contained 12% (v/v) glycerol. The same samples as in B were run on a separating gel which contained 40% (v/v) glycerol, as shown in C. Note that the slow-type MHC isoforms, SM1 and SM2, migrate ahead and behind, respectively, of the fast-type embryonic, neonatal, and adult MHC isoforms in the presence of 12% glycerol but both of the slow-type isoforms migrate ahead of the embryonic, neonatal, and adult PM MHC isoforms in the presence of 40% glycerol. SM2 migrates behind SM1 at both concentrations of glycerol as determined by electrophoresing samples from neonatal ALD (containing approximately equal amounts of SM1 and SM2) and adult ALD (predominantly SM2) (e.g., Ref. 27) on gels containing 12 and 40% glycerol (not shown). Note also that the migration order of isoforms in the fast MHC family is altered between 12 and 40% glycerol.

concentration. There is also a reversal of the adult fast and embryonic MHC isoform migration order between our standard separating gel with 12% glycerol and that reported by Talmadge and Roy (11) with 30% glycerol (not shown). These results clearly demonstrate that identification of specific MHC isoforms must not be based solely on electrophoretic migration order.

DISCUSSION

The present study details a simple and reliable protocol utilizing a uniform acrylamide concentration that allows consistent electrophoretic separation of four mammalian and seven chicken MHC isoforms. To our knowledge, most previous reports demonstrating MHC separation of this quality have included a time- and labor-intensive myosin extraction (9, 13, 18, 19) or myofibril isolation (11, 20). The sample preparation procedure outlined here can be completed in less than 20 min. The 8 M urea and 2 M thiourea, contained in the modified sample buffer of Fritz *et al.* (14), may be of sufficient strength to extract most muscle proteins without the need for further processing. Additionally, the presence of 8 M urea sufficiently increases sample density which facilitates loading the samples onto the gel.

Adequate reduction of protein disulfides has been found to be a key factor for the optimal resolution of very high molecular weight proteins, such as nebulin and titin (14). We have also found this to be extremely advantageous for the separation of MHC isoforms, especially during long gel runs (Fig. 1). As discussed by Fritz *et al.* (14), disulfide interchange from sulfhydryl oxidation is quite possible considering that many of the commonly employed resolving buffers are titrated to high pH values (8.8–9.3) (21). Therefore, 2-mercaptoethanol in the upper buffer chamber may break intramolecular disulfide bonds and prevent the formation of intermolecular disulfide bonds during electrophoresis, yielding increased band resolution, as we also observe.

Glycerol increases protein resolution in both the Laemmli and the Weber–Osborn gel systems (22–24). Huriaux and co-workers (15) reported a decreased migration of Barbel (*Barbus barbus* L.) slow and cardiac MHC isoforms with increasing glycerol concentration. We report in this investigation a decreased migration for members of the chicken fast MHC family, relative to the migration of slow MHC isoforms and altered migration order within the fast MHC family, with increasing glycerol concentration. The physicochemical mechanism underlying this phenomenon remains to be elucidated. This finding highlights a fruitious technique that may be employed in other myosin heavy chain separation problems.

Previous separation of the four mammalian MHC isoforms has been possible using acrylamide concentrations between 5 and 8% or gradient gels (8, 9, 11, 13, 20). Earlier studies have utilized ratios where the percentage of crosslinker is closer or equal to 5% (8, 9, 11, 13, 18, 20). It has been suggested that pore size reaches a minimum when the bisacrylamide represents about 5% of the total monomer concentration (24). The 0.5% bisacrylamide gels used in this study reduces crosslinking and results in a much more pliable gel which has been found to be much easier to silver stain and preserve than standard gels (16).

A variety of factors can affect band resolution including protein mobility, electrical field strength, duration of electrophoresis, diffusion coefficient, viscosity, tem-

perature, and molecular radius (25) as well as protein load and gel thickness. Since these factors are highly interdependent, the optimization process required for high-resolution MHC separation can be complex. We have, in summary, developed a protocol that can be utilized to prepare muscle samples in a relatively fast and simple manner and to electrophoretically separate MHC isoforms in adult avian and mammalian skeletal muscle with high resolution.

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