Purification of Native Myosin Filaments from Muscle

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ABSTRACT Analysis of the structure and function of native thick (myosin-containing) filaments of muscle has been hampered in the past by the difficulty of obtaining a pure preparation. We have developed a simple method for purifying native myosin filaments from muscle filament suspensions. The method involves severing thin (actin-containing) filaments into short segments using a Ca^{2+}-insensitive fragment of gelsolin, followed by differential centrifugation to purify the thick filaments. By gel electrophoresis, the purified thick filaments show myosin heavy and light chains together with nonmyosin thick filament components. Contamination with actin is below 3.5%. Electron microscopy demonstrates intact thick filaments, with helical cross-bridge order preserved, and essentially complete removal of thin filaments. The method has been developed for striated muscles but can also be used in a modified form to remove contaminating thin filaments from native smooth muscle myofibrils. Such preparations should be useful for thick filament structural and biochemical studies.

INTRODUCTION

Contraction of muscle is brought about by the cyclic interaction of thick (myosin-containing) with thin (actin-containing) filaments, generating filament sliding. To understand how myosin filaments function in contraction and its regulation, it is necessary to know their native structure, composition, and biochemistry. This requires a pure preparation of native filaments, isolated from actin filaments and other proteins. Homogenization of muscle tissue under relaxing conditions (ATP, low Ca^{2+}), which minimizes interaction between actin and myosin, readily releases separated actin and myosin filaments (Huxley, 1963; Kensler and Levine, 1982; Vibert and Craig, 1983; Crowther et al., 1985). Thin filaments can be purified from such homogenates by centrifugation, which sediments all the thick filaments, leaving substantial quantities of thin filaments in the supernatant (Hardwicke and Hanson, 1971; Lehman and Szent-Györgyi, 1975). The converse has not been so easy, however, because many thin filaments also sediment along with the thick filaments (Hardwicke and Hanson, 1971). Such co-sedimentation at first seems puzzling. It appears unlikely to be due to a similarity in sedimentation coefficient for the two types of filament, as actin filaments sediment at 33–50 S, depending on muscle (Hardwicke and Hanson, 1971), requiring long periods at high *g* forces to form a pellet, whereas myosin filaments (~132–300 S) (Hardwicke and Hanson, 1971; Emes and Rowe, 1978) sediment relatively readily. It seems more likely that actin filaments (one to several microns long) co-sediment with myosin filaments because they are mechanically trapped (Hardwicke and Hanson, 1971) in the mesh of sedimenting myosin filaments (1.6 to several microns long) or because multiple, short-lived, weak interactions between myosin and actin filaments occur even in relaxing conditions (Brenner et al., 1982, 1984; Xu et al., 1997) preventing effective separation of filaments during centrifugation. Such transient interactions, even if rare, could be problematic due to the numerous potential interactions between the hundreds of myosin heads on a thick filament and the hundreds of actin subunits in a thin filament.

A purified preparation of thick filaments would ideally preserve both biochemical and structural characteristics of the native filament. These would include enzymatic activity, the full complement of myosin and associated proteins, and the helically ordered arrangement of myosin heads known to exist in relaxed intact muscle. Although routine differential centrifugation of muscle filament suspensions under relaxing conditions does not separate thick from thin filaments, more specialized approaches have been reported to achieve this goal and have apparently preserved some of the above characteristics. Thick filament isolation by zone sedimentation on glycerol, sucrose, or D_2O/H_2O density gradients (Hardwicke and Hanson, 1971; Morimoto and Harrington, 1973; Trinick, 1982) has succeeded in some cases, but native helical structure was not demonstrated, and these methods have not come into routine use due to their difficulty. Moreover, the use of nonphysiological conditions, such as glycerol and sucrose gradients, is undesirable and may damage thick filaments (Hardwicke and Hanson, 1971).

Assuming that the difficulties in isolating thick from thin filaments arise largely for the reasons discussed above, a simple modification of the differential centrifugation approach would be to fragment or depolymerize the thin filaments first, reducing their mechanical and biochemical interactions with thick filaments and making separation from them straightforward. F-actin can be fragmented using the plasma protein gelsolin, an actin-binding protein that severs actin filaments by breaking the noncovalent bonds

