AN ELECTROPHORETIC STUDY OF THE SQUID ACTOMYOSIN

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As reported previously,¹,² efforts to isolate actomyosin from squid muscle by the ordinary method for rabbit and carp actomyosins failed to be successful. By a modified procedure, a protein fraction of the squid was obtained the solution of which showed high viscosity, and intense streaming birefringence (SB) and intense opalescence. This fraction was confirmed to be an actomyosin-like protein on the basis of the facts that it showed, on the addition of adenosinetriphosphate (ATP), not only a viscosity drop followed by recovery but also superprecipitation.³)

The present paper deals with some electrophoretic studies, performed to confirm the identity or non-identity of the squid actomyosin-like protein with actomyosins of the rabbit and carp. Some measurements were done on carp actomyosin as control.

Experimental

MATERIALS AND METHODS

Unless otherwise stated methods used were the same as in the previous reports.¹,²).

Materials: Both male and female carps, Cyprinus carpio (body weight 276∼400g.), were used in November and December. For the material, the dorsal lateral muscle was taken up immediately after killing the fish by cutting off the head.

Mantle muscle of squid, Ommastrephes sloani pacificus Steenstrup, including both sexes (body weight, 238∼516g.) was used. The experiments were carried out from January to July, and the squids were live or very fresh when they were brought in the laboratory.

Preparation of carp actomyosin:

To 30 g. of minced muscle, 270 ml. of 0.6 M KCl was added and homogenized for 1 min. by a baffle-plated Waring blender (∼10,000 r.p.m.)⁴). The mixture was centrifuged for 20 min. by 4,000 r.p.m. (∼2,800 g). To the supernatant, S₁, 2 volumes of water was added (0.2 M KCl), the mixture being centrifuged 30 min. by 4,000 r.p.m. After washing the precipitate, P₂, two times with 0.2 M KCl, the precipitate was

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brought to 0.6 M KCl by adding 2.4 M KCl solution and then subjected to dialysis.

Preparation of squid actomyosin:

The precipitate, P₂, obtained by the similar treatment with carp muscle was re-precipitated at 0.2 M KCl, and dissolved into ionic strength (I) 0.6 and dialysed. In the earlier stages of the study, the fractionation was repeated two times and the ionic strength of the last sample was brought to 0.4. However, an amount of the protein deposited occasionally when the solution was centrifuged after dialysis. Since it was suspected, that the insolubilization met with in the earlier stage of the study might be attributable to the repetition of precipitation, the reprecipitation was omitted and the last solution was brought to 0.6 ionic strength. Although these modifications seemed to make one successful in preparing the solution for electrophoresis, it was found, in the later stage of the study, that reprecipitation does not affect the solubility, when froth formation was avoided throughout the treatments.

Dialysis:

In cellophane bag, 20~50 ml. protein solution was dialysed against 1 l. of NaCl-phosphate buffer (I=0.3+0.1=0.4, pH~7.2) at 5°C, gently revolving the cellophane bag. Usually, the buffer solution was renewed after one night and two night dialyses respectively. After a definite period, protein solution was pipetted out and centrifuged for 20 min. by 4,000 r.p.m., the supernatant being subjected to electrophoretic analysis. The buffer solution used by Dubuisson was chosen in the present study.

Electrophoresis:

HITACHI HTB-type electrophoretic apparatus with Schlieren-diagonal system was used. The sectional area of the cell was 2×15 mm. and the conditions of electrophoresis were as follows: Electric field, 1.9~2.2 V/cm.; electric current, 12.0 mA; temperature, 1°C.; protein concentration, 2~6 mg./ml.; duration, 5 hr. To calculate the electrophoretic mobilities, conductivity of buffer solution was used for the ascending limb, and that of the protein solution for the descending limb.

Conductivity and pH:

A WHEATSTONE-bridge system conductance meter of ITO Electric Co. was used at 1°C., adopting 1 M KCl solution as standard. Value of pH was determined at 1°C., using BECKMAN G type glass electrode pH meter.

RESULTS

(A) Electrophoresis of carp actomyosin

To test the experimental system of the author, results with carp actomyosin were compared with those appearing in literature.

