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Transgenic expression and purification of myosin isoforms using the *Drosophila melanogaster* indirect flight muscle system

James T. Caldwell¹, Girish C. Melkani², Tom Huxford¹, and Sanford I. Bernstein^{2,*}

¹Department of Chemistry & Biochemistry, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-1030

²Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4614

Abstract

Biophysical and structural studies on muscle myosin rely upon milligram quantities of extremely pure material. However, many biologically interesting myosin isoforms are expressed at levels that are too low for direct purification from primary tissues. Efforts aimed at recombinant expression of functional striated muscle myosin isoforms in bacterial or insect cell culture have largely met with failure, although high level expression in muscle cell culture has recently been achieved at significant expense. We report a novel method for the use of strains of the fruit fly *Drosophila melanogaster* genetically engineered to produce histidine-tagged recombinant muscle myosin isoforms. This method takes advantage of the single muscle myosin heavy chain gene within the *Drosophila* genome, the high level of expression of accessible myosin in the thoracic indirect flight muscles, the ability to knock out endogenous expression of myosin in this tissue and the relatively low cost of fruit fly colony production and maintenance. We illustrate this method by expressing and purifying a recombinant histidine-tagged variant of embryonic body wall skeletal muscle myosin II from an engineered fly strain. The recombinant protein shows the expected ATPase activity and is of sufficient purity and homogeneity for crystallization. This system may prove useful for the expression and isolation of mutant myosins associated with skeletal muscle diseases and cardiomyopathies for their biochemical and structural characterization.

Keywords

Myosin; *Drosophila melanogaster*; Histidine tag; Transgenic expression; Indirect flight muscles

1. INTRODUCTION

Myosins comprise a large superfamily of molecular motor proteins that fulfill a variety of cellular functions [1]. Due to the vital role they play in functioning muscle and/or the high expression levels of some members of this class, the class II myosins are the most well characterized members of this superfamily and have been the focus of both biophysical and biochemical studies for decades [2]. X-ray crystallography has been instrumental in

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*Corresponding author. sbernst@sciences.sdsu.edu; Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4614. Telephone: (619) 594-5629. FAX: (619) 594-5676.

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elucidating the functional intermediates and reaction mechanisms of class II myosins from various organisms including: *Gallus gallus* (chicken) skeletal muscle myosin II [3] and smooth muscle myosin II [4], a modified version of *Dictyostelium discoideum* (slime mold) myosin II [5], and *Argopecten irradians* (scallop) muscle myosin II [6]. The chicken and scallop muscle myosin x-ray crystal structures were determined with myosin proteins purified directly from primary muscle tissue and the *Dictyostelium* protein was purified directly from a genetically modified version of the organism in culture [7]. Chicken smooth muscle myosin II was expressed in baculovirus-infected insect tissue culture cells [8].

One of the factors that limits our ability to carry out mechanistic studies on striated muscle myosin isoforms is the difficulty in preparation of purified functional myosins in quantities sufficient for biophysical and structural studies using recombinant expression systems. Some examples of striated muscle myosin proteins that would be interesting to study are *Drosophila* indirect flight muscle myosin [9], mouse cardiac myosin [10], and human beta cardiac myosin mutants responsible for hypertrophic or dilated cardiomyopathy [11]. Attempts to express these in *E. coli* or other bacterial recombinant protein expression systems have met with failure, as have efforts to use insect cells infected with recombinant baculoviruses. A recent study from the Leinwand laboratory [12], based upon methods developed in the Winkelmann laboratory [13, 14], reports the use of murine C₂C₁₂ myoblasts to produce adequate amounts of functional recombinant human skeletal muscle myosin II isoforms. However, generation of appreciable amounts of purified material by this approach is expensive.

The fruit fly *Drosophila melanogaster* represents a novel and inexpensive system in which to study muscle myosin function [15]. Unlike many organisms that contain multiple muscle myosin heavy chain genes, all the myosin heavy chain isoforms that are required throughout the fly life cycle are generated by alternative splicing of exons encoded by a single *Drosophila Mhc* gene [16, 17]. This gives rise to at least 15 different myosin heavy chain isoforms out of a possible 480 combinations [18, 19].

Muscle myosin is expressed at different times during the fruit fly life cycle [20]. In adult flies, significant amounts of myosin are expressed in the indirect flight muscles of the thorax. Use of transgenic flies expressing a specific myosin isoform in myosin-null indirect flight muscles allows the isolation of transgenically expressed myosins from dissected muscles [9]. Our dissection of dorsal longitudinal indirect flight muscles from individual flies routinely yields purified myosin at roughly one microgram per fly. Standard methods to solubilize and proteolytically process the myosin to produce the functional head domain (subfragment-1, S1) is feasible and provides adequate materials for some kinetic studies [21]. However, production of milligram quantities of S1 required for crystallography is not practical by this approach, in that isolation of 1 mg of S1 would require 1.9 g (about 2,000) flies to be dissected.

