On the Nature of the Streaming Birefringence Observed in the Aqueous Extracts of Squid Muscle—I. An Anomalous Component in the Aqueous Extracts of Squid Muscle

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OKADA and TADA (1954)¹⁾ in the authors' laboratory, have reported that a marked streaming birefringence (SB) is observed in the aqueous extracts of squid muscle. Many investigations have been made on SB of muscle extracts since the study by VON MURALT and EDSALL (1930)²⁾ on rabbit muscle myosin. However, SB of muscle extracts has been studied only in salt extractions (ionic strength 0.5~1.5) because it is generally believed that "myosin" is the only muscle protein which produce SB and it has a very low solubility in water or in dilute salt solutions.

What is then responsible for the SB observed in aqueous extracts of squid muscle? It may be due to the presence of something streaming birefringent and soluble in water or in very dilute salt solutions; or it may be due to the dissolution of myosins in aqueous extracts for some unknown reasons.

In order to clear up these questions, proteins of aqueous extracts of squid muscle, especially presence of "myosins"* in those aqueous extracts were examined with reference to the appearance of SB.

EXPERIMENTAL

(A) Methods

Fish samples. The animals studied were as follows: Squid (*surumeika*), *Ommastrephes sloani pacificus*; carp (*koi*), *Cyprinus carpio*; flat fish (*hirame*), *Paralichthys olivaceus* (TEMKIN & SCHLEGEL) and horse mackerel (*maaji*), *Trachurus japonicus* (TEMKIN & SCHLEGEL). All the samples employed were obtained from the Central Fish Market of Tokyo. When they were arrived at the laboratory, they were fresh and some were alive.

In the case of squid, a piece of muscle was cut from the mantle after removing the outer two of the four layers of skin. In fishes, the dorsal muscle was cut off, taking care not to conta-

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* In the present paper the word "myosins" was used in order to imply the unfractionated mixture of myosin homologues which includes the myosin proper or L-myosin as well as actomyosin.

minate it with red muscle. The piece of muscle was minced with knife, ground in mortar, and then the proteins were extracted.

Total proteins. From 1 g. minced muscle, the proteins were fixed and washed with trichloroacetic acid solution, keeping the concentration at 5 per cent throughout the experiment. The precipitate thus obtained was analysed for nitrogen by the semimicro-KJELDAHL method.

Determination of proteins in solution. Either the KJELDAHL method and/or the biuret colorimetric method was used. In the former, the proteins in solution were fixed with trichloroacetic acid; in the latter, a biuret reagent devised by one of the authors (MATSUMOTO and KANENITSU, 1954)³⁾ improving that of SOLS (1947)⁴⁾ was used. The reagent, containing 0.4 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 % NaOH , and 0.3 % glycerol, was added to an equal volume of the sample solution. After standing for 3 or more hours, the mixture was filtered through a glass filter, and then the absorption at 545 $\text{m}\mu$ was estimated spectrophotometrically.

Extraction. Minced muscle was mixed with the cooled solvent for ten minutes by use of a hand-made blender (2,500~4,000 r.p.m.), which was equipped with an insulating disc placed beneath the surface of the liquid for the purpose of preventing the denaturation of proteins due to froth formation.

Streaming birefringence. An apparatus reported by OKADA and TADA (1954)¹⁾ was used for detection and comparison of SB. This apparatus is composed of a pair of crossed nicols 20 mm. in diameter, and a rotary bench on which a small beacker containing the sample liquid is placed. Although it was originally devised for qualitative purpose, the authors succeeded in comparing the relative intensity of SB semi-quantitatively by observing the patterns of SB; the patterns were classified into the following four stages:

- + ; a distinct cross appears.
- $\pm$; bright and dark areas are moving like a cluster of clouds, but do not form into a distinct cross.
- $\pm$; the clouds are very faint.
- ; the field remains dark.