The present preparation of carp actomyosin which corresponded to 43~50% of total muscle proteins was homogeneous as far as the salting-in curve suggests. As Fig. 1 shows, the electrophoretic patterns of 6 runs for 3 samples suggested their homogeneity, either after 1 night dialysis or after 2 night dialysis. As shown in
Table 1. Electrophoretic mobilities of actomyosins of various animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ionic strength</th>
<th>pH</th>
<th>Ascending limb (×10^-5 cm^2/V·sec.)</th>
<th>Descending limb (×10^-5 cm^2/V·sec.)</th>
<th>Note</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp</td>
<td>0.4</td>
<td>7.2</td>
<td>-3.08±0.12*</td>
<td>-2.63±0.11*</td>
<td>1 night dialysis</td>
<td>The present author*</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>7.2</td>
<td>-3.10±0.07*</td>
<td>-2.75±0.15*</td>
<td>2 night dialysis</td>
<td>The present author*</td>
</tr>
<tr>
<td>Carp</td>
<td>0.35</td>
<td>7.1</td>
<td>-2.88±0.14</td>
<td>-2.62±0.21</td>
<td></td>
<td>HAMOIR7)</td>
</tr>
<tr>
<td>Cod</td>
<td>0.4</td>
<td>7.4</td>
<td>-3.05</td>
<td>-2.90</td>
<td></td>
<td>CONNELL8)</td>
</tr>
<tr>
<td>Cod</td>
<td>0.4</td>
<td>7.4</td>
<td>-3.30</td>
<td></td>
<td></td>
<td>DINGLE9)</td>
</tr>
<tr>
<td>Frog</td>
<td>0.35</td>
<td>7.2</td>
<td>-3.1</td>
<td>-2.9</td>
<td></td>
<td>GODEAUX10)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.4</td>
<td>7.2</td>
<td>-2.7</td>
<td></td>
<td></td>
<td>DUBUISSON5)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>7.3</td>
<td>-3.1</td>
<td>-2.5</td>
<td></td>
<td>DUBUISSON11)</td>
</tr>
</tbody>
</table>

* Average of three runs are presented.

Table 1, the mobility was slightly higher after 2 night dialysis than after 1 night dialysis, but the figures including both cases were practically equal to those of HAMOIR with carp actomyosin7) and found to be in the level obtained with cod8,9) rabbit5,11) and frog10) actomyosins. So the electrophoretic system of the present study was shown to be reliable.

(B) A preliminary electrophoresis of squid actomyosin

The squid actomyosin preparation containing 40~60% of the total muscle proteins...
was preliminarily subjected to electrophoresis after 2 night dialysis. The patterns of a few runs were essentially equal to Fig. 2, which reveals the presence of three components in almost equal amount with each other, different from carp actomyosin. On the one hand, it was suspected that incompleteness of purification might have resulted in such heterogeneity, and on the other hand, the heterogeneity might be related with the instability of the squid actomyosin-like protein, as it was found previously from the changes in solubility behaviors. Along the latter way of thinking, some change might have occurred on the actomyosin-like protein of the squid during electrophoresis. So, the salting-in curves were tested on the squid actomyosin preparation before and after 22.5 hr. dialysis against the buffer, \( I=0.4 \) and pH 7.2. As Fig. 3 shows, a protein fraction which is soluble at \( I=0.1 \) appears to have increased in amount during the dialysis. So the electrophoretic diagrams of Fig. 2 might have contained an artefact which was brought forth during the dialysis.

![Fig. 2. Electrophoretic diagram of a squid actomyosin preparation. 18,000 sec., 2.13 V/cm., 5.5 mg. protein/ml.](image)

![Fig. 3. Salting-in curves of a squid actomyosin preparation before and after dialysis preceding electrophoresis.](image)

- Curve A: Immediately after dissolution of the protein;
- Curve B: After dialysis for 22.5 hr.
- Protein concentration, 4.9 mg./ml.; pH, 6.7 ~6.9.
(C) Electrophoresis of squid actomyosin after various duration of dialysis

Although the above results suggested that electrophoresis will not allow to catch the intact state of the actomyosin preparation of the squid, it happened that an approach was made with success from the comparison of the electrophoretic diagrams obtained after various durations of dialysis.