In support of efforts aimed at producing large quantities of diverse muscle myosin proteins for structural and mechanistic studies, we have developed a method that employs *Drosophila melanogaster* as an *in vivo* expression system for tagged myosin proteins. Proof of concept was provided by Lowey *et al.* [22], who expressed and purified histidine-tagged myosin heavy chain in a mouse model for the study of familial hypertrophic cardiomyopathy. Our approach takes advantage of the single *Drosophila Mhc* gene and the ability to express transgenically encoded myosin heavy chain in the indirect flight muscles of a mutant lacking endogenous myosin in this muscle. Here we use the *Actin88F* promoter to drive high level expression of a histidine-tagged recombinant version of a *Drosophila* embryonic body wall myosin isoform (EMB). The protein can be purified by affinity chromatography on extracts from whole flies and the S1 motor domain can then be isolated

by proteolysis and size exclusion chromatography. Myosin prepared by this method retains its function, as evidenced by *in vitro* ATPase assays. The S1 fragment is of sufficient purity and homogeneity to support its crystallization. Herein we outline the methods employed to produce and isolate this functional myosin isoform, which should be applicable to other *Drosophila* muscle myosin isoforms and possibly to striated muscle myosins from other species.

2. MATERIALS AND METHODS

2.1. Construction of pattB6HisEmb plasmid

The 5' *Actin88F* promoter fragment (1406 bp) [23] was produced by PCR from the plasmid pL116-4 [24] with the forward primer act88fXba1 5'-AAT ATC TAG AAT GCA CAA TAG GCA AAT TTA GTT AAG-3' and reverse primer act88fPvu1 5'-ACG CCG ATC GGT CTG TCC TGC CTT TAT ATC-3'. A second fragment containing the 3' *Actin88F* promoter region, which also encodes the amino-terminal His-tag, tobacco etch virus (TEV) protease recognition site, and *Mhc* exon 2 5' region, was generated in three steps by standard PCR. First, the pL116-4 plasmid was amplified using the forward primer ActPvu1 5'-GAC AGA CCG ATC GGC GTG CCA T-3' and reverse primer ActHisTEV 5'-GAG GTT TTC GTG GTG GTG GTG GTG CAT CTT GGC AGT TGT TTA TCT GG-3' to produce the 3' *Actin88F* region, 6XHis-tag, and TEV cut site (695 bp). Next, the p5'emb plasmid [25] was amplified with the forward primer HisTEVMhc 5'-CAC CAC GAA AAC CTC TAC TTC CAA GGC CCG AAG CCA GTC GCA AAT CAG G-3' and reverse primer MhcPst1 5'-ACC CTG CAG ACC AAC GGA GAC G-3' to produce the His-tag, TEV cut site, and *Mhc* 5' exon 2 region (222 bp) and then the two overlapping 695 and 222 bp fragments were joined by amplification with the forward primer ActPvuI and reverse primer MhcPst1 for a final 890 bp fragment. These 5' *Actin88F* and 3' fragments were digested with Pvu I and ligated together. They were then digested with Xba I and Pst I before ligation with the similarly digested p5'emb vector. Genomic DNA from the Apa I site in exon 12 through exon 19 to the Kpn I site was next ligated with the similarly digested vector construct. Finally, the entire *Actin88F* promoter, His-tag, TEV site, EMB isoform, 3' *Mhc* genomic DNA construct (14,094 bp) was removed and ligated into the Xba I and Kpn I sites of the pattB vector (7411 bp) [26]. The pattB6HisEmb plasmid sequence was confirmed by the California State University MicroChemical Core Facility prior to shipment for injection into embryos. The resulting myosin isoform is expected to contain the EMB S1 region joined to the indirect flight muscle form of the myosin rod, which contains a subfragment-2 hinge region encoded by alternative exon 15a and a C-terminal tailpiece encoded by alternative exon 18 [27]. The pattB6HisEmb plasmid and its sequence are available from the authors.

2.2. Transformation of *Drosophila* with the embryonic *Mhc* isoform construct

BestGene Inc. (Chino Hills, CA) injected the pattB6HisEmb plasmid into 200 *Drosophila* embryos of FlyC31 strain 24485 (carrying a mutation in the white eye color gene) with the estimated cytosite 68E (chromosome 3). The PhiC31 integrase-mediated transgenesis system was selected for transformation due to the advantage of site-directed insertion of the transgene [26]. Five culture vials were received containing transformant flies with light orange eyes as the phenotypic marker for the transgene encoding the engineered myosin.

2.3. Culturing flies

The healthiest fly cultures were grown at 25 °C, which allowed for a 10 day generation time from egg to adult. Some stocks were kept at room temperature or 18 °C to slow growth for convenience. Fly food consisted of 30 g agar, 120 g sucrose, 75 g active dry yeast, 2.325 g calcium chloride dihydrate, 2.325 g ferrous sulfate heptahydrate, 12 g sodium potassium tartrate, 0.75 g sodium chloride, 0.75 g manganese chloride and 1.5 L water, which was

autoclaved for 30 min [28]. The food was then cooled to 50 °C before 12 mL of 10% Nipagen was added and then poured into the appropriate culture vials and bottles.

