ATP-Fueled Soft Gel Machine with Well-Oriented Structure Constructed Using Actin-Myosin System

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ABSTRACT: Fibrous actomyosin gel was obtained by applying an external shear stress to actomyosin solution injected from a syringe and then performing chemical cross-linking. The actomyosin gel shrunk when ATP was added, and the shrinkage ratio was proportional to its ATPase activity. The shrinkage of the gel was repeatable at least for four times. Furthermore, the shrinkage of the gel enhanced the regular orientation of the actin and myosin filaments in the gel, which in turn enhanced the shrinking velocity of the gel. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 2087–2092, 2009

Key words: actomyosin; chemically cross-link; ATP-fueled machine

INTRODUCTION

Many researchers have constructed small machines based on the motor proteins such as actin-myosin and microtubule-kinesin *in vitro*. For the past several years, considerable efforts have been made to control the motility of single motor proteins on microfabricated substrates. One directional motility has been realized by using a combination of a lithographic technique,[1–9] an external manipulation system, a flowing fluid,[10,11] an electric force,[12] a magnetic field,[13] etc.

The movement of a muscle tissue involves molecular changes triggered by actin-myosin interaction into the macroscopic deformation through its hierarchical structure in which actins and myosins are three dimensionally organized with a well-controlled size and ordered polarity.[14–16] Thus, high functionality in living systems are closely coupled to their well-organized complex structure. However, very few attempts have been made to enlarge the size, dimension, and complexity of motor-protein-based moving systems.[10,17]

Recently, several methods that involve passive[18,19] and active[20,21] self-assembly processes have been developed to form assemblies of motor proteins with well-controlled polarity. It has been demonstrated that these assemblies show preferential motion. These assemblies may generate a larger force as compared to single motor proteins. However, the number of motor proteins in the assemblies is limited since interactions between motor proteins occur only at a two-dimensional interface. This article describes a pilot study that was performed to expand the size, order, and dimension of the motor-protein-based motility system. Actomyosin, consisting of actin and myosin, is used as the protein complex. Since the discovery by Albert Szent-Gyorgyl that actomyosin superprecipitates during the hydrolysis of adenosine triphosphate (ATP),[22] this phenomenon has been used as an *in vitro* model of muscle contraction. Weber and Portzehl have also prepared filamentous actomyosin by pellet formation using centrifugation, and they successfully measured the tension of the filaments during superprecipitation.[23] This encourage us to proceed the above aim by using actomyosin.

In this study, we prepared actomyosin (AM) complex with a length and diameter of several centimeters and 4 mm, respectively, by applying a shear stress to AM complex injected from a syringe to induce an anisotropic structure; this was followed by chemical cross-linking to obtain AM gel. The system used for applying the shear stress is illustrated in Figure 1(a). The obtained gel showed anisotropic

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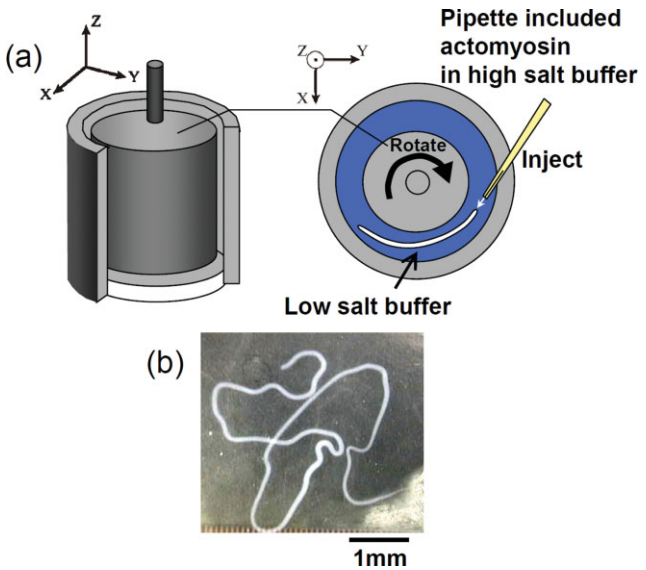

