SQUID TROPOMYOSINS

SHUICHIRO KUBO

Faculty of Fisheries, Hokkaido University, Hakodate, Japan

Contents

Introduction

Tropomyosin, which is one of the structure proteins of muscle fibril, was first discovered by Bailey[1] in 1946, and crystallized by him[2] from rabbit skeletal muscle dehydrated with organic solvents. The physico-chemical and chemical characteristics have been observed in detail on rabbit muscle tropomyosin[2-9]: it is an asymmetric protein and belongs to the α-keratin-epidermin-myosin-fibrinogen group according to the x-ray diffraction pattern. Its amino acid composition also resembles that of myosin. On the other hand, tropomyosin differs from actin not only in its behavior towards salts, but also in its amino acid composition.

Tropomyosins can be prepared in high purity more easily than other structural proteins in muscle. They have been prepared from different types of mammalian muscle[10-14], from lower classes of vertebrata[15-21] and from invertebrata[11-12,14,22-32]. They can be distinguished from other proteins by their high contents of acidic and basic amino acids. The ratio of lysine and arginine contents was found to change regularly with the phylogenetic level of the animal origin. They have been classified on the basis of their solubility and the ratio of lysine and arginine contents as water-insoluble and -soluble tropomyosins[27] or tropomyosin A and B[25,29].

Squid tropomyosin has already been isolated by Yoshimura from the mantle of *Ommastrephes sloani pacificus* STEENSTRUP[22], by Tsao et al. from *Sepia esculenta*[12,14] and by Kominz et al. from *Loligo pealeii*[29]. According to the amino acid composition and the diagram of salting-out with ammonium sulphate, all these tropomyosins belong to the water soluble tropomyosin or tropomyosin B.

In the present study, two kinds of crystalline proteins have been isolated from the tropomyosin fraction in the mantle of *Ommastrephes sloani pacificus* STEENSTRUP. Their solubility, amino acid composition, terminal residue and molecular size and shape have been elucidated. From these results, it is concluded that the one is identical to Yoshimura's tropomyosin or tropomyosin B whilst the other belongs to the water-insoluble or tropomyosin A group.

Acknowledgments

The author wishes to express his heartiest thanks to Professor Y. Ogura, Professor T. Ando and Professor H. Noda, Faculty of Science, Tokyo University, Tokyo, for their valuable criticisms of this study. He is greatly indebted to Professor K. Yoshimura, Faculty of Fisheries, Hokkaido University, Hakodate, and to Professor Y. Tonomura, Research Institute for Catalysis, Hokkaido University, Sapporo, for their invaluable advice in improving the manuscript and their constant encouragement during the investigation. The author's cordial thanks are also due to Professor S. Akabori and his collaborators, Faculty of Science, Osaka University, Osaka, for their instruction in the technique of amino acid analysis and terminal assay, and to Dr. T. Nihei, Department of Biochemistry, Dartmouth Medical School, Hanover, U.S.A., for his help in carrying out

the light-scattering measurements.

Experimental Results

I. Preparation of Protein

Bailey[24,27] developed the following two kinds of methods to extract and isolate tropomyosin from invertebrate muscle. The one is the extraction of tropomyosin from minced muscle, which was dehydrated beforehand with organic solvents, and the other the direct extraction from fresh minced muscle without dehydration, both being followed by the crystallization of tropomyosin by dialysis against KCl solution of low ionic strength. The former method was more convenient than the latter on squid muscle for the sake of the highly viscous actomyosin. In order to separate the two crystalline tropomyosins obtained in this study, it was, however, necessary to combine the dialysis method with the salting-out technique, since the two tropomyosins showed similar solubility in KCl solution of low ionic strength.

1) Methods

Isolation: Step 1. The muscle of fresh squid mantle, of which the cuticle has been rejected, was cut down to 10~20 mm width and was washed with ice cold water. The frozen muscle pieces, kept overnight at about −20°C, were passed through a meat mincer. This procedure was convenient for homogenizing the muscle. Step 2. The minced muscle was put with occasional stirring in 2 volumes of cold 0.05 M KCl-0.005 M K_2HPO_4-KH_2PO_4 solution at pH 6.8 and 0°C for 30 min. to remove the soluble proteins, and was centrifuged at 1,000×g. Step 3. After addition dropwise of 3 volumes of ethanol, which was cooled at −10°C, the residue was suspended for 30 min. at 0°C. Ethanol washing two times was followed by two ether washings. The drained residue from ether was allowed to dry at room temperature for about 2 hours. Step 4. The dehydrated fiber was extracted with 10 volumes of cold M KCl-0.05 M K_2HPO_4-KH_2PO_4 solution at pH 7 and 0°C. After standing for 24 hours, the suspension was pressed through cheese-cloth. The residue was re-extracted with the M KCl buffer for 4 hours and the liquid was squeezed out. The combined solution was dialysed thoroughly against 0.1 M KCl-0.005 M K_2HPO_4-KH_2PO_4 solution at pH 6.5 and 0°C. Crystals of needle shape thus obtained are shown in Fig. 1. Step 5. After the addition of 1% celite, the dialysed solution was filtered through on a Büchner funnel. The filter cake was extracted with stirring in 10 volumes of M KCl-K_2HPO_4-KH_2PO_4 solution at pH 7.5 for 4 hours. The solution of extracted protein was clarified by filtration whereupon 0.25 volume of saturated ammonium sulphate solution, which was kept cold and adjusted to pH 7.0, was added gradually. The precipitate was filtered with celite on a Büchner funnel. Step 6 (a). The cold saturated ammonium sulphate solution was added slowly to the clear filtrate in portions up to 39% saturation. The precipitate was collected, dissolved in 0.5 M KCl-K_2HPO_4-KH_2PO_4 solution at pH 7 and dialysed against the same solution. The salting-out procedure was repeated with this dialysed solution, and the 20~37% salting-out fraction was

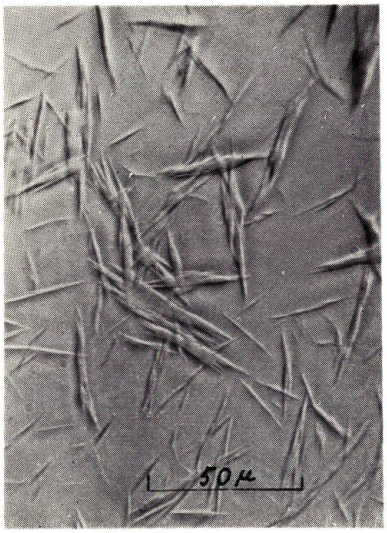


Fig. 1. Crystals of Step 4

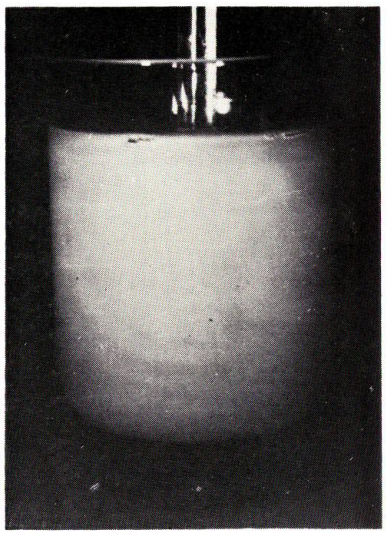


Fig. 2. Silky sheen of protein B

collected (fraction A). (b). The filtrate, which was obtained from the 39% salting-out fraction in Step 6 (a), was brought to 54% saturation. The precipitate was suspended in water, dialysed against the buffer, and once more subjected to precipitation at 41~54% saturation (fraction B).

Crystallization: Crystalline protein A. The precipitate of fraction A was dissolved in a small amount of 0.5 M KCl-0.005 M K_2HPO_4-KH_2PO_4 solution at pH 7.5. The protein solution was dialysed against 0.25 M KCl-0.005 M K_2HPO_4-KH_2PO_4 solution at pH 6.5, and the concentration of KCl of the dialysing solution was gradually decreased to 0.20, 0.15 and finally to 0.10 M. The protein was obtained as crystalline precipitates at the bottom in the cellophan bag. Recrystallization was carried out by repetition of the above procedure.

Crystalline protein B. After the filter cake of fraction B had been dissolved in water, the protein solution was dialysed progressively against 0.40-0.15 M KCl-0.005 M K_2HPO_4-KH_2PO_4 solution at pH 6.5. The crystalline protein deposited finally in 0.15 M KCl buffer solution. The crystalline protein was also obtained by the following procedure: cold distilled water was slowly added with constant stirring to about 1% protein solution in M KCl buffer at pH 7 and 0°C, and the solution was diluted to a final concentration of 0.15 M KCl. A silky sheen appeared as illustrated in Fig. 2, and crystals of needle-like shape deposited on the bottom after 24 hours at 0°C.

Salting-out analysis: The salting-out curves were obtained according to the method of Snellman and Tenow[33-34]. The protein solution was thoroughly dialysed against a buffer solution (0.5 M KCl-0.1 M K_2HPO_4-KH_2PO_4). Each 1 ml of the protein solution was added to 4 ml of the mixture of saurated ammonium sulphate solution and the buffer containing different amounts of saturated ammonium sulphate in a series. The mixtures were allowed to stand overnight at 0°C, and were filtrated. After washing and filling up to a constant volume

with the buffer solution, the extinction of the filtrate was measured at 260 and 277 mμ.

Dialysing analysis: Each 2 ml of the protein solution in 0.5 M KCl-0.1 M K_2HPO_4-KH_2PO_4 solution at pH 7.14 was dialysed at 0°C for 3 days against several change of the buffer solution, which contained K_2HPO_4-KH_2PO_4 at ionic strength 0.005 and KCl from 0 to 0.50 M. After washing and filling up to a constant volume with the buffer solution, the mixture was centrifuged at 15,000×g for 30 min at 0°C. The extinction of the supernatant was determined at 260 and 277 mμ.

2) Results

Bailey obtained pure tropomyosins from dehydrated adductor muscle of pinna and foot muscle of octopus by a procedure up to Step 4. In the case of squid, the crystalline protein obtained in Step 4 illustrated in Fig. 1 was, however, found to be contaminated with some impurities, even after several times re-crystallization. The results of salting-out and dialysing analyses on this crystalline protein in Step 4 are shown in Figs. 3 and 4. It is evident from Fig. 3 that the protein is composed at least of six components, viz., I 9~18%, II 18~37%, III 37~54%, IV 54~60%, V 60~69% and VI 69~75% saturated fraction. Since the extinction ratio at 277/260 mμ of fractions IV, V and VI is 0.69, 0.78 and 0.80, respectively, these fractions seem to be contaminated with rather large amounts of nucleic acid.