If a solution dialysed a night was analysed, patterns were obtained as shown in Fig. 4, A and B. While, as seen in the figure, only 2 components were found in a run within 4 hr. a middle-speeded component was found to separate from the faster component if electrophoresis was prolonged beyond 5 hr. The resulting pattern consisted

![Electrophoretic diagrams](image)

**Fig. 4.** Electrophoretic diagrams of a squid actomyosin preparation after dialysis of various durations.

<table>
<thead>
<tr>
<th>Dialysis</th>
<th>Electrophoresis</th>
<th>Electric field V/cm.</th>
<th>Concentration mg. protein/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 15 hr.</td>
<td>10,800 sec.</td>
<td>1.96</td>
<td>5.4</td>
</tr>
<tr>
<td>B 15 hr.</td>
<td>18,000 sec.</td>
<td>1.95</td>
<td>5.4</td>
</tr>
<tr>
<td>C 41 hr.</td>
<td>10,800 sec.</td>
<td>2.11</td>
<td>4.4</td>
</tr>
<tr>
<td>D 41 hr.</td>
<td>18,180 sec.</td>
<td>2.11</td>
<td>4.4</td>
</tr>
<tr>
<td>E 64 hr.</td>
<td>10,800 sec.</td>
<td>2.09</td>
<td>4.9</td>
</tr>
<tr>
<td>F 64 hr.</td>
<td>18,180 sec.</td>
<td>2.09</td>
<td>4.9</td>
</tr>
</tbody>
</table>
of 3 components whose electrophoretic mobilities corresponded each to the levels of three components of Fig. 2.

If solutions dialysed two nights or five nights long were analysed, all three components were found as shown in Fig. 4, C~F. Moreover, a calculation of the relative area for the three components suggested that, during dialysis, Component 1 decreased while Component 2 increased in amount (Table 2), and it was suggested that Component 1 changed into Component 2 during dialysis.

Although, in some cases, Component 3 was found to divide into two peaks, it appeared insignificant to distinguish these two from each other.

Throughout more than 20 runs with some 10 preparations, the results were substantially the same as the above. So it appeared that the actomyosin-like preparation of the squid, immediately after isolation, contains less than three components, that is, Component 1 only or Components 1 and 3 and the greater part of it (~60%)

### Table 2. Changes in the relative amount of the components of electrophoretic diagram of the squid actomyosin preparation during dialysis

<table>
<thead>
<tr>
<th>Component</th>
<th>15 hr.</th>
<th>41 hr.</th>
<th>64 hr.</th>
<th>15 hr.</th>
<th>41 hr.</th>
<th>64 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39</td>
<td>21</td>
<td>22</td>
<td>34</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>38</td>
<td>42</td>
<td></td>
<td>32</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>41</td>
<td>36</td>
<td>33</td>
<td>34</td>
<td>31</td>
</tr>
</tbody>
</table>

Figures denote the relative area of each component to the total area of the electrophoretic diagram.

### Table 3. Electrophoretic mobilities of the components of the squid actomyosin preparation.
Average of 6 runs are presented

<table>
<thead>
<tr>
<th>Component</th>
<th>Mobility (×10⁻⁵ cm²/V. sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 night dialysis</td>
</tr>
<tr>
<td></td>
<td>Ascending limb</td>
</tr>
<tr>
<td>1</td>
<td>$-4.78 \pm 0.85^*$</td>
</tr>
<tr>
<td>2</td>
<td>$-4.29 \pm 0.11$</td>
</tr>
<tr>
<td>3</td>
<td>$-3.02 \pm 0.12$</td>
</tr>
</tbody>
</table>

* $\sigma = \sqrt{\frac{\sum(U-U)^2}{N}}$ (Standard deviation), NaCl-phosphate buffer, $I=0.4$, pH~7.2; temperature, 1°C.; duration of electrophoresis, 3~5 hr.; electric field, 2.07~2.26 V/cm.
is occupied by Component 1.

The electrophoretic mobility of these components is shown in tabular form (Table 3). The figures obtained after 2 night dialysis seem slightly higher in ascending limb and slightly lower in descending limb compared with those obtained after 1 night dialysis.

It appears that the variation of mobility of each component is greater than the case with carp actomyosin. The turbidity migrated with Component 1, though its intensity was different in separate preparations.

In the last stage of the study, it happened that a preparation showing a single component as Fig. 5 was obtained. This preparation was gained by two-times reprecipitation at 0.2 ionic strength succeeded by centrifugation at 12,800 g using a very fresh squid muscle captured in November. Since the mobility of this peak corresponded to that of Component 1, it seemed most probable that the actomyosin-like protein of squid muscle is Component 1 (Fig. 5).

