ON FISH TROPOMYOSIN

I. Isolation and Amino Acid Composition

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Tropomyosin, which is one of the proteins to form part of muscle fibrils, was first discovered by Bailey[1] in 1946. It has been crystallized from muscles of several animals by dehydrating the minced muscle with organic solvents; the detailed properties[2)–7)] have been published on rabbit muscle tropomyosin.

In fish, Hamoir[8)] has isolated tropomyosin and nucleotropomyosin from carp muscle without preliminary dehydration and lately by means of electrophoresis has found two kinds of tropomyosins in salt extract.[9)] In the invertebrates, it has been also crystallized from squid,[10)] octopus and pinna.[11)] The amino acid composition, sedimentation and electrophoretic behaviors of squid muscle tropomyosin were described by Yoshimura.[10)]

Recently fish muscle proteins have been investigated electrophoretically from the view of point of comparative biochemistry.[9)] As fish represent the lowest classes of vertebrates, it should be permissible to draw general conclusions on the muscle proteins of vertebrates by comparison with fish muscle proteins and those of other animals'. In this study tropomyosin which can be obtained in high degree of purity from muscle fibrils is crystallized from haddock muscle by Bailey's method[2)] and its amino acid composition is reported.

EXPERIMENTAL

Isolation and purification

There have been found two kinds of methods to extract and isolate tropomyosin from muscle, Bailey's method[2)] and Hamoir's.[8)12)] The former method employs minced muscle for dehydration with organic solvents after which tropomyosin is extracted with high concentrated salt solution at neutral pH; and in the latter method, tropomyosin is extracted by the rather acid salt solution unfavarable for the extraction of myosin.

In the present experiment, tropomyosin is prepared from haddock muscle by Bailey's method.[2)] The fresh haddock muscle, in which tropomyosin contents comes to 0.34 per cent of fresh weight and the nitrogen is 2.99 per cent of total protein nitrogen, was kept in frozen state about −15 °C. to −20 °C. for two months. This treatment denatures actomyosin as completely as, and the isolation of tropomyosin is more easy. Minced muscle is washed with water and dehydrated in ethanol and ether. The fiber is extracted with M-KCl at pH 7 to give viscous extract. Step (1). The protein is precipitated at pH 4.6. Step (2). The precipitates are redissolved at pH 7 and impurity is removed by adding ammonium sulphate to 41 per cent saturation. Step (3). The mother liquid is further saturated to 75 per cent saturation, and the protein is salted out from the mother

liquor. The dialysed tropomyosin is purified by several method: the repetition of Step (1)-(3), heat treatment at neutral pH, crystallization and the treatment of ethanol and ether. The purity of several preparations is shown as Table 1.

Table 1. The purity of several preparations

Method	Nitrogen content (%)	Tryptophan content* (%)
Once performance of Step (1)-(3) and once crystallization	16.9	0.13
Once performance of Step (1)-(3) and twice treatment of ethanol and ether	16.8	0.04
Once performance of Step (1)-(3) and heat treatment at 100 °C., pH 7	16.7	trace
Thrice performance of Step (1)-(3)	16.8	0.04
Thrice performance of Step (1)-(3) and twice treatment of ethanol and ether	16.7	0.02
Thrice performance of Step (1)-(3) and thrice crystallization	16.7	0.01

* Tryptophan is estimated by means of the use of p-dimethyl amino benzaldehyd.

As tryptophan is not included in fish tropomyosin as well as in rabbit's, the tryptophan content is an index of the purity of the protein. The salted out protein of the first carrying out of Step (1)-(3) is easily crystallized by dialysis against a salt solution containing 16 g. ammonium sulphate per 1 liter and 0.01 M acetate buffer, pH 5.4 at 0 °C. This preparation contains some impurities and crystals in large plate are obtained as shown in Fig. 1 (a). However, the continued repetition of the Step (1)-(3) and thrice crystallization results in good purity and the form of elongated prisms as shown in Table 1 and Fig. 1 (b). Nine grams of this crystal are obtained from 1 kg. minced muscle. The elementary analysis is given in Table 2.

