STUDIES ON THE TROPOMYOSIN OF SQUID

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I. INTRODUCTION

The squid (*Ommastrephes sloani pacificus* STEENSTRUP) belongs to the cephalopoda mollusca. The squid muscle is soft and smooth and systematically, chemically and physicochemically is very peculiar as compared with those of the animals and fishes. The kinds and the amounts of amino acid which build up the muscle protein have been estimated by many persons. The author[1] also had determined the quantitative and qualitative differences of amino acids of squid muscle protein and extract and had found remarkable differences between squid and fishes.

Recently MIGITA[2] has studied on the squid muscle and found the differences of various properties between squid muscle and fish muscle and submitted some consideration on the origin of these differences.

The first difference is that the squid muscle can be readily torn off laterally in fibers, for the squid muscle fibers come together in bunches and are arranged in parallel.

The second is that the squid muscle shows marked contractiveness and the contractile character differs with direction. These facts are caused also by the fact that the muscle fibers are arranged in parallel and that contractile character differs with direction.

The third difference is that it is far more readily soluble in water than fish meat. Such a difference might be caused by the physicochemical and chemical properties of protein molecules and also by the existence of various substances other than protein

which are included in the muscle.

The fourth difference is that the squid muscle shows such a strong swelling ability in a salt solution as often to lead to peptisation under the condition in which fish meat does not exceed the swollen state, and such a strong swelling ability is caused by the excellent hydrochemical properties of squid muscle.

The fifth difference is that the isoelectric point of squid muscle protein measured from the minimum swelling or the maximum precipitation point, is lower than that of fish muscle protein. It is caused by the fact that the squid muscle protein includes a larger amount of acidic amino acids and less amount of basic amino acids than the fish muscle protein.

The sixth difference is that an aqueous extract from squid meat even of the highest freshness gives precipitation reaction with mercuric chloride, which is positive in fish meat, as a rule, only after spoilage has begun.

Seventh difference is that the squid muscle shows intense streaming birefringence not only in salt solution but also in aqueous extract. The salt solution of squid muscle and aqueous extract shows intense streaming birefringence. Ordinarily such a streaming birefringence appears only in neutral salt solution of fish muscle other than squid, but in the case of squid such a streaming birefringence is kept a long time at a low temperature. The cause of such a phenomena is due to the fibrous structure of such a protein molecule as myosin.

The aqueous extract of squid muscle included myosin and it remained still in the fibrous structure even after slight autolysis.

Various such differences between squid and other fish muscle were caused by the specific properties of protein and the various substances other than proteins in the squid muscle.

KITABAYASHI[3] has estimated the amount of myosin, myoalbumin, myogen and globulin-x of the squid muscle by the method of SNOW *et al.*,[4] the results are shown in Table 1.

Tabl 1. Protein-composition of squid muscle

Kinds of protein	Squid*	Cod**
Myosin	9	65
Myoalbumin	54	1
Myogen	24	10
Globulin-X	4	19
Stroma	5	5

* KITABAYASHI[3] ** SNOW *et al.*[4]

From the above table it is seen that the amount of squid muscle proteins differs much from that of fish, that is, the amount of myosin of cod's muscle is about 7 times as large as that of squid. More than a half part of the protein of squid muscle is myoalbumin but in the cod the amount of myoalbumin is only a trace.

The actin component of STRAUB,[5] studied in some detail by SZENT-GYÖRGYI[6] and the SZEGED school, must also be considered as part of the fibril. Indirect assay suggests that the proportions of myosin and actin are in the ratio 8 : 3 (BALENOVIĖ &

STRAUB[7]). Actin possesses the property of converting myosin sols into a thixotropic gel, an interaction which involves the sulphydryl groups of the myosin partner.[8][9]

KITABAYASHI[10] has estimated the amount of myosin, actin, and tropomyosin in the squid muscle and flat fish muscle; the proportions of mysoin and actin are in the ratio 1 : 1 for the fresh squid muscle and 5 : 1 for the fresh flat fish muscle. As mentioned above the amount of various kinds of protein in the squid muscle is much different from those of the fishes.

The myosin of the sarcoplasma is largely enzymic in function. ENGELHARDT & LJNBIMOVA[11] first showed that isolated myosin is associated with adenosine triphosphatase activity; subsequent attempts to separate the enzyme from myosin have been unsuccessful.

When the adenosine triphosphate was added to the myosin solution it caused variations in viscosity,[12] streaming birefringence,[13] and scattering of light[14] of myosin solution, and the variation in the physicochemical properties of myosin molecules was observed. At the time when the adenosine triphosphate is catalytically hydrolysed by the adenosine triphosphatase (myosin) energy produced and it is used to contract the myosin itself.

The fact that actomyosin was the filamentous gel of myosin contracted by the adenosine triphosphate and inorganic salt, was first observed by SZENT-GYÖRGYI.[15]

The converting of muscle or the mutual relationship between adenosine triphosphate and myosin, is the conversion of energy in the living body from the chemical to mechanical, and many students have given attention to a clarification of the relation between these phenomena.

Studies of the myosin as an enzyme became the object of researchers' attention.

Tropomyosin is a new fibrillar protein of asymmetric character and relatively low molecular weight, quite distinct in all its major properties from actin.

Tropomyosin has been crystallized from rabbit muscle by BAILEY[16] in 1948. From its amino acid pattern,[16] and X-ray and electromicroscopic investigation,[17] it is found to be prototype of a much larger myosin molecule and might conceivably be a unit utilized in the elaboration of myosin itself.