Figure 1 (a) Schematic illustration of the formation of fibrous AM. (b) Image of fibrous AM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shrinkage in the presence of ATP, and the shrinkage ratio and shrinkage rate were observed to be high. Chemical cross-linking also allows us to repeat shrinking of AM gel after elongation due to the increased resistance to stretching. It was found that repeated shrinkage increases the orientational ordering of the AM gel, which was detected from the change in the birefringence. The effect of the mixing ratio of actin and myosin on the shrinkage of the AM gel was also investigated.

MATERIALS AND METHODS

Sample preparation

Materials

Chemicals were purchased from Kanto, Tokyo Kasei, Wako, and Aldrich. All chemicals were used as receive, and all solutions used in experiments were prepared in deionized water purified with a MilliQ plus system (MilliporeTM, Eschborn, Germany).

Preparation of protein

G-actin was obtained from scallops and was purified by using the method of Spudich et al.[24] Myosin was obtained from scallops by using the method of Barany and Barany.[25] Actomyosin (later denoted as AM) was prepared by mixing actin and myosin. The actin/myosin weight ratio of obtained AM solution was electrophoretically estimated as 0.2 (g/g). The weight ratio of actin to myosin in AM was varied in the range of 0.6–4 (g/g) by adding actin.

Preparation of fibrous AM

Fibrous AM was obtained by applying a shear stress to AM solution [Fig. 1(a)]. Actomyosin solution (2 mg/mL) in which actin/myosin weight ratio of 0.2 or 1.6 (g/g) in high-salt buffer [0.6*M* KCl, 20 m*M* 3-(N-morpholino)propane sulfonic acid (MOPS); pH 7.0] was injected through a syringe (radius of exit wound was $\sim$ 4 mm) into a rotating cylindrical container with low-salt buffer (50 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* $CaCl_2$, 20 m*M* Tris-HCl; pH 7.4) at 13°C. (Actomyosin aggregates in low-salt buffer and dissolves in high-salt buffer.) The shear stress (Pa) was 0.178 Pa, which is calculated from the formula $F = \eta R\omega/d$, where F is the shear stress (Pa), η is the viscosity of the solution (N s/m^2), R is the radius of the rotator (m), ω is the angular velocity (m/s), and d is the distance between the container and the rotator. For the preparation of chemically cross-linked AM gel, fibrous AM was chemically cross-linked in 0.125*M* 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) in low-salt buffer at 13°C for 40 min. In this condition, about 90% of myosin monomers were cross-linked.[26]

EXPERIMENTS

Observation of shrinking behavior of fibrous AM

Fibrous AM and its gel were cut with cutter knife into $\sim$ 10 mm, and then they were shrank upon the addition of 5 m*M* adenocine 5′-triphosphate disodium salt (ATP). The shrinking was observed by using an optical microscope (Olympus BX 50, Japan). The images were captured using a cooled CCD camera (Olympus Color Camera HCC-3900, Japan) connected to a PC. The shrinkage ratio of AM was defined as the ratio of its contour length change resulting from the addition of ATP to its initial contour length (contour length is the long axis of AM). The contour length change of AM was analyzed using image analysis software (MetaMorph, Nippon Roper). The shrinkage ratio of AM (defined as average length) was determined as the average one of three samples. The shrinkage of AM could be repeated by performing the following procedure. After the shrinkage by the addition of 10 m*M* ATP, the AM gel was stretched to its initial length by picking its edges with two stainless-steel tweezers and was fixed in low-salt buffer without ATP for 10 min. Subsequently, 10 m*M* ATP was added to the stretched AM dipped in the buffer and the shrinking behavior was observed by using the method described below. Each presented experiments were performed at least for 10 times.