![Fig. 5. Electrophoretic diagram of a squid actomyosin preparation purified by reprecipitation twice and centrifuging at 12,800 g. 18,000 sec.; 1.94 V/cm.; 4.39 mg. protein/ml.; dialysis, 17 hr.](image)

Discussion

As reported previously, a globulin which was isolated from squid muscle was found to be an actomyosin or an actomyosin-like protein basing on its viscosity, streaming birefringence, responses of viscosity to ATP. Moreover, marked superprecipitation was observed with this preparation. However, the present results show that the squid actomyosin preparation behaves electrophoretically in a considerably different way compared with actomyosins of rabbit and carp: Firstly, the actomyosin-like protein of the squid migrated faster than others as Tables 1 and 3 show. Secondly,
it was usually accompanied with one or two impurities, while an electrophoretically homogeneous actomyosin was readily obtained in the case of the rabbit or carp muscle.

As regards the nature of Component 2, it is most probable that it was given rise to from Component 1 as a result of degradation or denaturation. Moreover, it is thought that Component 2 might be tropomyosin from the following reasons:

i) The results of the previous reports\textsuperscript{1,2} suggest that the component which is formed during storage of the solution of the actomyosin-like protein of the squid might be less viscous, might have no or, if any, low ATP-sensitivity, and might be less precipitable at lower ionic strengths.

ii) When salt-extract of the squid muscle was stored cold for 1~2 nights and then diluted to \( I=0.05 \), the amount of precipitating myosins\textsuperscript{*} was smaller than the amount obtained by diluting immediately after extraction. In the former cases, the precipitate increased markedly when the pH was brought to 6.5 while it was scanty at pH beyond 7.\textsuperscript{12}

iii) When actomyosin preparation of the squid was kept cold for a night at \( I=1.0, \) pH\textasciitilde10, no SB was found even after the solution had been brought to neutrality. When the solution was lead to an ionic strength of 0.1 and pH 7 by dialysis, a fine suspension with silky lustre was formed besides the solution showed an intense SB.\textsuperscript{13} These properties are reminiscent of tropomyosin.

iv) When the electrophoretic mobilities of Component 2 are compared with those of squid tropomyosin,\textsuperscript{18} the former appears to be higher than the latter (Table 4). However, the former are rather close to tropomyosins of the carp\textsuperscript{15} and the haddock\textsuperscript{16} and slightly lower than rabbit tropomyosin.\textsuperscript{14}

\textsuperscript{*} A globulin fraction which is precipitated by dilution of salt-extracts was called "myosins" as to include myosin, actomyosin and co-precipitable proteins.

\begin{table}[h]
\centering
\caption{Electrophoretic mobilities of tropomyosin of various animals}
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
Animal & Ion concentration & pH & Ascending limb & Descending limb & Reference \\
\hline
Rabbit & 0.35 & 7.3 & -5.6 & -4.9 & \textsuperscript{DUBUISSON\textsuperscript{14}} \\
Carp & 0.35 & 7.1 & -4.30 & -3.90 & \textsuperscript{HAMOIR\textsuperscript{15}} \\
Haddock & 0.35 & 7.1 & -4.90 & -3.90 & \textsuperscript{KUBO\textsuperscript{16}} \\
Venus (adductor) & 0.4 & 7.0 & -2.9 & -2.7 & \textsuperscript{KOMINZ \textit{et al.}\textsuperscript{17}} \\
Squid & 0.35 & 7.0 & -3.13 & -3.65 & \textsuperscript{YOSHIMURA\textsuperscript{18}} \\
Component 2 of squid actomyosin & 0.4 & 7.2 & -4.12 & -3.73 & \textsuperscript{Present author} \\
\hline
\end{tabular}
\end{table}
If there are two kinds of tropomyosin, tropomyosin A with lower mobility and tropomyosin B with higher mobility according to the notation of Kominz et al., Component 2 might belong to tropomyosin B as rabbit, carp and haddock tropomyosins do, while Venus tropomyosin shown in Table 4 belongs to tropomyosin A. Though content of tropomyosin of the squid was as low as 0.38% of fresh muscle, that of Component 2 might be higher (3%). The difference in yield between the two cases may make one suspect the ideenity of Yoshimura's tropomyosin to Component 2.