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between adjacent actin subunits (Yin and Stossel, 1979; Kwiatkowski et al., 1986; Janmey et al., 1987; Allen and Janmey, 1994). Gelsolin severs F-actin filaments stoichiometrically and with close to 100% efficiency (Selden et al., 1998). Thus, when treated with gelsolin at 1:1 gelsolin:G-actin ratio at high Ca^{2+} concentration, F-actin filaments are depolymerized (Schoepper and Wegner, 1991). Although the regulatory proteins troponin and tropomyosin, present on striated muscle thin filaments, partially inhibit severing by gelsolin, depolymerization can still be achieved under appropriate conditions (Fattoum et al., 1983; Ishikawa et al., 1989; Dabrowska et al., 1996). Thus, the use of gelsolin should be a fruitful approach to removing thin filaments, but with one proviso. Gelsolin requires either low pH (Lamb et al., 1993) or elevated Ca^{2+} concentration (1–100 μM) (Yin and Stossel, 1979; Chaponnier et al., 1986; Lamb et al., 1993; Allen and Janmey, 1994; Pope et al., 1997; Langer et al., 1998) for full F-actin severing activity. The calcium requirement decreases at more acidic pH and is $\sim$3 μM at pH 6.5. Below pH 6.0, gelsolin no longer requires Ca^{2+} and severs actin filaments in EGTA-containing solutions (Lamb et al., 1993). The treatment of filament suspensions with such acidic conditions or high Ca^{2+} concentrations could, however, cause several difficulties. Low pH ($<$6.0) could readily affect thick filament enzymatic activity or helical order, which is highly sensitive to ionic conditions (Wray et al., 1974). Micromolar Ca^{2+} levels activate filament preparations resulting in numerous thick-thin filament interactions and cross-bridge cycling. Activation may occur by Ca^{2+} binding to troponin on the thin filaments (actin-linked regulation, found in most striated muscles) or to the myosin heads (myosin-linked regulation, found in invertebrates, such as the scallop (Lehman and Szent-Györgyi, 1975)). In other myosin-linked systems, Ca^{2+} can bind to calmodulin, activating myosin light chain kinase (MLCK), resulting in phosphorylation of the myosin regulatory light chain (RLC) and consequent activation or potentiation of contraction in both smooth (Sweeney, 1998; Hori and Karaki, 1998) and striated muscles (Sellers, 1981; Craig et al., 1987; Sweeney et al., 1993). The heads of activated myosin filaments become disordered in a way that is not reproducibly reversible, making detailed structural studies of the head arrangement impossible (Vibert and Craig, 1985; Craig et al., 1987; Levine et al., 1996).

The requirement of Ca^{2+} for full severing activity can be overcome by limited proteolytic digestion of gelsolin (Kwiatkowski et al., 1985; Soua et al., 1985; Bryan and Hwo, 1986; Chaponnier et al., 1986; Sutoh and Yin, 1989; Helweg et al., 1993). This generates a Ca^{2+}-insensitive fragment that can sever thin filaments under low Ca^{2+} (relaxing) conditions. A simple version of this approach to removing thin filaments from filament suspensions has been used in ultrastructural studies of invertebrate thick filaments (Levine et al., 2000), but no attempt was made to purify the thick filaments. Purification of myosin filaments from rabbit psoas muscle by gel filtration after Ca^{2+}-insensitive gelsolin treatment has also been reported in abstract form (Rodgers and Davis, 1996), but preservation of helical order was not discussed and details of the method have not been published.

We describe here a simple method for purifying native thick filaments from muscle in near-physiological ionic conditions based on the above approach. A calcium-insensitive gelsolin fragment prepared from bovine serum is used to fragment thin filaments, and the thick filaments are then isolated by differential centrifugation. The native thick filaments obtained are biochemically pure and retain their native helical order. They are thus appropriate for biochemical, enzymatic, and structural studies. Tarantula muscle was used in this study because of our interest in the molecular organization of the myosin heads in this muscle in the relaxed state and in the structural and biochemical changes that occur in the myosin heads when they are phosphorylated (Crowther et al., 1985; Craig et al., 1987; Padrón et al., 1992, 1995; Offer et al., 2000; Hidalgo et al., 2001). These studies have been hampered by the numerous actin filaments in cryo-electron microscopy (cryo-EM) images of crude filament suspensions (much higher than in specimens examined by negative staining), which interfere greatly with thick filament image analysis. The purification method we describe solves this problem and reveals in addition three putative new nonmyosin components in these filaments. Preliminary experiments with other species suggest that this approach should be generally applicable.

MATERIALS AND METHODS

Preparation of filament suspensions from tarantula striated muscle

Relaxed filament homogenates were prepared from the leg muscles of brown tarantulas (family *Theraphosidae*), obtained from Carolina Biological Supply Co. (Burlington, NC) as previously described (Craig et al., 1987), using 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 5 mM PIPES, 1 mM NaN_3, 5 mM Mg ATP, pH 6.5, for the relaxing solution. Saponin-permeabilized muscle was homogenized for $\sim$1 s on ice in 3 ml of relaxing solution at setting 5 on a Polytron homogenizer. The homogenate was centrifuged at 15,000 $\times$ *g* for 2 min (Microspin 12S, Sorvall, Norwalk, CT) to remove large debris, and the supernatant containing the filament suspension was stored on ice and used the same day.

Preparation of Ca^{2+}-insensitive gelsolin

Gelsolin was purified from bovine serum (Pel-Freez Biologicals, Rogers, AR) according to Kurokawa et al. (1990), dialyzed against rigor solution (relaxing solution without MgATP), pH 6.5, and stored at $-$80°C. A Ca^{2+}-insensitive gelsolin fragment was prepared by incubating gelsolin (1 mg/ml) with protease type X (thermolysin) from *Bacillus thermoproteolyticus rokko* (Sigma, St. Louis, MO) at a weight ratio gelsolin:thermolysin of 5500:1. The gelsolin was in rigor solution, pH 6.5, and the thermolysin was in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4). The digestion was started by adding 2 mM $MgCl_2$ (to a final 5 mM) and $CaCl_2$ to 1.2 mM ($\sim$0.2 mM free Ca^{2+}). After 30 min at room

temperature, thermolysin was inhibited by adding freshly prepared phosphoramidon (Boehringer Mannheim Corp., Indianapolis, IN) to a final concentration of 100 μM (Suda et al., 1973) and by chelating Ca^{2+} by addition of EGTA to a final concentration of 2 mM (Ohta, 1967). Gelsolin fragments were fractionated by anion-exchange chromatography on macro-prep DEAE support at 4°C (Bio-Rad, Hercules, CA) (Bryan and Hwo, 1986). The solvent was 25 mM Tris-HCl, pH 7.0. A discontinuous gradient of NaCl (0, 0.1, 0.2, 0.3, and 1 M) was used to separate gelsolin fragments. The flow rate was 1 ml/min, and 1.2-ml fractions were collected and analyzed.