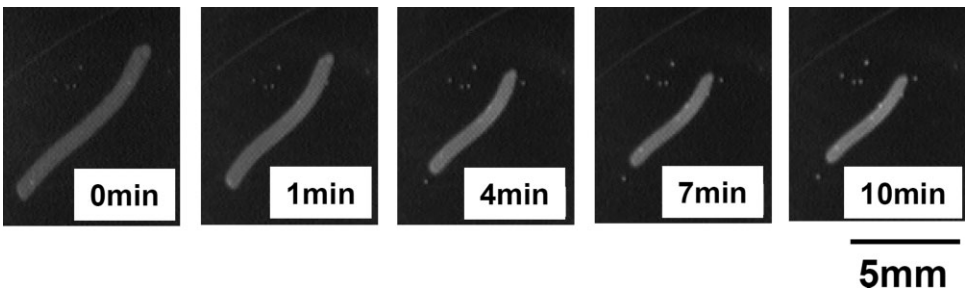

Figure 2 Time-lapse images of AM gel shrinking upon the addition of 5 m*M* ATP. The weight ratio of actin to myosin (g/g) in the gel is 0.2.

Measurement of ATPase activity of AM

The actin-activated ATPase activity (Mg-ATPase) was measured by determining the amount of inorganic phosphate liberated from ATP in low-salt buffer [50 m*M* KCl, 20 m*M* Tris(hydroxymethyl)aminomethane, (pH 7.4) containing 2 m*M* $MgCl_2$, 0.3 m*M* $CaCl_2$, and 1 m*M* ATP] in the presence of AM [myosin: 0.05 mg/mL, weight ratio of actin to myosin: 0.6 : 4 (g/g)] or 0.05 mg/mL of myosin [equal to the AM of weight ratio of actin to myosin = 0 (g/g)] in 8 min at 15°C. For the measurement of ATPase activity, actomyosin gel in a suspended form was used after homogenizing treatment to eliminate the effect of diffusion of actin and ATP. The reaction was terminated by adding 10% of trichloroacetic acid (TCA). The amount of inorganic phosphate liberated was determined by using the method of Youngburg and Youngburg.[27] The presented experiments were performed at least for five times.

Polarized light microscopy

The samples were observed under a polarized light microscope (Olympus BH-2, Japan) or a polarizer at room temperature. The presented experiments were performed for four times.

RESULTS AND DISCUSSION

Fibrous AM complex was obtained by injecting a solution containing a mixture of actin and myosin in high-salt buffer through a syringe into a rotating cylindrical container with low-salt buffer as shown in Figure 1(a). The shear stress applied to the AM complex was 0.178 Pa, which gives the highest shrinkage ratio (data not shown). Figure 1(b) shows an image of the AM complex. The change from the high-salt condition to the low-salt condition (in the cylindrical container) led to the self-assembly of myosins; the self assembly in turn resulted in the formation of a complex through interactions between the tails of myosin molecules (gel state). To provide structural strength, the AM complex was chemically cross-linked by using EDC. EDC is one of the zero-length cross-linkers and it modifies the amino acid side groups to permit cross-linking between reaction moieties without the involvement of carbon atoms.[28–30] The shrinkage of the AM complex gel (hereafter referred to as AM gel) was initiated by adding 5 m*M* of ATP in low ionic buffer (50 m*M* KCl). Figure 2 shows the anisotropic shrinkage of the AM gel [2 mg/mL; actin (g)/myosin (g) = 0.2] with time upon the addition of ATP. The shrinkage ratio (length after shrinking/length before shrinking) of the AM gel in the longitudinal and lateral directions 10 min after the addition of ATP were 57% and 84%, respectively. It has been confirmed that the AM gel did not shrink by adding an excess amount of KCl (~ 100 m*M*) as alternative of ATP. This result indicates that the contraction of AM gel was induced not by the decrease in osmotic pressure due to the increase in the ionic strength as observed in the general polyelectrolyte gel but by the contraction of actomyosin due to the hydrolysis of ATP. Figure 3(a) shows the time profile of the shrinking of the AM complex and gel with an initial size of 4.8 mm × 4 mm containing 20% of actin [actin (g)/myosin (g) = 0.2]. The shrinkage ratio was evaluated from the change in the longitudinal axis. As shown in Figure 3(a), there was no significant difference in the shrinking profiles between the complex and the gel.

