On Actomyosin of Squid Muscle from Salt-Extract —Preparation of Actomyosin*—

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In squid muscle, a water-soluble fibrous protein was found to exist which showed responses to ATP like actomyosin, and it was called M-actomyosin¹⁾²⁾. The amount of the water-soluble proteins by repeated extractions was as high as 50~55 % to total muscle proteins¹⁾. If it was assumed that a part of the water-soluble protein fraction was composed of non-myosins** as much as ~20 % of the total muscle proteins according to the fractionation of the salt-extracts¹⁾, the difference between the water-soluble fraction and non-myosins, about 30 %, might correspond to M-actomyosin which was extracted with water. On the other hand, the yield of M-actomyosin preparation from aqueous extracts was 1~9% of the total muscle proteins¹⁾²⁾³⁾, while fractionation of the salt-extracts of the squid muscle suggests that more than 60 % of the total muscle proteins are composed of myosin-like globulins⁴⁾. Hence, it was questioned whether the bulk of the structural proteins is composed exclusively of M-actomyosin, or it contains, besides M-actomyosin, other proteins, *e. g.*, an ordinary actomyosin as that of rabbit or carp.

If there exists, in squid muscle, an actomyosin which should have the same solubility behaviors as rabbit or carp actomyosin, it must be prepared by the same method from squid muscles.

By comparing the characteristic functional properties of these proteins, it will be tested whether the squid protein is identical with M-actomyosin or not. In the present report, trial for separating actomyosin of squid and some observation on the preparation obtained are described.

Experimental

METHODS

The most part of the methods employed are the same as the previous reports¹⁾²⁾.

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** The word "non-myosins" includes albumins such as myogens and myoalbumin, and, globulin X. The soluble fraction at low ionic strengths ($I=0.05\sim0.1$) in salt-extracts of muscle is believed to correspond to non-myosins.

Materials: Mantle muscle of common squid, *Ommastrephes sloani pacificus* STEENSTRUP, which were caught from October to February, were used. The body weight ranged from 310 to 640 g. and pH of the muscle was 6.2~6.9.

RESULTS

(A) The ordinary method to separate actomyosin

The method which has been used most commonly⁵⁾ is based on long extraction of SZENT-GYÖRGYI⁶⁾ followed by separation and purification of actomyosin as described by GREENSTEIN *et al.*⁷⁾ Thus, minced squid muscle was extracted with 5 times (v./w.) of WEBER-EDSALL'S solution (0.6 M KCl, 0.04 M Na₂CO₃, 0.01 M NaHCO₃) for 20 hours at cold place, with constant gentle stirring. The mixture was centrifuged for 20 min. by ~4,000 r. p. m. (1,500~2,800 g), giving an opalescent viscous extract, S₁, which showed an intense streaming birefringence (SB). When the extract was diluted with water to 0.1 M KCl concentration and adjusted to pH 6.5 with 6% acetic acid and centrifuged, the proteins of squid did not deposit well but some amount of loose, swollen precipitate, P₂, was found and the boundary between the supernatant and the precipitate was not sharp, though actomyosins of rabbit and carp always did separate sharply as the precipitate. Supernatant at 0.1 M KCl concentration of squid extract showed an intense SB which suggested that a considerable amount of the fibrous proteins

remained in solution. For it was suspected that the ionic strength (*I*) of precipitation was not proper, the extract S₁, was diluted to 0.05 ionic strength. The amount of the precipitate here, P₃, was larger than the precipitate P₂, and SB of the supernatant was less intense. However, when the precipitate, P₃, was dissolved in 0.6 M KCl and then diluted to 0.2 M (pH~6.3), no precipitate was formed. This suggests that the protein in P₃, is not the same as rabbit actomyosin. The yield per g. muscle was 77 mg. for S₁, 24 mg. for P₂, and 52 mg. for P₃, respectively. When the precipitate P₂ was dissolved in 0.6 M KCl, a salting-in curve as shown in Curve A of Fig. 1 was

Fig. 1. Salting-in curves at pH~6.8 of actomyosin preparations obtained by prolonged extraction with WEBER-EDSALL'S solution (Curve A) and with 0.6 M KCl solution (Curve B). Protein concentration in the mixture: Curve A, 0.38 mg./ml.; Curve B, 0.22 mg./ml.

obtained. This curve suggests that from the extract S₁ no protein might be obtained which resembles rabbit actomyosin.

