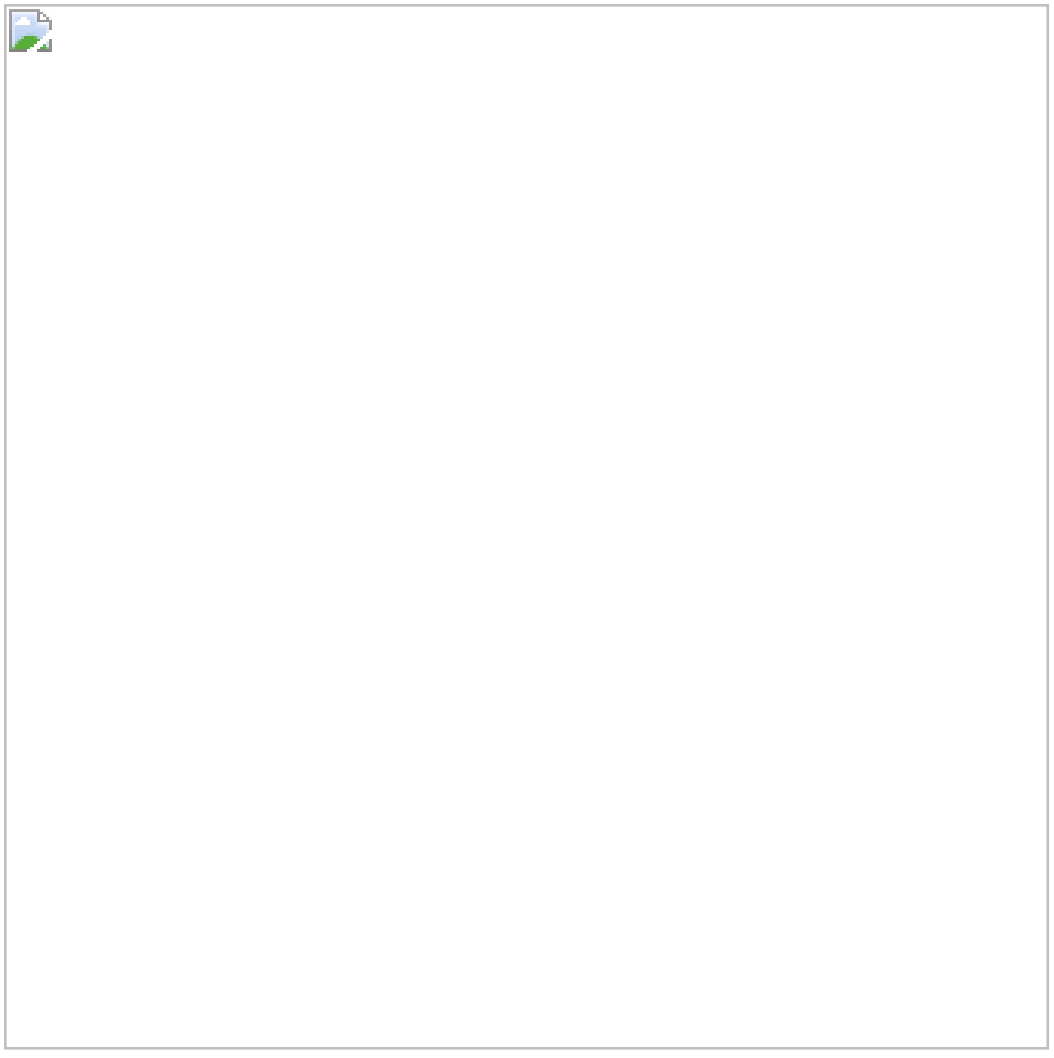

Isolation and Characterization of Sea Sponge Myosin

Authors: Kanzawa, Nobuyuki, Takano-Ohmuro, Hiromi, and Maruyama, Koscak

Source: Zoological Science, 12(6) : 765-769

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.765

ZOOLOGICAL SCIENCE 12: 765–769 (1995)