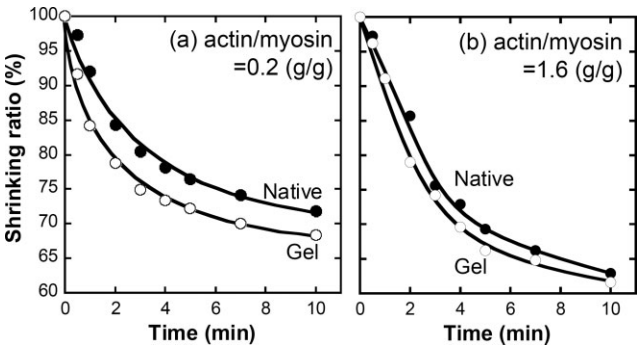

Figure 3 Time profile of shrinking behavior of AM complexes (closed circle) and AM gels (opened circle) resulting from the addition of 5 m*M* ATP. The weight ratio of actin to myosin (g/g) in AM is (a) 0.2 or (b) 1.6.

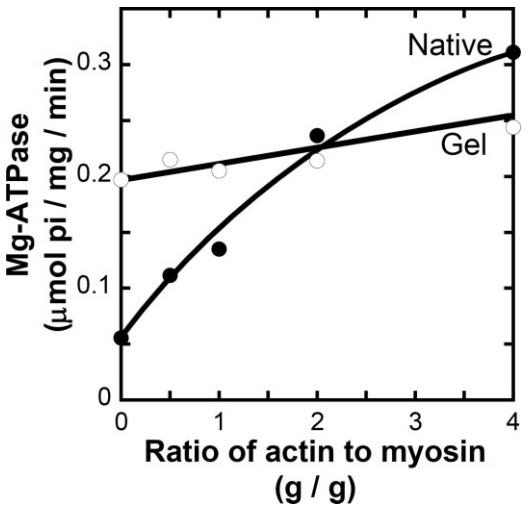

Figure 4 Mg-ATPase activity of AM complexes (closed circle) and AM gels (open circle). Assay was carried out in low-salt buffer [50 m*M* KCl, 20 m*M* Tris (pH 7.4)] containing 2 m*M* $MgCl_2$, 0.2 m*M* $CaCl_2$, and 1 m*M* ATP at 15°C.

On the other hand, the shrinkage ratio of AM gel and complex was 68% and 72%, respectively. Furthermore, the shrinkage rate was evaluated from the initial slope in the time range from 0 to 2 min in Figure 3(a), and it was found to be 16%/min and 8%/min for the AM gel and complex, respectively. Thus, a slight increase in the shrinkage ratio and rate was observed in the AM gel. This is rather surprising since cross-linking reaction might lead a denaturation of the active site on actin and myosin that responsible for the sliding motion.