2.4. Selection for homozygous EMB-expressing flies

Standard genetic crosses were made to generate a homozygous transgenic line in the *Mhc*¹⁰ background. *Mhc*¹⁰ is a myosin heavy chain null line for the indirect flight and jump muscles [29]. The heterozygous transgenic flies were crossed with A12 flies (*w*; *CyO/Bl*¹; *TM2,Ubx*¹³⁰/*TM6B,Tb,Antp*^{Hu}); the progeny were screened for curly-winged, light orange-eyed flies. The selected flies were crossed to the *Mhc*¹⁰ line; the selected progeny were self-crossed to produce a homozygous *Mhc*¹⁰, heterozygous transgenic myosin line which was self-crossed to produce the final true breeding homozygous transgenic line (darker orange eyes) in the homozygous *Mhc*¹⁰ background. The line we utilized expressed myosin at 76.4% of wild-type levels (relative to actin levels) in upper thoraces, as determined by densitometry [30]. The homozygous EMB-expressing flies are flightless in either the *Mhc*¹⁰ or *yw* background. This is expected, since EMB myosin alone does not support flight and it is dominant negative for flight [25].

2.5. Protein extraction

Myosin extraction has been done since the 1940s [31, 32]. The charged amino acid repeats in the myosin rod influence the solubility behavior of the myosin and consequently the ionic strength needed to form or dissolve myosin filaments. Low ionic strength allows for filament formation and precipitation of myosin, whereas high ionic strength favors myosin solubility. The methods developed for myosin extraction from transgenic *Drosophila* lines expressing His-tagged myosin are described here.

2.5.1 Bulk whole fly homogenization

1. Raise twenty-five 8 oz culture bottles of transgenic flies (*yw*, *Mhc*¹⁰, *68E:Act88F6HisEmb*) and clear the adults 24 h before collection of newly eclosed offspring.
2. On the day of the extraction, anesthetize the flies with CO₂, collect newly eclosed adults (less than 24 h old) and remove any pupae or larvae. Place the flies in a 50 mL Falcon tube. Flies may be flash frozen in liquid nitrogen and stored at -80°C for convenience.
3. Weigh the flies, 3.35 g is ~3500 flies at an average weight of 0.96 mg/fly.
4. Pour the flies into an ice cooled (4 °C) grinding chamber (Wheaton Potter-Elvehjem Tissue Grinders, 55 mL, Cat. No. 358054) with 45 mL homogenization buffer (12.5 % sucrose, 40 mM NaCl, 10 mM imidazole-Cl, 2 mM MgCl₂, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 % Triton X-100, and a Roche complete protease inhibitor cocktail tablet (Cat. No. 04 693 116 001)). Mount the Teflon pestle in a Wheaton overhead stirrer (Cat No. 903475), set the speed to 4 and insert into the grinding chamber containing the flies. Using constant pressure, raise the grinding chamber to pass the flies by the pestle at least once. Be careful to keep constant pressure to prevent the pestle from spinning at an increased rate and denaturing the proteins due to heating.

2.5.2 Clarification of the supernatant

1. Transfer the dark grey homogenate to two 26.3 mL ultracentrifuge tubes (Beckman #355618). Balance and centrifuge in a Beckman Type 60 Ti rotor at 16,000 rpm (18,178 x g) for 15 min at 4 °C in the Beckman Coulter L8-60M centrifuge.

2. Discard the supernatant by decanting into a beaker. The pellet consists of two layers formed along the tube's length, the inner one of whitish tissue and the outer black exoskeleton. Resuspend each pellet with a transfer pipet and 22 mL homogenization solution. Transfer solution into the glass 55 mL grinding chamber and homogenize using the glass pestle 2–4 times manually.
3. Pour the homogenate into clean ultracentrifuge tubes. Rinse the homogenizer with 5 mL homogenization solution and add to the tubes. Centrifuge the tubes in a Beckman Type 60 Ti rotor at 16,000 rpm (18,178 x g) for 15 min at 4 °C. Discard the supernatant.
4. Repeat step 6 with 50 mL wash buffer (homogenization buffer without protease inhibitor, 12.5 % sucrose, 40 mM NaCl, 10 mM imidazole-Cl, 2 mM MgCl₂, 0.2 mM EGTA, 1 mM DTT, 0.5 % Triton X-100).
5. Balance the tubes and centrifuge in a Beckman Type 60 Ti rotor at 23,500 rpm (39,214 x g) for 15 min at 4 °C. Discard the supernatant.