Recently, detailed accounts of amino acid analysis[18] with Dowex-50 resin column and of the investigations of N- and C- terminal residues[19][20] have been published with rabbit tropomyosin. It has been observed that tropomyosin contains a large amounts of polar amino acid groups and is composed of a large cyclic polypeptide chain.

In the present investigation, tropomyosin of squid muscle was newly crystallized.

It was found not to be nucleotropomyosin judging from the results of examination of total phosphorous content and ultraviolet absorption; remarkable differences were observed in amino acid composition, electrophoretic mobility and sedimentation constant, as a matter of course in crystallized condition.

From the squid muscle, which had remarkable differences in respect to the

systematical, chemical and physicochemical properties compared with other animal muscle, also could be separated the tropomyosin in pure crystalline state. From these facts the author considers it to be that the tropomyosin is a preform of myosin, and that the myosin had synthesized through the tropomyosin as previously stated by BAILEY.

To explain the chemical properties and physicochemical meaning of squid muscle myosin, it is very important to make studies of the physicochemical and chemical properties of squid tropomyosin.

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II. EXPERIMENTAL RESULTS

1. Preparation and Crystallization

Preparation of tropomyosin : Fresh squid muscle is minced, mixed with an equal volume of water, and homogenized for 1 min. and squeezed out in muslins after addition of equal volume of ethanol. The washings are repeated twice with 97% ethanol and then twice with ether. The fibre is dried in air at room temperature and, while still ether-damp, is immersed in M-KCl (700 ml/100 g fibre) and the pH of the suspension is adjusted to 7 with N-NaOH. After standing for 12 hrs. at 15–20°C, the suspension is pressed through muslin. The residue is now re-extracted as before with M-KCl for 1 to 2 hrs. and the liquid is squeezed out in a hand press.

The pH of the combined extracts is adjusted to 4.3 with N-HCl and after 1 hr. the whole is filtered and precipitate is dissolved in 5 vol. of water, then the pH is readjusted to 7 with N-NaOH. The total volume is measured and 0.7 vol. of saturated ammonium sulphate (containing 0.01 vol. of conc. liquid ammonia) is added with stirring. The flocculum of denatured protein is filtrated, leaving a clear filtrate to which solid ammonium sulphate is added in portions to 70% saturation. The precipitate of tropomyosin is filtered off under gravity continued overnight, dissolved in water and dialyzed for 24 hrs. against a large volume of water.

The dialyzed solution is treated with HCl to the point of maximum precipitation (near pH 5.3), and the precipitate centrifuged, dispersed in water and neutralized to pH 7. The salting out procedure described above is repeated.

Crystallization : Precipitate of tropomyosin obtained by twice-repeated salting

out to 70% saturation is suspended in small amount of water and dialyzed against water of pH 5.8 at 10°C ; it is then stored at 4°C for 2 days to increase white turbidity matter. Crude needle crystals are deposited on the cellophane membrane (Fig. 1) and collected by centrifugation. These crude crystals are dissolved in 0.2 M KCl of pH 7.2 and 2–3% protein solution then dialyzed at 0°C against 0.01 M phosphate buffer of pH 6.5 containing 12 g ammonium sulphate per liter. After standing 1 or 2 days needle crystals are deposited on cellophane membrane. Recrystallizations are repeated twice (Fig. 2).

Fig. 1. First crystals of tropomyosin × 1000

Fig. 2. Thrice crystals of tropomyosin × 400

Crystals are filtered on a sintered glass filter and dried through several changes of ethanol and ether, and stored in refrigerator. The squid tropomyosin is stable in organic solvents just as rabbit tropomyosin is.

The general properties of the tropomyosin are shown in Table 2.

Table 2. Composition of squid tropomyosin compared with rabbit and carp tropomyosin

	Total N	Tryptophane	Total phosphorus
Squid	16.68	0	0.07
Rabbit*	16.7	0	0.02
Carp**	16.7	—	0.20

* BAILEY[16] ** HAMOIR[21]

Tryptophane is not included in this protein ; tryptophane content is an index of the purity of tropomyosin according to BAILEY.[16] In this sample tryptophane is not estimated by means of the method of GOODWIN & MORTON,[22] and of p-Dimethyl amino benzaldehyd.[23] Differences according to animal kinds are not observed in nitrogen contents.

2. Assay of Tropomyosin

The minced muscle (100 g) is homogenized for 1 min. with an equal volume of water in a blender and squeezed out in muslin after addition of 200 ml ethanol. The residue is dried in ethanol and ether as before. It is then extracted with 100 ml M-KCl at pH 7, first for 12 hrs. at 15–20°C, with further change of KCl solution. The protein in the combined extracts is precipitated at pH 4.3 with N-HCl, centrifuged; the precipitate is dispersed in water (80 ml); the solution is neutralized to pH 7 and treated with saturated ammonium sulphate solution (containing 0.01 vol. conc. ammonia solution) to 41% saturation. The precipitate, after centrifuging, is triturated with 2 parts of 41% saturated ammonium sulphate solution, and again spun down. The combined supernatant fluid is treated with solid ammonium sulphate to 85% saturation, and the precipitated protein is filtered off under gravity. It is then dissolved in 100 ml water and dialyzed for 4 days against frequent changes of 0.1 M-KCl solution of pH 7.2. To a 10 ml sample of the dialyzed sol is added 0.2 ml M-phosphate buffer of pH 7.2, and the solution is heated in a boiling water bath for 10 min. After cooling for 1 hr. the slight precipitate of denatured protein is spun down, leaving tropomyosin in solution. The protein content is determined by estimating the total N and correcting for free NH_3 by distilling an undigested portion of solution at pH 9.5. The protein N content $\times$ 6 gives the yield of tropomyosin.

