A New Contractile Protein of Squid Muscle A Comparative Study with Carp Actomyosin*

Juichiro J. MATSUMOTO

(Tokai Regional Fisheries Research Laboratory)

From investigation of the behaviors of the streaming birefringent protein in the aqueous extracts of the squid muscle and of the quantitative relationships between the said protein and some protein fractions in the squid muscle, an assumption was brought about that the observed streaming birefringence (SB) may be due to dissolving-out of the myosins** into the aqueous extracts¹⁾. The assumption became more probable by comparing the intensity of SB of these fractions with each other²⁾.

When the extract was dialysed against the solutions of various ionic strengths (I), an amount of the streaming birefringent protein was found to precipitate at $I=0.3$ and pH 6.8³⁾. Thus, referring to the sofar described properties of muscle proteins of rabbit and carp⁴⁾⁵⁾, the existence herein of actomyosin-like protein was assumed. On the basis of that fact the author succeeded in separating and concentrating the actomyosin-like protein. Then experimental bases were requested to decide if it is indeed identical with actomyosin.

For this purpose, methods are given such as electrophoresis, ultracentrifuge, or comparison of some physico-chemical properties of the protein, as ROTH⁶⁾ and HAMOIR⁷⁾ did. They showed the respective identity of myosin and actomyosin of carp with those of rabbit which had been studied most profoundly. It was thought, however, more important to give attention upon their functional properties involved in the contraction of muscle, for these properties are the most specific ones to actomyosin.

For example, when adenosinetriphosphate (ATP) solution is added to actomyosin solution in 0.5~0.6 M KCl, a sudden decrease of viscosity and SB (the first phase), a duration of the low viscosity or weak SB (the second phase), and a recovery (the third phase) are observed successively. As to the mechanism of these changes, researchers share the view as follows⁴⁾⁸⁾⁹⁾ :

In the first phase, the shift to the right takes place in Equation (1) :

In the second phase, ATP is decomposed by the adenosinetriphosphatase (ATPase) activity of myosin or actomyosin as Equation (2) :

Received Feb. 16, 1957.

* Contribution B No. 259, from the Tokai Regional Fisheries Research Laboratory, Fisheries Agency.

Read before the Meeting of the Japanese Society of Scientific Fisheries on Oct. 28, 1955.

** The word "myosins" was used here to include both myosin or L-myosin and actomyosin.

As soon as ATP is removed from the system, shift to the left in Equation (1) which results in the recovery of the viscosity and SB occurs.

Besides the above changes in solution, addition of ATP to the suspension of actomyosin gel in the media of lower ionic strengths ($I < 0.15$) causes a kind of syneresis called "super-precipitation"¹⁰⁾. The ATPase activity to split ATP as Equation (2) is another functional property which is shared by both myosin and actomyosin.

In the present report, the ATP-response of SB, superprecipitation and some physico-chemical properties of the concentrated streaming birefringent protein fraction of squid muscle are treated and compared with those of carp actomyosin and the existence of actomyosin in the said fraction is discussed.

EXPERIMENTAL

METHODS

Unless otherwise stated, methods employed are same to the previous report¹⁾.

Samples Mantle muscle of both male and female of common squid, *Ommastrephes sloani pacificus* STEENSTRUP, (body weight, 340~600 g.) was used: Sample squids were purchased at wholesale markets. When they came to hand, they were very fresh and occasionally alive, being used in experiment as early as possible. Carps, *Cyprinus carpio*, including both sexes, were of weight 120~150 g. : Extraction was done immediately after killed by cutting off the head, and using the dorsal lateral muscle.

Extraction To avoid the injurious effect of heavy metals¹⁰⁾, glass-redistilled water was employed ; metallic vessels and equipments were avoided ; centrifuging tubes were covered with paraffin-paper during the centrifugation. The most part of the treatments including extraction, fractionation and centrifugation (3,500~4,000 r.p.m.) was done in a cold room (0~8°C.) and by using an ice-water bath. Thus the temperature of the liquid was not raised beyond 10°C. even just after the centrifugation. The obtained sample solutions were kept in an ice-box.