Purification of thick filaments from filament suspensions

Filament suspensions (200–250 μl) in relaxing solution, pH 6.5, were incubated at room temperature for 30 min with 50 μl of unfractionated calcium-insensitive gelsolin fragments (1 mg/ml) in rigor solution, pH 6.5, to fragment the thin filaments. The volume was then increased to 4 ml with relaxing solution, pH 6.5, and the samples were centrifuged at 18,700 $\times$ g for 15 min at 4°C (Beckman Optima TL ultracentrifuge and TLA-100.4 rotor, Beckman Instruments, Palo Alto, CA). After removing the supernatant, the original volume (200–250 μl) of relaxing solution, pH 6.5, was added and the pellet allowed to swell at least 1 h on ice. The pellet was then dispersed by brief, low-intensity vortexing to minimize damage to the thick filaments. The volume was again increased to 4 ml with relaxing solution, pH 6.5, and the procedure repeated, finally resuspending the second pellet (containing the purified thick filaments) in the original (200–250-μl) volume.

Gel electrophoresis

Gelsolin, gelsolin fragments, and filament preparations were analyzed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) slab gels by the method of Laemmli (1970). The apparent molecular weights of the peptides were estimated by comparison with the mobility of molecular weight standards (Bio-Rad). Gels were analyzed by densitometry using the Fluor-S MultiImager system (Bio-Rad).

Electron microscopy and image analysis

Filament suspensions were negatively stained at room temperature and 80% relative humidity with 1% uranyl acetate, as described by Craig et al. (1987), except that the holey carbon films were first coated with an additional film of thin carbon floated from mica, and observations were made where the thin film crossed the holes. For cryo-EM, a drop of suspension was applied to a grid coated with a holey carbon film that had been glow discharged in an amylamine atmosphere. The grid was then blotted and frozen in liquid ethane. The procedure was done at room temperature with ambient humidity 65–80%. Electron micrographs were recorded under low-dose (cryo-EM) or conventional-dose (negative stain) conditions on a Philips CM120 or CM10 microscope, respectively.

Images were digitized with a pixel size corresponding to 0.45 nm in the original specimen using a FlexTight scanner (Imacon, Fremont, CA). Fourier transforms from selected thick filaments were computed on a Silicon Graphics O_2 computer (SGI, Mountain View, CA) using the Brandeis helical package (Owen et al., 1996).

RESULTS

Preparation of Ca^{2+}-insensitive bovine serum gelsolin fragments

Our method for purifying native thick filaments from filament suspensions depended on an initial fragmentation of thin filaments under relaxing conditions (ATP, low Ca^{2+}). This was accomplished using a Ca^{2+}-insensitive fragment of gelsolin.

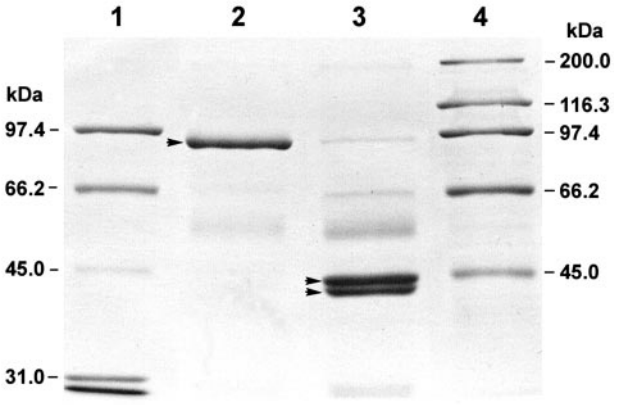

FIGURE 1 SDS-PAGE analysis of gelsolin and gelsolin fragments. The 10% gel was stained with Coomassie brilliant blue. Lane 1, low molecular weight markers; lane 2, gelsolin peak (~93 kDa, *arrow*) eluting from DEAE column; lane 3, gelsolin fragments (43 and 45 kDa, *arrows*) resulting from thermolysin proteolytic digestion of gelsolin; lane 4, high molecular weight markers.

Gelsolin was prepared by DEAE chromatography of the fraction of bovine serum precipitating between 35% and 50% ammonium sulfate saturation. A single peak eluted with a buffer containing 25 mM Tris-HCl, 30 mM NaCl, 2 mM $CaCl_2$, pH 8.0, and showed, by SDS-PAGE, a main band with apparent molecular weight of ~93 kDa, corresponding to gelsolin (Soua et al., 1985; Kurokawa et al., 1990), and three minor contaminant bands (Fig. 1, lane 2). The gelsolin was ~98% pure by gel densitometry.

Proteolytic digestion of bovine gelsolin with thermolysin at a weight ratio of 5500:1 resulted in loss of the 93-kDa gelsolin band and the appearance instead of similar amounts of two polypeptides with apparent molecular weights of 43 and 45 kDa (Fig. 1, lane 3). The other bands were not affected. This pattern remained the same for at least 3 days, consistent with complete inhibition of thermolysin after gelsolin digestion (data not shown). The 43-kDa fragment eluted with 1 M NaCl and the 45-kDa fragment with 0.2 M NaCl. Thus, thermolysin quantitatively cleaves bovine serum gelsolin into two halves, as has previously been demonstrated for human plasma gelsolin (Chaponnier et al., 1986; Sutoh and Yin, 1989).