Though the fractionation by the dialysing procedure is not so definite as that by the salting-out, in the dialysing curve three components were observed,

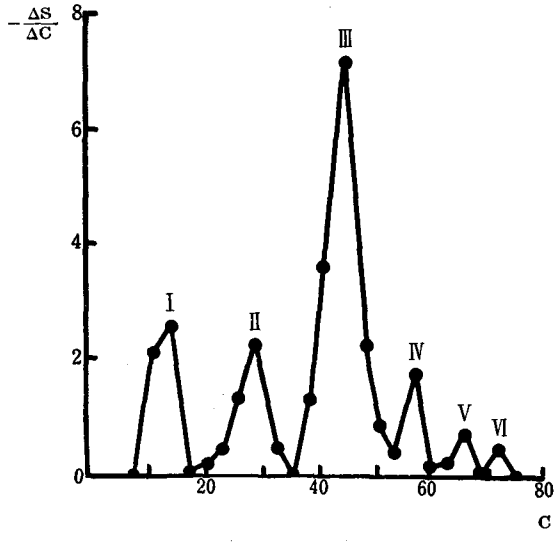


Fig. 3. Salting-out curve of the protein in Step 4; s, extinction at 277 mμ; c, per cent in volume of saturated $(NH_4)_2SO_4$

viz., 0.45~0.33 M KCl, 0.33~0.20 M KCl and 0.20~0.10 M KCl fraction*.

The chemical and physico-chemical properties of the proteins A and B will be described in the following sections.

II. General Properties of Crystalline Proteins A and B

In this section is described an examination of the purities of the crystalline proteins A and B by salting-out, electrophoretic and tryptophan analyses. The properties of the proteins will be described.

1) Methods

Salting-out and dialysing analyses: These methods have already been described above and applied to a protein solution of 0.5%.

Electrophoretic analysis: Electrophoresis was performed by a Hitachi Tiselius apparatus (HT-B type, cell size 2×15×50 mm) with a protein solution which was dialysed against a buffer (ionic strength 0.35 KCl and 0.1 K_2HPO_4-KH_2PO_4) at 0° for 48 hours.

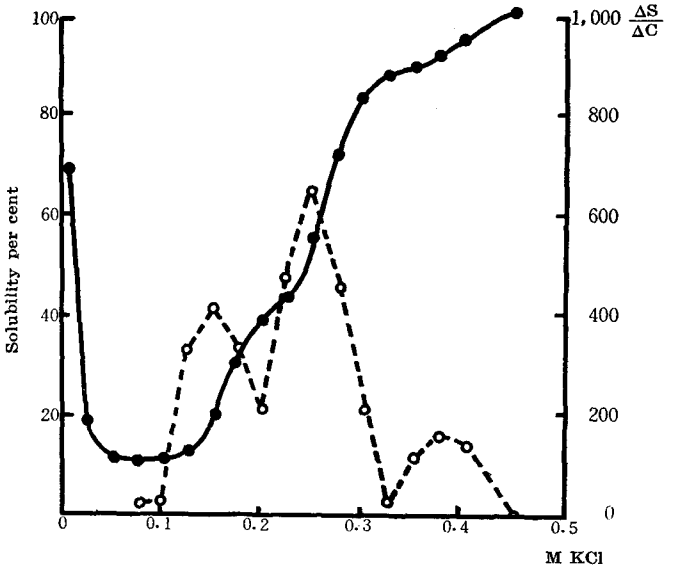


Fig. 4. Solubility curve of the protein in Step 4; s, soulubility per cent; c, concentration of KCl; ●, solubility; ○, $\Delta s/\Delta c$ Conditions: total protein concentration 1.02%, pH 7.14, 0°C

* The protein solution in Step 4 was separated into two portions by the dialysis against 0.20 M KCl buffer, and two crystalline proteins were obtained from 0.20 M KCl and 0.20–0.1 M KCl fractions by the successive dialyses. The salting-out curves of the 0.20 M KCl and 0.20 –0.10 M KCl fractions revealed five and the three components, respectively.

The procedure described unnder "*Isolation*" gives the best yields of crystalline proteins A and B.

Total nitrogen content: The protein was suspended in 0.005 M acetate buffer at pH 5.0 and washed several times with the same buffer, then with water, dried in ethanol and ether, and finally dried at 110°C for 12 hours. After decomposition of the dried protein under the condition according to Miller[35], total nitrogen was determined by the micro-Kjeldahl method.

Tryptophan content: Tryptophan content was determined by the use of p-dimethylaminobenzaldehyde[36-37].

Ultraviolet analysis: The ultraviolet absorption spectrum of the protein solution (about 2 mg/ml in 0.35 M KCl and 0.1 M K_2HPO_4-KH_2PO_4) was measured by a Shimadzu spectrophotometer (QB type 50).

Total phosphorus content: Total phosphorus content was determined by the method of Allen[38].

2) Results

The crystalline proteins obtained from the fractions of 20~37% and 41~54% saturation are illustrated, respectively, in Figs. 5 and 6.

The salting-out curves of the crystalline proteins A and B are given in Figs. 7 and 8. The differential salting-out curves of the proteins A and B showed one sharp peak in the range of 21~39% and 40~54% saturation, respectively. These salting-out ranges agree, respectively, with those of tropomyosins A and B[25,29] or water-insoluble and water-soluble tropomyosin[27]. The precipitation ranges against KCl solution were below 0.25 M KCl and 0.35 M KCl, respectively, for the proteins A and B (Figs. 9 and 10). Below 0.025 M KCl both these proteins formed a swollen gel. The solubility of protein B in KCl solution was increased by decreasing pH to 4.0 and by increasing pH to 9.0, while the protein A denatured at pH below 5.4.

Each of the proteins was homogenous electrophoretically at pH 7.15 as illustrated in Fig. 11, showing a single peak with the mobility of descending 1.65×10^{-5} cm^2/volt/sec and ascending 1.79×10^{-5} cm^2/volt/sec for protein A and

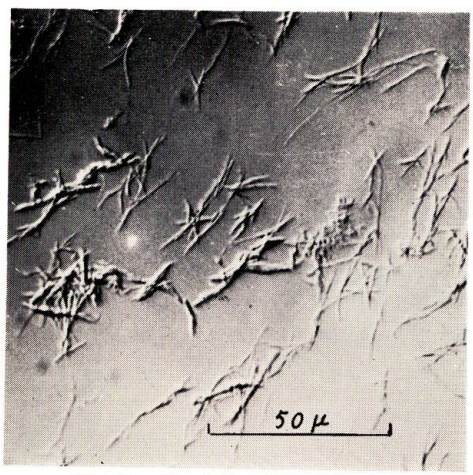


Fig. 5. Crystals of protein A
(2 times of recrystallization)

Fig. 6. Crystals protein B
(2 times of recrystallization)

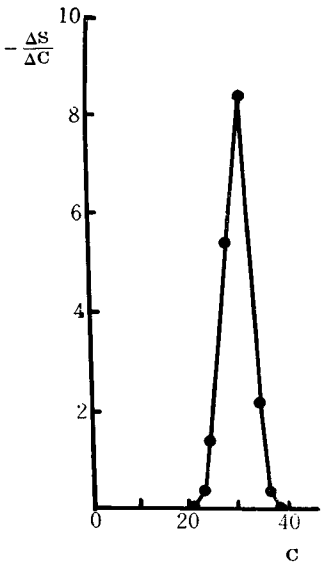


Fig. 7. Salting-out curve of protein A
s, extinction at 277 mμ; c, per cent in volume of saturated $(NH_4)_2SO_4$

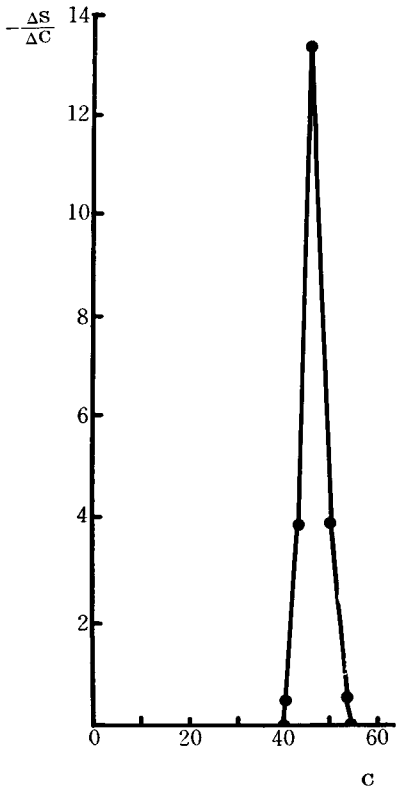


Fig. 8. Salting-out curve of protein B
s, extinction at 277 mμ; c, per cent in volume of saturated $(NH_4)_2SO_4$

of descending 2.10×10^{-5} cm²/volt/sec and ascending 2.25×10^{-5} cm²/volt/sec for protein B. The mobility of protein B was larger than that of A. This is consistent with the amounts of net negative charge estimated from their amino acid compositions.

The contents of total nitrogen, total phosphorus and tryptophan are recorded in Table 1. According to Bailey[2,27] tryptophan of the tropomyosin preparation is due to contaminations. The low contents of tryptophan (protein A 0.05, and protein B 0.02%) indicate the high purity of our preparations.

Both proteins showed the maximum absorption at 277 mμ and the minimum at 255 mμ, as given in Fig. 12. The extinction ratios of 277/255 mμ were 1.64 and 2.06, respectively for proteins A and B. The high values of extinction ratio of 277/255 mμ and their low phosphorus contents (protein A 0.06, and protein B 0.02%) indicate that these preparations are scarcely contaminated with nucleic acid.

