Studies on Adductor Muscle Proteins of the Scallop—I Isolation of Myosin

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(Received April 28, 1976)

Myosin was isolated from the smooth adductor of the scallop *Patinopecten yessoensis*. The natural actomyosin was first prepared as usual from a homogenate of the smooth adductor muscle and freed from the contaminating paramyosin effectively by Sepharose 2B gel filtration. The natural actomyosin was then kept in 2.5 M KCl·0.03 M MgCl₂ at 0°C for 3 hr, and the freed actin was removed by a 3 hr-ultracentrifugation at 80,000×*g*. From the supernatant containing myosin, the contaminating undissociated actomyosin, aggregated myosin, and tropomyosin were eliminated by ultracentrifugation at 70,000×*g* and repetition of a dilution-precipitation procedure. The myosin thus isolated was homogeneous in both ultracentrifugal and electrophoretic analyses and showed zero ATP sensitivity.

Unlike most of the bivalves, the scallop has the adductor composed of easily distinguishable two portions, the striated and the smooth ones. In this bivalve, the muscle proteins, such as myosin^{1,2)} and tropomyosin^{3,4)} have been isolated almost exclusively from the striated portion of the adductor and characterized extensively. Knowledge on the muscle proteins of the smooth portion, however, is still lacking, probably due to the difficulty in obtaining a given muscle protein completely freed from paramyosin that is present overwhelmingly in the smooth portion.⁵⁾ This led us to initiate the present study on the muscle proteins in the smooth portion of the scallop adductor.

This paper deals with the isolation of myosin by the method consisting of purification of the natural actomyosin by Sepharose gel filtration and subsequent separation of myosin from it.

Materials and Methods

Materials Living specimens of the scallop *Patinopecten yessoensis*, 11 cm in average shell length, were obtained at Tokyo Central Wholesale Fish Market. The smooth portion of the adductor including the translucent portion (designated hereafter 'the smooth adductor') was collected and used for preparation of myosin. The striated portion of the adductor was also used for preparation of actomyosin. All the extraction and separation procedures were performed in a cold room at 2-4°C, unless stated otherwise.

Analytical methods Protein concentration was determined mainly by the biuret method⁶⁾ using bovine serum albumin as a standard. The Folin-Lowry method⁷⁾ was

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also used when required.

The solubility of actomyosin was measured in 5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4 containing KCl at various concentrations. Actomyosin was dissolved in the above buffer containing 0.4 M KCl and aliquots of the solution were pipetted into test tubes. By using 5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4 and the same buffer containing 1 M KCl, the final volume and protein concentration of test solutions were adjusted to 2.5 ml and 1 mg/ml, respectively, and a series of different concentrations of KCl were provided. The test solutions were gently shaken, left overnight, and centrifuged at $15,000\times g$ for 30 min. Absorbances at 280 nm of the supernatants were measured to draw the salting-in curve.

The gel electrophoresis⁸⁾ was carried out in 7.5% gels containing 0.1% sodium dodecyl sulfate (SDS) for check of purity as well as for estimation of subunit molecular weight. After the run, the gels were stained with 2.5% coomassie blue in 9% acetic acid-45% methanol for 1 hr and destained in 7% acetic acid-25% methanol solution. γ -Globulin, bovine serum albumin, chymotrypsinogen a, and cytochrome c (Mann Res. Lab.) were used as standard proteins.

The ATPase activity of actomyosin was measured essentially according to the method of PERRY⁹⁾. The mixture consisting of 1 ml of 40 mM histidine buffer pH 7.4, 0.1 ml of 0.1 M CaCl_2 or MgCl_2 , 0.4 ml of 0.45 M KCl containing actomyosin in the final volume of 1.8 ml was put in a test tube and warmed to 25°C. After rapid addition of 0.2 ml of 0.05 M ATP, the whole mixture was incubated at 25°C for 5 min under stirring. The reaction was stopped by addition of 0.5 ml of 24% trichloroacetic acid. After centrifugation, the supernatant was analyzed for the inorganic phosphate (Pi) liberated by the method of NAKAMURA¹⁰⁾.