The two gelsolin fragments were separated by DEAE chromatography using a discontinuous NaCl gradient (data not shown). The 43-kDa fragment severed F-actin and thin filaments regardless of calcium concentration, whereas the 45-kDa fragment failed to sever F-actin or thin filaments at any calcium level. We conclude that the Ca^{2+}-independent severing activity resides in the 43-kDa fragment. Previous studies on human plasma gelsolin have shown that this corresponds to the N-terminal half of gelsolin (Chaponnier

et al., 1986; Sutoh and Yin, 1989). For simplicity, unfractionated fragments were used in thick filament purification.

Severing activity of gelsolin

The severing activity of intact gelsolin and gelsolin fragments on F-actin and thin filaments was assayed by negative staining electron microscopy. Intact gelsolin severed F-actin in the presence of Ca^{2+} (pCa ~2.9) at pH 7.0, but not in its absence, confirming the Ca^{2+} dependence of its severing activity (Yin and Stossel, 1979; Allen and Janmey, 1994; Langer et al., 1998). Native thin filaments (containing troponin and tropomyosin) present in the unpurified filament suspension responded similarly to gelsolin, although they were more resistant to fragmentation (Maciver et al., 2000), being unaffected in the absence of Ca^{2+} and requiring a pH lower than 6.5 for severing in its presence (pCa ~2.9). Thus, intact gelsolin is not useful for severing thin filaments in low Ca^{2+} (relaxing) conditions.

The severing activity of gelsolin was made insensitive to Ca^{2+} by digestion with thermolysin. Gelsolin fragments (unfractionated) completely fragmented F-actin (data not shown) and thin filaments (Figs. 2 *B* and 4 *B*) in relaxing solution (1 mM EGTA). This severing activity was strongly dependent on pH. Of the pH levels tested (6.0, 6.5, and 7.0), activity was highest at pH 6.0 and lowest (no activity on thin filaments) at pH 7.0. Thus, low pH aids severing activity. For our purification procedure, we chose pH 6.5 as a compromise that kept filaments close to neutrality and preserved thick filament helical order (Vibert, 1992) while maintaining thin filament severing activity.

Fragmentation of thin filaments in filament suspensions using Ca^{2+}-insensitive gelsolin

As found previously (Crowther et al., 1985; Craig et al., 1987), filament suspensions prepared from permeabilized tarantula striated muscle revealed thick (myosin-containing) and numerous thin (actin-containing) filaments, together with small vesicles and some crystalline fragments of sarcoplasmic reticulum (Scales and Inesi, 1976; Castellani and Hardwicke, 1983; Castellani et al., 1985) when analyzed by negative staining electron microscopy (Fig. 2 *Ai*). The thick filaments showed the typical helical ordering of cross-bridges characteristic of myosin filaments in relaxing conditions (Fig. 2 *A*) (Kensler and Levine, 1982; Vibert and Craig, 1983; Crowther et al., 1985). Although these images are generally adequate for analysis of thick filament structure (Kensler and Levine, 1982; Vibert and Craig, 1983; Crowther et al., 1985), they were significantly improved, providing thick filament images with no overlapping thin filaments, by brief treatment of the relaxed filament suspension with Ca^{2+}-insensitive gelsolin fragments. Filament suspensions treated with an approximately equimolar ratio of gelsolin fragment:actin subunits showed intact myosin filaments and essentially no thin filaments (Fig. 2 *Bi*). In addition to removing thin filaments, treatment with gelsolin fragments also resulted in improved negative staining of the thick filaments. Addition of inert, soluble protein (e.g., bovine serum albumin or bacitracin) is a common strategy to reduce surface tension and thus improve negative staining of biological specimens (Gregory and Pirie, 1973; Hayat, 2000), and the added gelsolin and consequent release of thin filament monomer components apparently performed this function here. As a result, after treatment with gelsolin fragments, a higher proportion of thick filaments showed good helical order, which is dependent on even spreading of stain. Preservation of helical order was apparent by eye and confirmed by the presence of a clear set of layer lines in thick filament Fourier transforms (Fig. 2 *Biii*) (cf. Crowther et al., 1985). By these criteria, the helical order of the thick filaments after severing of thin filaments was similar to that of thick filaments in the original suspension (Fig. 2 *A*).

Treatment of filament suspensions with gelsolin fragments in relaxing solution showed no observable effect on protein integrity when examined by SDS-PAGE. The major proteins in the original suspension were components of the thin filaments (actin, tropomyosin, and troponin subunits) and the thick filaments (myosin heavy chain, essential and regulatory light chains (ELC and RLC), and paramyosin) (Fig. 3 *A*; cf. Craig et al., 1987; Hidalgo et al., 2001). After gelsolin treatment, the same bands were present, with the same positions and relative intensities, as in the original filament suspension, together with bands attributable to the gelsolin fragments (Fig. 3 *B*). Thus, thermolysin-treated gelsolin has no proteolytic activity toward any of the proteins present in the filament suspension.

Cryo-EM analysis of the original filament suspension typically showed thick filaments and numerous thin filaments. The thick filaments were helically ordered but were usually crossed by multiple thin filaments, making image analysis difficult (Fig. 4 *A*). Filament suspensions treated with Ca^{2+}-insensitive gelsolin at a molar ratio of ~1:1 with actin subunits showed no thin filaments, and the thick filaments had excellent helical order (Fig. 4 *B*). However, background noise due to gelsolin fragments and thin filament subunits resulted in noisy filament transforms (Fig. 4 *Biii*) and hence difficulty in carrying out image analysis and reconstruction (cf. Crowther et al., 1985).