Isolation and Characterization of Sea Sponge Myosin

NOBUYUKI KANZAWA, HIROMI TAKANO-OHMURO* and KOSCAK MARUYAMA

Department of Biology, Faculty of Science, Chiba University, Chiba 263, and
**Department of Pharmacology, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan*

ABSTRACT—Myosin was purified to a homogeneity from sea sponge, *Halichondria okadai*. The myosin consisted of 220 kDa heavy chain, 18 kDa calcium binding light chain and 21 kDa phosphorylatable light chain. Rotary shadowed images showed the two headed myosin (myosin II) with a 160 nm tail. The myosin was less soluble in a KCl solution as compared to rabbit skeletal myosin.

The K^+-stimulated and Ca^{2+}-stimulated ATPase activities of sea sponge myosin were 0.46 and 0.07 μmol Pi min^{-1} mg^{-1}, respectively. The Mg^{2+}-activated myosin ATPase activity showed no significant enhancement by the addition of rabbit skeletal muscle actin despite that the light chain was phosphorylated by myosin light chain kinase from chicken gizzard. Sea sponge myosin 18 kDa light chain bound to Ca^{2+} ion but was not phosphorylated like *Physarum* plasmodia myosin light chains.

INTRODUCTION

Myosin molecule constituting of two heavy chains and of four light chains (myosin II) is a major element of energy transduction and force development in muscle or nonmuscle tissues. Myosin has been isolated and characterized from various organisms [1, 2]. Sea sponge, a lower eukaryotic animal, has not distinct muscle tissues. In this paper, for the first time, we report isolation and characterization of myosin from sea sponge, *Halichondria okadai*. Sea sponge myosin 18 kDa light chain could bind Ca^{2+} ion, and 21 kDa light chain was phosphorylated by myosin light chain kinase (MLCK) from chicken gizzard. *Physarum* plasmodia myosin is reported to have two species of light chains; one is phosphorylated by endogenous kinase and another binds to Ca^{2+} ion resulting in the Ca^{2+} inhibitory regulation [3, 4]. Although the regulation of myosin ATPase activity was obscure, sea sponge myosin contained phosphorylatable regulatory light chain and Ca^{2+} -binding light chain like as *Physarum* myosin.

MATERIALS AND METHODS

Materials

Sea sponge was collected in the sea near the Marine Ecosystem Center, Chiba University, Kominato. Sea sponges were cut into small pieces by hands removing mussels, small stones etc. and the pieces were well washed with a cooled Mg^{2+}- free artificial seawater. The washed sea sponges were added with a cooled salt solution consisting of 450 mM NaCl, 11 mM KCl, 2 mM $NaHCO_3$, 25 mM Na_2SO_4, and 2 mM EDTA and homogenized for 10 sec at 10, 000 rpm three times in a Waring blender. The resultant suspension was filtered through a nylon gauze. The filtrate was centrifuged for 5 min at 500 rpm to sediment large debris, and then the supernatant was further subjected to centrifugation for 5 min at 5,000 rpm. The precipitated sea sponge cells were suspended in the above solution, added with glycerol to a final concentration of 50%, and stored at −20°C until use. The yield of sea sponge cells were approximately 30 g from 100 g live sea sponges.

Preparation of proteins

Actin was prepared from acetone powder of rabbit skeletal muscle and purified by Sephadex G-150 column chromatography. Myosin light chain kinase (MLCK) and smooth muscle myosin were purified from chicken gizzard according to Nakamura and Nonomura [5] and Ebashi [6], respectively. Calmodulin was purchased from Sigma. Protein concentrations were determined by the method of Bradford [7] using bovine serum albumin as a standard.

Phosphorylation of myosin light chains

Purified sea sponge myosin (1 mg/ml) was treated with MLCK, 5 μg/ml, for 30 min at 25°C in a mixture of 8 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μg/ml calmodulin, and 20 mM Tris-HCl, pH 7.5. The reaction was stopped by the addition of EGTA to a final concentration of 10 mM. After dilution with 10 vol. of cold distilled water, the precipitate centrifuged for 10 min at 13,000 rpm was dissolved in a solution containing 0.6 M KCl, 1 mM $NaHCO_3$ and 0.5 mM DTT (dithiothreitol). Clarified sample was used for ATPase measurements.