The intensity of the sample solution was represented by the minimum concentration which will produce each stage of above patterns. Thus the minimum concentration which produced + was determined, and other stages were obtained by twofold serial dilutions, and then the values which produced + were compared with each other. If SB of the original solution was not so strong that it did not give SB pattern of + stage, the concentration for $\pm$ was adopted for comparison: if the data for $\pm$ was also unavailable, the same treatment was performed on the concentration for $\pm$. The intensity of SB is inversely related to the minimum concentration which produces any stage of the three patterns of SB.

(B) *Streaming Birefringence of the Aqueous Extracts in Repeated Extractions with Water*

When squid muscle was extracted repeatedly with distilled water, 2~20 times the weight of the muscle, it was found that the intensity of SB of each extract varied as the extraction was repeated. The first extract showed no or slight SB, the

second and the third showed it very markedly, and the following extracts decreased in SB in proportion to the concentration of the extracted proteins (Table 1 and Fig. 1).

Table 1. *The amounts of extracted muscle proteins by repeated extractions with distilled water*

(a) *Squid**

Extracts	SB	Extracted proteins		Appearance of precipitate
		mg. Pr.**./ml.	% per total proteins	
<i>S</i> ₁ ***	—	1.83	22.5	White compact mass
<i>S</i> ₂	±	1.09	14.3	} Translucent paste (C-phase) covered the precipitate and a part of it (D-phase) flowed out on decantation
<i>S</i> ₃	+	0.79	8.7 ₄	
<i>S</i> ₄	+	0.56	6.6 ₇	
<i>S</i> ₅	+	0.20	2.2 ₃	Translucent paste did not flow out
<i>S</i> ₆	—	0.06	0.8 ₂	White compact mass
Total extracted			55.3	

* pH 6.4; total proteins, 153 mg./g. muscle; protein-N/total-N, 70.8 per cent; date, November 16, 1953.

** Pr.; protein.

*** For the notations see Fig. 1.

(b) *Carp**

Extracts	SB	Extracted proteins		Appearance of precipitate
		mg. Pr./ml.	% per total proteins	
<i>S</i> ₁	—	1.79	19.1	White compact mass
<i>S</i> ₂	—	0.46 ₂	4.3 ₆	} Few ml. of white fluid suspension (D-phase) covered the surface of the precipitate
<i>S</i> ₃	—	0.16 ₂	1.7 ₈	
<i>S</i> ₄	—	0.13 ₁	1.5 ₇	
<i>S</i> ₅	—	0.05 ₆	0.6 ₉	Compact mass
Total extracted			27.5	

* pH 6.8; ♀; total proteins, 177 mg./g. muscle; protein-N/total-N, 89.7 per cent; date, December 21, 1953.

(c) *Horse mackerel**

Extracts	SB	Extracted proteins		Appearance of precipitate
		mg. Pr./ml.	% per total proteins	
<i>S</i> ₁	—	1.27	15.5	} White compact mass
<i>S</i> ₂	—	0.26 ₉	3.1 ₄	
<i>S</i> ₃	—	0.013	1.5 ₂	
<i>S</i> ₄	—	0.018	2.1 ₂	
Total extracted			22.3	

* pH 6.1; total proteins, 170 mg./g. muscle; protein-N/total-N, 79.0 per cent; date, May 18, 1954.

Fig. 1. SB of the extracts of the repeated extractions of squid muscle with water.

Also a difference in appearance after centrifugation was found between the first extract and the following ones. The first extract clearly separated into a liquid at the top (A-phase) and a compact precipitate at the bottom of the tube (B-phase). In the second and the following extracts, a translucent plastic paste (C-phase) was formed between the precipitate and the liquid. An upper part of this intermediate layer was apt to pass gradually into a dense suspension (D-phase) which flowed out on decantation together with the last part of the supernatant.* The apparent volume of the pasty layer decreased in the process of repeated extractions. It is interesting to note that the aqueous suspension of the intermediate layer, which formed an opaque liquid, gave a marked SB.

(C) Protein Contents in the Repeated Extractions with Water

When the squid muscle was washed repeatedly with water, 20~40 times the weight of the muscle as shown in Fig. 1, the amount of proteins in each extract varied as shown in Table 1. The total extractable proteins amounted to as much as 53 to 55.5 per cent of the total muscle proteins which was unexpectedly high, while in carp and horse mackerel muscle the total extractable proteins were 27.5 and 22.3 per cent respectively of the total muscle proteins.