To investigate the large shrinkage rate, the actin-activated ATPase activities (Mg-ATPase) of the AM gel and complex were monitored in the presence and absence of actin in the low ionic buffer (50 m*M* KCl; Fig. 4). Since myosin molecule itself is Mg-ATPase, the AM gels had Mg-ATPase even in the absence of actin [actin (g)/myosin (g) = 0 in Fig. 4].[30] As shown in Figure 4, the Mg-ATPase activity of the AM complex was enhanced in the presence of actin, and it increased with the actin concentration. In contrast, the Mg-ATPase activity of the AM gel was almost insensitive to the presence of actin. Furthermore, at the actin/myosin weight ratio of 0.6 (g/g), the Mg-ATPase activity of the AM gel was higher than that of the AM complex. This would directly lead to an increase in the shrinkage ratio and shrinkage rate of the AM gel. On the other hand, the Mg-ATPase of the AM complex became similar to that of the AM gel in the vicinity of actin/myosin weight ratio of 1.6 (g/g). To explore the relationship between the shrinking behavior and the Mg-ATPase activity in detail, we investigated the shrinking of the AM complex and gel (2 mg/mL) with this actin/myosin weight ratio. As shown in Figure 3(b), no significant difference was observed in the shrinkage ratios and shrinkage rates between the AM gel and the AM complex. This result strongly supports the argument that shrinking is directly associated with the Mg-ATPase activity. The large shrinkage ratio (~ 65%) observed might be attributed to the increase in the AM concentration due to the addition of actin. Thus, Mg-ATPase activity plays a crucial role in the shrinking behavior of AM. However, the low actin sensitivity of the Mg-ATPase activity of AM gel is still an open question.

The AM gel that was obtained by the chemical cross-linking of the AM complex showed resistance to repeated shrinking. After shrinking and stretching the AM gel to the initial length in high ionic buffer, the re-shrinking was performed by addition of ATP (hereafter this experiment is referred to as repeat test). The shrinkage of the gel was repeatable at least for four times. Figure 5 shows the result of the repeat test. It is evident that the initial slope of the shrinkage rate in the second run is much steeper than that in the first run, though the shrinkage ratio does not change significantly (73–74%). The shrinkage rates of the AM gel in the first and second runs were 6%/min and 15%/min, respectively. A cross-polarizer system equipped with an analyzer and a polarizer revealed that the repeat test increased the orientational ordering in the structure of the AM, which was detected as a change in the birefringence. Figure 6 shows cross-polarized-microscope (PLM) images of the AM gels before shrinking in the first (a) and second (b) runs. As shown in the figure, weak birefringence was observed in the sample in the first run, while strong anisotropic birefringence

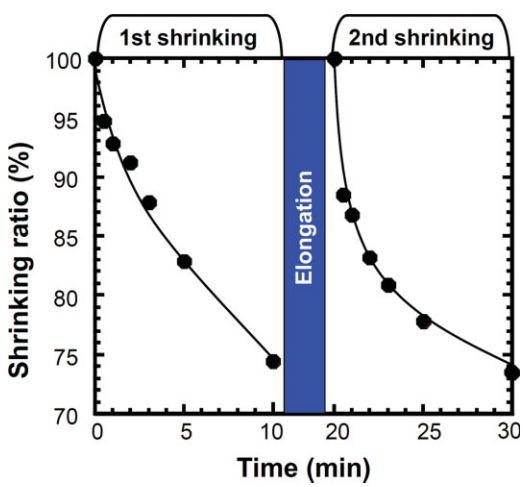

Figure 5 Time profile of repeat shrinking of AM gels upon the addition of ATP. The AM gels were stretched for 10 min to their original lengths in low-salt buffer without ATP after the first shrinking. The weight ratio of actin to myosin (g/g) in AM was 0.2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