Autoradiography

Sea sponge myosin was incubated for 30 min at 25°C with 6 μCi/ml of [γ-^{32}P] ATP (Amersham, 6000 Ci/mmol) in the above described MLCK solution. The reaction was terminated by the addition of 3 vol. of an SDS solution containing 10% SDS, 5% 2-mercaptoethanol and 20 mM Tris-HCl, pH 6.8. After SDS gel electrophoresis, gels were stained with Coomassie Brilliant Blue, and dried on a Whatman No. 5 filter paper. Autoradiography was carried out at −80°C for 1 day with Kodak X-omat film and Cornex Lightening-plus intensifying screen (DUPONT).

ATPase measurements

The reaction mixture for ATPase assay was as following. K^+

Accepted September 11, 1995
Received July 31, 1995

-EDTA-activated: 1 M KCl, 10 mM EDTA, 20 mM Tris-HCl, pH 7.5 and 1 mM ATP; Ca^{2+}-activated: 0.1 M KCl, 3 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5 and 1 mM ATP; Mg^{2+}-activated: 30 mM KCl, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, rabbit skeletal muscle F-actin, 1/3 by weight to myosin and 1 mM ATP. The reaction was started by adding ATP to a final concentration of 1 mM and the mixture was incubated for 5 min at 25°C. After terminating the reaction by adding malachite green solution, inorganic phosphate was assayed as described by Kanzawa *et al.* [8].

SDS gel electrophoresis

An SDS gel electrophoresis was performed according to Laemmli [9] using 15% (for myosin light chain) and 7.5–18% (for myosin purification step) polyacrylamide gels. Two dimensional gel electrophoresis was carried out as described by Mikawa *et al.* [10]. Ca^{2+} binding assay was performed as described by Maruyama and Nonomura [11] after two dimensional gel electrophoresis. Stained gels with Stains-all were photographed through a red filter.

Solubility test of myosin

Sponge myosin and rabbit skeletal muscle myosin dissolved in 0.6 M KCl were dialyzed overnight against various concentrations of KCl buffered with 10 mM potassium phosphate buffer, pH 7.0. The supernatant after centrifugation for 30 min at 15,000×*g* was assayed for protein concentrations. The relative solubility is given as % protein concentration relative to the one before dialysis.

Electron microscopy

Samples in 50% glycerol was sprayed onto freshly cleaved mica. The samples were rotary shadowed with platinum and carbon at 7.5°C and then with carbon at 90°C on the specimen stage of a JEOL JFD-9000 freeze-fracture apparatus. The specimen was observed under a JEOL 100S electron microscope operated at 80 kV.

RESULTS

Purification of myosin

Stored sea sponge cells were washed with a low salt buffer (40 mM KCl, 20 mM Tris-HCl, pH 7.0, 0.5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine and 5 mM EGTA), and centrifuged for 5 min at 10,000 rpm (TOMY, No. 4 rotor). Precipitate was resuspended in the same buffer and homogenized in a teflon homogenizer. This procedure was repeated 3 times. All the procedures were carried out at 4°C.

The washed precipitate was extracted for 30 min with an ATP solution containing 10 mM ATP, 0.3 M KCl, 40 mM Tris-HCl, pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 5 mM benzamidine, 0.2 mM $CaCl_2$, leupeptin, 10 μg/ml and 0.01% casein. After centrifugation for 15 min at 13,000 rpm, the supernatant was filtrated through Whatman No. 3 filter paper and diluted with 10 vol. of 0.5 mM DTT solution. The precipitate collected by centrifugation for 10 min at 10,000 rpm was dissolved in 0.15 M potassium phosphate buffer, pH 7.5 containing 0.5 mM DTT and dialyzed against the same buffer (Fig. 1c).