Preparation of the squid protein The squid muscle was minced in a mortar and extracted repeatedly with cold water. From the extracts, ones which showed an intense SB were chosen and combined, added with KCl to 0.3 M and dialysed overnight against 3~6 volumes of 0.3 M KCl in a cold place. When the dialysate was centrifuged, some amount of the precipitate was obtained which, by adding an equal volume of 1.2 M KCl, becomes into an opalescent viscous solution showing an intense SB (Fig. 1). Table 1 shows the yield of protein in each fraction to the total muscle proteins. After the preparation was finished on the second day of the experiment, various tests were done within next 2 days using Fraction S₇. Dialysis against 0.3 M KCl as in the present work did not always give a good amount of the precipitate P₄ and a lower concentration of KCl such as 0.2 M might be more favorable as will be reported later.

Preparation of actomyosin of carp muscle According to the method of SZENT-GYÖRGYI¹⁰⁾ which was improved by WATANABE¹¹⁾¹²⁾, minced carp muscle was extracted with WEBER-EDSALL's solution (0.6 M KCl, 0.04 M NaHCO₃, and 0.01 M Na₂CO₃) for 24 hours ; after centrifuging-off the residual proteins, actomyosin was precipitated three

Fig. 1. Preparation of the streaming birefringent protein fraction in the aqueous extracts of squid muscle.

Table 1. A quantitative aspect of the repeated extraction of squid muscle* with water

Extract	Volume (ml.)		pH	SB	Concentration mg. protein/ml.	Yield	
	Extract	Ppt.				mg. protein/ g. muscle	% of total protein
S_1	227	66	6.1	—	3.66	16.6	11.2
S_2	172	88	6.2	—	1.79	6.2	4.2
S_3	167	116**	6.3	+	1.62	5.4	3.7
S_4	134	165**	6.5	+	2.27	6.1	4.2
S_5	125	193**	6.8	+	1.27	3.2	2.2
S_7 ***	56	1****	6.7	+	4.42	6.1	4.2

* ♀; pH 6.1; total proteins, 14.8%; Protein-N/total-N: 0.829; Sept. 26.

** These figures include the amount of the translucent paste described previously (C- and D-phases).

*** Concentrated fraction.

**** Probably denatured proteins.

times at 0.1~0.2 M KCl, being washed twice with 0.05 M KCl; the resultant white mass of the protein, which amounted to 26~29 per cent of total muscle proteins, was dissolved into 0.6 M KCl solution. It showed an opalescent turbidity, a high viscosity and a marked SB; the protein concentration, 12~62 mg./ml. pH 6.5~6.9.

Determination of protein in solution Either semimicro-KJELDAHL method or biuret method¹³⁾ was used. Nitrogen content in the proteins was conveniently assumed as 16.0 %.

Streaming birefringence As reported by MIGITA and MATSUMOTO¹¹⁾, a semiquantitative estimation was done using the instrument of OKADA and TADA¹⁴⁾. For, in this method, to classify the intensity of SB into 3 stages, +, $\pm$, and -, was found more reliable than to classify into 4 stages, +, $\pm$, $\pm$, and -, as described in the previous report, the classification into 3 stages was adopted as the routine method, in place of the other. To detect the ATP-response of SB, protein concentration was adjusted to nearly the lowest level which showed + pattern of SB in a series of repeating two-fold dilutions of the sample solution with 0.6 M KCl. To 8~10 ml. of such protein solution, 0.5~0.8 ml. of 0.025~0.055 M ATP solution was added with or without the addition of 0.16~0.2 ml. of M/20 $MgCl_2$, and SB was pursued.

Superprecipitation Protein solution was diluted with water to $I=0.1$, where the protein was lead to a turbid suspension. To 1~2 ml. of the suspension, different amounts of 0.025 M ATP solution were mixed with or without adding 0.05 M $MgCl_2$ of 1/50 of its volume, the changes that follow during the storage at room temperature being observed and photographed.

ATP sample ATP- Na_2 of National Biochemical Corporation and ATP- Na , of Zellstoffabrik were used. The purity estimated by $\Delta 7P$ was 72~100 %.

Salting-in range Phosphate buffer ($I=0.125$, pH 6.8) 2 ml., KCl solution of various concentrations 7 ml., and protein solution in 0.5~0.6 M KCl solution 1 ml. were mixed so that the resultant ionic strength of the mixtures varied from 0.05 to 0.5 and pH was kept near 6.8. After 2 hour cold storage, tubes were centrifuged (3,000 r.p.m., 20 min.) and protein concentration was determined.