Tropomyosin content in squid muscle is 0.38 g per 100 g fresh muscle and tropomyosin nitrogen content is 1.76% of total nitrogen. According to BAILEY's report[16], tropomyosin content is 0.47 g per 100 g fresh rabbit muscle and 0.21 g fresh pig cardiac muscle.

3. Ultracentrifugation

The solutions are examined by the ultracentrifuge (Spinco, Model E) at 60000 r.p.m./min. The tropomyosin is dissolved in a phosphate buffer of ionic strength 0.1 containing 0.25 M-sodium chloride. The pH of the solution is 7.1. The values of the sedimentation constants are corrected to 2.0°C and to a water basis.

The preparation of tropomyosin appears homogeneous as a result of ultracentrifugation. It sediments as a single fairly symmetrical peak whatever the concentration (Fig. 3). The values of the sedimentation constants at different concentrations are shown in Fig. 4 and Table 3; the sedimentation constant extrapolated to zero concentration is $3.20\times10.^{-13}$

When the sedimentation constant of squid tropomyosin is compared with that of crap and rabbit tropomyosins, it is seen that this tropomyosin sediments faster and shows higher sedimentation constants than the others.

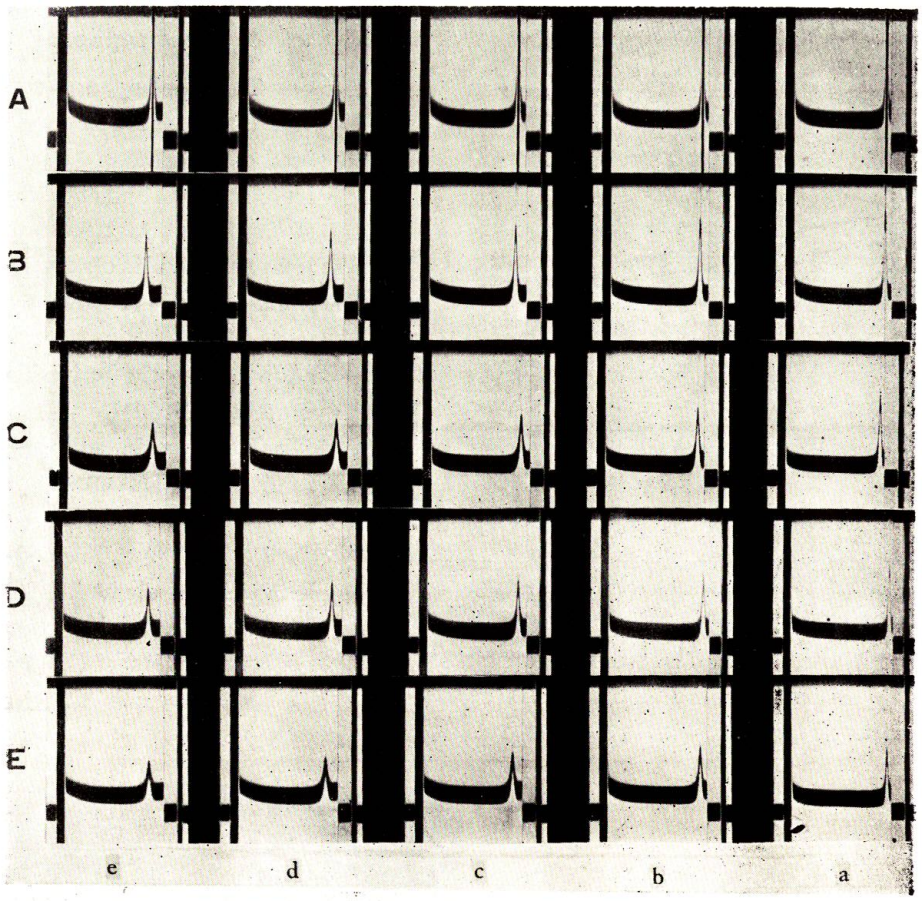

Fig. 3. Sedimentation diagram of squid tropomyosin

A. 0.75% protein concentration B. 0.50% protein concentration C. 0.37% protein concentration D. 0.33% protein concentration E. 0.25% protein concentration
a. 10 min.after full speed, **b, c, d,** and **e** are after time intervals of 8 min. (Migration to left)

Table 3. Sedimentation data for tropomyosin at different concentrations in solution (I=0.35: pH 7.1)

Squid		Carp*		Rabbit**	
Protein conc. (g/100ml)	S_{20} corr. $\times 10^{-13}$	Protein conc. (g/100ml)	S_{20} corr. $\times 10^{-13}$	Protein conc. (g/100ml)	S_{20} corr. $\times 10^{-13}$
0.25	3.16	0.22	2.59		
0.33	2.97	0.34	2.73		
0.37	2.77	0.44	2.52	0.60	2.60
0.50	2.81	0.58	2.57	0.653	2.55
0.75	2.36	0.89	2.22	0.665	2.51
Extrapolate	3.20	Extrapolate	2.85		

* HAMOIR[21] ** BAILEY[16]

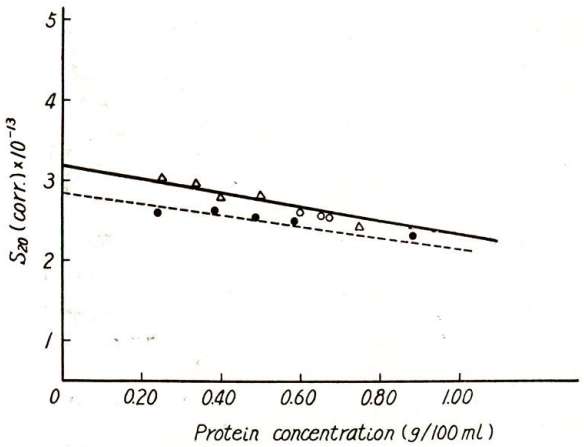

Fig. 4. Variation of S_{20} (corr.) for tropomyosin

Upper curve: squid tropomyosin Lower curve: rabbit and carp tropomyosin △ squid, ○ carp, ● rabbit, carp and rabbit values taken from HAMOIR[21] and BAILEY[16]

