Studies on Myosin of Spiny Lobster-I Isolation and Physico-chemical Properties

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A method for preparation of purified myosin from the abdominal muscle of spiny lobster was devised and the physico-chemical properties of the myosin obtained were examined. Intrinsic viscosity, sedimentation constant ($s_{20,w}^0$), and[†] diffusion constant ($D_{20,w}^0$) of the myosin were 2.05 dl/g, 6.65 S, and $1.35 \times 10^{-7} \text{cm}^2 \text{sec}^{-1}$, respectively. The molecular weight was determined to be $445,000 \pm 15,000$ ($M_{s,D}$) and $500,000 \pm 50,000$ (M_e). The myosin was precipitated with ammonium sulfate between 40 and 50% saturation at pH 7.0, and salted in with more than 0.15 M KCl at pH 6.5. The above physico-chemical properties as well as amino acid composition of spiny lobster myosin resemble, as a whole, those of other myosins so far reported.

A number of studies on myosins, especially on rabbit skeletal muscle myosin, have been published but the progress in the corresponding research work on aquatic animals has been hampered by difficulties in isolating the pure myosins, the difficulties originating from their instability and readily aggregating property. This holds true, in particular, to the myosin of crustacea. Woods *et al.*¹⁾ isolated lobster myosin and examined some properties in comparison to rabbit myosin. Except that, no reliable paper seems available at present. Such a situation prompted us to undertake a study on the myosin of spiny lobster, one of the most useful marine invertebrates in Japan. The present paper deals with a method of isolation that was devised by modifying PERRY's method²⁾ for rabbit myosin, and also deals with physico-chemical properties of the myosin obtained.

Experimental

Isolation of myosin After various preliminary experiments the method of preparation shown in Fig. 1 was devised: The abdominal muscle of the live spiny lobster *Panulirus japonicus* was minced, extracted with 0.55 l KCl-phosphate buffer (0.3M KCl, 0.1M KH_2PO_4 , and 0.05M K_2HPO_4 ; pH 6.5)³⁾ for 10 min, and centrifuged. The supernatant was filtered through gauze and brought to 2.5 l with addition of solid KCl at the same pH to dissociate the actomyosin present into actin and myosin. The solution was saturated with ammonium sulfate at pH 6.5 and centrifuged to remove the resultant precipitate that was confirmed to consist mainly of actin and undissociated actomyosin. The supernatant was dialyzed against 0.05M KCl at pH 6.5. The precipitate formed was collected by

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Fig. 1. Isolation procedure of spiny lobster myosin.

centrifugation, and the volume was measured. The precipitate was dissolved by addition of solid KCl to bring the ionic strength to 0.5, and centrifuged. The supernatant was diluted to 0.25 I for precipitating actomyosin and centrifuged. The myosin was further purified by repeating four times the precipitation in 0.05 M KCl and dissolution in 0.5 M KCl to remove the contaminating water-soluble proteins. During this process some aggregated myosin appeared. It was removed by centrifugation at 0.25 I and pH 6.5. In the final step, the still remaining water-soluble proteins were removed by adsorption onto the DEAE-cellulose previously equilibrated with 0.25 M KCl at pH 6.5. All the operations were carried out at 0 to 4°C and the purified myosin obtained was kept at 0°C until used.

Analytical methods *Protein concentration:* A modified biuret method of UMEMOTO³⁾ was applied. The protein nitrogen concentration (mg/ml) was calculated by multiplying the color intensity reading at 545 nm by 0.94 which was determined for spiny lobster myosin.

Viscosity: Viscometry was done as usual with an Ostwald type viscometer.

ATP sensitivity: The method of PORTZEHL *et al.*⁴⁾ was applied.

Superprecipitation: The observation was performed according to the method of KASAI.⁵⁾

Ultracentrifugal analysis: A Hitachi model UCA-1A ultracentrifuge was employed for sedimentation and diffusion analyses. The routine sedimentation analyses were carried out at 55,430 rpm and around 20°C on the protein dissolved in 0.5 M KCl, pH 6.5.

Sedimentation ($s_{20,w}^0$) and diffusion constant ($D_{20,w}^0$) were obtained by extrapolation of the apparent constants, $s_{20,w}$ and $D_{20,w}$ at different protein concentrations. The molecular weight ($M_{s,d}$) was calculated on the basis of both constants and its partial specific volume, 0.730, as measured with a pycnometer. The molecular weight determination was performed also by sedimentation-equilibrium with the above ultracentrifuge at 10°C.