It is noteworthy that although the supernatant of aqueous extracts of carp or horse mackerel muscle showed no SB, the precipitates or residues did if they were mixed into an unstable aqueous suspension by stirring up the contents of the tube with a glass rod; or, when the centrifugation was incomplete, we could observe SB. This may suggest that the fraction present in the aqueous extract of squid muscle and responsible for SB might be related to myosins.

* For the sake of convenience in study, the notations, A-, B-, C-, and D-phase, were given to these phases.

(D) *Extractions with Varying Volumes of Water*

Squid muscle was extracted with water, wherein the ratio of the water to the muscle was varied from 2.5 to 160 times the weight of muscle. From the protein concentration and the volume of the supernatant after centrifugation, the amount of the extracted proteins per total muscle proteins was determined. The value was found to increase as the ratio of water/muscle weight increased. When the ratio of water/muscle weight reached 5~2.5, 10 per cent of the total proteins were extracted, and below this ratio the per cent of extracted proteins did not seem to decrease.

In the case of muscle of flat fish or of horse mackerel, the amounts of extracted proteins did not vary widely with the change of the ratio of water/muscle, and SB was never detected (Fig. 2). In squid muscle, extracted proteins increased steadily with the increasing ratio of extracting water to muscle, while the minimum concentration for the detectable SB was found to decrease, that is, the intensity of SB increased as the ratio of water to muscle was raised (Table 2).

Fig. 2. The variation of the amount of extracted proteins to the ratio of extracting water to muscle.

pH of the muscle; squid, pH 6.2; flat fish, pH 6.4 (in both cases); horse mackerel, pH 6.4.

Total proteins in muscle: squid, 155 mg. Pr./g. muscle; flat fish, 204 and 197 mg. Pr./g. muscle; horse mackerel, 173 mg. Pr./g. muscle

Date of experiment: squid, Oct. 15, 1953; flat fish, Oct. 10, 1953 and April 13, 1954; horse mackerel, Sept. 28, 1952.

*Table 2. The change in the relative intensity of SB of aqueous extracts of squid muscle with increasing ratio of extracting water to muscle**

Water/muscle		5	20	40	100
Extracted Pr./total Pr. (%)		18.0	21.9	24.6	30.0
Original extract.	mg. Pr./ml.	5.44	1.73	0.99	0.53
	SB	±	±	±	+
Diluted solutions	Minimum concn. ** for SB +				0.53
	" " ±	5.4	1.7	0.50	0.13
	" " ±	1.4	0.87	0.26	0.07

* pH 6.4; total proteins, 150 mg./g. muscle; date February 26, 1954.

** The concentrations are represented by mg.Pr./ml.

In view of these facts, the authors were led to the following opinion. The water-extractible proteins of squid muscle might be, in a rough measure, divided into two fractions, the first of which is readily soluble in water but does not show SB like the water extractible fraction of teleosts and mammals, and the second fraction, which may occupy the most part of the second or the following extracts, is less soluble than the first fraction, and shows SB and may be the very fraction responsible for the characteristic of squid muscle.

(E) *The Amounts of Non-Myosins in Squid Muscle*

Salt solution extracts of muscle proteins were fractionated into "myosins" and "non-myosins" by the following method based upon a scheme* developed by DYER et al.⁵⁾ in 1950 (Table 3). Although it is not yet concretely proved that the structural globulins of squid muscle is identical with myosins of mammalian and fish muscles, the fraction which precipitated from salt extracts on dilution was defined here as "myosins" and the supernatant fraction as "non-myosins".

Ten gram minced muscle was extracted for 10 minutes with 200 ml. of cold KCl solution of 1.0 ionic strength buffered with 0.03 M KHCO_3 or with phosphates* to pH 7.2~7.5 and centrifuged. The extracts was then diluted to the ionic strength of 0.05 (or 0.1) and pH of the diluted extract was adjusted approximately to pH 6.5. After 4~20 hour cold storage myosins are centrifuged off from the extract.