4. Electrophoresis

The electrophoretic behavior of protein is greatly effected of the kind of buffer, pH, ionic strength and electric field strength. It is necessary at least that results obtained over a range of pH values and varied buffer ionic strengths be adopted as the criterion of protein purity.[24]

The experiments are performed with TISELIUS apparatus (Hitachi Seisakujo, cell size; 2×15×50 mm). They should be continued about 2–3 hrs. at 4°C and potential gradient is 3.23 volt/cm. pH is determined by glass electrode, Beckman Model-G. Preparations are homogeneous electrophoretically, showing a single component with no impurities (Fig. 5).

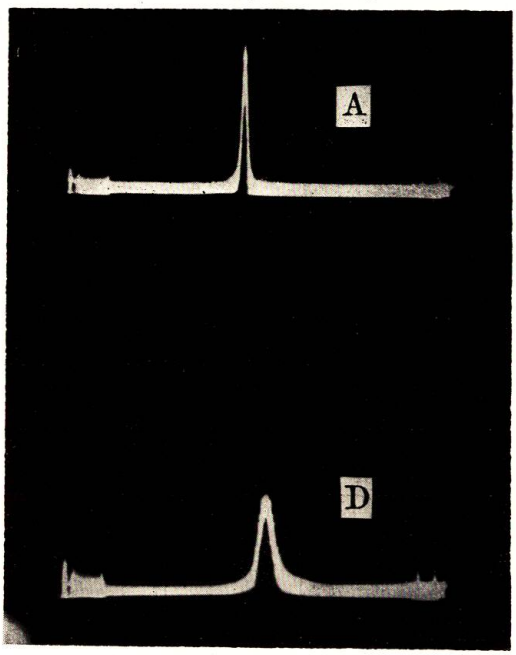

Fig. 5. Electrophoretic diagram of tropomyosin

1% protein concentration, 8640 sec., pH 7.4, I=0.15 (0.1 for phosphate buffer and 0.05 for NaCl), 3.25 volt/cm. A. ascending migration to left, D. descending migration to right

The mobilities found are given in Table 4.

Table 4. Electrophoretic mobilities of tropomyosin (protein concentration 1.0%)

Conditions of electrophoresis		Mobility (10^{-5} cm²/volt/sec)			
		I = 0.15		I = 0.35	
pH		As.	Ds.	As.	Ds.
3.6	Acetate-buffer-NaCl	+6.29	+5.82	+4.22	+3.81
3.8	I=0.15(I=0.1 for acetate buffer and 0.05 for NaCl)	+4.94	+4.48	+3.34	+3.01
4.0		+4.29	+3.90	+2.43	+2.91
4.2	I=0.35(I=0.1 for acetate buffer and 0.25 for NaCl)	+3.62	+3.29	+1.08	+1.52
6.4	Phosphate-buffer-NaCl	−0.35	−2.73	−2.22	−1.99
7.0	I=0.15 (I=0.1 for phosphate buffer and 0.05 for NaCl)	−5.02	−4.50	−3.13	−2.80
7.4		−6.37	−5.71	−4.10	−3.65
7.9	I=0.35 (I=0.1 for phosphate buffer and 0.25 for NaCl)	−8.01	−7.91	−5.56	−4.98

The mobility values obtained differ from those of carp and rabbit tropomyosin at I=0.35 and pH 7.1 (Ascending, −4.30, Descending, −3.90 of carp[21]: Ascending, -5.6, Descending, −4.9 of rabbit[25]) and at I =0.15 and pH 7.4 (Ascending -6.90 Descending, −6.20 of carp[21]) and moreover on the acid side I=0.15 and pH 3.5 (Ascending, +6.15, Descending, +5.76 of carp). The mobilities of squid tropomyosin are small in alkaline side and great in acid side. This should be due to the amino acid contents of protein constituent, having basic polar amino acid such as histidine, arginine, lysine and having acidic polar amino acid amide-NH_3 combining with carboxyl radical of acidic polar group such as glutamic acid and aspartic acid.

pH-mobility curve found is given in Fig. 6, but the range from pH 4.2 to 6.4 was not used in the experiment on account of isoelectric zone.

Isoelectric point lies near pH 5.4 in this figure and from the obtained maximum turbidity of solutions by A. K. A.-Type photonepherometer it lies near pH 5.6. Isoelectric point lies somewhat to the alkaline side comparing with rabbit tropomyosin pH 5.1.[16]

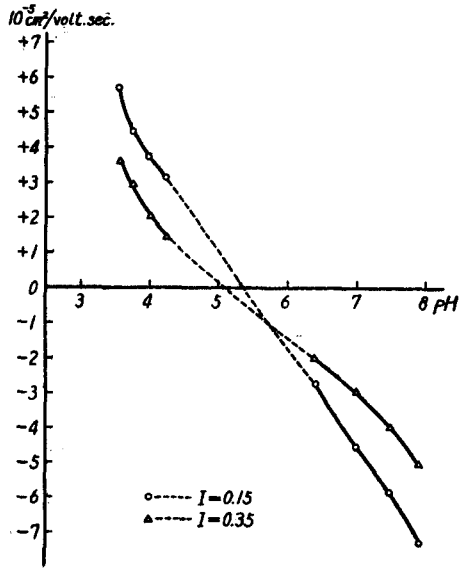

Fig. 6. pH–mobility curve for tropomyosin from descendings

5. Phosphorus and Ultraviolet Absorption

Tropomyosin and nucleotropomyosin have been crystallized from carp muscle by HAMOIR[21] both proteins have the same electrophoretic mobility, however, are different in phosphorus contents and ultraviolet absorption.