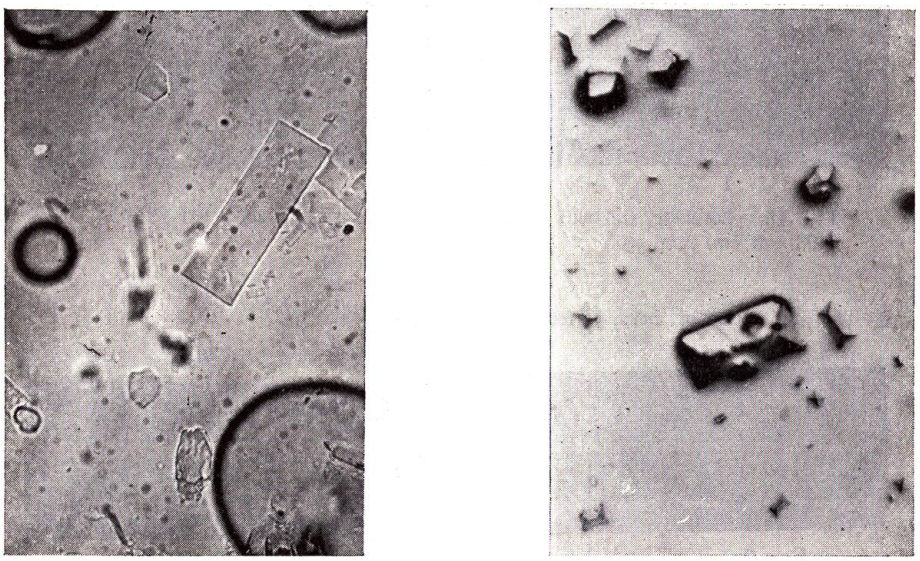
Electrophoretic analysis

The electrophoretic test is performed by Tiselius apparatus (cell size, 2 × 15 × 50 mm.) on the preparations as described above.

The obtained results are shown as Table 3 and Fig. 2. The preparation obtained from once carrying through of Step (1)-(3) and crystallization includes more impurity than that of thrice repeated Step (1)-(3) like the results obtained from the test of tryptophan contents. This impurity migrates with a mobility of about 70 per cent of that of tropomyosin. The preparation derived from thrice repetition of Step (1)-(3) and thrice crystallization has a high purity and a single boundary in glycine-HCl buffer and phosphate buffer, I=0.15, I=0.35, pH 2.5-3.5 and pH 5.9-8.0. The pH-mobility curve found is graphed in Fig. 3; the iscelectric point lies near pH 5.1 from the extrapolation.

(a) Crystals of once performance Step (1)-(3) and crystallization

(b) Crystals of thrice performance Step (1)-(3) and thrice crystallization



(× 180)

Fig. 1. Tropomyosin crystals from haddock skeletal muscle

Table 2. Elementary analysis of haddock tropomyosin

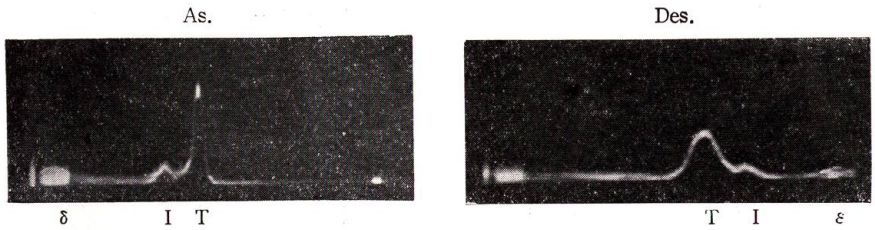
Nitrogen (%)	Sulphur (%)	Phosphorus (%)
16.7	0.76	0.03

Table 3. Electrophoretic analyses of preparations

Preparation		Tropomyosin		Impurity	
		ratio (%)	mobility*	ratio (%)	mobility*
Once performance of Step (1)-(3) and crystallization	Descending	83	-6.50	17	-4.44
	Ascending	83		17	
Thrice performance of Step (1)-(3)	Descending	95	-6.53	5	-4.39
	Ascending	92		8	

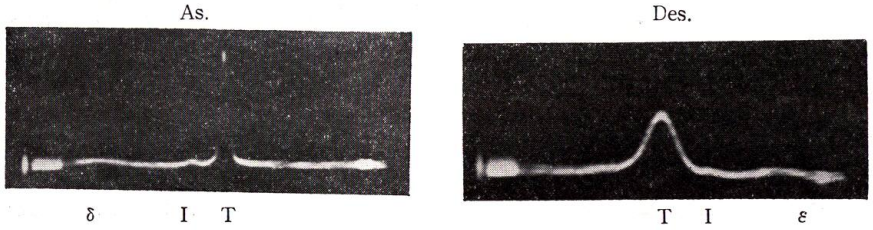
* × 10^5 cm^2/sec. volt.