2.5.3 High salt solubilization of myosin

1. Resuspend each pellet in 22 mL myosin extraction buffer/Nickel column binding buffer (500 mM NaCl, 20 mM sodium phosphate buffer, 30 mM imidazole-Cl, pH 7.4) by repeated pipetting with a transfer pipet. Transfer the solution to the grinding chamber. Homogenize with the glass pestle manually and decant into the centrifuge tubes. The solution will be black with fine particulates.
2. Sonicate the solution in the 26.3 mL tubes on ice with 20 quick pulses at 50% duty cycle, setting of 5, using a micro tip (Fisher Scientific Sonic Dismembrator Model 100).
3. Wait three min and sonicate for another 20 quick pulses in order to break up the tissue and possibly solubilize the thick filaments.
4. Incubate tubes on ice for 30 min to allow for a more complete dissolution of the thick filaments.
5. Centrifuge the tubes in a Beckman Type 60 Ti rotor at 50,000 rpm (177,520 x g) for 35 min at 4 °C. The pellet contains the exoskeleton and some unextracted myosin along with the remainder of the cellular components.

2.5.4 Low salt precipitation of myosin

1. Divide the supernatant equally (8.3 mL each) into six 70 mL ultracentrifuge tubes (Beckman Cat. No. 355622). Fill the tubes to capacity with 4 mM DTT. Leave the tubes in an ice bucket overnight at 4 °C.
2. Centrifuge the tubes with the diluted myosin solution in a Beckman Type 45 Ti rotor at 45,000 rpm (158,420 x g) for 35 min at 4 °C. Discard the supernatant, shake out the remaining solution and place the tubes upside down on a paper towel to dry for a few min. in a 4 °C coldroom (note that sections 2.5.5 and 2.6 should be performed in a coldroom as well).

2.5.5 Solubilization of the myosin pellets

1. Remove/resuspend the enriched myosin pellets (~1 cm diameter) with 3 mL of pellet resuspension buffer (1.5 M NaCl, 30 mM imidazole-Cl, 20 mM sodium phosphate buffer, pH 7.4) being careful to collect all of the sticky pellet material with the pipet tip.

2. Transfer the solution to a chilled 50 mL beaker with a stir bar and cover with Parafilm. Wash/rinse/collect the remaining pellet from the tubes with a 1 mL pipet and 3.0 mL pellet resuspension buffer.
3. Place the beaker on a stir plate set at ~90 rpm for 45 min, and mix the settled pellet with a 1 mL pipet periodically. Add 20 mL binding buffer to lower the ionic strength and dilute the protein. The optimal salt concentration for the Ni column is 500 mM. After an additional 45 min, decant and pipet the remainder of the solution into a 60 mL syringe. Resuspension of the myosin pellets is difficult and takes patience, sometimes requiring a few hours; this time can be used to ready the column.
4. Filter the protein solution with a 0.45 μ m syringe filter (Fisher Scientific Cat. No. 09-719D). The filter may clog and need to be replaced once or twice.

2.6 Myosin purification by Ni-affinity chromatography

The general principle of Ni-affinity chromatography is that His-tagged recombinant proteins bind specifically while allowing other proteins to flow through, permitting elution of highly purified His-tagged proteins [33].

1. Prepare solutions for column run. The buffers should be mixed and filtered with either the Corning 500 mL Vacuum Filter/Storage Bottle System with 0.22 μ m membrane (Cat. No. 430769) or the Millipore Steriflip 50 mL Disposable Vacuum Filtration system with 0.22 μ m Millipore Express PLUS Membrane (Cat. No. SCGP00525), depending on the volume needed.
2. Prepare a GE Healthcare HisTrap HP 5 mL column (Cat. No. 17-5248-02) using Luer Lock syringes and attempt a 5 mL/min flow rate by counting the drops to a timer (100 drops/min).
3. Wash the column with 25 mL filtered water using a 30 mL syringe.
4. Equilibrate the column with 30 mL binding buffer (500 mM NaCl, 20 mM sodium phosphate buffer, pH 7.4, 30 mM imidazole-Cl). Position the column in a clamp on a stand with a 500 mL beaker placed underneath for collection of waste.
5. Load the column with the filtered enriched myosin solution.
6. Wash the column with 60 mL binding buffer.
7. Elute the column with a single step elution buffer (500 mM NaCl, 20 mM sodium phosphate buffer, pH 7.4, 250 mM imidazole-Cl). Collect the eluate in two Amicon Ultra-15 concentrators (Cat. No. UFC905024).
8. Centrifuge the concentrators in a Sorvall Legend RT centrifuge at 3000 x g for up to 30 min at 4 °C, in order to reduce the volume to 2.5–3.0 mL.
9. Determine concentration of the purified myosin solution spectrophotometrically by quantifying the absorbance at 280 nm using an absorbance coefficient of 0.53 cm⁻¹ for 1 mg·mL⁻¹ [9, 34].

2.7. Limited Proteolysis: Subfragment-1 preparation and purification

2.7.1 Solution exchange and contaminant removal

1. Divide the concentrated purified myosin solution equally into six polycarbonate ultracentrifuge tubes (13 × 51 mm, 3.5 mL, Beckman Cat. No. 349622), then dilute with 3.0 mL 4 mM DTT (final NaCl concentration ~70 mM).