The total phosphorus content is determined by the method of CHAIN modified by BINENBLUM.[26]

The ultraviolet absorption spectrum of protein dissolved in the phosphate sodium chloride buffer of pH 7.2 and I=0.35 is measured with a Shimadzu spectrophotometer.

Table 5. Total phosphorus content of squid tropomyosin compared with carp and rabbit tropomyosin

	Squid	Carp*	Rabbit**
Tropomyosin	0.07%	0.20%	0.02%
Nucleotropomyosin	—	0.47%	—

* HAMOIR[21] ** BAILEY[16]

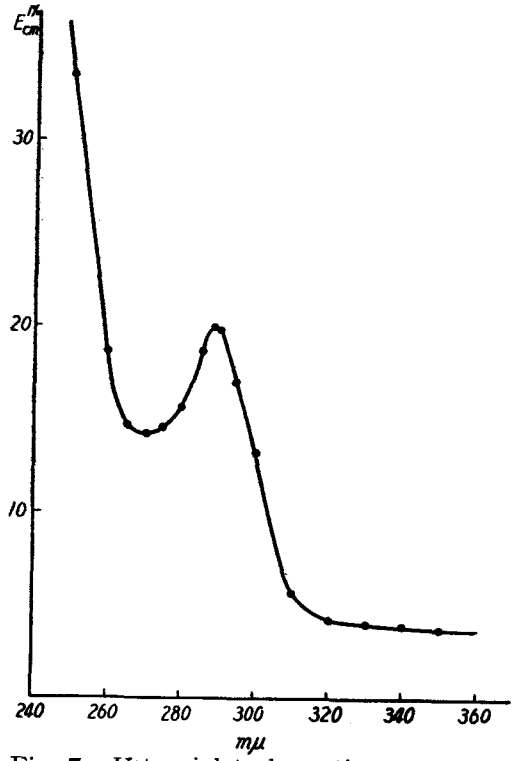

Fig. 7. Ultraviolet absorption spectrum of tropomyosin in a phosphate NaCl buffer of pH 7.1 and I=0.35

The phosphorous content and ultraviolet spectrum found are given in Table 5 and Fig. 7.

The squid tropomyosin has a small total phosphorus content compared with carp tropomyosin and does not show the absorption spectrum of nucleic acid at 260 mμ.

6. Amino Acid Composition

The hydrolysis of the protein is performed in sealed glass tubes evacuated to about 12 mm pressure in an oven at 105°C with 100 volume of 5 N-HCl, to prevent the formation of a large amount of insoluble black humin[28] which is due to the presence of air during hydrolysis. The hydrolysate is filtered by glass filter, and excess HCl is removed by repeated distillation in vacuo. The hydrolysate is made up to 10 ml.

Cystine and cysteine are determined chromatographically as cysteic acid using the perfomic acid oxidation.[29] Sample is

oxidized at 0°C for 4 hours and perfomic acid is removed in vacuo at 30–40°C. The residue is hydrolyzed by 6 N-HCl for 20 hrs. Almost all of HCl is removed by distillation in vacuo and finally neutralized by N-NaOH.

Micro-Kjeldhal determination on the hydrolysates gives the nitrogen value on which percentile values are based. To convert to terms of protein weight, the nitrogen value of 16.68% is used.

The chromatography of the hydrolysate is performed on column of Dowex-50 and Dowex-2. A 0.9×100 cm column of Dowex-50 resin is used for the acidic and neutral amino acid and a 0.9×15 cm of the same resin for the basic amino acid. The 0.5 ml hydrolysate as 5 mg original protein is employed. On the determination of tyrosine and phenylalanine, the fraction of tyrosine plus phenylalanine is obtained by 0.9× 15 cm column chromatography owing to the difficulty of regulating temperature.

Tyrosine is analyzed by the ultraviolet spectrometer, and phenylalanine is determined by subtraction of the tyrosine value from the tyrosine plus phenylalanine value obtained by chromatography.

A 0.9×15 cm column of Dowex-2 resin is used for the cysteic acid and the 1 ml hydrolysate as 5 mg original protein is worked by 0.1 N chloracetic acid for effluent buffer.

Fractions (1 ml) are collected using a Mitamura automatic fraction collector equipped with a drop-counting device. The samples are analyzed by the photometric ninhydrin procedure of MOORE & STEIN.[30] Reading of optical density is made with a Shimadzu spectrophotometer at 570 mμ (proline at 440 mμ). The color intensities are corrected for asparic acid, serine, alanine, valine, tyrosine and phenylalanine according to MOORE & STEIN,[27] for glutamic acid, methionine, glycine, lysine, histidine, ammonia, arginine and cysteic acid according to SCHRAM, MOORE