An Ostwald type viscometer having a flow time of about 90 sec at 10°C for distilled water was used. The measurements were carried out at 10°C. ATP sensitivity was determined according to the method of PORTZEHL *et al.*¹¹⁾

The sedimentation analyses were carried out at 55,430 rpm and 20°C, with a Hitachi UCA-1A analytical ultracentrifuge.

Guaranteed reagents and the deionized glass-distilled water were used throughout the present experiments.

Results

Preparation of paramyosin-free natural actomyosin To each of the striated and smooth adductor muscles was added 10 volumes of 0.04 M KCl adjusted at pH 7.0. The mixture was blended in an Ultra-Turrax TP 18/10 homogenizer (Janke Co.) and centrifuged at $3,000\times g$ for 15 min. The supernatant was discarded. This operation was repeated four more times, and the residue finally obtained was extracted with 7 volumes of Weber's

solution (0.6 M KCl·0.04 M NaHCO₃·0.01 M Na₂CO₃, pH 9.2) for about 20 hr. After centrifugation at 8,000×*g* for 15 min, the supernatant was diluted with 5 volumes of cold 5 mM 2-mercaptoethanol at pH 7.0. The resultant precipitate was collected at 900×*g* for 15 min and dissolved in 0.6 M KCl·0.025 M Tris-HCl buffer pH 7.4. This dilution-precipitation procedure was repeated two more times, and the final solution was centrifuged at 80,000×*g* for 30 min with a Hitachi 55P-2 preparative ultracentrifuge to eliminate some insoluble matters. The yield of the crude natural actomyosin from an individual scallop was about 200 and 20 mg from the striated and the smooth adductor, respectively. As shown in Table 1, the crude natural actomyosin of striated adductor is higher in both Ca²⁺- and Mg²⁺-ATPase activity than that of smooth adductor.

Table 1. Ca²⁺- and Mg²⁺-ATPase activity of crude natural actomyosins obtained from both scallop adductors (μmole Pi/min/mg protein)

Adductor	Ca ²⁺ -ATPase activity	Mg ²⁺ -ATPase activity
Striated	0.77	0.16
Smooth	0.32	0.10

The salting-in curve of the crude natural actomyosin from striated adductor is presented in Fig. 1. It was soluble, partially at about 0.25 M and perfectly at above 0.33 M KCl. This crude actomyosin was then purified by gel filtration on Sepharose 2B columns (2.5×45 cm) equilibrated with each of 0.35, 0.4, 0.45, and 0.6 M KCl in 5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4. The flow rate was fixed at 20 ml/hr. Three peaks appeared irrespective of the molarity of KCl, as shown in Fig. 2. From the positions of elution, SDS-gel electropherograms, and Ca²⁺- or Mg²⁺-ATPase activity (Fig. 3), the fastest peak I was judged to be the pure actomyosin, peak II supposedly to be myosin for the most part, and peak III to be a mixture of actin and tropomyosin. From the yield and electrophoretic homogeneity of peak I, the use of 0.45 M KCl was found best.

Then, the crude natural actomyosin from the smooth adductor was subjected to the same gel filtration at a KCl level of 0.45 M. Again three peaks appeared roughly at the same positions as with the striated adductor actomyosin (Fig. 4a). The fastest peak I showed both Ca²⁺- and Mg²⁺-ATPase activity. By SDS-gel electrophoresis, the presence of some tropomyosin in peak I was recognized. Peak II was conceived to be almost

Fig. 1. Salting-in curve of crude natural actomyosin from striated adductor of scallop in 5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4.

Fig. 2. Sephadex 2B gel filtration of crude natural actomyosin from the striated adductor of scallop in 5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4 containing KCl at different concentrations.

* Abbreviations: NAM=natural actomyosin, MHC=myosin heavy chain, MLC=myosin light chain, AC=actin, TM=tropomyosin, PM=paramyosin.

Fig. 3. Sephadex 2B gel filtration of crude natural actomyosin from the striated adductor of scallop in 0.45 M KCl·5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4.

exclusively occupied by paramyosin from the electropherogram and the absence of Ca^{2+} - and Mg^{2+} -ATPase activity, and peak III to be a mixture of actin and tropomyosin. Fractions of peak I as shown by both arrows in Fig. 4a were combined and subjected to the second gel filtration. The elution curve is shown in Fig. 4b. Fractions indicated by

both arrows were combined and used for preparation of myosin as described below.