2. Incubate for one h on ice for myosin precipitation. This also removes soluble contaminants that bound to the Ni-affinity column.
3. Centrifuge the solution in a Beckman TL-100 ultracentrifuge, in a pre-cooled TLA-100.3 rotor at 50,000 rpm (106,120 x g) for 35 min at 4 °C. Discard supernatants and resuspend each ~2 mm wide pellet with 250 µL digestion buffer (120 mM NaCl, 20 mM sodium phosphate pH 7.0, 1 mM EDTA, 4 mM DTT). Myosin pellets do not readily dissolve at 120 mM salt. Use the pipet tip to collect and remove the sticky pellet and pipet the remainder of the pellet and solution into two pre-cooled ultracentrifuge tubes (Beckman microfuge tube polyallomer, Cat. No. 357448).
4. Carefully homogenize the whitish-colored clear pellets with an Eppendorf tube pestle (Research Products International Corp., Cat. No. 199226). The solution is milky colored and translucent.

2.7.2 Time-limited proteolysis

1. Place the two tubes in a 20 °C water bath for 3 min to equilibrate.
2. Perform proteolysis based upon previously published methods [21, 35, 36]. Prepare a 10 µg/µL α -chymotrypsin solution by gently mixing water with the lyophilized powder (Worthington Biochemical Corp., Cat. No. LS001475). Add 15 µL chymotrypsin solution to the temperature-equilibrated myosin solution. Final chymotrypsin concentration is approximately 0.20 mg/mL.
3. After 6 min, quench the reaction by adding 5 µL of 200 mM phenylmethanesulfonyl fluoride (PMSF) in ethanol. Mix in the PMSF/ethanol solution carefully so as not to precipitate the protein. Final PMSF concentration is approximately 1.5 mM. Immediately place the tubes on ice and then into the TLA 100.3 rotor.
4. Centrifuge the solution to precipitate the insoluble myosin rod and any remaining undigested full-length myosin at 68,000 rpm (196,280 x g) for 25 min at 4 °C.
5. Carefully pipet the enriched S1 supernatant into an Eppendorf tube and place on ice.

2.7.3 Purification by liquid chromatography

1. Perform size exclusion chromatography with isocratic elution of protein using an Amersham Biosciences AKTA FPLC with a 16/60 column with Superdex 200, a flow rate of 1 mL/min, collecting 2 mL fractions. Cover the protein-rich fractions, as determined by 280 absorbance (see chromatogram, Figure 2C), with parafilm and place on ice overnight for convenience.
2. Perform SDS-PAGE analysis of protein peak fractions.
3. Concentrate the S1 fractions (6–8 mL total volume) using two 0.5 mL Millipore Biomax 5K NMWL centrifugal filter devices (Cat. No. UFV5BCC00). Six to ten 15 min centrifugations at 12,000 rpm (10,701 x g) in the Beckman Microfuge are needed to reduce the volume down to 200 µL.
4. Determine the concentration of the S1 solution spectrophotometrically by quantifying the absorption at 280 nm using an absorbance coefficient of 0.75 cm⁻¹ for 1 mg·mL⁻¹ [21, 34].

2.8 Guidance and avoiding potential pitfalls

The time period required for injection of DNA into embryos to the receipt of transgenic lines from BestGene, Inc. is approximately six weeks. Once the flies are received, crosses to generate homozygous lines in an *Mhc*¹⁰ background, assessment of expression levels and culturing of gram quantities of flies takes approximately four months. The myosin isolation protocol takes two days.

When resuspending the myosin pellet, which can be very viscous and sticky, careful pipetting and mixing with the pipet tip is required. The pellet can stick to anything it touches. This can lead to significant loss of material. Section 2.5.5, step 1 and section 2.7.1, step 3 require that the myosin pellets be transferred within a pipet tip. To accomplish this, pipet the appropriate solution against the edge of the pellet to free it from the side of the tube. Then gently draw the pellet into the tip or hold it at the tip with the vacuum pressure created by the pipet. Then perform the transfer. Low-binding pipet tips, either treated polymer or siliconized glass, may prevent the pellet from sticking to the inside of the tip. If glass pipets are used, tips should be heat treated to produce rounded edges.

2.9 Myosin preparation from microscopic dissection of the indirect flight muscle

In addition to the His-tagged myosin purification procedure as described above, preparation of myosin from dorsolongitudinal indirect flight muscle was done from 180–200 EMB or His-Tagged EMB transgenic flies. Briefly, dorsolongitudinal indirect flight muscles were removed by microscopic dissection and purification of myosin was carried out at 4 °C using methods essentially as described previously [9, 37, 38]. Concentration of the purified myosin was determined spectrophotometrically by measuring the absorption at 280 nm. Purified myosin was used within 2 h for ATPase assays.