Isoelectric point (pI) The precipitation optimum was measured by the following method, where the ionic strength was kept at 0.4 and 0.6 by adding KCl solution to increase the solubility of the protein in the neighborhood of pI . The mixtures were composed of acetate buffer of various pH values ($I=0.1$) 2 ml., 0.7 M KCl 2 ml., and protein solution (in 0.4 M KCl) 4 ml.; after 1~2 hour cold storage, the protein concentration and pH of the centrifuged solutions (3,000 r.p.m., 20 min.) were determined.

RESULTS

(A) Response of SB to ATP

ROTH⁶⁾ and HAMOIR⁷⁾ have reported the decrease in viscosity of carp actomyosin upon adding ATP, but the whole course of the ATP-response of viscosity or SB of fish actomyosin has not yet been reported. Thus the change of SB of the carp actomyosin was studied both at 20°C. and at 0°C. At the room temperature, SB disappeared immediately after the addition of ATP and meanwhile it was found again (Fig. 2, Curves B and C.). These courses suggest that changes in Equations (1) and (2) have taken place there. As the ATPase activity of rabbit actomyosin which catalyzes the reaction in Equation (2), is inhibited by Mg ion at this ionic strength, Curves D and E of Fig. 2 show that there

Fig. 2. Changes in SB of the actomyosin of carp muscle in 0.6 M KCl upon addition of ATP.

Date : Dec. 3 ; pH of fresh muscle : 7.0.

Curve	Protein concn. (mg./ml.)	ATP (M)	MgCl ₂ (M)	pH	Temperature (°C.)
A	1.6	—	—	6.6	20
B	3.2	0.0012	—	6.6	20
C	1.6	0.0012	—	6.6	20
D	1.6	0.0012	0.001	6.8	20
E	1.6	0.0012	0.001	6.6	20
F	1.6	0.0012	—	6.8	~0*
G	1.5	0.0023	—	6.6	~0*→20
H	1.6	0.0012	0.001	6.8	~0*
I	1.6	0.0012	0.001	6.8	~0*→20

* The solution was occasionally taken out from the ice both for the observation of SB.

was working a similar effect also in the case of carp actomyosin. When ATP was added at 0°C., Curves F and G, where Mg ion was absent, resembled to Curves B and C, but the second phase of Curve F was longer and that of Curve G did not finish until the mixture was brought to 20°C. The fact suggests that the ATPase activity was depressed at the low temperature. When Mg ion was added (Curves H and I), the once-disappeared SB did not restore as in the cases at room temperature (Curves B and C). The difference between Curves B and C, which is shown in the duration of the second phase, might be due to that the protein concentration, and therefore the concentration of ATPase, was higher in Curve B than in Curve C. In the case of Curve G, SB which did not restore in the cold did reappear as soon as the reaction mixture was brought to the room temperature : In Curve G, more ATP was used than in Curve F. These results would

Fig. 3. Changes in SB of the streaming birefringent fraction in the aqueous extracts of squid muscle upon addition of ATP in 0.6 M KCl.

Date : A~F, Sept. 1 ; G~I, Sept. 27.

pH of fresh muscle : A~F, 6.4 ; G~I, 6.1.

Curve	Protein concn. (mg./ml.)	ATP (M)	MgCl ₂ (M)	pH	Temperature (°C.)
A	2.1	—	—	6.6	24
B	2.1	—	—	6.6	~0
C	2.7	0.0012	—	6.8	24
D	2.7	0.0012	0.001	6.4	24
E	2.7	0.0012	—	6.7	~0*
F	2.7	0.0012	0.001	6.7	~0*
G	2.0	0.0024	—	6.2	23
H	2.0	0.0024	0.001	6.2	23
I	2.0	0.0024	0.001	6.2	~0*

* The solution was occasionally taken out from the ice-bath for the observation of SB.

prove that carp actomyosin responds to ATP in a similar manner to rabbit actomyosin. It would be also shown that the method estimating SB by use of this apparatus deserves to treat the ATP-response of actomyosin.