III. Molecular Size and Shape of Proteins A and B

The size and shape of the proteins A and B were determined by the light-

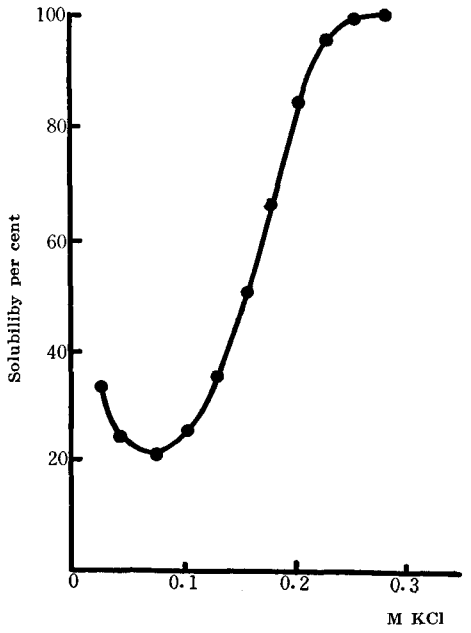


Fig. 9. Solubility curve of protein A; Conditions: total protein concentration 0.57 %, pH 7.14, 0°C

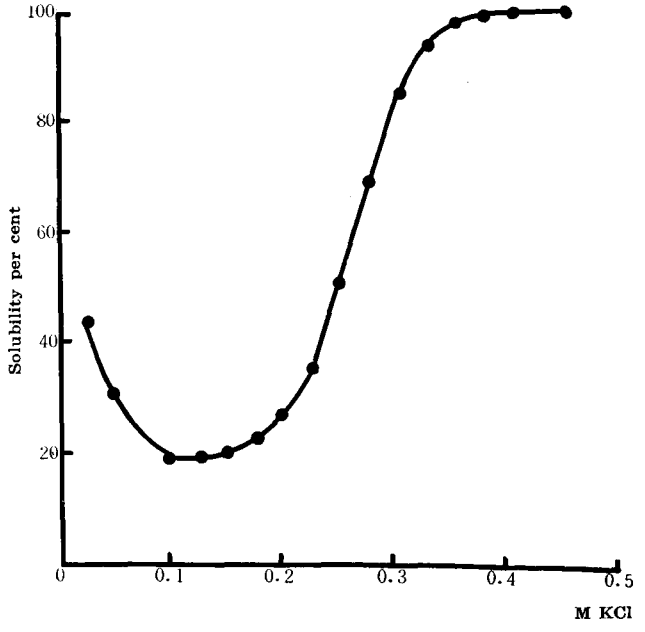
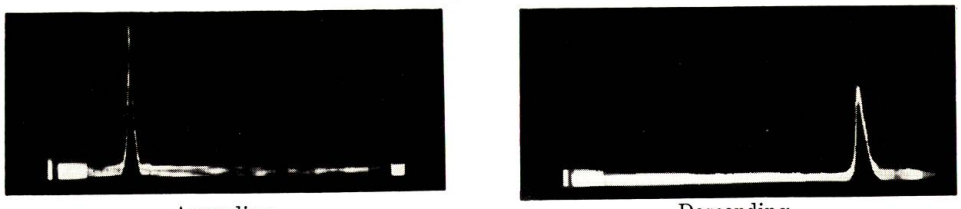


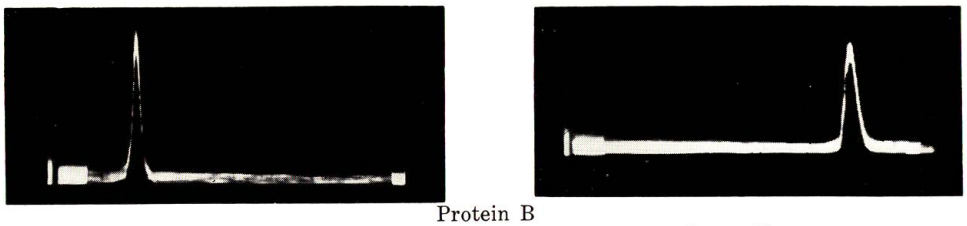
Fig. 10. Solubility curve of protein B; Conditions: total protein concentration 0.55 %, pH 7.11, 0°C

Protein A



Ascending Descending

I=0.45 (KCl 0.35 and K_2HPO_4–KH_2PO_4 0.1)
pH 7.15 protein concentration 0.57 %
2.47 volt/cm 8100 sec



Protein B

Ascending Descending

I=0.45 (KCl 0.35 and K_2HP_4–KH_2PO_4)
pH 7.15 protein concentration 0.70 %
2.47 volt/cm 8100 sec

Fig. 11. Electrophoretic diagrams of proteins A and B

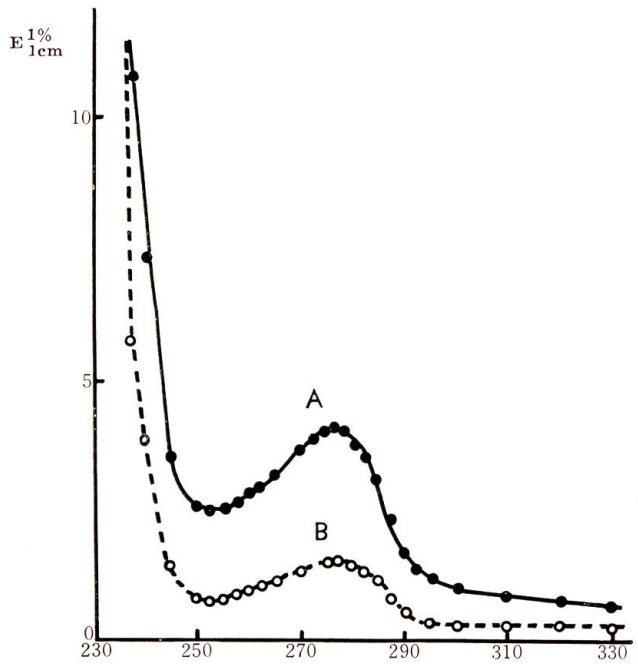


Fig. 12. Ultraviolet absorption curves of A and B; A, protein A; B, protein B; in ionic strength 0.45 KCl–K_2HPO_4–KH_2PO_4 solution

Table 1. Contents of total nitrogen, total phosphorus and tryptophan

Protein	Nitrogen (%)	Phosphorus (%)	Tryptophan (%)
Protein A	18.1	0.06	0.05
Protein B	18.1	0.02	0.02

scattering method. Tsao et al.[6] have found by observations of osmotic pressure and viscosity that the particle weight and viscosity increment of rabbit skeletal tropomyosin are remarkably affected by the ionic strength of solution and that the values of them at 0.6 M KCl reduce to about half by an increase of KCl concentration from 0.1 to 0.6 M.

Therefore, the intensities of light-scattering of the proteins A and B have been measured at high ionic strength of the solution. The size and shape of rabbit and fish (haddock) tropomyosins were also determined.

1) Methods

The protein solutions, dissolved in KCl-K_2HPO_4-KH_2PO_4 of ionic strength 1.25 (KCl 1.20 and buffer 0.05) at pH 7.05, were clarified two centrifugations at $10^5 \times g$, each for 1.5 hours, by means of a Hitachi preparative ultarcentrifuge. The light-scattering intensities of the protein solution were measured in a Brice-Phoenix apparatus at the wave length of 546 mμ and room temperature. The measurements of refractive index increment of the solutions, which were dialysed against the solvent, were carried out by means of a Brice-Phoenix differential refractometer under the same condition as the measurements of light-scattering. The values of the refractive index increment were 0.188, 0.187, 0.188 and 0.188 for squid A, squid B, rabbit and fish tropomyosins, respectively.

The molecular weight and the radius of gyration were derived from a graphical procedure of Zimm[39-40]: the molecular weight was given as the reciprocal of the intercept of the Zimm-plot with the $(Kc/R_\theta)c=o$, where R_θ is the intensity of light-scattering at the angle Θ, and K is given by the following equation:

$$K=2\pi^2 n_0^2 (dn/dc)^2/N\lambda^4$$

The refractive index of the solvent is indicated by n_0, that of the solution by n, N is Avogadro's number and is the wave length of light in vacuum.

The square of the radius of gyration was obtained from the slope of the tangent which one was able to draw to the curve $(Kc/R_\theta)c=o$ as a function of $\sin^2(\Theta/2)$ at the point where $\Theta=o$.

Bcause of the presence of large aggregations, the intensities of light-scattering at low angles increased remarkably. Therefore, the intensities above 45° were adopted, to determine the molecular weight and the radius of gyration.

2) Results

In Figs. 13 and 14, the reduced intensities are presented as an angular envelope respectively for the proteins A and B. In Table 2, the molecular weight and radius of gyration are listed, together with those of rabbit and fish tropomyosins.

Both the molecular weight (125,000) and the length (rod 1,560 Å, coil

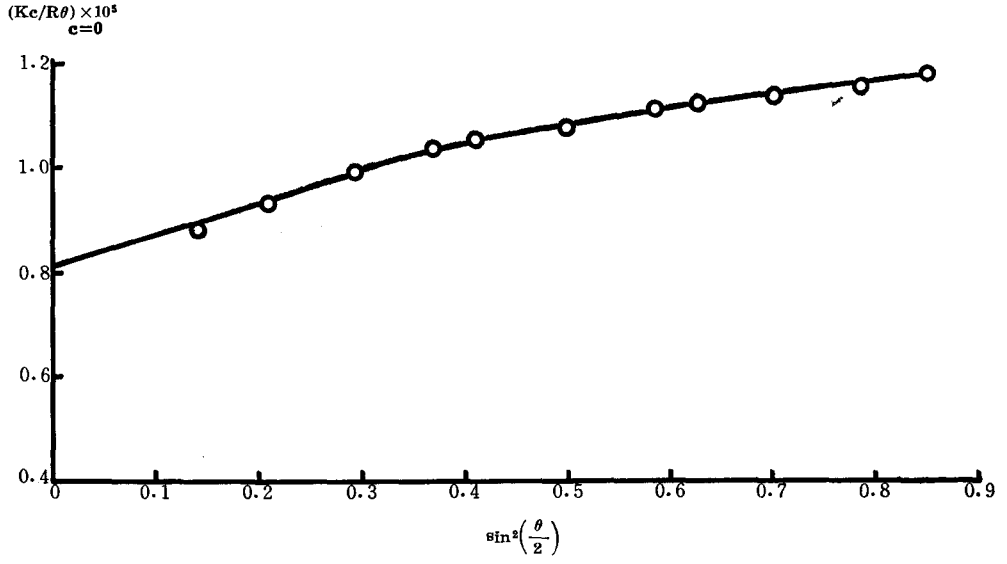


Fig. 13. Reduced intensity of light-scattering as an angular envelope of protein A
Condition: ionic strength 1.25 KCl-K_2HPO_4-KH_2PO_4, pH 7.05

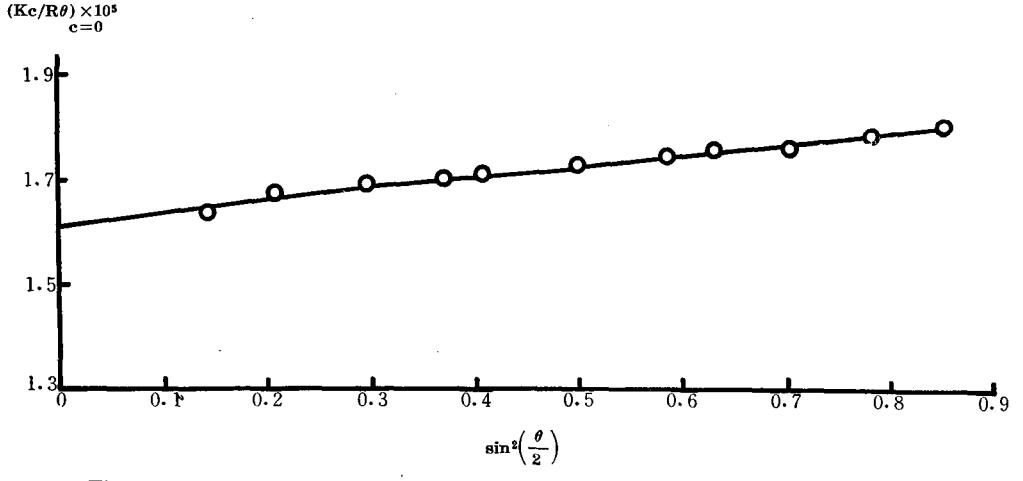


Fig. 14. Reduced intensity of light-scattering as an angular envelope of protein B
Conditions: ionic strength 1.25 KCl-K_2HPO_4-KH_2PO_4, pH 7.05

1,100 Å) of protein A are about twice as large as these of protein B (molecular weight 62,000, rod 720 Å, coil 510 Å). The values of protein B are somewhat larger than these of fish tropomyosin (molecular weight 55,000, rod 520 Å, coil 370 Å) and of rabbit tropomysin (molecular weight 53,000, rod 490 Å, coil 350 Å).