(B) Long extraction with 0.6 M KCl

It was reported by WEBER⁸⁾⁹⁾ and BAILEY¹⁰⁾, that at higher pH and at higher ionic strength, rabbit actomyosin is apt to be denatured and to dissociate irreversibly into actin and myosin, and also that actin is denatured during the extraction with alkaline media and remains in supernatant when the extract was diluted for separating actomyosin. It was found by the author, that if the salt-extract of squid muscle was stored for a period myosins* are apt to become less precipitable at the lower ionic strength¹¹⁾ ($I=0.05$). As the pH value of the above extract of squid muscle with WEBER-EDSALL's solution was found to be 8.2, the alkaline reaction might be responsible for the failure in separating actomyosin. Thus, 0.6 M KCl solution was used in place of the ordinary WEBER-EDSALL's solution. To remove the soluble proteins more effectively, precipitations were done at 0.2 M KCl concentration from the first step, and repeated twice at the same ionic strength.

The extract S_1 , whose pH was 6.3, was diluted and centrifuged, supernatant being readily separated from the precipitate. The 0.6 M KCl solution of the precipitate P_1 obtained after 3 times reprecipitations gave a salting-in curve shown in Curve B of Fig. 1. While a larger part of the precipitate showed salting-in at $I=0.2\sim0.3$ and seemed to be the proteins corresponding to actomyosins of rabbit and carp, a considerable amount of another component was found which is soluble in lower ionic strengths. Probably, the actomyosin of squid was caught but the impurity was not removed. The yield per g. muscle was 94 mg. for the extract S_1 , and 32 mg. for the precipitate P_1 (concentration 2.2 mg./ml.), and those of the dissolved proteins in the supernatants were 22 mg. for S_2 , 20 mg. for S_3 , and 14 mg. for S_4 , respectively. It must be noted that SB was found in these three supernatants obtained by repeating precipitations. This suggests that some fibrous proteins have remained dissolved in the supernatant.

(C) Short extraction with 0.6 M KCl

The inhomogeneity of the preparation obtained by the long extraction with 0.6 M KCl might be due to some change or denaturation of the protein during extraction described above⁸⁾¹⁰⁾. So the duration of extraction was reduced to 10 min.**, using the same extractant. For this extraction, a blender equipped with an anti-foam baffle plate⁴⁾ was used to help the degradation of tissue. Method of preparation and its result were shown in Fig. 2 and in Table 1, respectively. On dilution succeeded by centrifugation, the precipitates were more compact compared with the cases of long extraction, and the supernatants were easily separated from the precipitates. The solution in 0.6 M KCl of the precipitate P_1 was opalescent, highly viscous, and intense-

* As in the previous report¹⁾, the word "myosins" was used here to comprehend the structural globulins of muscle, such as myosin and actomyosin.

** The later experiences show that the duration of homogenizing suffices with 30 sec.~3 min. by ca. 10,000 r. p. m.

Fig. 2. Preparation of actomyosin of squid muscle.

Table 1. A quantitative aspect of the preparation of actomyosin of squid muscle*

Fraction	pH	SB	Concentration	Yield	
			mg. protein/ml.	mg. protein per g. muscle	% of total muscle proteins
S_1	6.4	+	13.7	142.6	95
		(intense)			
S_2	6.3	—	1.36	31.9	21
S_3	6.3	—	0.39	7.4	5
S_4	6.2	—	0.18	4.3	3
S_5	6.3	+	8.70	89.4	60
		(intense)			

* pH 6.7.

** Total muscle proteins were assumed as 150 mg./g. muscle which is a rough average of many data.

ly streaming birefringent. The salting-in curve of P_4 was shown in Curve A of Fig. 3, which suggests that it was composed of a considerably homogeneous component which has its salting-in range at $I=0.15\sim0.4$ at pH ~6.8 . So this fraction might correspond to the actomyosins of rabbit and carp, though the salting-in range appears a little lower than those of actomyosins of rabbit ($I=0.24\sim0.5^{(9)(10)(12)}$) and carp ($I=0.2\sim0.5^{(13)(14)(15)}$). Since the yield of the preparation P_4 amounted to about 60 % of the total muscle proteins as Table 1, and since the supernatants of precipitations at 0.2 ionic strength did

not show SB, it seems that the most part of the fibrous proteins, *i.e.* myosins was brought down in this fraction.

Thus a preparation like actomyosins of rabbit and carp was prepared by a modified method, while the ordinary method could not yield such preparation.