Some discrepancies did existed upon repeated measurments, but the amounts of non-myosins of flat fish, horse mackerel, and carp muscle were found to be approximately the same amount as water-extractible muscle proteins of the respective fishes, while in squid muscle the amount of non-myosins was as little as one fifth to one

* This method was adopted for the sake of convenience, since a number of different methods are recommended in literature on this subject, and because the values obtained by the method here chosen were in close agreement for different samples of each species.

Table 3. The amount of non-myosins of squid muscle and some teleost muscles determined by precipitating myosins in the salt solution extracts

Species	Non-Myosins (% per total Pr.)	Condition of precipitation			Sample muscle		
		pH	Ionic strength	Salt	pH	Total proteins, mg.Pr./g.	Date
Squid	17.1	7.1	0.05	KCl-KHCO ₃	6.8	167	Oct. 26, '53
	9.6	7.3	0.1	KCl-KHCO ₃	6.1	144	Nov. 27, '53
Flat fish	18.3	7.4	0.05	KCl-KHCO ₃	6.4	197	Apr. 14, '54
	24.2†	6.5	0.05	KCl-phosphate	6.4	197	July 5, '54
Horse mackerel	23.2†	7.2	0.05	KCl-KHCO ₃	6.1	170	May 18, '54
	21.3	6.5	0.05	KCl-phosphate	6.1	173	July 8, '54
Carp	30.1	7.2	0.05	KCl-KHCO ₃	7.2	173	June 1, '54
	23.8		0.09	NaCl-NaHCO ₃	6.9	177	Oct. 9, '53
	23.2~24.5	6.5	0.05	KCl-phosphate	6.8	166	June 28, '54

The true amount of non-myosins in each case would be slightly lower than these figure since myosins remaining in diluted solutions are not taken into account.

† An approximate determination of water extracted proteins of the same muscle gave the value of 23.2% in flat fish, and 20.3% in horse mackerel.

third of the maximum amount of water-extractible proteins obtained by repeated extractions.

Therefore, we may conclude that squid muscle myosins, which have the property of SB and are highly soluble in the concentrated salt solution, dissolve considerably into the aqueous extracts.

(F) *Dialysis of Aqueous Extracts of Squid Muscle*

In order to separate the protein producing SB, the first and the second aqueous extracts of the repeated extractions were dialysed separately against distilled water.

The cellophane film available in the market was used. The volume of the outer liquid was 20~40 times the volume of sample liquid, renewed four to six times in 24 hours, and kept at 4~7°C. The extracts were then centrifuged.

A part of proteins in the extracts was laid down whereas the control samples left standing for the equal period gave but slight precipitate on centrifugation (Table 4).

Now it would be rightly said, that the components of the aqueous extracts of squid muscle can be roughly divided into two fractions: albumins and a fraction depositing on dialysis.

It is probable that the former fraction is the truly water-soluble one, while

* The KCl-phosphate extractant was composed of KCl solution of 0.85 ionic strength and phosphate buffer of 0.15 ionic strength (pH 7.2~7.3).

Table 4. *Dialysis of the aqueous extracts of squid muscle**

	Extract	Before dialysis	After dialysis	Control**
Concentration† (mg. Pr./ml.)	<i>S</i> ₁	1.14 (±)	0.82 (±)	1.03 (±) ††
	<i>S</i> ₂	0.64 (+)	0.10 ₆ (±)	0.43 (+)
Soluble proteins per total muscle proteins (%)	<i>S</i> ₁	16.5	11.6	14.3
	<i>S</i> ₂	9.6	1.6	6.1
	<i>S</i> ₁ + <i>S</i> ₂	26.7	13.2	20.4

* pH 6.1; total muscle protein, 132 mg. Pr./g. muscle; protein-N/total-N, 63.1%; date, February 23, 1954.

** Supernatant obtained by centrifuging the same extract after standing for the equal duration with that of dialysis.

† The correction was given for the change of volumes during the dialysis.