With the streaming birefringent fraction of squid muscle obtained by the method of Fig. 1, the results were as shown in Fig. 3. As Curve C in the figure shows, the change in the absence of Mg ion gave the same course with the case of rabbit and carp actomyosin. Thus it is suggested that the streaming birefringent fraction of squid muscle shows a similar SB response to ATP and also shows an ATPase activity. If the solution was ice-cooled, a change as Curve E was observed, wherein no difference was found except less quick recovery of SB than Curve C at the room temperature. The addition of Mg ion to the

squid protein gave no appreciable effect as shown in Curves D, F, H, and I, while Mg ion prevented the restoration of SB of carp actomyosin. Nevertheless, if SB was restored once, it resumed meantime to disappear which was not the case in the absence of Mg ion. Curves G, H and I were obtained with another lot of sample solution. It might involve a difference in ATPase activity of both preparations, that SB restored after a longer period there than in Curves C~F.

In these experiments 38~150 g. moles ATP per 10^5 g. carp actomyosin and 45~120 g. moles per 10^5 g-squid protein were used.

(B) Superprecipitation

As to the superprecipitation of fish actomyosin, no observation has yet been reported. In the present study, the carp actomyosin showed a typical superprecipitation as shown in Fig. 4 and Table 2 : If ATP was added in the absence of Mg ion to the protein suspension which was obtained by diluting the original solution to 6 times of its volume and to 0.1 M KCl concentration, the protein aggregated immediately, the aggregate shrinking, and forming a stopper-like precipitate within 3 minutes. When 0.001 M Mg ion was present, the turbidity of the protein suspension decreased just after the addition of ATP, and the protein gradually aggregated into a suspension of fine particles within 30 minutes, and separated from the mother liquid within 1 hour, forming a compact precipitate a night after, but it remained in a fine-particled state. While a stopper-like precipitate was formed in the room temperature and without Mg ion, a fine-particled superprecipitate was obtained even without Mg ion, if cooled with ice. Thus the carp actomyosin was found to make super-

(A) Three min. after adding ATP.

(B) Three hours after adding ATP.

Fig. 4. Superprecipitation of carp actomyosin.

Date : Dec. 3 ; pH of fresh muscle : 7.0.

Tube o contains water as a control.

With tubes a~h see Table 2.

Table 2. Superprecipitation of carp actomyosinDate : Dec. 3 ; pH of the mixtures : ~ 6.8 .

	a	b	c	d	e	f	g	h
Protein concn. (mg./ml.)	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
ATP (M)	—	—	0.005	0.00125	0.000125	0.005	0.00125	0.000125
MgCl ₂ (M)	—	0.001	—	—	—	0.001	0.001	0.001
KCl (M)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

(Observation after adding ATP)

Immediately after	Turbid suspension	Marked contraction stopper-like ppt.	Clear supernatant separates	Becomes transparent	
10 min.		Contraction proceeds	Stopper-like ppt.	Fine particled ppt. aggregates	Fine-particled suspension
30 min.					
3 hr.		Stopper like ppt.			
20 hr.				Fine particled compact ppt.

(A) Three min. after adding ATP.

(B) sixteen hours after adding ATP.

Fig. 5. Superprecipitation of the streaming birefringent fraction of the aqueous extracts of squid muscle.

Date : Sept. 26 ; pH of fresh muscle : 6.1.

Tude o contains water as a control.

With tubes a~g see Table 3.

precipitation similar to rabbit actomyosin in literatures^{10)*}.

With the streaming birefringent fraction of squid muscle the results in Fig. 5 and

* In studying the superprecipitation, the author owed a detailed knowledge on the superprecipitation of rabbit actomyosin to the private communication of Prof. E. MIYAZAKI.

Table 3. Superprecipitation of the streaming birefringent fraction in the aqueous extracts of squid muscle

	a	b	c	d	e	f	g	
Protein concn. (mg./ml.)	0.74	0.74	0.74	0.74	0.74	0.74	0.74	
ATP (M)	—	—	0.0042	0.00025	0.0042	0.0011	0.00024	
MgCl ₂ (M)	—	0.001	—	—	0.001	0.001	0.001	
KCl (M)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
(Observation after adding ATP)								
Immediately after	Turbid suspension			Wool like suspension	Turbidity decreases	Turbid Suspension		
10 min.			Clear supernatant separates	Pptn. begins	Suspension			
60 min.			Supernatant separates	Ppt. sets down				
16 hr.			Voluminous ppt.	Fine compact ppt.				

Table 3 were obtained, showing that the said fraction of squid does superprecipitate as carp actomyosin does. In this case a stopper-like precipitate was not formed even in the absence of Mg ion. While the sedimentation was more rapid in the absence of Mg ion, the precipitate when sedimentation finished was more compact in the presence of Mg ion.