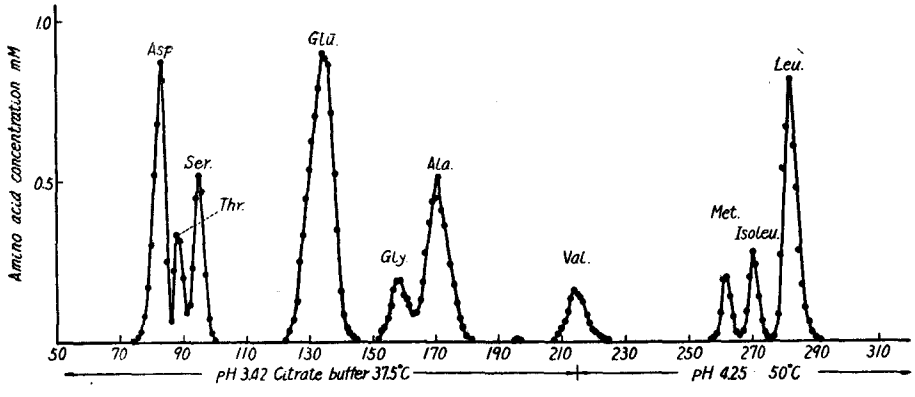

Fig. 8. Tropomyosin Dowex-50 0.9×100 cm

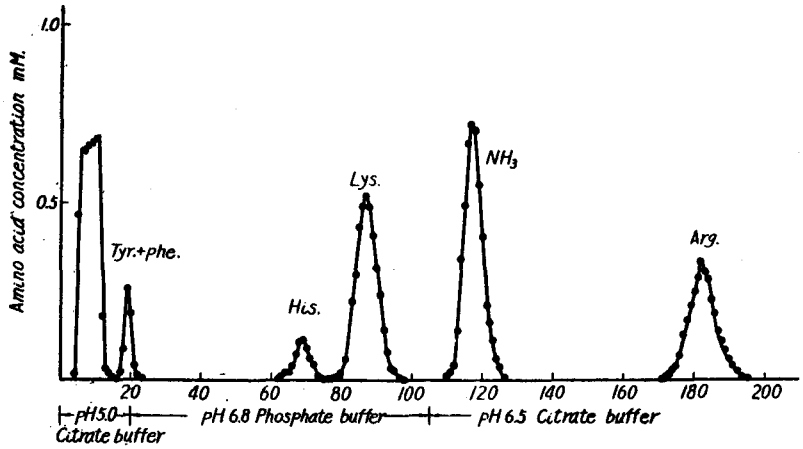

Fig. 9. Tropomyosin, Dowex-50 0.9×15 cm

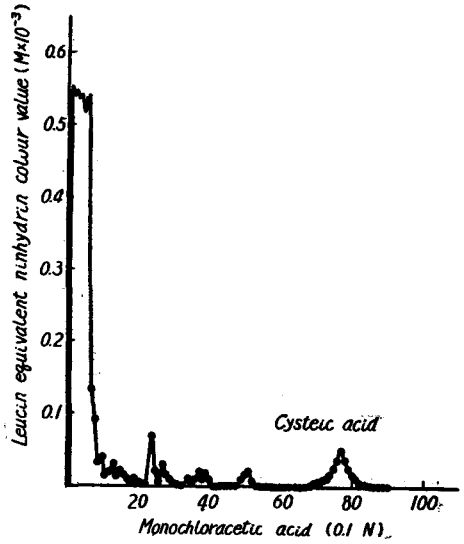

Fig. 10. Tropomyosin Dowex-2 0.9×15 cm

& BIGWOOD,[29] and MOORE & STEIN.[30] The obtained results are shown in Figs. 8, 9, and 10.

The separation of aspartic acid, threonine and serine is imperfect because of no addition of detergent to buffer. The variation on the base line of effluent curve was caused by the incompletness of the hydrolysis; small artifact peaks are recognized before the valine.

Compounds in which the carboxyl group of isoleucine or valine is coupled in peptide linkage are not easily hydrolyzed by HCl,[31][32][33] these peptides are effluted before the valine peak.[28] The small amount of valine and isoleucine in Table 6 should be due to this effect.

Certain amino acids, such as aspartic acid, threonine, serine and lysine are labile to acid hydrolysis and especially threonine and serine, and the amounts of ammonia increase with hydrolysis arise from these amino acids.

The corrections are applied to aspartic acid (+5%), threonine (+7%), serine (+10%) and lysine (+8%), moreover to ammonia (−18%). The recovery of glutamic

acid and cysteic acid by chromatography analysis are respectively about 97% and 90%,[13] and corrections are applied to these amino acids. Obtained result is shown in Table 6.

Table 6. Amino acid composition of squid tropomyosin

	Amino acid N %	Amino acid weight %	Amino acid residue %	Amino acid mol number into 10^5 g protein	
				Squid	Rabbit**
Cystine/2	0.46	0.66	0.56	5.5	6.5
Aspartic acid	7.69	12.20	10.54	92	89
Threonine	2.54	3.61	3.07	30	28
Serine	4.28	5.35	4.43	51	40
Glutamic acid	16.42	28.80	25.28	196	211
Proline	0	0	0	0	0
Glycine	2.20	1.97	1.50	26	12.5
Alanine	7.36	7.82	6.24	88	110
Valine	1.92	2.69	2.28	23	38
Methionine	1.34	2.38	2.09	16	16
Isoleucine	1.52	2.38	2.06	18	29
Leucine	7.32	11.49	9.90	88	95
Tyrosine	0.62	1.34	1.21	7	15
Phenylalanine	0.60	1.18	1.05	7	3.5
Histidine	2.89	1.78	1.56	11.5	5.5
Lysine	13.09	12.13	10.63	83	110
Arginine	21.19	10.99	9.85	63	42
Tryptophane	0	0	0	0	0
Amide-NH_3	7.31	1.48*	1.39*	(87)	(64)
Total	99.56	106.77	92.25	805	851

* Omit. from the total value ** KOMINZ *et al.*[18]

As shown in Table 6 in spite of good recovery of amino acid nitrogen, 99.56%, the recovery of amino acid residue is only 92.25 per cent and these results should be caused by the fact that the oxyamino acid is decomposed to ammonia.