Clarified solution by centrifugation for 30 min at 13,000 rpm was subjected to a DEAE-Sephadex A-50 column (1.5×10 cm) equilibrated with 0.15 M potassium phosphate buffer, pH 7.5, and 0.5 mM DTT. Crude myosin was eluted with 0.3 M KCl, 0.17 M potassium phosphate buffer, pH 7.5, and 0.5 mM DTT. The eluted fraction containing crude myosin was dialyzed against 0.5 M KCl, 2 mM ATP, 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.0, and 0.5 mM DTT. Clarified dialyzate was concentrated by Amicon Centriprep 30 and subjected to TOSOH HPLC using a TSK gel G-4,000SW column. The main peak was collected. The yield was approximately 1 mg starting from 40 g of sea sponge cells.

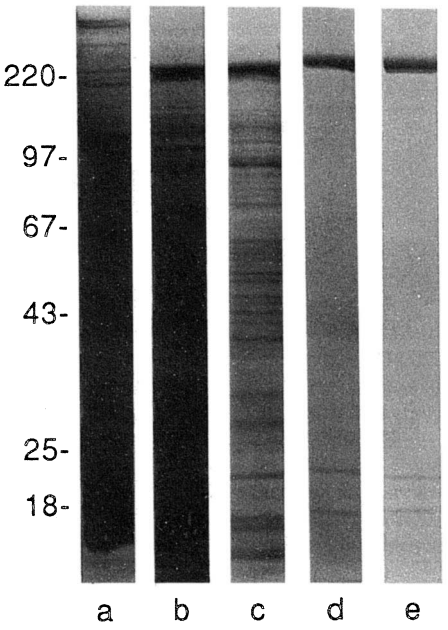

FIG. 1. Purification of sea sponge myosin. a, whole SDS extract; b, extract with ATP-KCl solution; c, dissolved fraction with 0.15 M potassium phosphate buffer; d, eluate of DEAE Sephadex A-50 column chromatography; e, after HPLC. Numerals show molecular mass in kilodalton.

Figure 1a is an SDS extract of dissociated sea sponge cells. Figire 1b is the first ATP-KCl extract and Figure 1d is the eluate of DEAE Sephadex A-50 column. Myosin was effectively concentrated in this step. HPLC gel chromatography largely removed contaminants. Sea sponge myosin consisted of 220 kDa heavy chain and two species of light chains (Fig. 1e).

Molecular shape of sea sponge myosin

Rotary shadowed image of sea sponge myosin was conventional two headed myosin (Fig. 2a). The myosin tail length was about 160 nm like that of rabbit skeletal muscle myosin (Fig. 2b). Both myosins were not morphologically distinguishable.

Solubility of sea sponge myosin

Sponge myosin was less soluble in KCl as compared to rabbit skeletal muscle myosin (Fig. 3). In the presence of 0.25 M KCl and 10 mM potassium phosphate buffer, pH 7.0, rabbit skeletal myosin was soluble by 80%, whereas 80% of

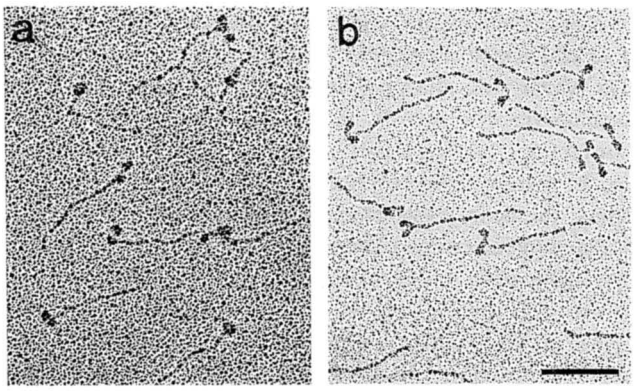

FIG. 2. Rotary shadowed images of sea sponge myosin. a, sea sponge myosin; b, rabbit skeletal muscle myosin. Bar, 100 nm.