Preparation of myosin To the par-myosin-free actomyosin dissolved in 0.45 M KCl·5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4 was added KCl and MgCl_2 up to 2.5 M and 0.03 M respectively. The solution was kept at 0°C for 3 hr and then centrifuged at $80,000\times g$ for 3 hr. The supernatant was dialyzed against 11.5 volumes of 2 mM 2-mercaptoethanol·10 mM K-phosphate buffer pH 6.6 to decrease the molarity of KCl down to 0.2 and centrifuged at $70,000\times g$ for 1 hr. The precipitate mainly consisting of the undissociated actomyosin and aggregated myosin was discarded. The supernatant was diluted with 3 volumes of cold 2 mM 2-mercaptoethanol·7.5 mM K-phosphate buffer pH 6.2 and centrifuged at $900\times g$ for 15 min. The supernatant containing tropomyosin was discarded. The myosin was subjected again to the above procedure after being dissolved in 0.6 M KCl·2 mM 2-mercaptoethanol·10 mM K-phosphate buffer pH 7.0. The yield was about 60 mg from 100 g of the wet smooth adductor. The myosin thus isolated showed an almost

Fig. 4. a: Sepharose 2B gel filtration of crude natural actomyosin from the smooth adductor of scallop in 0.45 M KCl·5 mM 2-mercaptoethanol·0.025 M Tris-HCl buffer pH 7.4. b: The second gel filtration of Peak I (Fr. 6 ~ 11).

* Refer to the footnote in Fig. 2 for the abbreviations.

Fig. 5. Sedimentation pattern of smooth adductor myosin of scallop. Photograph was taken at 20 min after reaching the top speed, 55,430 rpm. Protein concentration: 0.8 mg/ml.

Fig. 6. SDS-gel electrophoresis of smooth adductor myosin of scallop. MHC=myosin heavy chain; MLC=myosin light chain.

symmetrical peak in ultracentrifugal analysis (Fig. 5). The ATP sensitivity was found to be zero. As shown in Fig. 6, it did not reveal any band other than the heavy and

the light subunit chain in SDS-gel electrophoresis. All these results demonstrated that the scallop myosin was obtained in a high purity.

Discussion

BÁRÁNY and BÁRÁNY¹⁾, and RÜEGG¹²⁾ attempted to isolate the paramyosin-free natural actomyosin from the adductor muscle of bivalve. In order to eliminate the contaminating paramyosin they adopted a fractional precipitation with KCl at $I=0.27-0.3$. Since the salting-in ranges of both proteins overlap each other, they were not able to prepare the paramyosin-free natural actomyosin in a high yield. In addition, the collection of the natural actomyosin by ultracentrifugation usually results in formation of a large amount of gel component. These defects of the conventional method for isolation of actomyosin were also experienced with the smooth adductor of scallop in our preliminary experiments. The application of Sepharose 2B gel filtration was found to be satisfactory for isolation of the paramyosin-free actomyosin, as described above.

In preparation of myosin, SZENT-GYÖRGYI *et al.*¹³⁾ noticed the contamination of an unknown component sedimenting more slowly than myosin in the smooth adductor myosin of scallop *Aequipecten irradians*, but did not succeed in eliminating it. As shown in Fig. 5, this component was perfectly eliminated by Sepharose 2B gel filtration. On the other hand, RÜEGG¹²⁾ and BÁRÁNY and BÁRÁNY¹⁾ observed the formation of aggregated myosin during the purification of the striated adductor myosin of scallops, *Pecten maximus* and *P. irradians*, respectively, though they did not make any effort to remove it. In the present study as well, there appeared a considerable amount of the aggregated myosin during the purification. The exclusion of this aggregated myosin was another difficulty, but it was overcome ultimately by repetition of the dilution-precipitation.

Acknowledgements

The authors wish to express their heartfelt thanks to Honorary Professor F. MATSURA of our university for valuable suggestions and encouragements in the course of the present work.

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