ATP employed here amounted to 6~200 g. mole per 10⁵ g. protein in the case of carp actomyosin, and 33~570 g. mole per 10⁵ g. protein in the case of the squid preparation.

Experiments described in (A) and (B) will show that an actomyosin or an actomyosin-like protein is dissolved in the streaming birefringent fraction of squid.

(C) Superprecipitation in the crude aqueous extracts of squid muscle

The foregoing results showed that the aqueous extracts $S_3 \sim S_5$ of Fig. 1 contained an actomyosin-like protein. A superprecipitation study was then tried on the crude aqueous extracts (Fig. 6). To extracts $S_1 \sim S_5$ of Fig. 1, KCl and ATP were added to 0.1 M

Fig. 6. Superprecipitation of the aqueous extracts of squid muscle. Picture 22 hours after adding ATP (0.00092 M) ; $I=0.1$ (KCl) ; pH~6.5 ; Date : Sept. 26 ; pH of fresh muscle : 6.1. P_5 is the fluent suspension covering the surface of the centrifuged residue of the fifth extraction.

and 9.2×10^{-4} M respectively, and mixed. While, 10 minutes after mixing, only S_4 and S_5 showed separation of precipitate from the mother liquid, a night after, $S_2 \sim S_5$ gave precipitates which were distinguishable from the controls. This suggested that an actomyosin-like protein has been extracted as early as into S_2 . Though, in S_5 , the precipitate after sedimentation was less compact than the control, the supernatant was more clear in the tube added with ATP, and it was suggested that a mild superprecipitation might occur also in S_5 . The fluent suspension, P_5 , that covered the residue of the fifth extraction (D-phase of the previous report¹⁾) shrank to a stopper-like precipitate upon addition of ATP.

(D) Salting-in range and isoelectric point (pI)

To get further information, the salting-in range and pI were studied both on carp actomyosin and on the streaming birefringent fraction of squid.

Fig. 7. The salting-in range of the carp actomyosin (Curve A) and the actomyosin like fraction of squid muscle (Curves B and C*).

Date ; A, Dec. 3 ; B, June 23 ; C, Sept. 1.

pH of fresh muscle : A, 7.0 ; B, 6.2 ; C, 6.4.

* The solution used in the experiment of A~F in Fig. 3.

As shown by Curve A of Fig. 7, the carp actomyosin began to dissolve at $I=0.25$ and at $pH \sim 6.8$, reaching a maximum solubility at $I=0.4$. Such behavior is in accordance with the results of ROTH and HAMOIR, and the preparation treated here appears to be pure actomyosin.

The salting-in curve of the squid protein was as Curves B and C of Fig. 7 : These curves suggest that, besides the major fraction which have the salting-in range at $I=0.25 \sim 0.4$, there is a minor fraction which is soluble at the ionic strength as low as 0.15 and occupies some 30 % of the total protein in the solution. Thus the preparation is apparently not homogeneous. This point will be discussed later.

The pI of carp actomyosin was near $pH 4.8 \sim 4.9$ at $I=0.4$ and near $pH 4.2$ at $I=0.6$, while that of the squid protein was found near $pH 4.7$ at $I=0.4$: There is no significant difference between pI values of carp actomyosin and of the squid protein.

The pI value found on carp actomyosin, however, seems rather lower than pI of

actin-containing myosin of carp (pH 5.1~5.4) reported by several investigators^{8,15,16}. But with myosin of rabbit, it was suggested that pI is dependent on the ionic strength¹⁷, and the present data were obtained at the higher ionic strengths. Thus, the problem of the pI would be dealt with after an additional study.

DISCUSSION

(1) From the above results, the streaming birefringent fraction of squid appears to be similar to rabbit or carp actomyosins, in two respects, the ATP-response of SB and the superprecipitation. For these properties reflect the most important characteristics of actomyosin, it would be reasonable to say that the streaming birefringent fraction studied here contains an amount of actomyosin or an actomyosin-like protein, which plays a role in the contraction of squid muscle in vivo, and also shows mechanochemical responses on addition of ATP in vitro.