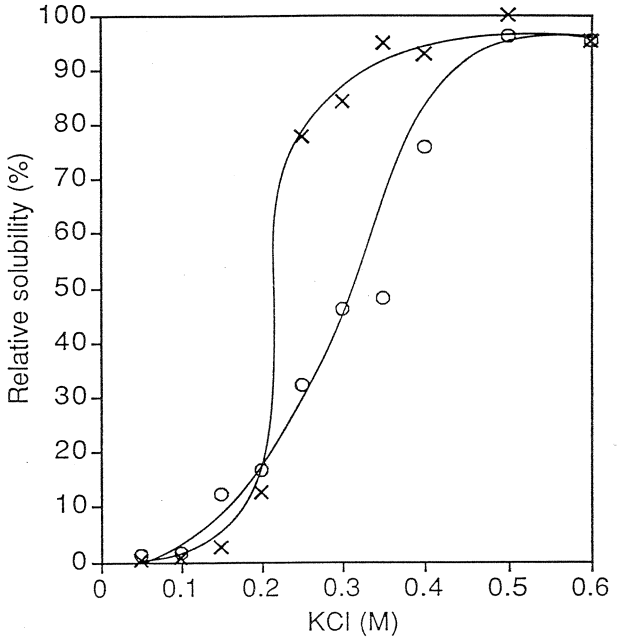

FIG. 3. Solubility in KCl of sea sponge myosin. After dialysis, myosin was centrifuged and the protein concentration in supernatant was assayed. ○, sea sponge myosin; ×, rabbit skeletal muscle myosin.

sea sponge myosin was precipitated (Fig. 3). At 0.5 M KCl, sponge myosin was maximally soluble.

Sponge myosin regulation

Two dimensional gel electrophoresis revealed that the myosin has two species of light chains (Fig. 4a). Relative molecular masses of the myosin light chains compared with chicken gizzard smooth muscle myosin light chains were approximately 21 and 18 kDa, respectively (data not shown). Figure 4b shows that the sea sponge myosin light chain was phosphorylatable by chicken gizzard MLCK. Only the basic 21 kDa myosin light chain was phosphorylated. Chicken gizzard smooth muscle myosin and sea sponge myosin light chains were stained with cationic carbocyanine dye "Stains-

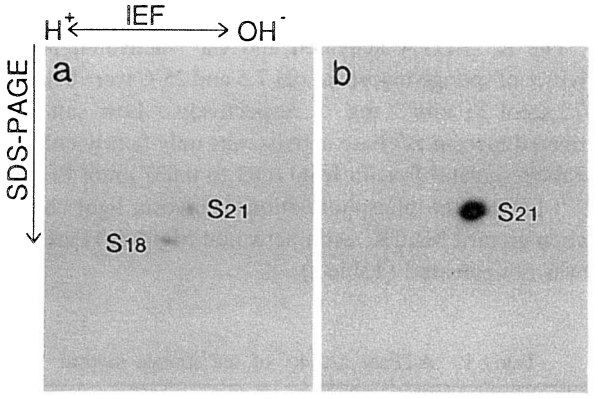

FIG. 4. Phosphorylation of sea sponge myosin light chains by chicken gizzard myosin light chain kinase. a, two dimensional gel electrophoresis pattern; b, autoradiogram. S_{21} and S_{18}, 21 kDa and 18 kDa light chains from sea sponge myosin. IEF, isoelectricfocusing; SDS-PAGE, SDS gel electrophoresis pattern.