(a) The preparation of once performance Step (1)-(3) and crystallization



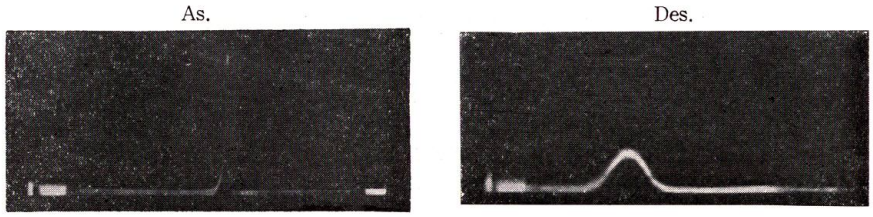
I=0.15 (phosphate, 0.1 and NaCl, 0.05) protein conc., 1.25% pH 7.40
3.98 volt/cm. 7200 sec.

(b) The preparation of thrice performance Step (1)-(3)

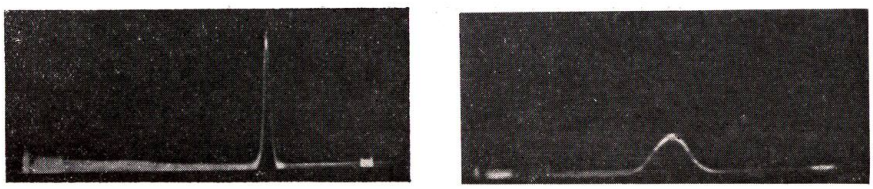


I=0.15 protein conc., 1.25% pH 7.40 3.98 volt/cm. 6308 sec.

(c) The preparation of thrice performance Step (1)-(3) and thrice crystallization



I=0.15 protein conc., 0.6% pH 7.70 4.30 volt/cm. 7230 sec.



I=0.15 (glycine-HCl, 0.1 and NaCl, 0.05) protein conc., 0.06% pH 2.91
4.44 volt/cm. 7200 sec.

Fig. 2 Electrophoretic diagram of haddock tropomyosion
(T : Tropomyosin I : Impurity)

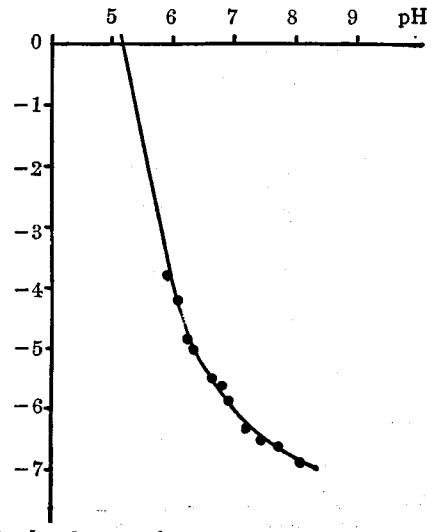


Fig. 3 pH-Mobility curve of haddock tropomyosin
[I=0.15 (phosphate, 0.1 and NaCl, 0.05), and calculated from descending boundary]

Amino acid composition

In the amino acid analysis of protein, the values of serine and threonine have been corrected by 10 per cent and 5 per cent for decomposition during hydrolysis according to the report of Rees.[13] The destruction rate of these amino acids is not only due to kinds of proteins, but the other amino acids are also decomposed by hydrolysis in 6 N HCl. The use of two periods of hydrolysis has been proved desirable.[14)–17]

The tropomyosin, which is purified by the thrice repetition of Step (1)–(3) and crystallization, is dialysed against water and dried in freezing. After disolution of the protein in pyrex tube in 200 volumes of re-distilled 6 N HCl, the solution is frozen in a bath of solid carbon dioxide and ethanol: the tube is evacuated to 10 mm. pressure and sealed. Hydrolysis is performed in an oven at 110 ± 2 °C. for 22 and 72 hours. The slight yellow hydrolysate is filtered on glass filter. Excess HCl is removed by repeated drying in vacuo; the residue is washed into volumetric flask and made up to desired volumn.