Tropomyosin, which contains a large amount of acidic and basic polar amino acid residues and no tryptophane and proline, is an unique protein.

7. The Adenosine Triphosphatase (ATP-ase) Activity of Tropomyosin

Since ENGELHARDT & LJUBIMOWA[11] indicated the mutual relationship between adenosine triphosphate (ATP), which possesses a large amount of chemical energy, and myosin, which is a contractile material, the studies in this field have been active.

Myosin and actomyosin have the ATP-ase activity which splits off the terminal phosphate of ATP. The chemical energy which produced by this reaction is used for contraction of myosin or actomyosin.

There are divergences of opinion as to whether myosin is identical with ATP-ase, or whether the ATP-sae activity is combined tightly with myosin or actomyosin. The ATP-ase activity of myosin depends on its charge which in turn depends on the ions. Though in the absence of salts, myosin is inactive as ATP-ase, it is inactive also if there is too large a quantity of them.

Ca-ion strongly promotes and Mg-ion strongly inhibits the ATP-ase activity of myosin, while both Ca-ion and Mg-ion promote the same enzymic activity of actomyosin. For the existence of Ca-ion there are two optimum pH of ATP-ase ; they are pH 6.5 and 9.2. On the other hand, it is suggested that the tropomyosin is a prototype of the much larger myosin molecule and may conceivably be a unit utilized in the elaboration of myosin itself.

An attempt was made to ascertain whether the crystalline tropomyosin has ATP-ase activity or not, under the addition of Ca- or Mg-ions to reaction medium as the activator of ATP-ase. Range of hydrogen ion concentration of experiment is chosen pH 6.5–pH 9.5 and as the buffer solution borate-boric acid buffer are chosen. The concentration of adenosine triphosphate solution is 978γ/1 ml.

As the tropomyosin solution 0.1% crystalline squid tropomyosin solution in 0.1% KCl (pH 7.2) is prepared. Following instructions are to be observed : the reaction temperature is 30°C, after 5 min add trichloracetic acid solution and stop enzymic action.

The free phosphoric acid of this solution is estimated by the FISKE-SUBARROW method.[37]

The obtained results are shown in Table 7.

Table 7. ATP-ase activity of squid tropomyosin

pH	Mg-ion		Ca-ion	
	Optical density measured	Optical density corrected	Optical density measured	Optical density corrected
6.71	0.170	−0.025	0.195	−0.010
7.61	0.179	−0.016	0.220	+0.010
7.93	0.192	−0.003	0.225	+0.020
8.27	0.190	−0.005	0.220	+0.015
8.67	0.180	−0.015	0.239	+0.024
8.87	0.210	+0.025	0.224	+0.019
9.07	0.170	−0.025	0.205	+0.000
9.23	0.169	−0.026	0.200	−0.005

From the above results the author is able to recognize the following facts :

Mg-ion as the activator of the ATP-ase activity of squid tropomyosin shows negative effect, while Ca-ion as the activator shows positive effects but within the range of experimental error.

From the above results it is deduced that the squid tropomyosin has no ATP-ase activity.

III. DISCUSSION

In the previous chapter the results of the present studies on the squid tropomyosin are described. In comparing the physicochemical and chemical properties of squid

tropomyosin with those of rabbit tropomyosin, which have been already reported by BAILEY, there are many differences between these two tropomyosins. To have resistance against denaturation, and to separate in crystalline form are common properties. However, in detail, there are also differences in many points, for example, in the process of crystallization and the form of crystal. The crystalline form of rabbit tropomyosin which was isolated by BAILEY[16] is a quadrilateral plate or hexagonal plate, but the squid tropomyosin is a needle. The sedimentation velocity of tropomyosin was measured by ultracentrifugal method, and from this value sedimentation constant is calculated. The squid tropomyosin has a higher value of sedimentation constant than the rabbit. This means that the apparent molecular weight of the squid tropomyosin is higher than the rabbit.

The electrophoretic mobility of the squid tropomyosin is small on the alkaline side and great on the acid side, and the isoelectric point lies somewhat to the alkaline side as compared with rabbit tropomyosin. These facts mean that the amino acids which compose tropomyosin are different in squid from those in rabbit muscle.

The amino acid composition of squid and rabbit tropomyosins are shown in Table 8: both tropomyosins do not have tryptophane and proline, and the other amino acids contents are obviously different between them.

Table 8. Comparative amino acid value of tropomyosin from squid and rabbit muscle

	Amino acid N%		Amino acid weight %		Amino acid mol numbers into 10^5g protein	
	Squid	Rabbit*	Squid	Rabbit*	Squid	Rabbit*
Cystine/2	0.46	0.55	0.66	0.78	5.5	6.5
Aspartic acid	7.69	7.46	12.20	11.84	92	89
Threonine	2.54	2.35	3.61	3.33	30	28
Serine	4.28	3.36	5.35	4.20	51	40
Glutamic acid	16.42	17.70	28.80	31.00	196	211
Proline	0	0	0	0	0	0
Glycine	2.20	1.05	1.97	0.94	26	12.5
Alanine	7.36	9.20	7.82	9.78	88	110
Valine	1.92	3.18	2.69	4.48	23	38
Methionine	1.34	1.34	2.38	2.38	16	16
Isoleucine	1.52	2.43	2.38	3.80	18	29
Leucine	7.32	7.46	11.49	12.44	88	95
Tyrosine	0.62	1.26	1.34	2.72	7	15
Phenylalanine	0.60	0.29	1.18	0.58	7	3.5
Histidine	2.89	1.38	1.78	0.85	11.5	5.5
Lysine	13.19	18.40	12.13	16.06	83	110
Arginine	21.19	14.10	10.99	7.30	63	42
Tryptophane	0	0	0	0	0	0
Amide-NH_3	7.31	5.35	1.48	—	(87)	(64)
Total	95.56	96.86	106.77	112.45	805	851