Purification of thick filaments after treatment with Ca^{2+}-insensitive gelsolin

After treatment with calcium-insensitive gelsolin fragments, it became possible to purify the thick filaments by differ-

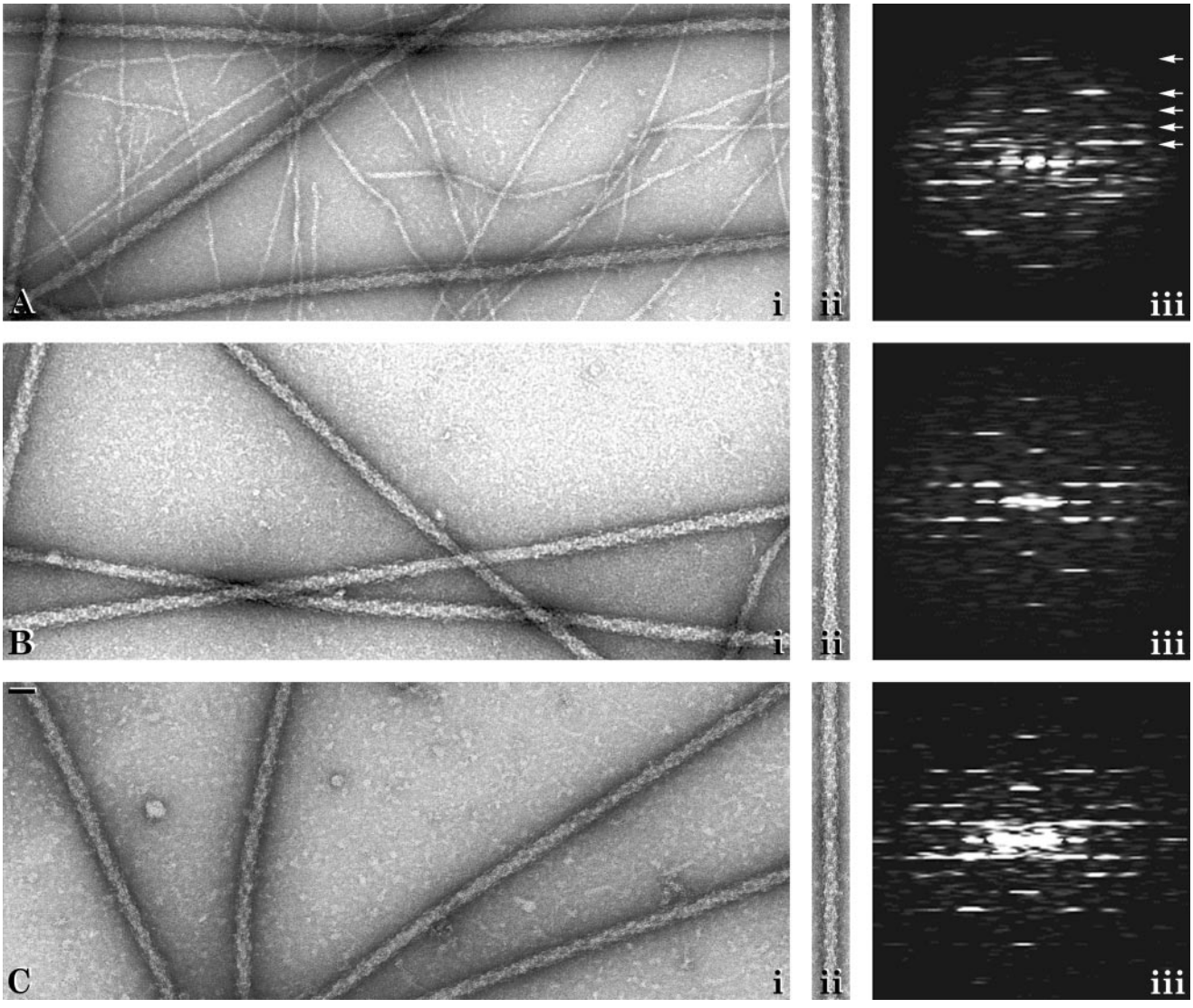

FIGURE 2 Negative staining electron microscopy and image analysis of tarantula thick filament purification. (*A—C*) Electron micrograph of a filament field (*i*), a selected thick filament (*ii*), and its computed Fourier transform (*iii*). (*A*) Original filament suspension in relaxing solution, pH 6.5, showing thin filaments and helically ordered thick filaments. (*B*) Filament suspension after gelsolin fragment treatment. Thin filaments are removed, and thick filaments show excellent helical order. (*C*) Resuspended pellet after treatment of filament suspension with gelsolin fragments followed by first differential centrifugation. In all micrographs, the appearance of helical order of thick filaments is enhanced by viewing obliquely along the filament length or by turning them horizontally. Optical diffraction patterns are marked with arrows pointing to the approximate position of layer lines 1 (43.5 nm), 2 (21.7), 3 (14.5 nm), 4 (10.8 nm), and 6 (7.25 nm). Note that purification of the thick filaments by centrifugation removes most soluble proteins, whose presence would normally aid stain spreading (Gregory and Pirie; 1973; Hayat, 2000) and hence preservation of helical order (see text). To demonstrate the presence of helical order in the purified filaments, soluble protein (such as gelsolin fragments, cytosol (supernatant from high-speed centrifugation of the original suspension) or ultrafiltrate of the original filament suspension) was therefore added to the filament suspension or used to rinse the grid before staining. Scale bar, 50 nm.