However, it was stated by Bailey and other workers that tropomyosin can be isolated in high yield (25~30% of the total muscle proteins) from muscles of Pinna and other mollusks, and that tropomyosin is a dominant protein of molluscan muscle. Very recently, Yoshimura et al. isolated by a similar way from the dehydrated squid myofibril a crystalline tropomyosin with a good yield corresponding to a greater part of the myofibrillar proteins.

It is inferred from the experimental bases of the present paper that a dominant protein, which is isolated as an actomyosin-like complex protein from squid muscle, gives rise to an artefact which is alike to tropomyosin. The isolation methods of Bailey and other treatments include treatment at high ionic strength for some period or treatment with KI solution, both of which should promote the degradation of the actomyosin-like protein of the squid. Therefore, one might think of the secondary formation of tropomyosin during isolation procedures, e.g. dialysis for long duration, from an actomyosin-like complex protein, if it might have been functioning as a contractile element in situ and isolated once in vitro. A tropomyosin, which neither has ATPase activity nor shows ATP-response of viscosity, might not carry by itself the contractile function.

Concerning other molluscan muscles, actomyosin-like proteins were isolated in terms of myosin B from Pecten and other shellfishes by Yagi and Tonomura et al. So, some actomyosin-like proteins might exist in molluscan muscles. As pointed out by Kominz et al. it might be due to the lability of these actomyosin-like proteins of Molluscs that tropomyosins which were contained as a sub-unit were readily isolated from the molluscan muscles.

On the other hand, Rögg has brought forth another explanation of the abundance of tropomyosin in Pinna adductor: Tropomyosin molecules exist in a polymerized form, which is connected with viscous tonus in situ, besides actomyosin plays a role of the contractile element. However, as far as the squid proteins studied by the present author are concerned, tropomyosin appears to come from the actomyosin-like protein because the falls in viscosity and in ATP-sensitivity of the actomyosin preparation parallel the changes as well in solubility behaviors as in electrophoretic properties.

If it is reflected that the squid mantle is composed of the smooth muscles, it
might be interesting that a contractile protein isolated from the uterus smooth muscle has some similarity to the squid protein. The uterus protein which was referred to as actotropomyosin by Snellman et al.\textsuperscript{30)} showed an electrophoretic pattern consisting of three peaks, \(-7.7\times\), \(-4\times\) and \(-2.8\times10^{-5}\) cm\(^2\)/V. sec. In viscometric study,\textsuperscript{30-33)} the uterus protein was found to respond to ATP, its contractile function in situ being suggested.

In either hypothesis of Kominz et al.\textsuperscript{17)} or of Snellman,\textsuperscript{30)} actin should be found after the complex protein was decomposed. Nevertheless, none of three electrophoretic components of the squid actomyosin preparation can not be identified as actin according to the present interpretation. Further informations are needed before proposing a conclusion.

Further study on the relationship of the actomyosin-like protein and tropomysin of molluscan muscles will be of significance in the comparative biochemistry of muscle contraction.

**Summary**

1) The actomyosin preparation isolated from the squid mantle muscle was subjected to electrophoresis at an ionic strength of 0.4 and pH 7.2 (NaCl-phosphate buffer), three components with the mobilities, \(-5.1\times\), \(-4.1\times\), and \(-3.2\times\) (ascending limb), \(-4.4\times\), \(-3.7\times\), and \(-3.0\times10^{-5}\) cm\(^2\)/V. sec. (descending limb), respectively, being observed usually. It appeared a characteristic of the squid actomyosin-like protein that the electrophoretic homogeneity is less readily attained by isolating procedure, while actomyosins of the rabbit and carp readily give a single pattern. However, in few examples of the squid, preparations showing a single peak of the fastest were obtained.

2) By comparing the electrophoretic patterns of different samples obtained after different periods of dialysis, and results of various experiments, a tentative interpretation was presented as follows:

Component 1, the fastest, is the actomyosin-like protein of the squid muscle, which shows high viscosity and its ATP-response. The actomyosin-like protein of the squid is higher in mobility than the ones of rabbit and carp actomyosins.

Component 2, the middle-speeded, is an artefact which is derived from Component 1 during dialysis and electrophoresis. Component 2 might correspond to tropomysin.

With the nature of Component 3, the slowest, no reasonable explanation is obtained.

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References