The molecular weight of rabbit tropomyosin obtained by the light-scattering method agreed well with the results obtained by the osmotic pressure method by

Table 2. Molecular weight and length of protein A, B, rabbit and fish (haddock) tropomyosins

Protein	$(Kc/R_\theta)_{c=0}\times10^5$	M	r_g	Length (Å)
Protein A	0.802	125,000	449	1,560 (rod)
				1,100 (coil)
Protein B	1.610	62,000	208	720 (rod)
				510 (coil)
Rabbit tropomyosin	1.890	53,000	142	490 (rod)
				350 (coil)
Fish tropomyosin	1.810	55,000	151	520 (rod)
				370 (coil)

Solution, ionic strength 1.25 (1.20 KCl, 0.05 K_2HPO_4–KH_2PO_4); n_0=1.340; r_g, radius of gyration; M, molecular weight.

Tsao et al.[6] (53,000 in 6.7 M urea) and by Doty and Sanders by the light-scattering method cited in Bailey[41] (53,000~55,000 at ionic strength 1.01 and 100,000 at ionic strength 0.1)

IV. Amino acid Composition of Proteins A and B

In the usual procedure of determination of the amino acid composition of protein, certain amino acids, such as serine and threonine, are susceptible to decomposition[42]. Beside these amino acids, glutamic and aspartic acid also suffer loss to some extent[43-44]. The destruction rates of these amino acids depend not only upon the duration of hydrolysis, but also upon the kind of protein. On the other hand, compounds in which the carboxyl group of isoleucine and valine are coupled in peptide linkage are not easily hydrolysed[43-46].

Therefore, to overcome these difficulties, two different length-periods of hydrolysis have been adopted in the following determinations.

1) Methods

The dried protein was suspended with about 200 volumes of three times glass-distilled HCl in a pyrex tube, and the solution was frozen in a bath of solid carbon dioxide and ethanol. After having been evacuated to about 2 mm pressure, the tube was sealed. Hydrolysis was performed in an oven at 105±1°C for 22 or 72 hours. After hydrolysis of the protein during the appropriate hours, the pale yellow hydrolysate was filtered on a glass filter and excess HCl was removed by repeated drying in vacuo. The residue was dissolved in water and made up to a desired volume. Total nitrogen of the hydrolysate was determined by the micro-Kjeldahl method and the nitrogen value of 18.1% was used to calculate the weight of protein.

The chromatography of the hydrolysate from 2.5~3.0 mg protein was performed on Dowex 50-X 8 column by the method of Moore and Stein[47], except for no addition of detergent to all the buffers employed: a 0.9×100 cm column

was used for acidic and neutral amino acids and a 0.9×15 cm column for basic amino acids and amide nitrogen. Eluted fractions were analysed by the ninhydrin procedure according to Moore and Stein with slight modifications[48]. To determine tyrosine and phenylalanine, the fraction of tyrosine plus phenylalanine was obtained by 0.9×15 cm column chromatography while the tyrosine value was directly measured on the hydrolysate by the method of Ceriotti and Spandrio[49]. Since the proline content of the proteins was low, it was independently measured by the method Chinard[50] on the fraction of glutamic acid and its adjacent in 15~18 mg protein hydrolysate. After the oxidation of protein with performic acid according to the method of Schram et al.[51], cystine and cyteine were determined as cysteic acid on 25~40 ml fraction of the long column with 15~18 mg protein hydrolysate. Amide nitrogen was determined independently by the method of Bailey[52] in order to check the values calculated from the chromatographic results.

2) Results

The elution curves of 22- and 72-hour-hydrolysates on the long column are given in Figs. 15 and 16 for protein A and in Figs. 17 and 18 for protein B.

In the case of the 22-hour-hydrolysates of both these proteins, unknown peaks were recognized before and after the valine fraction and in front of the methionine. In 72-hour-hydrolysates several unknown peaks were revealed only in front of methionine. Since the amounts of valine and isoleucine in 72-hour-hydrolysate are higher than those of 22-hour, the peaks before and after valine may be due to some peptides which contain valine and/or isoleucine. On the other hand, the peaks eluted before methionine seem to be artifacts due to the change of the buffer.

The elution curves of 22- and 72-hour-hydrolysates in the short column are shown in Figs. 19, 20 (protein A) and Figs. 21, 22 (protein B). After ammonia peaks, several other peaks were revealed, which may be due to the artifacts of the change of pH 6.5 buffer.

The analytical results obtained from three pairs of samples of 22- and 72-hour-hydrolysates of proteins A and B are presented in Table 3. A comparison of the analytical values of each hydrolysate indicates that certain amino acids, such as valine and isoleucine, and ammonia are extremely increased, but serine and threonine are decreased by increase in the length of hydrolysis. The decomposition of aspartic acid, glutamic acid, lysine and methionine, which has been reported by several previous investigators[18,43-44], was not observed in the present analysis.

The values for threonine and serine were obtained by extrapolating the results of 22- and 72-hour-hydrolyses to zero time, assuming that the losses of these amino acids occurred linearly with the time of hydrolysis. As the value of ammonia obtained by chromatography fluctuated greatly, the value gained independently by the method of Bailey[52] was adopted as valid. For the values of isoleucine and valine were taken those of 72-hour-hydrolysis. The average values of 22- and 72-hydrolysates were adopted for the amounts of other amino acids. The amino acid compositions of proteins A and B thus obtained are summarized in Tables 4 and 5, respectively. The results of the present amino

Table 3. Effect of hydrolysis time on the amounts of amino acids eluted from proteins A and B
(Nitrogen as per cent of total protein nitrogen)

Amino acid	Protein A		Protein B	
	22 hour	72 hour	22 hour	72 hour
Glycine	2.01±0.05	2.03±0.03	1.84±0.07	1.83±0.02
Alanine	6.89±0.03	6.88±0.01	7.24±0.02	7.26±0.05
Valine	2.76±0.10	2.82±0.04	2.31±0.06	2.42±0.03
Leucine	7.45±0.11	7.35±0.15	6.68±0.17	6.76±0.09
Isoleucine	2.32±0.03	2.70±0.04	1.82±0.01	2.16±0.02
Proline	0.25±0.05	0.25±0.03	0.16±0.03	0.18±0.02
Phenylalanine	0.77±0.20	0.73±0.18	0.92±0.25	0.95±0.20
Methionine	0.92±0.06	0.98±0.05	1.69±0.04	1.61±0.05
Arginine	23.98±0.11	23.92±0.05	20.02±0.13	20.03±0.13
Histidine	2.94±0.07	2.95±0.08	2.27±0.06	2.23±0.09
Lysine	9.78±0.10	9.74±0.05	12.20±0.11	12.26±0.09
Aspartic acid	7.80±0.04	7.78±0.05	7.97±0.13	7.81±0.11
Glutamic acid	13.69±0.28	13.73±0.30	16.68±0.28	16.58±0.32
Serine	3.76±0.02	3.56±0.03	3.54±0.05	3.22±0.04
Threonine	2.99±0.07	2.81±0.05	1.70±0.03	1.65±0.04
Tyrosine	0.81±0.04	0.78±0.02	0.49±0.05	0.49±0.03
Amide Nitrogen	8.10±0.41	8.40±0.29	7.66±0.35	8.02±0.46

acid analysis seem to be satisfactory, as the recovery of amino acid nitrogen per total nitrogen was 98.03 and 95.72% respectively in proteins A and B. On the basis of the results exhibited in Tables 4 and 5, the side chain properties of proteins A and B are given as in Table 6. The amino acid patterns of these two proteins are rather unique: the amounts of glutamic acid, aspartic acid, alanine, leucine, arginine and lysine are remarkably high, but the amounts of histidine, tyrosine, phenylalanine, half cystine and proline are low. As shown in Table 6, the amounts of free acidic, basic and polar side chain groups are very high.

Some differences in these two proteins are obvious in respect to the contents of alanine, leucine, methionine, threonine, glutamic acid, lysine, arginine and amide nitrogen. Especially amongst the basic amino acids, the ratio of the contents of lysine and arginine of protein A replaces that of protein B and the content of glutamic acid of protein A is lower than that of protein B. The sum of free acidic and basic groups and net negative charge at pH 8 are also distinguishably different from each other.

V. Terminal Residues in Proteins A and B

To demonstrate difference in the chemical structures of proteins A and B, the terminal residues of these proteins were examined.