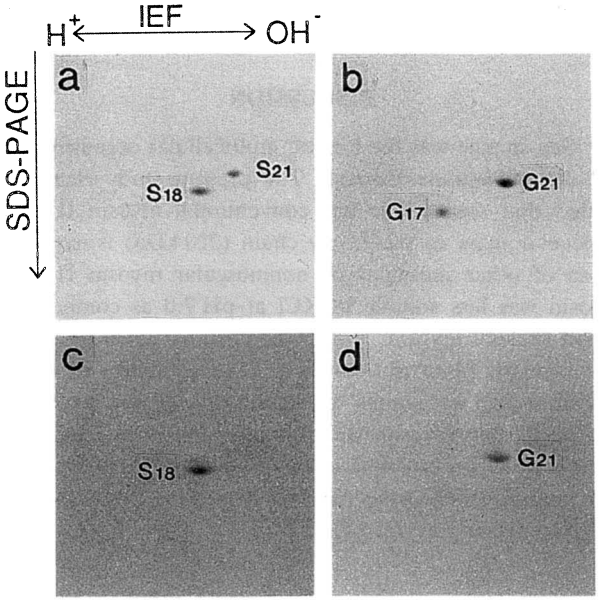

FIG. 5. Ca^{2+} binding ability revealed by staining with Stains-all after two dimensional gel electrophoresis. a, b, Coomassie Brilliant Blue stain of sea sponge and chicken gizzard myosin light chain respectively. c, d, Stains-all stain of sea sponge and chicken gizzard myosin light chain respectively. c and d are photographed though a red filter after Stains-all staining. S_{21} and S_{18} are the same as Fig. 3. G_{21} and G_{17} are 21 kDa and 17 kDa chicken gizzard myosin light chain. IEF, isoelectricfocusing; SDS-PAGE, SDS gel electrophoresis pattern.

all" after the two dimensional gel electrophoresis. Gizzard myosin 21 and 17 kDa light chains were stained blue and red, respectively (data not shown). Because of photography through a red filter the gizzard 21 kDa light chain spot was enhanced and the gizzard 17 kDa light chain spot was faded out (Fig. 5d). It is evident that the 21 kDa light chain bound to Ca^{2+} ion. On the contrary, in sea sponge myosin, the 18 kDa light chain was a Ca^{2+} binding light chain (Fig. 5c).

ATPase activity of myosin

The K^+-EDTA activated and Ca^{2+}-activated ATPase activities of sponge myosin at pH 7.5 and 25°C were 0.462 and 0.075 μmol Pi min^{-1} mg^{-1}, respectively. Low salt Mg^{2+}-activated myosin ATPase activity was only faintly enhanced by rabbit skeletal F-actin from 0.03 to 0.037 μmol Pi min^{-1} mg^{-1}. After the phosphorylation of myosin light chain by chicken gizzard MLCK, actin-activated Mg^{2+}-ATPase activity was not elevated (Table 1).

TABLE 1. ATPase activity of sea sponge myosin

	ATPase activity (μmol Pi $min^{-1}mg^{-1}$)	
	unphosphorylated	phosphorylated
K^+-EDTA	0.462	
Ca^{2+}	0.075	
Mg^{2+}	0.030	0.028
Mg^{2+} + actin	0.037	0.038

DISCUSSION

Sea sponge was the highest multicellular organism without distinct muscle tissues. The present study clearly revealed that sea sponge has conventional myosin II. The molecular mass of the heavy chain (220 kDa) is similar to those of other muscular or nonmuscular myosin II. The myosin was less soluble in KCl at pH 7.0 as compared to rabbit skeletal myosin, and formed bipolar filaments at low salt strength (data not shown). The solubility of sponge myosin in KCl was similar to that of sea anemone myosin [8]. It appears that myosin from animals of the seawater is less soluble in KCl solution as compared to myosins from terrestrial animals and myosins from animals of the freshwater (for crayfish myosin, see ref [12]).

Sea sponge myosin contained two species of light chains of 18 kDa which showed Ca^{2+} ion binding ability and of 21 kDa which was phosphorylated by MLCK, although the phosphorylatable myosin light chains of chicken gizzard showed Ca^{2+} binding ability (Fig 5d). In *Physarum* plasmodia, the myosin with phosphorylatable and Ca^{2+} binding light chains, the actin-activated myosin Mg^{2+}-ATPase activity is inhibited by micromolar levels of Ca^{2+} and the Ca^{2+} binding light chain plays a key role in the inhibitory regulation of myosin ATPase [3]. However, calcium ions had no influence on Mg^{2+} ATPase activity of sea sponge myosin in the presence of actin (data not shown). It is to be noted that actin-enhanced ATPase activity is not observed with scallop myosin [13] and insect muscle myosin [14, 15]. Any significant enhancement in actin-activated Mg^{2+} ATPase activity of sponge myosin was not caused by phosphorylation with MLCK and also by direct Ca^{2+} binding. It is desirable to do the above mentioned experiments using actin isolated from sea sponges. Table 2 summarizes the regulation and the light chains of several invertebrate myosins.