Besides these similarities, the contractile protein of squid differs in some respects from rabbit actomyosin. Firstly, it is extracted easily with water at a definite condition, being held in the extract even after centrifugation, but precipitated if KCl was added to the concentration of 0.05~0.3 M. Secondly, in the SB-response on adding ATP, the once-disappeared SB did restore even in the presence of Mg ion, which is not the case with rabbit and carp actomyosins. Thus the ATPase activity of the actomyosin-like protein appears to be not inhibited by Mg ion. However, the protein preparation might be contaminated with "the water-soluble, Mg-activated ATPase¹⁸", for it was prepared according to an scheme (Fig. 1), which was designed in view of getting a good yield of the actomyosin-like protein, and not in view of making an enzymatically pure preparation.

Another difference was observed that, if ATP was once added together with Mg ion, the once-restored SB of the actomyosin-like protein of squid, resumed to disappear within 2 hours (Fig. 3, Curves D, F, H and I) : The once-restored SB was stable if Mg ion was absent (Fig. 3, Curves C, E, and G). The cause of this difference is obscure, but it might be a characteristic of the squid protein, because a similar course of the ATP-response of viscosity was reported on myosin B extracted from the adductor of scallop¹⁹ which is not so remote from squid in biological classification. However, result of YAGI *et al.*²⁰ should also be taken into account that myosin B fraction of scallop and other shellfishes appear similar to myosin B of rabbit in ATP-response of viscosity, superprecipitation and in ATPase activity.

As shown in Fig. 5, the actomyosin-like protein did not make a stopper-like superprecipitate but a fine-particled one which was observed with carp actomyosin only in the presence of Mg ion. It might be due to that the test of the squid protein was done at a lower protein concentration than that of carp actomyosin, or to that a small quantity of Mg ion was contained in the preparation of the actomyosin-like protein of squid.

(2) The salting-in curve of the fraction S_2 used in this work contained one or two minor fractions as much as some 30 % of the total proteins (Fig. 7, Curves B and C). Then a question arises which component will be responsible for the actomyosin-like properties. It seems most probable that the major component which begins salting-in at the ionic

strength higher than 0.25 is it, as expected also from the salting-in behavior of rabbit or carp actomyosin. If a solution of the streaming-birefringent fraction of squid in 0.6 M KCl was stored in cold, the function to superprecipitate diminished day after day. In parallel to the diminution of this activity, the salting-in curve of the solution changed, suggesting that the major component is decreasing in amount while the minor ones are increasing²¹⁾. This fact will favor the above view that the major component is the actomyosin-like protein.

(3) It is true that the actomyosin-like protein of squid showed functional characteristics of actomyosin as a contractile protein of muscle. However, it is also true that the protein has some properties deviating from actomyosins of rabbit or carp. Among these deviations a fact that the actomyosin-like protein is readily extracted from fresh muscle with water is predominant. Since this property was found to be shared by the muscles of mollusks²²⁾, the author proposes to call the protein tentatively by the name of "M-actomyosin" which means that there are some deviations from the properties of actomyosin of the striated muscles of rabbit and other animals.

SUMMARY

1) To prove the assumption that the streaming birefringence (SB) observed in the aqueous extracts of squid muscle is due to the dissolving-out of actomyosin or an actomyosin-like protein, the streaming birefringent protein was separated as a concentrated solution by precipitating it through dialysis against 0.3 M KCl. Then the properties of the protein were compared experimentally with those of carp actomyosin.

2) Whereas SB of carp actomyosin, on addition of ATP, showed a very similar response to that reported on rabbit actomyosin (Fig. 2), the streaming birefringent protein of squid behaved itself in a similar but distinct way, if Mg ion was present (Fig. 3).

3) Both carp actomyosin and the streaming birefringent protein of squid showed a typical superprecipitation (Figs. 5 and 6, Tables 2 and 3).

4) From salting-in curves, it was found that the used preparation of the streaming birefringent protein of squid contained one or two minor components, while that of carp actomyosin was homogeneous (Figs. 7).

5) It was inferred that, in the aqueous extracts of squid muscle, an amount of actomyosin-like protein is dissolved which has the functional properties of the contractile proteins. However, for some differences from actomyosins of rabbit and carp were found, and for these differences may be shared by mollusks, it was proposed to call this protein "M-actomyosin".

The author is indebted to Dr. M. MIGHTA for his valuable advice, to Prof. S. MIZUSHIMA for the encouragement to this work, and to Miss. A. YAMASHITA and Miss. M. AKIBA for their technical assistance.

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