†† The signs in the parentheses denote the intensity of SB.

most of the latter fraction may be composed of another protein, the solubility of which varied depending on the extracting conditions as shown in Table 1 and Fig. 2.

It is interesting that after dialysis the amount of proteins remaining in solution per total muscle proteins (13.2 %) was close to the level of non-myosins (9.6~17.1 %). In addition, SB of the supernatant of the second extract became less marked after dialysis and centrifugation, and the precipitate formed by centrifugation showed an intense SB when it was suspended in distilled water.

By similarly treating the precipitate from the first extract which failed as usual to show SB, the presence of SB could not be detected.

(G) *The Solubility of Water-Extractible Proteins of Squid Muscle in Solutions with Different Concentrations of KCl*

When a series of the aqueous extracts of squid muscle was mixed with KCl solutions of different concentrations* and then centrifuged after standing in cool place for about 20 hours, it was found that varying amounts of the proteins were deposited, as shown in Fig. 3.

It is instructive to find that the curves for the second or the third extract (Curves B and C) reveal that the solubility of the proteins as well as SB in the supernatant fell to a minimum value at the ionic strength of 0.05~0.1, increasing again as the ionic strength was raised. These curves resemble the solubility curve of "the crystalline myosin" or "myosin A"*** of SZENT-GYÖRGYI (1947)⁶⁾. On the other hand protein which precipitated at the ionic strength of 0.03~0.2 showed a marked SB when suspended in water.

These facts would offer a strong support to the assumption that it might be

* The final protein concentration was kept constant.

*** Generally referred to "myosin proper" in recent papers.

Fig. 3. The change in solubility of the water extracted proteins of squid muscle with increasing concentrations of KCl.

Curve A: the first extract (S_1) of squid, *Ommastraphes sloani pacificus*, containing 19.9% of total muscle proteins.

Curve B: the second extract (S_2) of the same sample containing 9.5% of total muscle proteins.

Curve C: the mixture of the third (S_3) and the fourth (S_4) extracts of squid (*yariika*), *Loligo bleekeri*, containing 15.8% of total muscle proteins.

pH value of the supernatant after centrifugation: A, ~6.4; B, 6.5~6.7; C, 6.7~7.0.

pH of muscle: A and B, 6.4; C, 6.1.

Date: A and B, July 19, 1954; C, March 30, 1954.

myosin proper which is responsible for SB in the aqueous extracts of squid muscle.

According to H. H. WEBER and PORTZEHL (1952)⁷⁾ myosin proper or L-myosin does *not dissolve in water* but only *disperses into a colloidal suspension* by taking up water and swelling due to the DONNAN effects. This seems to be in agreement with the observations in the present experiment that the precipitate in 0 ionic strength solution was more transparent and voluminous than the precipitates in a solution of an ionic strength of 0.005 or more, and that the turbidities of the supernatant liquids corresponding to the points on the left side of the minimum point in the curves were more intense than those on the right side (Fig. 4).

The presence of myosin proper in aqueous extracts was thus suggested. *Actomyosin* also should not be neglected, since the concentration of proteins remaining in

Fig. 4. The change in turbidity of the supernatant after centrifugation of a squid extract† mixed with varying amounts of KCl.

From left, original extract, control (0.000), 0.001, 0.0025, 0.005, 0.02, 0.05, 0.10, 0.20, 0.30, 0.50, and 1.0 ionic strength.

Protein concentration in each tube, 0.5 mg. Pr./ml.; pH of muscle, 6.3; date, January 6, 1954.

† The third extract of the repeated extractions with water 10 times the weight of the muscle.

solution did not stop increasing when the ionic strength was raised beyond 0.3.

In curve A of Fig. 4 there existed only a shallow depression, and at this point here remained a considerable amount of protein in solution. This may suggest that most of the first aqueous extract is composed of non-myosins which does not show SB.