When the salting-in curve of the preparation was compared before and after each precipitation in Fig. 2, it was shown that by each precipitation, the amount of the presumably impure component which was soluble in lower ionic strengths had decreased. However, this component could not be removed completely by three or four times repeated precipitations and also by additional washings with 0.05~0.2 M KCl solutions.

This preparation will be tentatively assumed as "actomyosin" of squid in the present study.

(D) Effect of pH on solubility behaviors of squid actomyosin

Salting-in curves at various pH's of actomyosin of squid obtained by the above method were as shown in Fig. 4, and its solubility behavior over a wide range of pH

Fig. 3. Salting-in curve at pH ~6.8 of actomyosin preparation obtained by short extraction with 0.6 M KCl solution and its change during storage of the preparation.

Curve A, immediately after preparation;
Curve B, 1 day after preparation;
Curve C, 3 days after preparation;
Curve D, 8 days after preparation.

Fig. 4. Salting-in curves at various pH's of actomyosin preparation.

Fig. 5. Solubility of actomyosin preparation at various pH's and at $I=0.4$.

curve was found to occur. This suggests that the protein changed into a readily soluble one (Fig. 3).

at $I=0.4$ was as Fig. 5.*

Fig. 4 shows that the salting-in range of squid actomyosin shifts to the lower ionic strength with increase in pH value, and shifts to the reverse side with decrease in pH value. Also it was shown that, at a considerably low pH (pH~6), a little part of the preparation was soluble at lower ionic strengths. It was also suggested from Fig. 5 that the isoelectric point of squid actomyosin at $I=0.4$ is near pH ~4.6.

(E) The instability of squid actomyosin

If actomyosin of squid was stored in dissolved state in 0.5~0.6 M KCl (pH ~6.5) in cold place, a gradual change of the salting-in

Discussion

With the present study, a globulin fraction, presumably "actomyosin" of squid was separated and purified to a considerable degree. Although it must be examined experimentally if this preparation is nothing else but the structural protein which is responsible for the contraction of squid muscle, this protein was regarded as actomyosin for the following reasons. Thus, the protein which occupied the larger part of the muscle proteins, was in dissolved state, opalescent, highly viscous and also intensely streaming birefringent. It appears, however, to have three as properties distinct from actomyosins of rabbit and carp. Thus, i) this protein cannot be prepared by the ordinary method for actomyosin but can be obtained by a scheme with some modification; ii) it changes readily in 0.5~0.6 M KCl solution into another protein or another state which is soluble in lower ionic strengths, while no such change has been found on actomyosins of rabbit¹²⁾ and carp¹⁵⁾; and iii) its salting-in appears to begin at a lower ionic strength.

It appears that the second property is responsible for the first, that is, actomyosin of squid was not separated by the ordinary method because it was changed during the long extraction with salt solution. The change of solubility behaviors of squid myosins during the storage of salt-extract¹¹⁾ might be due to the same property as studied here.

Curve A of Fig. 3 and the curves of Fig. 4 show that a small part of the preparation is soluble in lower ionic strengths, and so even at pH ~5.8. Whether the

* The method used here is the same with that which was used to estimate the iso-electric point in the previous report²⁾.

soluble fraction is an impurity, or the dissolved part of a homogeneous preparation, cannot be answered in the present stage of knowledge. Some different mechanisms may also be possible. However, it would be worth while pointing out that it did precipitate completely also at $I=0.4$, when pH was as low as near 4.7 (Fig. 5). In Fig. 5, a small valley is found on the curve near pH 7. A similar pattern was found in the swelling curve of whole muscle of squid and fishes which were worked out by OKADA *et al.*¹⁶⁾

The time is not ripe for answering if actomyosin of squid in the present study is identical with M-actomyosin recovered from aqueous extract or not. However, it would be interesting to note that squid actomyosin and M-actomyosin are very similar as far as their lability is concerned.

Summary

1) To separate actomyosin of squid, the ordinary method used for rabbit actomyosin was tried, but the result was not successful.

2) By extracting squid muscle for 10 min. with 0.6 M KCl, followed by precipitating 3 times at 0.2 M KCl concentration, a preparation which is in all probability actomyosin of squid was obtained. The preparation was, if dissolved in 0.5~0.6 M KCl solution, opalescent, highly viscous and intensely streaming birefringent, and also showed its salting-in range at $I=0.15\sim0.4$ (pH ~ 6.8).

3) If the protein was stored in dissolved state in 0.5~0.6 M KCl and at neutrality, a change occurred so that it becomes readily soluble at lower ionic strengths. The change appears to be responsible for the fact that squid actomyosin is not separated by the ordinary method.

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