The chromatography of the hydrolysates is performed on Dowex 50 (X-8) column by the procedure of Moore and Stein,[18] except for no addition of detergent to all buffers and the elution of tyrosine and phenylalanine at 50°C. instead of 75°C. to avoid cracking the column. A 0.9 × 100 cm. column is used for acidic and neutral amino acids and 0.9 × 15 cm. column for the basic amino acids and amide nitrogen. In preliminary experiment, each amino acid in synthetic mixture is sufficiently eluted. Cystine and cysteine are determined on Dowex 2 column, 0.9 × 15 cm., as cysteic acid after oxidation of the protein by performic acid.[19] Micro Kjeldahl analysis on the hydrolysates give the nitrogen value on which percentile values are based. To convert of terms of protein weight, the nitrogen value of 16.7 per cent is used for tropomyosin. Amide nitrogen is determined independently in order to check the values calculated from the chromatographic results.

The elution curves of long column of 22 and 72 hours hydrolysates are given in Figs. 4 and 5. In the case of 22 hours hydrolysate, the variations in the base line of the elution curves are remarkably in comparison with 72 hours', especially before and after the valine: unknown asymmetric peaks obtained are shown in Table 4.

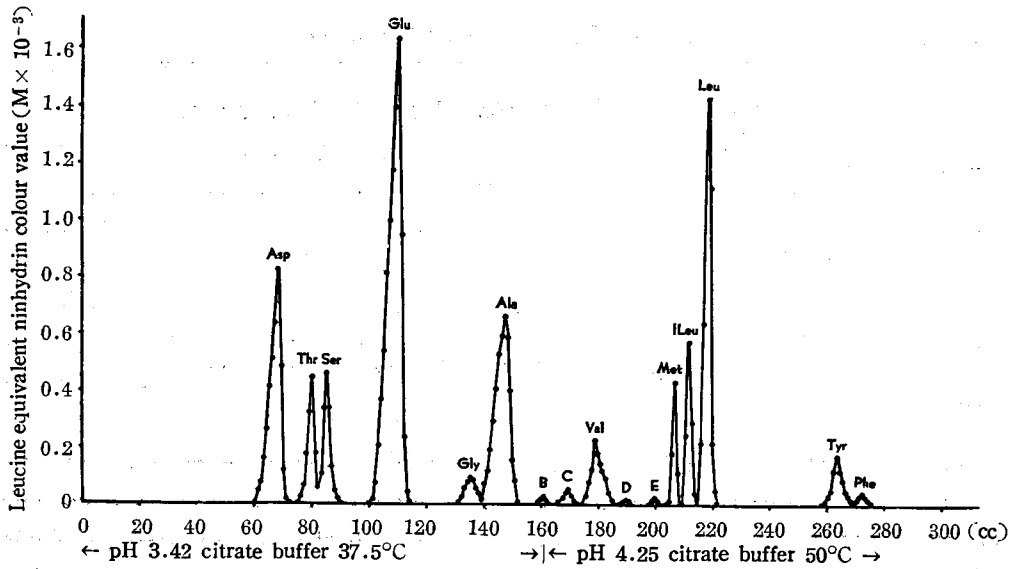


Fig. 4 Elution curve for a 22 hours hydrolysate of tropomyosin on a 0.9×100 cm. Dowex-50 (x-8) column (4.24 mg. of protein hydrolysate is used)