2.10. Determination of Steady-State ATPase Activity

ATPase activities of myosin from dissected muscles or Ni-affinity column chromatography were determined using [γ -³²P] ATP as previously described in detail [9, 37, 38]. After the nickel column, eluate was concentrated, diluted with 4 mM DTT and centrifuged in a Beckman TL-100 ultracentrifuge as described in section 2.7. This step removes soluble contaminants. Myosin pellets were dissolved to a concentration of 2 μ g/ μ L in myosin storage buffer (20 mM MOPS pH 7.0, 500 mM KCl, 20 mM DTT, 2 mM MgCl₂). For CaATPase assessment, 1 μ L of the freshly prepared myosin solution was added to 125 μ L CaATPase buffer (10 mM imidazole pH 6.0, 0.1 M KCl, 10 mM CaCl₂). CaATPase activity of myosin was initiated at 25 °C by addition of [γ -³²P] ATP. After a 15 min incubation the reaction was quenched by adding 100 μ L of 1.8 M HClO₄. Extraction and scintillation counting were done essentially as described previously [9]. Basal MgATPase assessment was carried out in a similar fashion, except using 18.2 μ L of MgATPase buffer (10 mM imidazole pH 6.0, 20 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 1 mM [γ -³²P] ATP). Actin-stimulated MgATPase assays were performed in MgATPase buffer in the presence of increasing concentrations of chicken F-actin (0.1 to 2 μ M). For assays in MgATPase buffer, the reaction was quenched after 25 min with 50 μ L of 1.8 N HClO₄. Basal Mg²⁺-ATPase activities obtained in the absence of actin were subtracted from actin-activated data points. Mean values from at least three different experiments were used to determine average ATPase values of each myosin. Maximal actin-activated ATPase ($V_{\max}$) was obtained by fitting all data points from several preparations (minimum of three data points) of EMB and His-tagged EMB myosin with the Michaelis-Menten equation using Sigma Plot software. Values were averaged to give the mean $\pm$ SD. Statistical comparisons of ATPase data were carried out using Student's *t* tests.

2.11. Crystallization

Peak S1 protein fractions from size exclusion chromatography were pooled and concentrated to 7.28 mg/mL. S1 crystals were grown by the hanging drop, vapor diffusion method. Crystallization conditions were found using Hampton Research crystal screens (Cat. No. HR2-110 and HR2-112). One microliter of concentrated S1 was mixed with 1 μ L mother liquor on a glass coverslip which was then inverted and sealed over the reservoir containing 1 mL mother liquor. Microcrystals were grown by incubation at room temperature with mother liquor containing 0.1 M HEPES pH 7.5, 20% w/v polyethylene glycol 10,000. Larger rod-shaped crystals formed when using mother liquor containing 0.1 M HEPES pH 7.5, 17.5% w/v polyethylene glycol 8,000.

3. RESULTS

3.1 Design and construction of indirect flight muscle protein expression system

In order to provide a cost effective and robust method for high level expression of class II myosin motor proteins (Figure 1A), we designed a *Drosophila melanogaster* expression system in which the endogenous indirect flight muscle myosin heavy chain isoform is replaced by an engineered transgenic isoform. This involves expressing the transgene in the *Mhc¹⁰* strain, which lacks myosin heavy chain in its flight and jump muscles [29]. Placement of the target gene under transcriptional control of the *Actin88F* promoter [23] insures that the myosin of the indirect flight muscle in the fly is composed entirely of the target myosin protein. Our system also allows for the addition of epitopes, such as a hexahistidine tag (His-tag), to aid in detection and purification of the recombinant protein from whole organism homogenates (Figure 1B).

The fly line we generated is designed to express a skeletal muscle myosin II embryonic body wall muscle isoform (EMB) S1 domain (Figure 1C) attached to the indirect flight muscle rod region. The pattB6HisEmb plasmid that we constructed to express the modified EMB isoform (Figure 1B) was built into the PhiC31 injection vector pattB, which allows targeted and efficient germ line transposition [26]. The insert contains the *Actin88F* promoter followed by coding regions for a His tag and a recognition site for the TEV protease. This permits Ni-based chromatographic isolation as well as clipping of the His tag from the encoded myosin. Myosin heavy chain cDNA containing exons 2–12 of EMB and genomic DNA for the exons 12–19 region encode the expressed myosin isoform. While the cDNA encodes the EMB S1 head, the genomic DNA is expected to encode transcripts that produce the indirect flight muscle version of the rod. This has isoform-specific domains at the subfragment-2 hinge and in the C-terminal tailpiece [27], which may provide higher stability to the myofibrils in the transgenic organisms [9, 24, 30]. The 3' non-coding region of the genomic DNA contains necessary transcription termination and polyadenylation sites. The pattB6HisEmb plasmid was injected into *D. melanogaster* attP 68E *yw* embryos to produce transgenic lines with the PhiC31 targeted insertion. Selection based on eye color allowed for production of flies that are homozygous for the modified EMB gene.

The function of the EMB isoform has been studied by comparison to the indirect flight muscle isoform and various chimeric isoforms with regard to biophysical, enzymological, mechanical and ultrastructural properties [9, 21, 25, 37, 39–47]. Hence isolation of large quantities of EMB S1 for further studies, particularly for production of high-resolution crystal structures, should provide deep insights into myosin structure-function relationships.