ential centrifugation. As discussed in the Introduction, we reasoned that the thick filaments should sediment with minimal contamination by thin filaments as long as the thin filaments were relatively short; i.e., full depolymerization of thin filaments with high gelsolin levels, as described in the previous section, was probably not necessary. We therefore used a gelsolin:actin ratio of ~1:5, which shortened thin filaments from a mean length of ~2.5 μm (cf. Levine et al., 1983) to ~0.5 μm, both conserving gelsolin and minimizing potential gelsolin contamination of the purified thick filaments. The filament suspension was centrifuged twice after gelsolin fragment treatment, and the pellet from each step was resuspended in relaxing solution and analyzed by negative staining and SDS-PAGE. The first centrifugation produced an enriched thick filament fraction (pellet 1) with a relative amount of actin five times less than the original

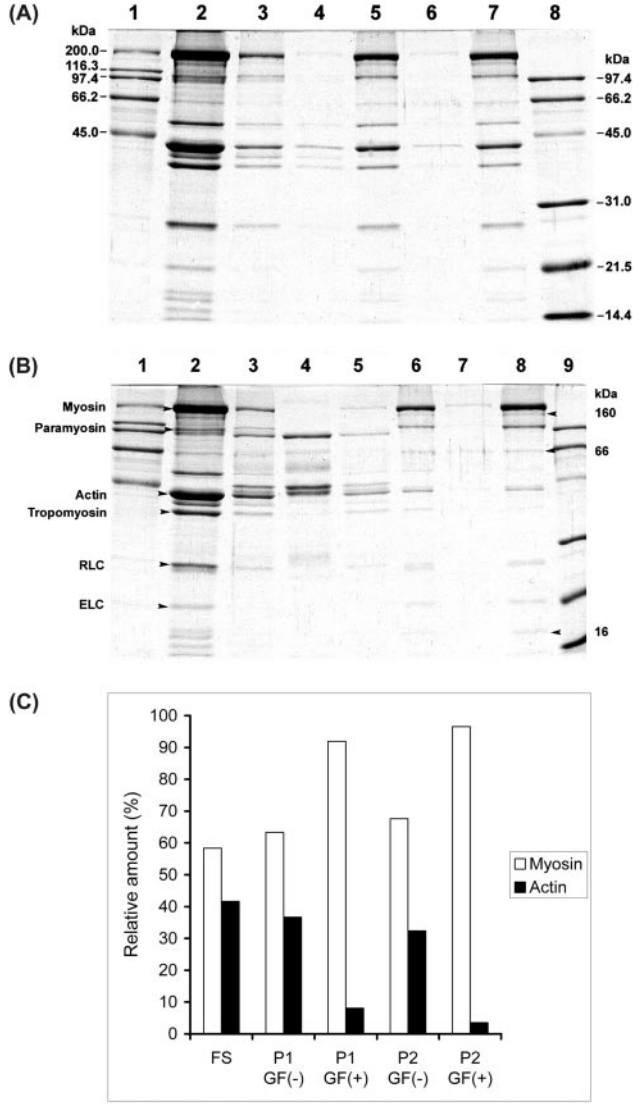

FIGURE 3 SDS-PAGE analysis of the purification of thick filaments. The 13% gel was stained with Coomassie brilliant blue. (*A*) Control, centrifugation without gelsolin treatment: lane 1, high molecular weight markers; lane 2, crude filament suspension; lane 3, filament suspension plus relaxing solution; lane 4, supernatant 1 (from first centrifugation); lane 5, pellet 1; lane 6, supernatant 2 (from second centrifugation); lane 7, pellet 2; lane 8, low molecular weight markers. (*B*) Centrifugation of filament suspension after gelsolin fragment treatment: lane 1, high molecular weight markers; lane 2, crude filament suspension (*arrows* indicate myosin, paramyosin, actin, tropomyosin, RLC, and ELC); lane 3, filament suspension after gelsolin fragment treatment; lane 4, gelsolin fragments (some undigested gelsolin is also present); lane 5, supernatant 1; lane 6, pellet 1; lane 7, supernatant 2; lane 8, pellet 2 (*arrows* indicate nonmyosin components at ~160, 66, and 16 kDa); lane 9, low molecular weight markers. (*C*) Densitometric analysis of the myosin heavy chain/actin ratio in the original filament suspension (FS) and in pellets 1 (P1) and 2 (P2) after centrifugation with and without gelsolin fragment (GF) treatment.

filament suspension (Fig. 3, *B* and *C*). Analysis by negative staining electron microscopy showed thick filaments with good helical order (Fig. 2 *C*) and a few short thin filament fragments and occasional shortened I-segments (Huxley, 1963). The second centrifugation (pellet 2) resulted in a more highly purified fraction of thick filaments with higher relative amount of myosin (myosin heavy chain 96.5% of total actin plus myosin heavy chain) and a relative amount of actin 12 times less (3.5% of actin plus myosin heavy chain) than in the original filament suspension (Fig. 3, *B* and *C*). SDS-PAGE analysis of this last fraction showed bands corresponding to the myosin heavy chains, the regulatory and essential light chains, paramyosin, and unidentified components with apparent molecular weights of ~160, 66, and 16 kDa, respectively (Fig. 3 *B*). Gelsolin fragments (43 and 45 kDa) and tropomyosin and troponin bands were not detected. Control preparations (no gelsolin treatment) showed high levels of thin filament components, even after two centrifugations, demonstrating the critical need for fragmentation of the thin filaments into short segments to make their removal possible (Fig. 3, *A* and *C*).