1) Methods

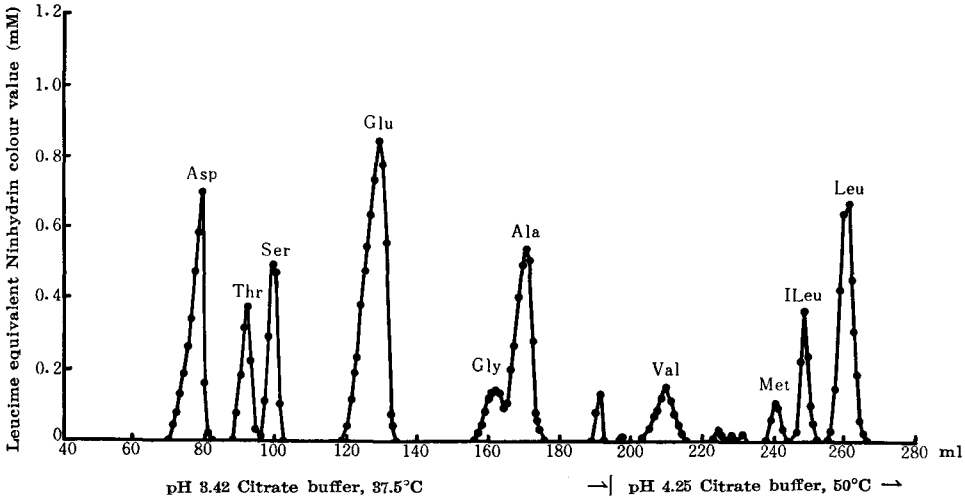


Fig. 15. Elution curve for a 22-hour-hydrolysate of protein A on a 0.9×100 cm column Dowex-50 (X-8), 3.02 mg protein

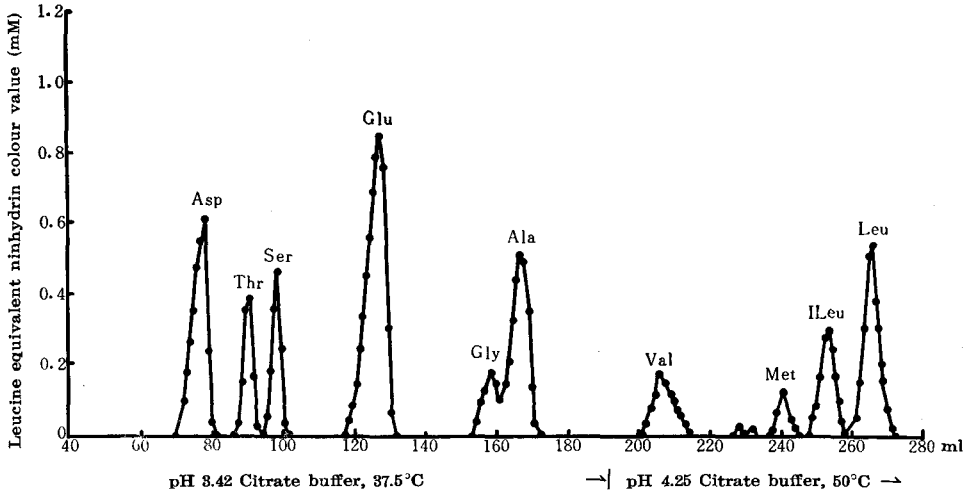


Fig. 16. Elution curve for a 72-hour-hydrolystae of protein A on a 0.9×100 cm column Dowex-50 (X-8), 2.90 mg protein

N-terminal assay: The dinitrofluorobenzene (DNFB) method of Sanger[53] was applied for the examination and estimation of N-terminal residue in these two proteins. The dinitrophenyl-protein (DNP-protein), which was prepared under the conditions recommended by Sanger, was hydrolysed in a sealed glass tube by 6 N HCl at 100°C during appropriate hours. The hydrolysate was extracted with ether, and separated into ether soluble and water soluble fractions.

After sublimation of dinitrophenol, dinitrophenyl-amino acids (DNP-amino

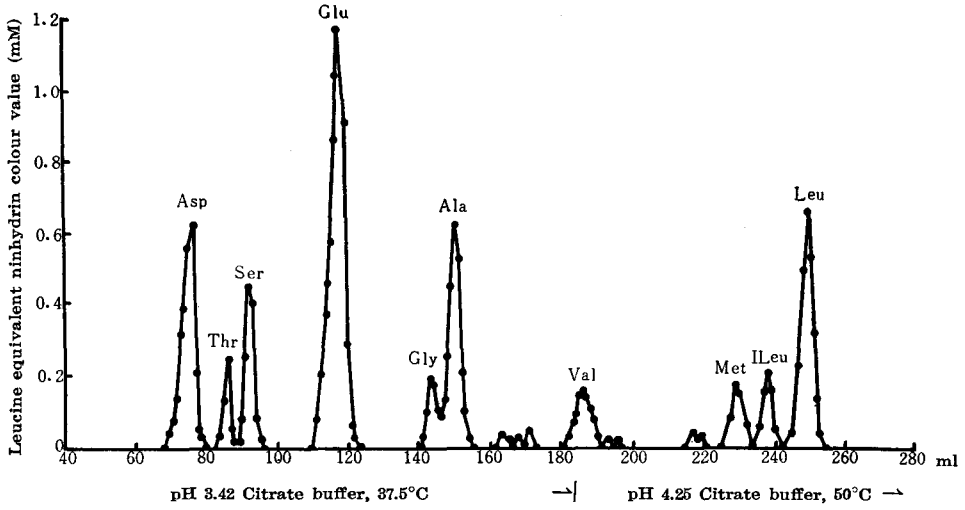


Fig. 17. Elution curve for a 22-hour-hydrolystae of protein B on a 0.9×100 cm column Dowex-50 (X-8), 2.92 mg protein

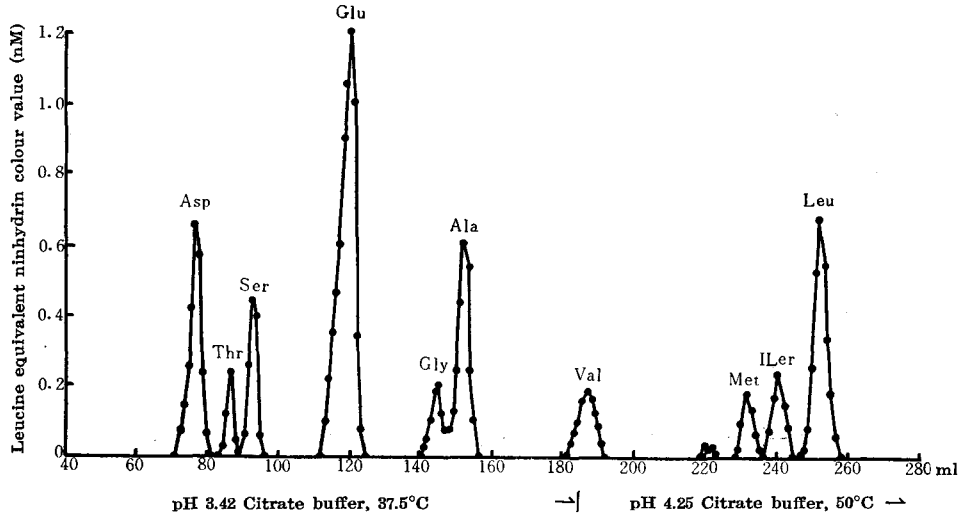


Fig. 18. Elution curve for a 72-hour-hydrolystae of protein B on a 0.9×100 cm column Dowex-50 (X-8), 2.94 mg protein

acids) in the ether soluble fraction were chromatographed on No. 51 Toyo filter paper by the method of Levy[54-55], and eluted with 1% $NaHCO_3$. Their amounts were measured colorimetrically at 360 mμ, adopting the molar extinction coefficient of 1.70×10^4. DNP-amino acids in the water soluble fraction were absorbed in a talc column[56]. After elution with N HCl-ethanol (1 : 4), it was chromatographed in tert-amyl alcohol[55].

For the inspection of the terminal cysteyl residue[57], the protein oxidized

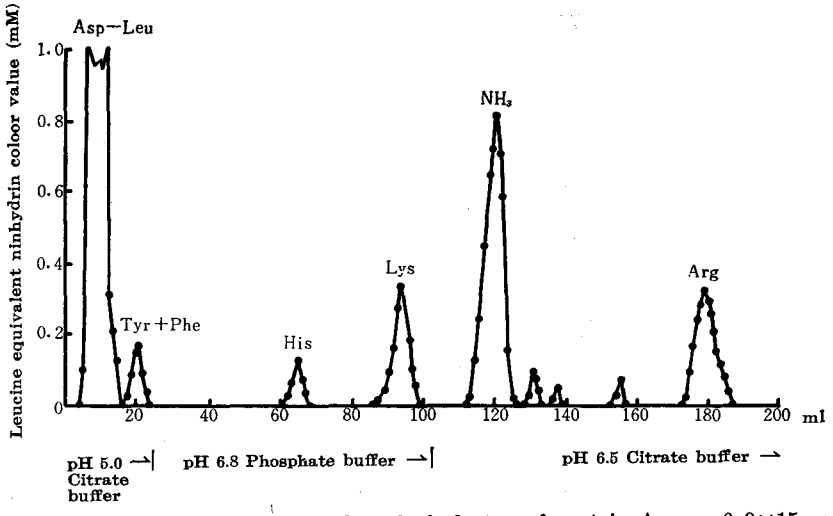


Fig. 19. Elution curve fro a 22-hour-hydrolystae of protein A on a 0.9×15 cm column Dowex-50 (X-8), 3.02 mg protein

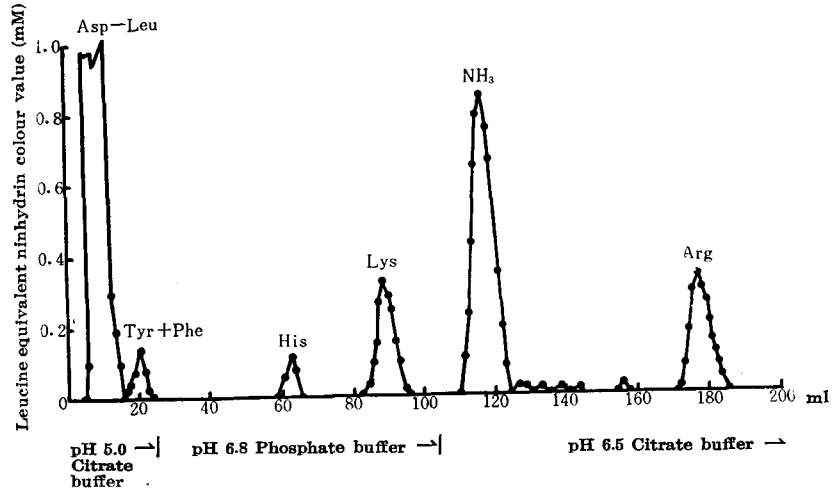


Fig. 20. Elution curve for a 72-hour-hydrolystae of protein A on a 0.9×15 cm column Dowex-50 (X-8), 2.90 mg protein

by the method of Schram et al.[51] was treated with DNFB: DNP-amino acid in the water soluble fraction was examined by the same method as described above. In this assay, the amounts of the original protein were calculated from the amide nitrogen in the DNP-protein.