3.2 Purification of His-tagged EMB

Myosin can be purified from whole flies by microdissection and removal of the dorsolongitudinal indirect flight muscles and homogenization in high salt myosin extraction

buffer [9]. Solubilized myosin heterohexamers (two heavy chains and two each of the essential and regulatory light chains) can then be polymerized into myosin filaments at low salt and collected via centrifugation. Treatment with limiting amounts of protease yields S1 fragments that contain the myosin motor domain, as well as myosin rods (Figure 1A) [21]. The rods reassemble into high molecular weight filaments in low salt buffer and can be separated from S1 by centrifugation.

By virtue of its His tag, isolation of engineered myosin that is expressed in the indirect flight muscle system does not require microdissection of individual muscle fibers from flies. Placement of the His-tag at the amino-terminus (Figure 1C) avoids its interference with myosin assembly and its catalytic mechanism. Lowey *et al.* [22] showed this to be the case for mouse cardiac myosin. Whole flies can be harvested in gram amounts and myosin can be isolated following homogenization and various precipitation steps (Figure 2A). An initial homogenization of flies yields a protein mixture (Figure 2B, lane 2) from which the myosin can be enriched (Figure 2B, lane 7). His-tagged myosin can then be purified by Ni-affinity chromatography (Figure 2B, lane 10); typically ~10 mg of protein is produced. Similar treatment of wild-type *yw* control flies yields little or no myosin after affinity chromatography (Figure 3, top). The presence of the His tag is confirmed by anti-His Western blots (Figure 3, bottom), and no detectable His-tagged protein is isolated from control flies (Figure 3, bottom). Low-salt precipitation of His-tagged proteins removes soluble contaminants, notably a protein of ~ 75 kDa (compare Figure 2B, lanes 10 and 11).

Digestion of myosin isolated by Ni-affinity chromatography using α -chymotrypsin permitted preparation of the EMB S1 fragment and the rod (Figure 2B, lane 12). Note that the α -chymotrypsin treatment clipped the His tag, likely at sites in the TEV protease recognition site (data not shown). Removal of myosin rod and undigested myosin by centrifugation under low salt conditions (Figure 2B, lane 14) improves the purity of S1 (Figure 2B, lane 13). Size exclusion chromatography (Figure 2C) yields an EMB S1 fragment (Figure 2B, lane 15) that is sufficiently pure to be crystallized (Figures 2C, inset).

Our procedure allows us to isolate milligram amounts of full-length transgenic myosin. S1 fragment can be readily produced from this myosin and isolated by size exclusion chromatography. In the purification shown in Figure 2B, we obtained 171 μ g of S1. We note that S1 produced from flash-frozen flies can be crystallized, as can frozen S1. Hence collection of sufficient quantities of S1 for x-ray diffraction studies of crystals should be feasible using our system.

3.3 Basal and Actin-activated ATPase Activities

We measured the CaATPase, basal MgATPase, and actin-stimulated MgATPase activities of untagged EMB and His-tagged EMB recombinant transgenic myosins (Figure 4). As the ATPase activity and site of actin binding are in the S1 head, it is expected that the presence of the indirect flight muscle domains in the rod will not affect these properties. Indeed, we observe that His-tagged EMB recombinant myosin showed ATPase activity essentially identical to EMB myosin, which had previously been expressed without a His tag [9, 45]. This is true when the His-tagged EMB myosin is isolated from dissected indirect flight muscles or by Ni-affinity chromatography. CaATPase and basal MgATPase activities of EMB myosin are not statistically different from His-tagged EMB myosin (4.83 ± 0.83 vs. 4.58 ± 0.93 and 0.083 ± 0.029 vs. 0.091 ± 0.033 sec^{-1} , respectively). The measured actin-stimulated V_{max} for EMB is also not significantly different ($p > 0.05$) from His-tagged EMB (0.77 ± 0.05 vs. 0.73 ± 0.08 sec^{-1}). These results confirm that His-tagged EMB recombinant transgenic myosin possesses enzymatic activity like that of EMB transgenic myosin lacking the His tag.

DISCUSSION

We have described a novel method for expression and purification of functional class II striated muscle myosin motor proteins using whole fruit flies and have illustrated its utility by generating highly pure preparations of the recombinant EMB isoform in quantities sufficient for crystallization. This approach has several advantages over the classical microdissection and extraction techniques. First, the need for time consuming and tedious microdissection is eliminated. Second, the amount of full-length His-tagged myosin that can be purified from gram quantities of fruit flies exceeds the amount that is typically purified through the microdissection technique. Third, the fact that His-tagged myosin can be extracted from frozen whole flies eliminates the need for synchronizing the eclosion of several thousand flies prior to the start of the protocol. Fourth, the Ni-affinity column can effectively interact with an amino-terminal His-tag on myosin, thereby eliminating the endogenous myosin during the affinity purification step. Finally, concentrated preparations of S1 derived from this method can be aliquotted, flash frozen, thawed, and crystallized.