DISCUSSION

i) It was reported by SNELLMAN and BJÖRNSTÄHL (1941)⁹⁾ that hemocyanin of mollusks shows SB. However, since native hemocyanin molecule is not longitudinal but rather globular* and hemocyanin content** of squid muscle is very low (1.5% of total muscle proteins), the contribution of hemocyanin molecule to the SB observed by the primitive apparatus here employed would be left out of consideration.

ii) It is noteworthy that, among a number of the extracts of the fish muscle with salt solutions, there were found some exceptional cases that failed to show SB, while SB in salt solution of squid muscle was very intense and *never* failed to be detectable.

* The axial ratio of the hemocyanin molecule of various animals is 2.3~7.3 for the native state and 14.4~18.5 for the dissociation components (see MIZUSHIMA and SHIMANOUCHI, 1949)¹¹⁾, while it is 100 for myosin proper and 50~250 for natural actomyosin (see WEBER and PORTZEHL, 1952)¹²⁾.

** Copper in squid muscle: 5.6 mg./kg. (HATAGOSHI, 1941)⁹⁾; Cu content of hemocyanin, 0.24~0.26% for squid, *Loligo*, and other mollusks (HERNLER and PHILIPPI, 1933)¹⁰⁾.

iii) The reason why the myosins of squid muscle can *disperse* into the aqueous suspension remains obscure. It is said that the muscles of mollusks are composed of smooth muscles, and recent papers report that the myosins contained in the smooth muscle of some vertebrates are distinct in several properties from the myosins of the skeletal muscles (see DUBUISSON, 1952,¹³).

Moreover TANAKA (1954)¹⁴ in the authors' laboratory found that the tissue structure of squid muscle is very specific and less organized than the skeletal muscles of fishes.

In fact, the most part of the precipitates of teleost muscles formed after extraction by water and centrifugation were, usually, rather coarse to the touch, while the precipitates of squid muscle were soft and pasty.

Further experiments are necessary to decide whether the above discrepancies are due to the characteristics of myosins themselves or to the tissue structure.

iv) Streaming birefringence in aqueous extracts was observed not only in the muscle of many kinds of squid, but also in the muscle of mollusks such as small clams, abalone and snail, and so far as the authors' experiences, it seems that this characteristic phenomenon is *common to the mollusks*. SB was also observed in aqueous extracts of sea cucumber, though it has not yet been decided whether it is attributable to the same cause.

v) The present opinion, that the proteins in question are myosin proper and actomyosin, is based mostly on their solubility characteristics and the marked SB.

By the use of modern techniques, further evidence and more detailed information should be obtained before any definite conclusion can be reached.

SUMMARY

1) When squid muscle were repeatedly extracted with 2~20 times the amount of water, streaming birefringence (SB) was observed in the second and the following extracts (Fig. 1).

2) When squid muscle were extracted repeatedly the total amount of proteins passing into solution amounted to 55.5 per cent of total proteins. In carp and horse mackerel muscle, however, the total extractible proteins was not so much beyond the amount of non-myosins which was to be expected from the literature on the subject (Table 1).

3) The amount of water soluble proteins of squid muscle increased with the increase of the ratio of extracting water to muscle, while those of flat fish and horse mackerel muscle attained a constant value with the increasing ratio (Fig. 2).

4) In squid, the amount of non-myosins was as small as one fifth to one third of the water extractible fraction, while in flat fish, horse mackerel, and carp, the amount of non-myosins were found to coincide roughly with that of the water extra-

ctible fraction (Tables 1 and 2).

5) If the aqueous extracts of squid muscle were dialysed against distilled water, some of the proteins precipitated on centrifugation. The precipitate from the second extract showed SB if it was mixed into an aqueous suspension, while the precipitate of the first extract did not.

6) Some of the proteins precipitated out if KCl was added to aqueous extracts of squid muscle so as to make up mixtures of varying ionic strengths ranging from 0.0005 to 1.0. The relationship of solubility to ionic strength seems to suggest the presence of both myosin proper and actomyosin in the aqueous extracts (Figs. 3 and 4).

7) In view of these facts it was suggested that in the case of squid muscle, myosin might be dispersed into the aqueous suspension and give rise to the characteristic SB.

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