C-terminal assay: C-terminal residue was measured by the method of Ohno and Akabori[58-61], use being made of about 50 mg of the protein which was previously dried over P_2O_5 at 110°C for 12 hours. The protein was hydrazinolysed with anhydrous hydrazine at 100°C for an appropriate time, and excess

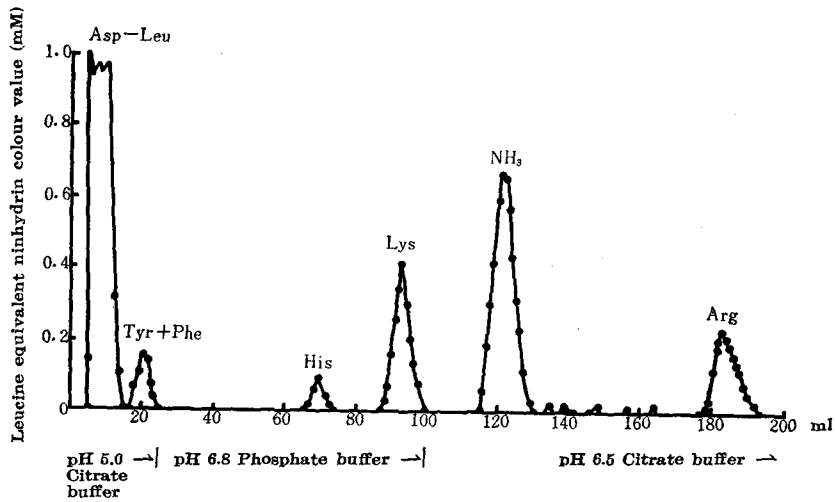


Fig. 21. Elution curve for a 22-hour-hydrolysate of protein B on a 0.9×15 cm column Dowex-50 (X-8), 2.92 mg protein

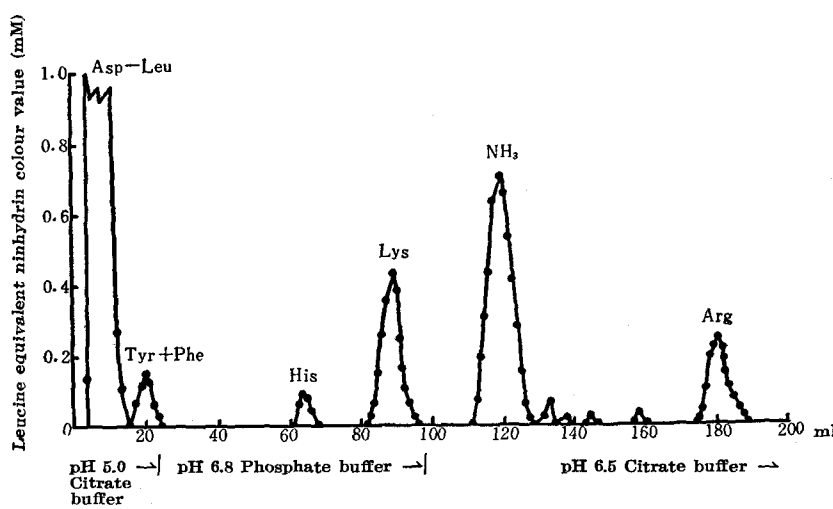


Fig. 22. Elution curve for a 72-hour-hydrolysate of protein B on a 0.9×15 cm column Dowex-50 (X-8), 2.94 mg protein

hydrazine was removed in vacuo over conc. H_2SO_4. Amino acid hydrazide was removed by twice repetition of treatment with isovaleraldehyde. After removal of isovaleraldehyde with ethyl acetate, the water soluble fraction was treated with DNFB. The mixture was acidified with 2 N HCl, and extracted with ethyl acetate. The ethyl acetate fraction was washed with 2% $NaHCO_3$*. The $NaHCO_3$

* Most of DNP-amino acids and a part of isovaleral derivatives of DNP-aspartic and glutamic monohydrazides are transferred into $NaHCO_3$ fraction, while all of di-DNP-tyrosine and a part of DNP-tryptophan remain in the ethyl acetate layer. Di-DNP-tyrosine in this acetate fraction could not be detected in proteins A and B. Tryptophan was not included among these proteins.

Table 4. Amino acid composition of protein A

Amino acid	Amino acid N per total N (%)	Amino acid weight per 100 g protein (%)	Amino acid residue weight per 100 g protein (%)	Amino acid moles per 10^5 g protein
Glycine	2.02	1.95	1.48	26
Alanine	6.89	7.94	6.34	89
Valine	2.82	4.27	3.61	37
Leucine	7.40	12.54	10.82	96
Isoleucine	2.70	4.58	3.95	35
Proline	0.25	0.37	0.31	3
Phenylalanine	0.75	1.60	1.43	10
Half Cystine	0.37	0.57	0.48	5
Methionine	0.95	1.83	1.61	12
Tryptophan	—	—	—	—
Arginine	23.95	13.50	12.11	78
Histidine	2.95	1.97	1.74	13
Lysine	9.76	9.21	8.07	63
Aspartic acid	7.79	13.40	11.59	101
Glutamic acid	13.71	26.04	22.86	177
Serine	3.86	5.26	4.36	50
Threonine	3.08	4.75	4.03	40
Tyrosine	0.80	1.87	1.69	10
Amide Nitrogen	7.98	(1.45)	—	(104)
Total	98.03	111.65	96.48	845

( ), omitted from the total value

Table 5. Amino acid composition of protein B

Amino acid	Amino acid N per total N (%)	Amino acid weight per 100 g protein (%)	Amino acid residue weight per 100 g protein (%)	Amino acid moles per 10^5 g protein
Glycine	1.84	1.78	1.36	24
Alanine	7.25	8.34	6.65	94
Valine	2.42	3.66	3.10	31
Leucine	6.72	11.37	9.81	87
Isoleucine	2.16	3.66	3.16	28
Proline	0.17	0.25	0.22	2
Phenylalanine	0.94	2.00	1.78	12
Half Cystine	0.22	0.34	0.29	3
Methionine	1.65	3.17	2.79	21
Tryptophan	—	—	—	—
Arginine	20.03	11.29	10.13	65
Histidine	2.25	1.50	1.33	10
Lysine	12.23	11.54	10.12	79
Aspartic acid	7.84	13.42	11.61	101
Glutamic acid	16.63	31.60	27.74	215
Serine	3.68	5.10	4.15	48
Threonine	1.79	2.76	2.34	23
Tyrosine	0.49	1.15	1.04	6
Amide Nitrogen	7.42	(1.34)	—	(96)
Total	95.72	112.38	97.15	849

( ), omitted from the total value

Table 6. Side chain properties of proteins A and B

Group	Protein A (%)	Protein B (%)
Free acid group	20.6) 38.8	26.6) 44.1
Basic group	18.2	18.1
Hydroxy group	11.8	9.2
Amide group	12.3	11.3
Polar group	63.5	64.8
Nonpolar group	36.9	35.2
Net negative charge at pH 8	33	76

Table 7. DNP-amino acids as N-terminals of proteins A and B*

Protein A				Protein B			
Amino acid	Hydrolysis time (hour)			Amino acid	Hydrolysis time (hour)		
	4	8	12		4	8	12
Leu or ILeu	0.0_6	0.0_7	0.0_4	Thr	0.1_2	0.1_1	0.0_9
Val	0.1_0	0.1_0	0.0_6				
Ala	0.0_2	0.0_3	0.0_2				

* All amounts are expressed as moles per mole of protein

fraction was acidified and extracted with ether. C-terminal DNP-amino acid in the ether fraction was estimated by the method described above in the paragraph on N-terminal assay. Further to identify the DNP-amino acids, the eluted solution of DNP-amino acids was hydrolysed with HCl for 4 hours, and was re-chromatographed.

In these assays, 125,000 and 62,000 were adopted as the molecular weights of proteins A and B, respectively.

2) Results

N-terminal residue: In the ether soluble fraction of protein A, DNP-alanine, -valine and -leucine or -isoleucine were detected, and in the ether soluble fraction of protein B, DNP-threonine was detected, as shown in Table 7. The amount of each DNP-amino acid, however, was not more than 0.1 mole per mole of protein. Therefore, these DNP-amino acids seem to arise from impurities of the protein preparations. In the water soluble fraction, only ε-DNP-lysine was detected in these two proteins and also in the performic acid-oxidized proteins A and B. Therefore, it may be concluded that by the DNFB method no N-terminal residue can be detected in the proteins A and B.

C-terminal residue: Certain amounts of DNP-serine, and traces of DNP-valine, -alanine and -isoleucine or -leucine were detected in protein A. DNP-isoleucine or -leucine, and a trace of DNP-threonine were dected in protein B. To identify DNP-isoleucine or -leucine, the eluted spot was hydrolysed with

Table 8. DNP-amino acids as C-terminals of proteins A and B*

Protein A			Protein B		
Amino acid	Hydrazinolysis time (hour)		Amino acid	Hydrazinolysis time (hour)	
	5	10		5	10
Ser	0.6_7	0.5_9	ILeu	0.6_8	0.5_7
Leu or ILeu	trace	trace	Thr	0.1_0	0.0_4
Val	0.0_5	0.0_8	Glu or Asp	trace	trace
Ala	trace	trace			

* All amounts are expressed as moles per mole of protein

conc. ammonia at 100°C for 2 hours[62], and only isoleucine was detected by chromatography in tert-amyl alcohol at pH 6.0[55].

The amounts of the DNP-amino acids are given in Table 8. It is obvious from this table that protein A has one C-terminal reisdue, viz., serine, and protein B has one isoleucine as C-terminal residue. Other amino acids may be due to impurities.

Discussion

All the tropomyosins, whose amino acid compositions have been presented, show several specific patterns in their respective compositions as given in Table 9, in which the amino acids of vertebrata and invertebrata tropomyosins are tabulated in regular sequence according to their amounts. They show high contents of glutamic acid, aspartic acid, alanine, leucine, lysine and arginine, but have small amounts of histidine, methionine, proline, phenylalanine, tyrosine and half cystine. They have no tryptophan. Beside these characteristics of amino acid compositions, they all show a particularly high content of polar side chain (62~66%) and of ionic side group (35~47%), as reported in Table 10. The amino acid compositions and the side chain properties of proteins A and B, listed in Table 6, also show these specific patterns common to all tropomyosins.

In spite of these common properties of the tropomyosins, there are several differences in their amino acid compositions and side chain properties. It is sight obvious from Table 9 that in vertebrate tropomyosin the content of lysine is much higher that that of aspartic acid, but, on the contrary, in invertebrate tropomyosin the content of lysine is lower than that of aspartic acid. It is also interesting to note that in the vertebrate, arthropod and some of the mollusc tropomyosins the content of lysine is higher than that of arginine, but in other mollusc and annelide tropomyosins, the lysine content is lower than the arginine content. As shown in Table 10, tropomyosins can be divided into two groups, the one having comparatively higher content (43~47%) and the other having lower content (35~37%) of ionic side groups.