It should be noted that the endogenous indirect flight muscle versions of the essential and regulatory myosin light chains are expected to be present in the myosin isolated in our system. The regulatory light chain appears to have a single encoded isoform [48], but there is an indirect flight muscle specific isoform of the essential light chain [49]. Presence of the indirect flight muscle essential light chain could affect enzymatic and mechanical properties of an isoform that is normally produced in a different tissue. Furthermore, the presence of the *Drosophila* light chains needs to be kept in mind should efforts be made to express myosin heavy chains from other organisms using the *Drosophila* system. Of course, RNAi or genetic approaches can be used to prevent fly light chain expression and additional transgenes encoding light chains from another organism can be introduced.

Currently, the method we developed is being employed to produce milligram quantities of His-tagged indirect flight muscle myosin for structural and biophysical studies and comparison to the EMB isoform. In the future, various other isoforms and mutant versions of the protein can be generated that should yield additional insights into structure-function relationships in the myosin molecule.

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ABBREVIATIONS

EMB	embryonic body wall muscle isoform of myosin heavy chain
Mhc	myosin heavy chain
S1	proteolytic subfragment-1 of myosin
TEV	tobacco etch virus

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Figure 1.

Myosin protein, transgene and encoded isoform. A) Schematic diagram of a functional class II myosin protein dimer. The two identical myosin heavy chain subunits are depicted in different shades of grey. The amino-terminal “Motor Domain”, which contains the actin-binding sites and ATPase activity, is labeled as are the carboxy-terminal coiled-coil “Rod” regions. The proteolytically stable subfragment-1 region is labeled as “S1”. One copy each of the essential and regulatory light chains, labeled ELC and RLC, respectively, are associated with each myosin heavy chain creating an overall functional hexamer. B) Graphic map for the PhiC31 pattB injection plasmid that was used to create the His-tagged EMB recombinant expressing transgenic fly line. Expression of the His-tagged EMB recombinant myosin isoform is driven by the *Actin88F* promoter primarily in the indirect flight muscle [50–52]. In addition to the N-terminal His-tag (HT), the transgene encodes a TEV protease recognition site. A cDNA encoding the S1 region of EMB (with alternative exons highlighted in color) is followed by genomic DNA containing exons and introns (coding for the indirect flight muscle version of the myosin rod) and the 3' termination sites of *Mhc*. C) Ribbon diagram representation of a homology model of the *D. melanogaster* EMB motor domain based upon the x-ray crystal structure from scallop (PDB ID:1QVI). Alternative exons specific to fly EMB (3a, 7a, 9b, and 11c) are colored and labeled. The amino-terminal end to which the His-tag is attached is also highlighted. The homology model was produced by loading the EMB sequence into SWISS-MODEL [53] and running the SwissModel Automatic Modelling Mode followed by figure rendering using PyMOL software [54].

Figure 2.

Expression and purification of His-tagged EMB recombinant myosin in the indirect flight muscle system. A) Flowchart of the sequence of steps in purification. Horizontal arrows that shift down or up represent centrifugation steps in which the protein remains in either the pellet or the supernatant, respectively. B) Coomassie Blue stained 10% SDS PAGE analysis of samples taken during the course of purification. The initial homogenate (lane 2) is pelleted. Pelleted material is homogenized and sonicated in high salt buffer (lane 5) and centrifuged. The supernatant (lane 6) is filtered (lane 7) prior to loading on a Ni column. The concentrated eluate (lane 10) is highly enriched in myosin heavy and light chains, typically yielding ~10 mg of protein. This is further purified by low salt precipitation prior to proteolysis (lane 11). Proteolysis with α -chymotrypsin yields myosin rod and S1 (lane 12). Much of the remaining full-length myosin and rod can be removed by centrifugation (lanes 13 vs. 14). C) Chromatogram from size exclusion chromatography on mixture of products of

proteolyzed His-tagged EMB (lane 13 in panel B). The major peak represents an S1 sample (lane 15, panel B) of sufficient purity for crystallization (inset; bar, 0.2 mm).

Figure 3.

Identification of His-tagged EMB recombinant myosin. A Coomassie-Blue stained 10% SDS PAGE analysis of separate preparations of myosin produced by microdissection of the dorsal longitudinal indirect flight muscles from the transgenic fly line expressing His-tagged EMB (lane 2; upper panel) and a control (*yw*) wild-type fly line (lane 3; upper panel). Lanes 4 and 5 (upper panel) reveal the protein that eluted from Ni-affinity chromatography on the His-tagged EMB and wild-type lines, respectively. Note that no myosin was isolated by this approach from wild-type flies. The lower panel is a Western blot of samples in the same order that has been probed with anti-His antibody (Qiagen). Note that the His-tag is only present in myosin isolated from the transgenic lines expressing the recombinant EMB isoform.

Figure 4.

ATPase activity of full-length embryonic myosin isoforms. Histograms of Ca , basal Mg , and actin-stimulated MgATPase activities are shown from four experiments for EMB (maroon) and His-tagged EMB recombinant myosin (green).