* KOMINZ *et al.*[18]

About the non-polar amino acid in the squid tropomyosin there is a large content of serine, glycine and phenylalanine and less content of isoleucine and tyrosine in comparison with rabbit. Regarding the amino acids which form the acidic group in

the squid tropomyosin, there is much aspartic acid and less glutamic acid comparing with rabbit, but the differences of the respective amino acids contents are not very great.

Regarding to the amino acids which form the basic group in the squid tropomyosin, there is much content of arginine and histidine and less of lysine in comparison with rabbit.

As the amount of any one amino acid has so little significance for the interpretation of both tropomyosins and of biogenetic relationships, it is most profitable to compare whole groups of amino acids expressed as percentage of the total residues as recorded in Table 9.

Table 9. Tropomyosin amino group percent of total residue

	Squid	Rabbit*
Free-acid group	25.0	27.8
Base group	19.6) 44.6	18.5) 46.3
Hydroxy group	10.9	9.8
Amide group	10.8	7.4
Polar group	66.9	64.3
Non-polar group	33.1	35.7

* BAILEY[16]

In both tropomyosins, the amino acids with non-polar side chain comprise 33% (squid) and 35% (rabbit) of the total; the polar side chains are calculated as 67% (squid) and 64% (rabbit). In terms of charges of both negative and positive type, the values of 44.6% (squid) and 46.3% (rabbit) for tropomyosins are higher than any other protein (myosin 34%, fibrinogen 26%, edestin 27% lactoglobulin 28%, casein 23%, insulin 23%, ovalbumin 20% and zein 3%).

Comparing after the subtraction of free acidic and basic amino group in both tropomyosins, squid tropomyosin is calculated as 5.4% and rabbit 9.3%. According to these values, the differences of isoelectric point and electrophoretic mobility seem to be obvious.

From the result of examination of amino acid content of squid tropomyosin, if one molecule of tropomyosin contains 3 molecular residues of half cystine which is present in the smallest amounts, the amounts of amino acid residues and calculated molecular weight are as shown in Table 10 and average molecular weight is 54300 ± 200.

The molecular weight of rabbit tropomyosin obtained from osmotic pressure by TSAO *et al.*[34] is 53000.

The molecular weight of rabbit tropomyosin calculated from the result of amino acid content is 53000 according to KOMINZ *et al.*[18]

Thus squid tropomyosin shows different behavior in respect to many properties such as crystalline forms, sedimentation constant, electrophoretic mobility, isoelectric point, phosphorus and amino acid composition when comparisons are made with rabbit and carp tropomyosin.

Table 10. Molecular weight and molecular number of tropomyosin

	Amino acid residue	Minimum molecular weight	Number of residue	Molecular weight
Cystine/2	0.56	18204	3	54600
Aspartic acid	10.54	1091	50	54500
Threonine	3.07	3300	16	52800
Serine	4.43	1964	28	55000
Glutamic acid	25.28	551	107	54700
Proline	0	0	0	0
Glysine	1.50	3811	14	53400
Alanine	6.24	1139	48	54700
Valine	2.28	4355	12	52300
Methionine	2.09	6269	9	56400
Isoleucine	2.06	5511	10	55100
Leucine	9.90	1142	48	54800
Tyrosine	1.21	13521	4	54100
Phenylalanine	1.05	13999	4	56000
Histidine	1.56	8719	6	52300
Lysine	10.63	1205	45	54200
Arginine	9.85	1585	34	53900
Tryptophane	0	0	0	0
Amide-NH_3	1.39	1151	47	54100
Mean				54300±200

V. CONCLUSION

Tropomyosin which has more stability to denaturation is newly crystallized from squid muscle. It appears homogeneous upon electrophoresis and ultracentrifugation. Comparing with BAILEY's rabbit tropomyosin,[16] there is no difference in nitrogen content (squid 16.68, rabbit 16.70%), but there is difference in phosphorus content, (squid 0.07 %, rabbit 0.02 %).

As the squid tropomyosin is low in amount of total phosphorus content and does not show ultraviolet absorption of nucleotropomyosin at 260 mμ, it is concluded that it is not a nucleotropomyosin.

Squid tropomyosin shows higher sedimentation constant than rabbit and carp tropomyosins; the sedimentation constant extrapolated to zero concentration is 3.20×10^{-13} (rabbit and carp, 2.85×10^{-13}).[21]

The electrophoretic mobility of squid tropomyosin is smaller on the alkaline side and greater on the acid side than corresponding qualities of rabbit and carp tropomyosins; the pH of isoelectric point of squid tropomyosin is 5.6 (rabbit, 5.1).[16]

In amino acid composition, there is difference in the content of each amino acid, especially in acidic amino acid and basic amino acid: squid tropomyosin is the more rich in content of aspartic acid, arginine and histidine and is less rich in glutamic acid and lysine than the rabbit tropomyosin.

The average molecular weight of squid tropomyosin calculated on the bases of amino acid composition, is 54300±200, and the molecular weight of rabbit tropomyosin obtained from osmotic pressure and from amino acid composition, both, is 53000.[34]

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