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

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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.

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 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 *Escherichia 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 C_2C_{12} 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

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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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 fight 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 2000) 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 *D. 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 GTG CAT CTT GGC AGT TGT TTA TCT GG-3′ to produce the 3′ *Act88F* 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 subfragement-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 trangenic 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].