Gel filtration: To check purity of the myosin obtained, gel filtration was carried out on a column (2.5 × 50 cm) of Sepharose 4 B using 0.5 M KCl solution, pH 6.5.

Salting-out curve: The measurement was done according to the method of TENOW *et al.*⁶⁾ using Toyo filter paper No. 5 C.

Salting-in curve: The measurement was done according to the method of MATSUMOTO⁷⁾ using Toyo filter paper No. 5 C.

Amino acid analysis: The myosin was hydrolyzed with 6 N HCl in a sealed evacuated tube and analyzed with a Hitachi model KLA-3 B amino acid analyser.

Analytical grade reagents and deionized glass-distilled water were used throughout the present experiment.

Results and Discussion

Isolation and purity of spiny lobster myosin In the preparation of myosin from cold-blooded animals are encountered some difficulties which usually do not occur in warm-blooded ones. The first problem is that a quantity of actomyosin will come out even after a very short time extraction, which makes the yield of myosin extremely low. The second problem is that myosins of cold-blooded animals are highly susceptible to aggregation. With respect to the first problem, WEBER⁸⁾ and TSAO⁹⁾ devised a method featuring in the dissociation of actomyosin into actin and myosin by high concentrations of ATP and Mg^{2+} . BÁRÁNY¹⁰⁾ applied a high concentration of Mg^{2+} for the same purpose. On the other hand, TORIMOTO¹¹⁾ found that actomyosin is also dissociated in the KCl solution at the concentration more than 2 M. Referring to his finding, solid KCl was added to the extract of spiny lobster muscle with 0.55 *I* KCl-phosphate buffer, pH 6.5 until the ionic strength was raised up to 2.5 *I*. It was shown by ultracentrifugal analysis that the actomyosin in the resultant solution was for the most part dissociated into actin and myosin. Subsequent addition of solid ammonium sulfate to saturation was found to precipitate almost selectively the actin and the remaining actomyosin. It was proved that complete removal of the still remaining actomyosin was attained by centrifugation in 0.25 M KCl solution at pH 6.5.

The second problem is the formation of aggregated myosin during the repeated dilution-precipitation procedures for removal of the water-soluble proteins. It was confirmed by ultracentrifugal analysis that this aggregated myosin was removed also by centrifugation in 0.25 M KCl solution at pH 6.5.

On the basis of the above examinations the purification procedure as shown in Fig. 1 was worked out. The myosin obtained was found to be zero in ATP sensitivity value and to cause no superprecipitation at all, evidencing that it was entirely free from actomyosin. The myosin preparations were found to be almost monodisperse at least until one day after isolation both in ultracentrifugation (Fig. 2) and in gel filtration (Fig. 3). Yield was approximately 0.5 g per 100 g of muscle.

Physico-chemical properties The spiny lobster myosin was analyzed for several physico-chemical properties to give the following results.

Intrinsic viscosity: Intrinsic viscosity

Fig. 2. Ultracentrifugal patterns of spiny lobster myosin.

Photographs were taken 50 min after the top speed 55,430 rpm was reached at 20°C. The myosin was dissolved in 0.5 M KCl, pH 6.5, at 1.71 mg/ml (top) and 2.84 mg/ml (bottom).

Fig. 3. Gel filtration of spiny lobster myosin on Sepharose 4 B column (2.5 × 50 cm) with 0.5 M KCl, pH 6.5.

Fig. 4. Reduced viscosity of lobster myosin in 0.5 M KCl, pH 6.5, as a function of protein concentration.

Fig. 5. Concentration dependence of sedimentation coefficient of spiny lobster myosin in 0.5 M KCl at pH 6.5.

Fig. 6. Concentration dependence of diffusion coefficient of spiny lobster myosin in 0.5 M KCl at pH 6.5.

$[\eta]$ of spiny lobster myosin was measured to be 2.05 dl/g as shown in Fig. 4. This value is comparable with 2.2¹²⁾ and 2.35¹³⁾ for rabbit, 2.0 for frog¹⁴⁾ and 1.8 dl/g for cod¹⁴⁾ myosin.

Sedimentation constant: A concentration dependence of $s_{20,w}$ value was observed in this myosin, as seen in Fig. 5. By extrapolation, $s_{20,w}^0$ was found to be 6.65 S. As for $s_{20,w}^0$ values of skeletal muscle myosins, 6.11 to 6.50 for rabbit^{13,15,16)}, 6.2 for rat¹⁷⁾, 6.33 for cod,¹⁸⁾ and 6.03 S for mullet¹⁹⁾ are found in literature, roughly agreeing with the value determined here for spiny lobster myosin. In this connection WOODS *et al.*¹⁾ reported a value of 5.82 S for lobster myosin.