DISCUSSION

The aim of this study was to find a simple and rapid method for purifying native thick filaments for biochemical and structural studies, using a fresh, crude filament suspension from muscle as starting material. Simplicity and speed were important because thick filaments are structurally labile. A method that did not require Ca^{2+} was needed because calcium activates cross-bridge cycling and causes loss of the helical order of myosin filaments, which is not fully reversible (Vibert and Craig, 1985; Craig et al., 1987). Our method combines thin filament fragmentation in relaxing solution, using a calcium-insensitive gelsolin fragment, with differential centrifugation to separate thick filaments from the suspension.

The thick filaments purified from the filament suspension showed two heavy chains, corresponding to myosin (~201 kDa) and paramyosin (~111 kDa), and two light chains, the regulatory light chain (~27 kDa) and essential light chain (~20 kDa) (Fig. 3 *B*, lane 8) (Levine et al., 1983; Craig et al., 1987; Hidalgo et al., 2001). There were also small amounts of three other protein bands with apparent molecular weights of ~160, 66, and 16 kDa, respectively. All of these components were present in the original, untreated filament suspension and therefore do not result from gelsolin- or thermolysin-induced breakdown of larger polypeptides. In fact, no change was detectable in any of the proteins of the filament suspension after treatment with either gelsolin or uninhibited thermolysin (data not shown). The purified thick filaments were intact (mostly full length and unbroken) and showed native helical structure in relaxing solution, similar to that observed in the initial filament suspension (Figs. 2 and 4). Thus, the protein components and the labile helical organization of myosin heads are preserved after gelsolin treatment and differential centrifugation and resuspension.

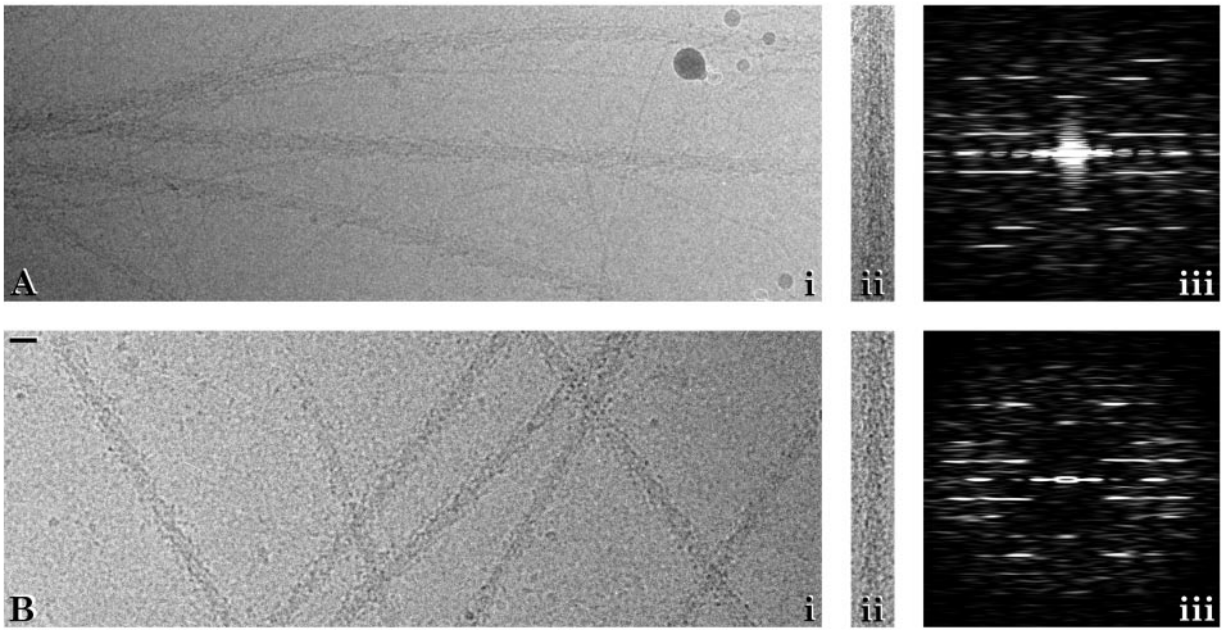

FIGURE 4 Cryo-electron microscopy and image analysis of filament suspension from tarantula striated muscle. (*A* and *B*) An electron micrograph of a filament field (*i*), a selected thick filament (*ii*), its computed Fourier transform (*iii*). (*A*) Original filament suspension in relaxing solution, pH 6.5, showing thin filaments and helically ordered thick filaments. This was a rare image where relatively few thin filaments were present. (*B*) Filament suspension after gelsolin fragment treatment. Thin filaments are removed, and thick filaments show excellent helical order (judged both by eye and by the layer lines visible in the Fourier transform), but background is high. Centrifugation removes the background, and clear helical order is visible by eye, but Fourier transforms are less strong (data not shown). It is possible that, as with negative staining, some soluble protein is required to create a specimen layer over the holes in the support film that is optimal for cryo-preservation of the labile array cross-bridges. Scale bar, 50 nm.