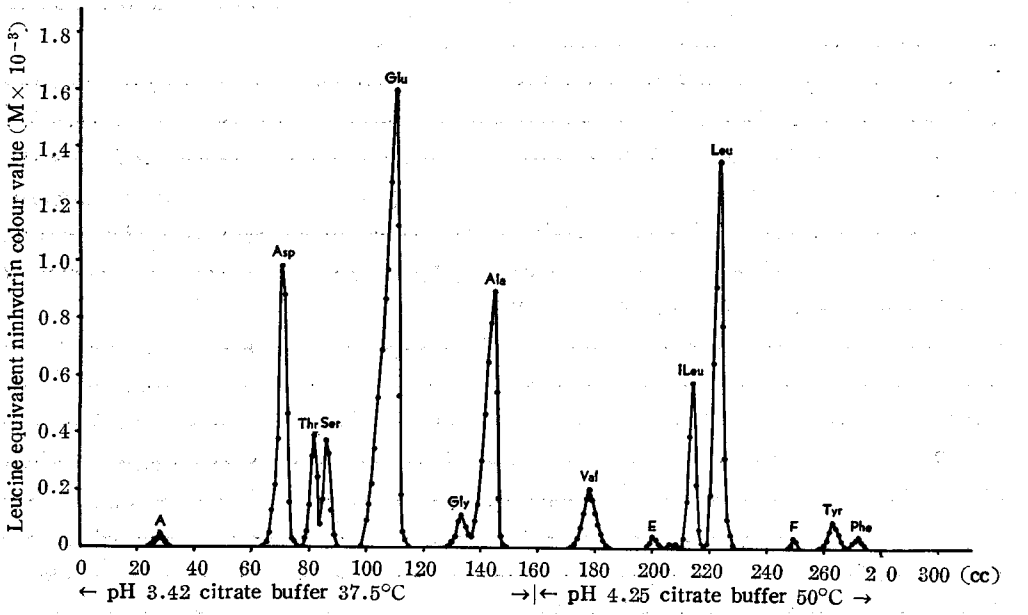


Fig. 5 Elution curve for a 72 hours hydrolysate of tropomyosin on a 0.9×100 cm. Dowex-50 (x-8) column (4.23 mg. of protein hydrolysate is used)

All peaks in Table 4 are not identified, but C and D, which are eluted before and after the valine in 22 hours hydrolysate and disappeared in 72 hours', should be certain peptides in view of the facts that compounds in which the carboxyl group of isoleucine

Table 4. Unknown peaks in hydrolysates

Fraction no. (cc.)	22 hours hydrolysate (N%)*	72 hours hydrolysate (N%)*
25 (A)		0.24
160 (B)	0.17	
170 (C)	0.38	
190 (D)	0.10	
200 (E)	0.20	0.22
250 (F)		0.15

* Nitrogen as per cent of total protein nitrogen in hydrolysate and the average value of three analyses

or valine are coupled in peptide linkage are not easily hydrolyzed by HCl and these peptides are eluted near the valine peak, On the other hand, peak E shows no variation in amounts of nitrogen per cent. As the change of buffer is performed at fraction number 160 cc. and the replacing of the buffer in column is completed at 200 cc., it seems to be an artifact which is eluted out.

On the short column, the variation of the base line after ammonia is both extreme in 22 and 72 hours hydrolysates as shown in Figs. 6 and 7.

In Table 5 are presented the analytical results calculated from three pairs of samples of 22 and 72 hours hydrolysates of tropomyosin on Dowex 50 columns. A comparison of the analytical values of each hydrolysate indicates certain decomposition of serine, threonine, tyrosine and lysine as the hydrolysis time is lengthened. Expecially, methionine is almost decomposed by hydrolysis and irregular peaks appears at the original position of methionine. On the other hand, amounts of valine, leucine, isoleucine and ammonia are increased by prolonged hydrolysis.

Assuming that the loses of amino acids accompany with the first order kinetic, the extrapolation to zero-time is used for the values of serine, threonine, tyrosine and lysine. But no calculation is performed for glutamic acid and ammonia because of their large average deviation. For the value of ammonia is applied that of independent analysis. For aspartic acid, glutamic acid, glycine, alanine, phenylalanine, histidine and arginine are adopted the average values of 22 and 72 hours hydrolysis, and the resuts of 72 hours for the values of valine, isoleucine and leucine. Then, for the value of methionine is applied the result of 22 hours' hydrolysis. The amino acid composition of tropomyosin is summarized in Table 6 from these results.