Yoshimura[22] and Bailey[23,27] have already reported some specificity in the arginine and lysine contents of mollusc tropomyosins. Not only in mollusc tropo-

Table 9. Amino acid compositions of various tropomyosins
(Amino acid moles per 10^5 g of tropomyosin)

Vertebrate								Arthropod		Mollusc												Annelid	
Rabbit skeletal (8)		Frog (21)		Carp (21)		Haddock (18)		Homarus (29)		Protein B		Squid mantle (22)		Loligo mantle (29)		Verus foot, adductor tropomyosin B (29)		Protein A		Verus foot, adductor tropomyosin A (29)		Pinna adductor (27)		Octopus (27)		Arenicola (29)	
1. Glu	211	1. Glu	201	1. Glu	201	1. Glu	237	1. Glu	200	1. Glu	215	1. Glu	212	1. Glu	195	1. Glu	205	1. Glu	177	1. Glu	169	1. Glu	173	1. Glu	160	1. Glu	156
2. Lys	110	2. Lys	112	2. Lys	113	2. Lys	114	2. Asp	101	2. Asp	101	2. Asp	99.5	2. Asp	103	2. Asp	110	2. Asp	101	2. Asp	114	2. Asp	111	2. Leu	104	2. Asp	120
3. Ala	110	3. Ala	106	3. Leu	107	3. Ala	101	3. Ala	90	3. Ala	94	3. Ala	95	3. Ala	93	3. Ala	101	3. Leu	96	3. Ala	108	3. Leu	103	3. Asp	91	3. Leu	106
4. Leu	95	4. Leu	95	4. Ala	106.5	4. Leu	101	4. Leu	88	4. Leu	87	4. Leu	95	4. Leu	86	4. Leu	88	4. Ala	89	4. Leu	106	4. Ala	102	4. Ala	78.5	4. Ala	87
5. Asp	89	5. Asp	91	5. Asp	96	5. Asp	85	5. Lys	78	5. Lys	79	5. Lys	85	5. Lys	78	5. Lys	78	5. Arg	78	5. Arg	81	5. Arg	79	5. Arg	70	5. Arg	85
6. Arg	42	6. Arg	42	6. Arg	41	6. Ser	40	6. Arg	63	6. Arg	65	6. Arg	69	6. Arg	63	6. Arg	67	6. Lys	63	6. Lys	59	6. Lys	64.5	6. Lys	60	6. Lys	56
7. Ser	40	7. Ser	34	7. Val	37	7. Arg	38	7. Ser	43	7. Ser	48	7. Ser	55	7. Ser	45	7. Ser	43	7. Ser	50	7. Ser	39	7. Ser	49	7. Ser	52	7. Ser	44
8. Val	38	8. Val	32	8. Ser	33	8. Thr	34	8. Val	33	8. Val	31	8. Thr	33	8. Thr	38	8. Val	40	8. Thr	40	8. Thr	36	8. Val	35.5	8. Thr	37	8. Thr	33
9. ILeu	29	9. ILeu	29	9. ILeu	30	9. ILeu	32	9. Thr	27	9. ILeu	28	9. Gly	28	9. ILeu	25	9. Thr	35	9. Val	37	9. Val	28	9. ILeu	28	9. ILeu	34	9. Val	30
10. Thr	28	10. Thr	25	10. Thr	24	10. Val	29	10. Met	22	10. Gly	24	10. Val	25	10. Val	24	10. ILeu	27	10. ILeu	35	10. ILeu	22	10. Thr	22	10. Val	32	10. ILeu	25
11. Met	16	11. Met	25	11. Met	23	11. Tyr	21	11. Gly	14	11. Thr	23	11. ILeu	20	11. Gly	17	11. Gly	20	11. Gly	26	11. Thr	18	11. Met	15.5	11. Gly	16	11. Gly	25
12. Try	15	12. Tyr	18	12. Tyr	18.5	12. Met	18	12. ILeu	12	12. Met	21	12. Met	17	12. Met	12	12. Tyr	20	12. His	13	12. Gly	15	12. Tyr	14	12. Met	13	12. Met	13
13. Gly	12.5	13. Gly	14	13. Gly	18	13. Gly	12	13. Tyr	12	13. Phe	12	13. His	12	13. Phe	12	13. Met	8	13. Met	12	13. Met	11	13. Gly	12	13. His	11	13. Tyr	10
14. Cys/2	6.5	14. His	7	14. His	6	14. Cys/2	6.5	14. Phe	11	14. His	10	14. Phe	8	14. Tyr	10	14. Phe	6	14. Tyr	10	14. Phe	6	14. Phe	8.5	14. Tyr	8	14. Phe	6
15. His	5.5	15. Cys/2	6	15. Phe	6	15. Phe	6	15. His	3	15. Tyr	6	15. Thr	8	15. Pro	4	15. His	3	15. Phe	10	15. His	4	15. His	4.5	15. Phe	6.5	15. His	6
16. Phe	3.5	16. Phe	4	16. Cys/2	5	16. His	3.5	16. Pro	3	16. Cys/2	3	16. Cys/2	6	16. His	3	16. Pro	2	16. Cys/2	5	16. Pro	1.5	16. Cys/2	2.5			16. Pro	4
		17. Pro	3	17. Pro	3					17. Pro	2							17. Pro	3								
Total	851	Total	844	Total	868	Total	878	Total	800	Total	849	Total	867.5	Total	803	Total	853	Total	845	Total	817.5	Total	824	Total	779	Total	806

myosins but also in several animals' tropomyosins, Kominz and Laki[25,29] have observed that the number of net negative charge and the ratio of lysine to arginine decrease regularly as the phyla of animal origin have a lower position from the view-point of evolutionary theory. They have classified tropomyosins into the two groups: tropomyosins A and B. Tropomyosin A salts out at 26~35% ammonium sulphate saturation; its net negative charge is 25~35 at pH 8, and its content of arginine is higher that that of lysine. Tropomyosin B salts out at 41~55% saturation; its net negative charge is about 80, and its content of lysine is higher than that of arginine. On the other hand, Bailey[27] has divided tropomyosins into water-insoluble and -soluble. These two tropomyosins perhaps correspond to the groups A and B of Kominz and Laki, respectively. It is apparent

Table 10. Side chain properties of various tropomyosins

Group	Vertebrate			
	Rabbit skeletal (8) (%)	Frog (21) (%)	Carp (21) (%)	Haddock (18) (%)
Free acid group	27.8	28.1	27.0	29.7
Basic group	18.5) 46.3	19.1) 47.2	18.5) 45.5	18.0) 47.7
Hydroxy group	9.8	9.1	8.8	10.8
Amide group	7.4	6.5	7.2	7.0
Polar group	64.3	63.5	62.1	66.2
Nonpolar group	35.7	36.5	38.9	33.8

Group	Arthropod	Mollusc		
	Homarus (29) (%)	Squid mantle (22) (%)	Loligo mantle (29) (%)	Verus foot, adductor tropomyosin B (29) (%)
Free acid group	26.4	25.0	26.2	27.1
Basic group	18.0) 44.4	19.1) 44.1	17.9) 44.1	17.4) 44.5
Hydroxy group	10.2	11.0	11.6	11.5
Amide group	11.2	10.9	10.6	9.8
Polar group	65.8	66.6	66.5	65.9
Nonpolar group	34.2	33.4	33.5	34.1

Group	Mollusc			Annelid
	Verus foot, adductor tropomyosin A (29) (%)	Pinna adductor (27) (%)	Octopus foot (27) (%)	Arenicola (29) (%)
Free acid group	21.2	19.3	18.4	20.2
Basic group	17.6) 38.8	17.0) 36.3	16.9) 35.3	18.2) 38.4
Hydroxy group	11.4	10.3	12.4	10.8
Amide group	13.5	13.3	10.6	14.0
Polar group	63.7	63.1	63.6	63.3
Nonpolar group	36.3	36.9	36.4	36.7

from the results presented (Figs. 7, 8, Tables 4, 5, 6 and 9) that the salting-out range, the amounts of negative charge and the ratio of lysine to arginine of protein A correspond to tropomyosin A of Kominz and Laki or to the water-insoluble tropomyosin of Bailey and that all properties of protein B to the tropomyosin B of Kominz and Laki or to the water-soluble tropomyosin of Bailey.

Squid tropomyosins have already been prepared by Yoshimura[22] from the mantle of the animal used in this report (*Ommarstrephes sloani pacificus* STEENSTRUP), by Tsao et al.[12] from *Sepia esculenta,* and by Kominz and Laki[29] from *Loligo pealeii.* All these tropomyosins belong to tropomyosin B group or water-soluble tropomyosin group in respect to their solubility, salting-out range, amino acid composition and net negative charge. This seems to be the first time that two types of tropomyosin have been isolated from the muscle of squid mantle, cephalopoda, as described in this report. In the isolation of these proteins, the previous investigators have applied Bailey's procedure which was originally adopted for rabbit skeletal muscle[2]. In the procedure, protein A would be denatured by the treatment of acid and removed by fractionation with 41~55% ammonium sulphate saturation. On the other hand, Bailey's method[24,27] for isolation of water-insoluble tropomyosins from adductor muscle and octopus foot muscle might be unsuitable for the squid mantle, as the proteins A and B have similar solubility against dilute KCl solution.

The protein B has the same salting-out range, C-terminal amino acid, and amino acid composition pattern, as those of Yoshimura's tropomyosin[22]. Differences are observed in nitrogen contents, and in solubility in KCl of these two proteins. However, these two tropomyosins may be identical, because these differences are not substantial. The difference in solubility might be due to its change by subjection to high (about 9.0) or low (below 4.5) pH. The low nitrogen content in Yoshimura's tropomyosin might reflect its incomplete dryness.

N-terminal residue could not be detected in the two proteins A and B by the usual DNFB method, but one serine residue and one isoleucine residue respectively were detected as C-terminal by the use of anhydrous hydrazine. Similar results have already been observed in other tropomyosins: no N-terminal residue has been detected in any of the tropomyosins isolated at present (the adductor muscle of pinna[27], the foot muscle of octopus[27], rabbit skeletal muscle[5], pig heart[14], duck gizzard[14], frog[19], fish[19] and the body muscle of prawn[14]). On the other hand, Locker[7] established the existence of one isoleucine as C-terminal residue in rabbit skeletal tropomyosin by the use of carboxypeptidase and of anhydrous hydrazine. He has found isoleucine-serine-threonine-methionine-isoleucine-alanine- as the C-terminal sequence. The present author has previously found one C-terminal isoleucine residue in the tropomyosins isolated from frog and fish skeletal muscle by the use of anhydrous hydrazine[19]. By the use of the method of carboxypeptidase A, Kominz and Lake have reported two C-terminal residues, viz., isoleucine and serine in rabbit skeletal and bladder muscle, asparagine and leucine in uterus muscle tropomyosin[10], isoleucine and probably serine in frog tropomyosin[21]. However, these descrepancies in the number of C-terminal residue may be attributable to the inherent difficulty of application of the carboxypeptidase method. To identify C-terminal residue by this method, it is necessary

to check up time sequence and the effect of the ratio of substrate to enzyme. It is interesting that all these tropomyosins have no detectable N-terminal amino acid, in connection with the recent observation of the existence of masked N-terminal amino acid in tobacco mosaic virus protein[63].