The success of our method supports our suggestion that a major reason for previous difficulties in separating myosin and actin filaments was mechanical trapping of thin filaments in the matrix of sedimenting thick filaments and/or transient interactions between myosin heads and thin filaments under relaxing conditions. When the thin filaments were shortened to ~0.5 μm in mean length by treatment with gelsolin, differential centrifugation resulted in actin contamination of <3.5%. This compares with an initial actin level of 41.6% in the filament suspension and reduction to only 36.7% without gelsolin treatment (Fig. 3). Simple shortening of the thin filaments thus has a major impact on their removal by centrifugation. When higher levels of gelsolin were used, sufficient to completely depolymerize the thin filaments, actin contamination was undetectable. This dependence on filament length presumably underlies our ability to remove almost all the thin filaments from homogenates of scallop (*Placopecten magellanicus*) striated muscle by the same procedure, but without gelsolin treatment (results not shown; cf. Hardwicke and Hanson, 1971), as scallop myosin filaments ~2.0 μm in length (Vibert and Craig, 1983) are about half the length of those in tarantula muscle (~4.5 μm) (Levine et al., 1983; Crowther et al., 1985), and the thin filaments are correspondingly shorter. Other factors must also play a part, however, as vertebrate filaments, similar in length to those from scallop, are not easily isolated (Hardwicke and Hanson, 1971; Morimoto and Harrington, 1973).

The finding of protein bands that co-purify with the thick filaments, in addition to the myosin heavy chain, light chains, and paramyosin, is consistent with the presence of additional, nonmyosin, thick filament components (Fig. 3). The possibility remains, however, that some of these bands may originate from elements of the sarcoplasmic reticulum, which persist in small quantities through the purification procedure as vesicles of various kinds (Hardwicke and Hanson, 1971; Scales and Inesi, 1976; Castellani and Hardwicke, 1983; Castellani et al., 1985), or from Z-line components, which are not totally removed. However, ~80% of the total membrane protein in the vesicles is a Ca^{2+}-ATPase of ~115 kDa (Hasselbach, 1974; LeMaire et al., 1976; Scales and Inesi, 1976), and the main protein band of the Z-disc is α-actinin with a subunit molecular weight of 94–103 kDa (Blanchard et al., 1989). These proteins are both quite different in mobility from any of the three co-purifying bands, making it more likely that the latter do have a thick filament origin. This is also suggested by the fact that the three bands appear to occur in a constant ratio to the myosin heavy chain in different preparations. N-terminal sequencing or development of antibodies to these components should reveal their source definitively.

The final method that we adopted for thick filament purification followed several unsuccessful attempts. Gel filtration after gelsolin treatment (Rodgers and Davis, 1996) was unsuccessful in our hands. Filtration through a large-pore Millipore filter also failed, apparently because the pores became clogged with filaments. We also tried cycles of low ionic strength treatment, which aggregates thick filaments, followed by sedimentation and resuspension in normal ionic strength, which disperses them. This again failed to separate thick from thin filaments. Our studies were carried out on a small scale, which was more than adequate for the SDS-PAGE and ultrastructural analysis needed. For enzymatic and other studies requiring larger quantities, it should be straightforward to increase the scale of the preparation.

Our purification method was developed using a filament suspension from tarantula muscle, which we had failed to purify by any other method. There appears to be no reason why it should not also be successful on other types of muscle as it depends on only two simple steps that should be generally applicable. The report of purification of rabbit skeletal muscle thick filaments by a similar approach supports this view (Rodgers and Davis, 1996), and other studies have shown that Ca^{2+}-insensitive gelsolin fragment can depolymerize insect muscle thin filaments (Levine et al., 2000). Application of this method to smooth muscle thick filaments would be particularly valuable, as the thick filaments in smooth muscle are outnumbered by thin filaments much more than in striated muscle (e.g., Xu et al., 1996). There are two possible problems, however. First, smooth muscle myosin filaments are unstable in relaxing solution (Suzuki et al., 1978), and special methods are therefore necessary to minimize their disintegration during preparation (Cooke et al., 1989; Xu et al., 1996). Second, the presence of caldesmon on smooth muscle thin filaments could interfere with their fragmentation by gelsolin (Ishikawa et al., 1989; Dabrowska et al., 1996). Despite these potential difficulties, we have found that a simple method for improving ultrastructural studies of smooth muscle thick filaments, when subsequent purification is not required, is merely to wash the EM grid, with adherent rigor smooth muscle fibril suspension (Cooke et al., 1989; Xu et al., 1996), with a low concentration of calcium-insensitive gelsolin fragments in relaxing solution for 30 s. This simple, brief treatment completely removes thin filaments, leaving well separated, well ordered smooth muscle myosin filaments (data not shown). This simplified approach is equally successful with tarantula muscle filament suspensions and is therefore likely to be generally applicable.

We conclude that, using calcium-insensitive gelsolin to fragment thin filaments, and differential centrifugation to separate thick filaments from the rest of the suspension, it is possible to obtain a highly purified fraction of thick filaments that retain their native biochemical and structural properties. These filaments are suitable for enzymatic, protein composition, and structural studies. Such preparations should be useful, for example, in producing images of pure, frozen-hydrated, helically ordered thick filaments. These are necessary to calculate high-resolution three-dimensional maps required to produce a near-atomic model of the organization of myosin heads on the thick filament (Padrón et al., 1998; Offer et al., 2000).

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