DISCUSSION

Haddock tropomyosin is easily crystallized in the form of a long prism from the salted out precipitates, depending upon the presence of salts to break down the large

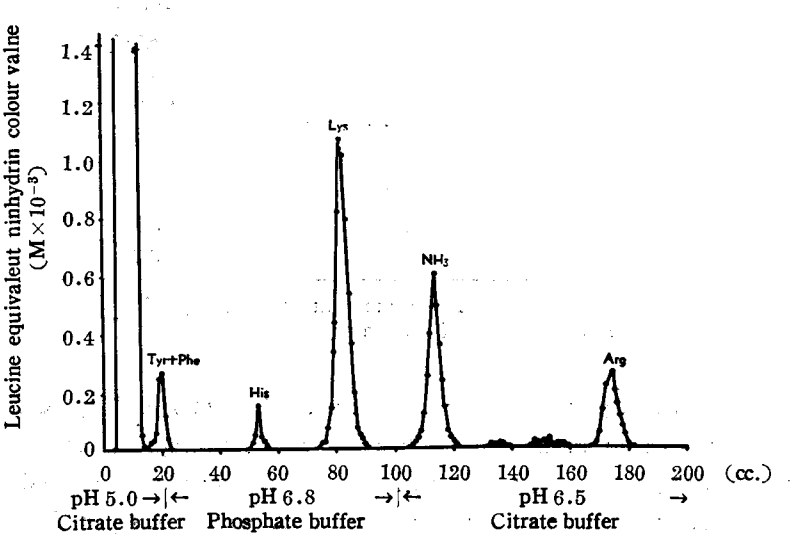


Fig. 6. Elution curve for a 22 hours hydrolysate of tropomyosin on a 0.9×15 cm. Dowex-50 (x-8) column (4.24 mg. of protein hydrolysate is used)

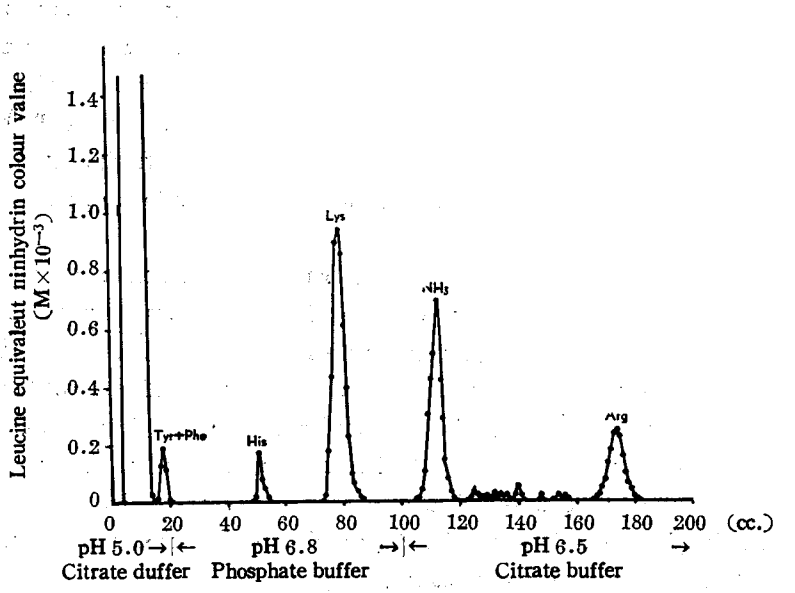


Fig. 7. Elution curve for a 72 hours hydrolysate of tropomyosin on a 0.9×15 cm. Dowex-50 (x-8) column (4.23 mg. of protein hydrolysate is used)

Table 5. Effect of hydrolysis time on amino acid composition of tropomyosin

Amino acid	Nitrogen as per cent of total protein nitrogen	
	22 hours hydrolysate	72 hours hydrolysate
Asp	7.16±0.17	7.14±0.11
Thr	2.69±0.02	2.28±0.02
Ser	2.96±0.04	2.24±0.04
Glu	20.04±0.30	19.63±0.40
Gly	0.99±0.05	1.01±0.03
Ala	8.55±0.01	8.38±0.05
Val	2.06±0.10	2.44±0.04
Met	1.48±0.06	
Ileu	2.31±0.04	2.69±0.04
Leu	7.48±0.01	8.45±0.00
Tyr	1.36±0.03	0.74±0.02
Phe	0.30±0.01	0.28±0.02
His	1.57±0.09	1.64±0.08
Lys	17.68±0.02	16.65±0.05
Amide-N	5.57±0.33	6.10±0.32
Arg	12.54±0.11	12.67±0.15

Analytical values are obtained from 4.23-4.24 mg. of protein hydrolysate and from 5.01 mg. protein hydrolysate for Gly., Tyr. and Phe.