Diffusion constant: The $D_{20,w}$ values at three myosin concentrations are shown in Fig. 6. The extrapolation gave $D_{20,w}^0 = 1.35 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. $D_{20,w}^0$ values of rabbit²⁰⁾ and rat²¹⁾ myosins were reported to be 1.04 and $1.56 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$, respectively. In addition, MOMMAERTS *et al.*²²⁾ reported $1.04 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ as $D_{20,w}$ at 0.5% myosin and MACKIE *et al.*²³⁾ $1.16 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ as a mean of $D_{20,w}$ values at different concentrations. The value for spiny lobster myosin is in the range found by other workers.

Molecular weight: Based on $s_{20,w}^0$ and $D_{20,w}^0$ obtained, the molecular weight ($M_{s,d}$) of spiny lobster myosin was calculated as $445,000 \pm 15,000$. The sedimentation equilibrium gave a value of $500,000 \pm 50,000$. Many values have been reported for the molecular weight of rabbit myosin; 4.2×10^5 ²²⁾, 4.9×10^5 ²⁴⁾, 5.46×10^5 ¹³⁾, 5.5×10^5 ²⁵⁾, 6.0×10^5 ¹⁾, *etc.*

In other animals the following values were reported: 4.5×10^5 ²¹⁾ for rat; $5.0 \sim 5.5 \times 10^5$ ²¹⁾ for cod; and 6×10^5 ¹⁾ for lobster. More recent data, however, fall very near to 500,000: *e.g.*, 5.04×10^5 ²⁰⁾ and $4.8 \sim 5.1 \times 10^5$ ²⁸⁾, irrespective of animal species.

Fig. 7. Salting-out curve of spiny lobster myosin. The myosin previously dissolved in 0.5 I KCl-phosphate buffer at pH 7.0 was used.

Fig. 8. Salting-in curves of spiny lobster myosin.

Salting-out curve: As given in Fig. 7, spiny lobster myosin was precipitated with ammonium sulfate in the range from 40 to 50% saturation at pH 7.0. This is coincident with the range reported for rabbit myosin by TSAO⁹⁾ and DUBUISSON.²⁷⁾

Salting-in curve: As shown in Fig. 8, the salting-in curve of the myosin varied greatly with pH, indicating the importance of pH adjustment during the preparation. The range of salting-in for chicken myosin was reported as 0.10 to 0.18 μ at pH 7.0,²⁸⁾ being close to that found here at the similar pH.

Table 1. Amino acid composition of spiny lobster myosin in comparison with those of some other myosins

	Gram moles/10 ⁵ protein			
	Spiny lobster	Scallop ¹⁰⁾	Cod ²⁹⁾	Rabbit ¹⁰⁾
Aspartic acid	96	103	85	85
Threonine	27	34	36	41
Serine	68	50	45	36
Glutamic acid	165	158	145	155
Proline	29	23	25	22
Glycine	58	46	44	43
Alanine	75	69	71	75
Valine	49	40	44	47
Methionine	24	27	20	23
Isoleucine	51	41	35	43
Leucine	82	78	77	78
Tyrosine	20	17	15	19
Phenylalanine	32	28	23	27
Lysine	81	81	72	89
Histidine	10	14	13	16
Arginine	44	53	38	43
Cystine/2	5	10.3	7.2	8.8
Tryptophan	—	7.1	3.7	6.2
(Amide NH ₂)	(93)	(93)	(99)	(96)

Amino acid composition: The amino acid composition of the myosin is given in Table 1, together with those of scallop,¹⁰⁾ cod,²⁹⁾ and rabbit¹⁰⁾ myosins for comparison. Although there is a great resemblance among them, some characteristic differences are noticed between the spiny lobster myosin and these other myosins: In the former, the contents of serine, glycine, and isoleucine are significantly higher while those of threonine, half-cystine, and histidine are much lower.

Thus, it may be concluded that physico-chemical properties of spiny lobster myosin are very similar to those of rabbit and other vertebrate myosins except some small differences. This seems to support the recent finding by BRIVIO *et al.*³⁰⁾ that the large subunits of myosin, in contrast to the small subunits, did not undergo fundamental changes during the evolutionary process.

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