Not only do the proteins A and B differ very much from each other in their molecular weights and lengths, but also they differ from the tropomyosins of rabbit and fish which have similar molecular sizes nad shapes. The molecular weights of the tropomyosins vary greatly from one another; rabbit 53,000, duck gizzard 150,000[12], pig cardiac 89,000[12], fish 55,000, prawn 77,0000[12], sepia 68,000[12], squid B 62,000, squid A 125,000 and pinna 137,000[30]. However, all these tropomyosin molecules are highly asymmetric and their molecular weights increaes generally in proportion to the increase in molecular length or axial ratio. Furthermore all tropomyosins show several common patterns in their amino acid compositions. These observations may suggest that tropomyosins are similar in their molecular pattern and that the difference in their size is attributable to the different lengths along the long axis.

The close relationship between paramyosin filament and tropomyosin has recently been confirmed by many workers[24,28,64-66], especially in respect to the periodical pattern of paramyosin filament in x-ray diffraction and the molecular length of water-insoluble tropomyosin. It is now generally accepted that paramyosin is identical to water-insoluble tropomyosin or that it is composed mainly of water-insoluble tropomyosin. Therefore the paramyosin filament may be contained in squid mantle, since tropomyosin A can be isolated from the mantle.

Note Added in Proof

Two kinds of tropomyosins have been isolated in homogeneous, crystalline form from the adductor muscle of *Pecten maximus* by Bailey and Rüegg. Their solubility, electrophoretic characteristics and amino acid composition have been elucidated. (cf. Bailey, K. and Rüegg, J. C. (1960). Further chemical studies on the tropomyosins of lamellibranch muscle with special reference to *Pecten maximus*. *Biochim. et Biophys. Acta* 38, 239.)

Summary

1. Two kinds of crystalline proteins in the tropomyosin fraction have been isolated from the dehydrated muscle of squid mantle by the purification procedure with ammonium sulphate fractionation.

2. The crystalline protein A salted out in the range of 21~39% ammonium sulphate saturation and the crystalline protein B in 40~54% saturation.

3. The electrophoretic mobilities of these proteins were measured in KCl-phosphate buffer of pH 7.15 and ionic strength 0.45 at 0°C. The mobility of the protein A (desc. 1.65 and asc. 1.79×10^{-5} cm^2/volt/sec) was lower than that of the protein B (desc. 2.10 and asc. 2.25×10^{-5} cm^2/volt/sec).

4. These proteins showed a characteristic tropomyosin pattern in respect

to amino acid contents and especially in side chain properties of amino acids, though they obviously differed from each other in the sum of free acidic and basic groups, net negative charge and the ratio of contents of lysine and arginine.

5. The molecular weight of proteins A and B were determined respectively to be 125,000 and 62,000 by the technique of light-scattering. These values are higher than that of rabbit, 53,000 and of fish (haddock) tropomyosins, 55,000. The molecular lengths of proteins A (rod 1,560 Å, coil 1,100 Å) was higher than those of protein B (rod 720 Å, coil 510 Å) and also those of rabbit (rod 490 Å, coil 350 Å) and fish tropomyosins (rod 520 Å, coil 370 Å).

6. No N-terminal residue was detected in these two proteins by the use of DNFB method, but one C-terminal serine was obtained in the protein A and one C-terminal isoleucine in the protein B by the use of anhydrous hydrazine.

7. From the salting-out ranges and the patterns of amino acid composition, it was concluded that the protein A belongs to water-insoluble or A group and the protein B to water-soluble or B group.

References

1) Bailey, K. (1946). *Nature* **157**, 368.
2) ——— K. (1948). *Biochem. J.* **43**, 271.
3) ———, Gutfreund, H, & Ogston, A. G. (1948). *Ibid.* **43**, 279.
4) Astbury, W. T., Reed, R. & Spark, L. C. (1948). *Ibid.* **43**, 282.
5) Bailey, K. (1951). *Ibid.* **49**, 23.
6) Tsao, T. C., Bailey, K. & Adair, G. S. (1951). *Ibid.* **49**, 27.
7) Locker, R. H. (1954). *Biochim. et Biophys. Acta* **14**, 533.
8) Kominz, D. R., Hough, A., Symonds, P. & Laki, K. (1954). *Arch. Biochem. Biophys.* **50**, 148.
9) Laki, K. (1957). *J. Cell. and Comp. Physiol.* **49**, 249.
10) Kominz, D. R., Saad, F., Gladner, J. A. & Laki, K. (1957). *Arch. Biochem. Biophys.* **70**, 16.
11) Sheng, P. K. & Tsao, T. C. (1954). *Acta Physiol. Sinica* **19**, 203.
12) Tsao, T. C., Tan, P. H. & Peng, C. M. (1955). *Ibid.* **19**, 389.
13) Sheng, P. K., Tsao, T. C. & Peng, C. M. (1956). *Ibid.* **20**, 151.
14) Jen, M. H. & Tsao, T. C. (1957). *Ibid.* **21**, 91.
15) Hamoir, G. (1950). *Biochem. J.* **48**, 146.
16) ——— (1951). *Ibid.* **50**, 140.
17) ——— (1955). *Advances in protein chemistry* **10**, 249.
18) Kubo, S. (1957a). *Bull. Fac. Fish. Hokkaido Univ.* **8**, 147.
19) ——— (1957b). *Ibid.* **8**, 214.
20) Dingle, J. R. & Odense, P. H. (1959). *J. Fish. Res. Bd. Canada* **16**, 53.
21) Saad, F., Kominz, D. R. & Laki K. (1959). *J. Biol. Chem.* **234**, 551.
22) Yoshimura, K. (1955). *Mem. Fac. Fish. Hokkaido Univ.* **3**, 159.
23) Bailey, K. (1956a). *Biochem. J.* **64**, 9 p.
24) ——— (1956b). *Pubbl. Staz. Zool. Napoli* **29**, 96.
25) Kominz, D. R., Saad, F. & Laki, K. (1957). *Nature* **179**, 206.
26) Laki, K. (1957). *Arch. Biochem. Biophys.* **67**, 240.
27) Bailey, K. (1657). *Biochim. et Biophys. Acta* **24**, 612.
28) Hanson, J., Lowy, J., Huxley, H., Bailey, K., Kay, C. M. & Rüegg, J. C. (1957).

Nature **180**, 1134.
29) Kominz, D. R., Saad, F. & Laki, K. (1958). *Proceeding of the Conference on the Chemistry of Muscular Contration.* [p. 66]. Tokyo; Igaku Shoin Ltd.
30) Kay, C. M. (1958). *Biochim.* et *Biophys. Acta* **27**, 469.
31) Laki, K., Horváth, B. & Klatzo, I. (1958). *Ibid.* **28**, 656.
32) Kay, C. M. & Bailey, K. (1959). *Ibid.* **31**, 21.
33) Snellman, O. & Tenow, M. (1954). *Ibid.* **13**, 199.
34) Tenow, M. & Snellman, O. (1954). *Ibid.* **15**, 395.
35) Miller, G. L. & Miller, E. E. (1948). *Anal. Chem.* **20**, 48.
36) Spies, J. R. & Chambers, D. C. (1949a). *Ibid.* **20**, 30.
37) ——— & ——— (1949b). *Ibid.* **21**, 1249.
38) Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
39) Zimm, B. H. (1948). *J. Chem. Phys.* **16**, 1099.
40) ——— & Stockmayer, W. H. (1949). *Ibid.* **17**, 130.
41) Bailey, K. (1954). *The Proteins* (Neurath, H. & Bailey, K. editors) Vol. 2, part B, [p. 1021]. New York; Academic Press Inc.
42) Rees, M. W. (1946). *Biochem. J.* **40**, 632.
43) Smith, E. L. & Stockell, A. (1954). *J. Biol. Chem.* **207**, 504.
44) ———, ——— & Kimmel, J. R. (1954). *Ibid.* **207**, 551.
45) Hirs, C. H., Stein, W. H. & Moore, S. (1954). *Ibid.* **211**, 941.
46) Harfenist, E. J. (1953). *J. Am. Chem. Soc.* **75**, 5528.
47) Moore, S. & Stein, W. H. (1951). *J. Biol. Chem.* **192**, 663.
48) Narita, K. (1954). *Tanpakushitsu Kagaku* (The Chemistry of Protein) (Akabori, S. & Mizushima, S. editors) Vol. 1, [p. 201]. Tokyo; Kyoritsu Shuppan Co. Ltd.
49) Ceriotti, G. & Spandrio, L. (1957). *Biochem. J.* **66**, 607.
50) Chinard, F. P. (1952). *J. Biol. Chem.* **199**, 91.
51) Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.
52) Bailey, K. (1937). *Ibid.* **31**, 1406.
53) Sanger, F. (1945). *Ibid.* **39**, 507.
54) Levy, A. L. (1954). *Nature* **174**, 126.
55) Frankel-Conrat, H., Harries, J. I. & Levy, A. L. (1955). *Methods of Biochemical Analysis* (Glick, D. editors) Vol. 2, [p. 359]. New York; Intersience publ.
56) Sanger, F. (1949). *Biochem. J.* **45**, 563.
57) Betteheim, F. R. (1955). *J. Biol. Chem.* **212**, 235.
58) Akabori, S., Ohno, K., Ikenaka, T., Nagata, A. & Haruna, I. (1953). *Proc. Japan Acad.* **29**, 561.
59) Ohno, K. (1953). *J. Biochem.* (*Tokyo*) **40**, 621.
60) ——— (1954). *Ibid.* **41**, 345.
61) ——— (1956). *Tanpakushitsu Kagaku* (The Chemistry of Protein) (Akabori, S. & Mizushima, S. editors) Vol. 4, [p. 209]. Tokyo; Kyoritsu Shuppan Co. Ltd.
62) Lowther, A. G. (1951). Nature **167**, 767.
63) Narita, K. (1958). *Biochim. et Biophys. Acta* 28, 184.
64) Rüegg, J. C. (1958). *Biochem. J.* **69**, 46p.
65) Eliott, F., Hanson, J. & Lowy, J. (1957). *Nature* **180**, 1291.
66) Kahn, J. S. & Szent-Gyorgyi, A. G. (1959). *Science* **130**, 160.