Table 6. Amino acid composition of tropomyosin

Amino acid	Amino acid per 100g. of protein	Amino acid residue per 100 g. of protein	N as per cent of protein N	Moles of amino acid per 10^5 g. of protein	
Asp	11.36	9.82	7.15	85.4	85
Thr	4.11	3.49	2.89	34.4	34
Ser	4.19	3.47	3.34	39.8	40
Glu	34.82	30.57	19.84	236.6	237
Pro	0	0	0	0	0
Gly	0.90	0.68	1.00	11.9	12
Ala	9.00	7.18	8.47	101.0	101
Val	3.41	2.89	2.44	29.1	29
Met	2.64	2.32	1.48	17.6	18
Ileu	4.22	3.64	2.69	32.1	32
Leu	13.26	11.44	8.45	101.0	101
Tyr	3.88	3.49	1.79	21.4	21
Phe	0.58	0.52	0.29	3.5	3.5
His	0.99	0.88	1.61	6.3	6
Try	0	0	0	0	0
Lys	16.64	14.59	19.05	113.8	114
Amide-N	0.86*		5.15*	61.0*	61*
Arg	6.57	5.89	12.61	37.7	38
Cys/2	0.78	0.65	0.55	6.5	6.5
Total	117.35	101.52	98.80		878

* Omit from the total value

aggregates. The tropomycsin has a unique property toward salts: it can be depolymerized and fragmented by salt concentration and the crystals have to make enormus hydration just as in the case of rabbit tropomyosin. Because of this, the form of crystals and the purity are extremely effected by impurities; it is necessary to crystallize tropomyosin from precipitates which have been purified as completly as possible.

In Table 7, the electrophoretic mobility of haddock tropomyosin is compared with that of other origins. The mobility of haddock tropomyosin is rather in agreement with that of carp and is closer to rabbit's to squid's. These facts should be expected from the amino acid composition.

The amino acid composition of haddock, squid and rabbit tropomyosin is shown in Tables 8 and 9. Like rabbit and squid protein, haddock tropomyosin contains no tryptophan and proline. In the tropomyosins of invertebrates,[10,11] aspartic acid and the amide nitrogen is much higher and arginine appers to have replaced part of lysine, but these facts do not hold in the

Table 7. Electrophoretic mobilities* of tropomyosins

Buffer	Haddock	Carp[8]	Squid[10]	Rabbit[20]
I=0.35 (phosphate 0.1, NaCl 0.25) pH 7.1	-4.09	-3.90	-2.80	-4.9
I=0.15 (phosphate 0.1, NaCl 0.05) pH 7.4	-6.58	-6.20	-5.71	

* $\times 10^5 cm^2/sec.$ volt.: calculated from descending boundary

Table 8. Comparative amino acid composition of tropomyosins

Amino acid	Moles of amino acid per 10^5g. of protein		
	Haddock	Squid*	Rabbit[7]
Asp	85	100	89
Thr	34	32.5	28
Ser	40	55	40
Glu	237	212	211
Pro	0	0	0
Gly	12	28	12.5
Ala	101	95	110
Val	29	25	38
Met	18	17	16
Ileu	32	19.5	29
Leu	101	95	95
Tyr	21	8	15
Phe	3.5	8	3.5
His	6	12	5.5
Try	0	0	0
Lys	114	90	110
Amide-N	(61)	(94)	(64)
Arg	38	68	42
Cys/2	6.5	6	6.5
Total	878	871	851

* Being corrected on data of Yoshimura[10] by the private communication of Dr. K. Bailey.
( ) Omit from the total value.

Table 9. Amino acid group per cent of tropomyosins

Group	Haddock	Squid[10]	Rabbit*
Free acid group	29.7	25.0	27.8
Base group	18.0	47.7	19.6	44.6	18.5	46.3
Hydroxy group	10.8		10.9		9.8	
Amide group	7.0		10.8		7.4	
Polar group	66.2		66.9		64.3	
Non-polar group	33.8		33.1		35.7	

* Calculated from data of Kominz et al.[7]

case of haddock tropomyosin. Hoddock tropomyosin is also more close to rabbit tropo myosin in amino acid composition than to squid, although some differences are recognized with respect the number moles of amino acid.

SUMMARY

Haddock tropomyosin is crystallized in high purity. The electrophoretic mobility is more close to that of mammalian tropomyosin than to invertebrate's, and simular facts are also recognized in the amino acid composition.

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