REFERENCES

1 Harrington WF, Rodgers ME (1984) Myosin. Annu Rev Biochem 53: 35–73

2 Warrick HM, Spudich JA (1987) Myosin structure and function in cell motility. Annu Rev Cell Biol 3: 379–421

3 Kohama K, Takano-Ohmuro H, Tanaka T, Yamaguchi Y, Kohama T (1986) Isolation and characterization of myosin from amoebae of *Physarum polycephalum*. J Biol Chem 261: 8022–8027

4 Kohama K, Kendrick-Jones J (1986) The inhibitory Ca^{2+}-regulation of the actin-activated Mg-ATPase activity of myosin from *Physarum polycephalum* plasmodia. J Biochem 99: 1433–1446

5 Nakamura S, Nonomura Y (1984) A simple and rapid method to remove light chain phosphatase from chicken gizzard myosin. J Biochem 96: 575–578

TABLE 2. Myosin light chains of eukaryote myosin II

Species	Source	Myosin light chain		Regulation	Reference
		Essential	Regulatory		
Myxomycota					
Physarum polycephalum	Whole cell	14	18	Inhibitory Ca regulaton	Kohama *et al.* [4]
Porifera					
Halichondria okadai	Whole cell	18	21	?	This paper
Coelenterata					
Actinia equina	Tentacle	19	18	Phosphorylation	Kanzawa *et al.* [8]
Actinia equina	Body	17	22		
Annelida					
Urechis unicinctus	Bodywall	19	24	Ca binding	Kanzawa *et al.* [16]
Mollusca					
Aequipecten irradians	Adductor	15	18	Ca binding	Kendrick-Jones [17]
Arthropoda					
Drosophila melanogaster	Tubular	20	30	Phosphorylation	Takano-Ohmuro *et al.* [14]

6 Ebashi S (1976) A simple method of preparing actin-free myosin from smooth muscle. J Biochem 79: 229–231

7 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

8 Kanzawa N, Sato O, Takano-Ohmuro H, Maruyama K (1993) Sea anemone (*Actinia equina*) myosin. Comp Biochem Physiol 104B: 509–514

9 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

10 Mikawa T, Takeda S, Shimizu T, Kitaura T (1981) Gene expression of myofibrillar protein in single muscle fibers of adult chicken: micro two dimensional gel electrophoretic analysis. J Biochem 89: 1951–1962

11 Maruyama K, Nonomura Y (1984) High molecular weight calcium binding protein in the microsome of scallop striated muscle. J Biochem 96: 859–870

12 Sakurai Y, Kanzawa N, Maruyama K (1995) Characterization of myosin and paramyosin from crayfish fast and slow muscles. Comp Biochem Physiol : in press

13 Kendrick-Jones J, Lehman W, Szent-Györgyi AG (1970) Regulation in molluscan muscles. J Mol Biol 54: 313–326

14 Takano-Ohmuro H, Takahashi S, Hirose G, Maruyama K (1990) Phosphorylated and dephosphorylated myosin light chains of *Drosophila* fly and larva. Comp Biochem Physiol 95B: 171–177

15 Takahashi S, Takano-Ohmuro H, Maruyama K (1990) Regulation of *Drosophila* myosin ATPase activity by phosphorylation of myosin light chain-I. Wild-type fly. Comp Biochem Physiol 95B: 179–181

16 Kanzawa N, Kawamura Y, Matsuno A, Maruyama K (1991) Characterization of myosin isolated from bodywall smooth muscle of the Annelid *Urechis unicinctus*. Proc Japan Acad 67: 176–180

17 Kendrick-Jones J (1976) Regulatory light chain in myosins. J Mol Biol 104: 747–775