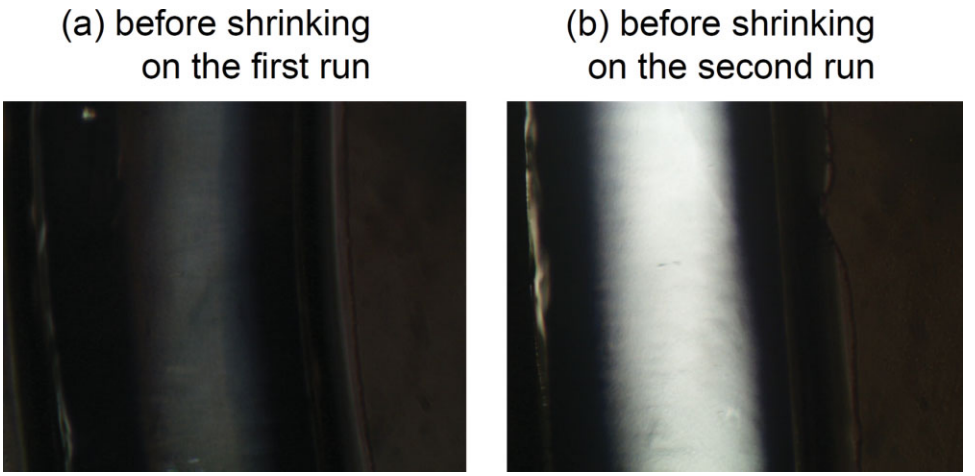

Figure 6 PLM images of AM gels (a) before shrinking in the first run and (b) before shrinking in the second run. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

was detected in the sample after the repeat test. Thus, the shrinking and relaxing processes led to the orientational ordering of the AM structure. This ordering might be related to the reorientation of actin filaments and myosin fibrils in the AM during a relative sliding motion, as schematically illustrated in Figure 7. This is analogous to myofibrillogenesis, where contraction and relaxation lead to the formation of a well-ordered structure (sarcomere).[31] These results are capable of providing an insight into the still-unknown process of myofibrillogenesis and can be applied for designing a motor-protein-based motility system with a well-ordered and high-dimensional structure. The stress generated by the shrinking of the AM gel was also estimated by using a rheometer (ARES-2 KFRT, TA Instruments) and it was found to be around 3.5×10^{-3} N/cm^2, which corresponded to approximately 7.0×10^{-3} N/cm.

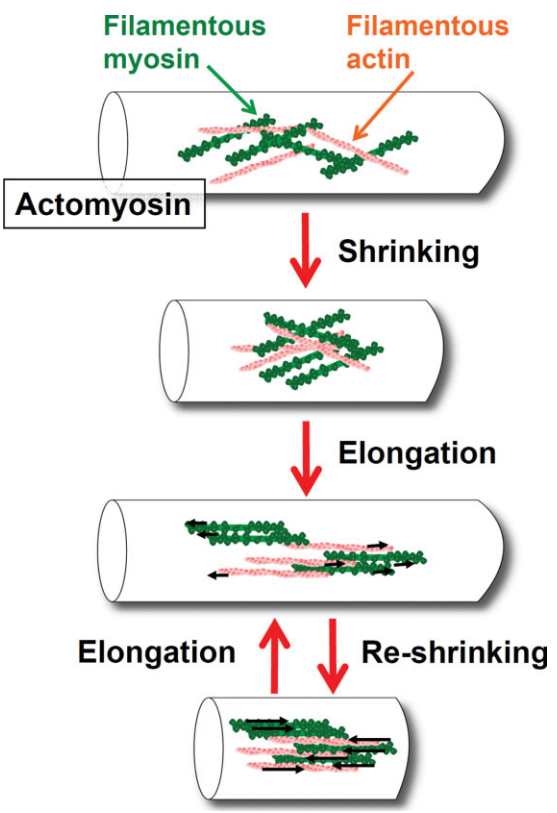

Figure 7 Schematic illustration of the orientation mechanism by ATP-driven shrinkage of AM gel. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSIONS

Fibrous AM gel was obtained by applying an external shear stress to the AM complex and performing chemical cross-linking. The AM gel shrunk when ATP hydrolysis was performed, and the shrinkage rate was proportional to its ATPase activity. The chemical cross-linking of the AM complex made it possible for repeating shrinkage. Furthermore, the ordering of the orientation of filamentous actin and myosin in the AM gel was increased by the shrinking–stretching process as shown in the birefringence change of Figure 6, and the repeating shrinkage also led to a slight increase in the shrinkage rate which would be originated from the enhanced orientation of actin and myosin filaments in the AM gel.

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