ON FISH TROPOMYOSIN

II. Terminal Residue in Tropomyosins

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Bailey[1] has obtained negative results for N-terminal residue in rabbit skeletal tropomyosin and suggested that the tropomyosin is composed a large cyclic peptide chain, together with the report of physico-chemical results.[2] On the other hand, the C-terminal residue in rabbit skeletal tropomyosin has been proved by Locker[3] to possess one isoleucine residue, who used the carboxypeptidase method and the hydrazine method. The C-terminal sequence has been also found to be isoleucine-serine-threonine-methionine-isoleucine-alanine-. It seems to be of interest in respect to protein structure that the number does not coincide in N- and C-terminal residue.

As results of that the amino acid composition has been investigated on tropomyosin from the different types of mammalian muscle, from lower classes of vertebrates and invertebrates phyla. The differences according to animal origin have been remarkably observed in the contents of lysine, arginine and amide nitrogen.[4)-7)]

In this study, assay of N-terminal residue in fish tropomyosin is carried out by Sanger's DNFB (dinitrofluorobenzene) method[8] and of C-terminal residue by the standard hydrazine method of Akabori and Ohno.[9)-12)] It is shown that fish tropomyosin contains no N-terminal residue and possesses one isoleucine residue for C-terminal residue. In addition to fish, the same results are obtained in bull frog and squid tropomyosin.

EXPERIMENTAL

Protein preparation

Haddock and bull frog tropomyosin, which was purified by repeating the precipitation and salting out as described in previous paper,[7] were crytallized three times, using Bailey's method.[13] Squid tropomyosin is crystallized three times by Yoshimura's method.[4] These tropomyosins dissolved in 0.1 M KCl at pH 7.5 are dialyzed against water at 2°C for two days. Viscous solution obtained after filteration is freezing-dried to fiber. Molecular weight of tropomyosin adopts 53,000 for haddock and bull frog and 54,300 for squid.

Assay for N-terminal residue

The DNFB method is applied for the detection and estimation of N-terminal residue in proteins. Since the most of DNP-amino acids are very sensitive to light in dilute solution,[14] the procedure of all analysis is carried out in dark room.

Haddock tropomyosin is treated with DNFB under the conditions recommended by Sanger.[8] After the DNP-protein is hydrolyzed in sealed glass tube by 6 N HCl at 100°C

for desired time, hydrolysate is extracted by ether to divide ether soluble fraction and water soluble fraction. Amide nitrogen in DNP-protein is determined in order to check amounts of original protein.

Ether soluble fraction: After evaporating ether, dinitrophenol in residue is sublimated under the conditions of 80°C and 1 mm. pressure. This sublimation of dinitrophenol is repeated three times; the ether solution of residue is chromatographed. Each spot, which is separated by two dimensional paper chromatography of Levy,[15] is eluted in 5 cc. of 1 per cent $NaHCO_3$ at 60°C for 15 min. and estimated at 360 mμ. Obtained results are shown in Table 1.

Table 1. DNP-amino acids in ether soluble fraction from molar haddock tropomyosin

DNP-amino acid	Hydrolysis time		
	4 hrs.	8 hrs.	16 hrs.
	(mol)	(mol)	(mol)
Asp or Glu	trace	trace	trace
Ala	trace	trace	trace
Di-Lys	trace	trace	trace
Leu or ILeu	0.05	0.06	0.08

In the detected four types of DNP-amino acid, DNP-leucine or DNP-isoleucine is obtained to the extend of no more than 0.1 mol per molar haddock tropomyosin and in trace for the other DNP-amino acids. The total molar of these DNP-amino acids is only about 0.1 mol per molar protein, these quantities seem to be arisen from some impurities. Four types of ether soluble DNP-amino acid attributable to impurities have been also observed in rabbit tropomyosin.[1]

The results obtained from bull frog and squid tropomyosin at 16 hours hydrolysis are shown in Table 2. In these cases, each DNP-amino acid which seems to be due to impurities, does not reach to 0.1 mol per molar protein.

Table 2. DNP-amino acids in ether soluble fraction from molar bull frog and squid tropomyosin

DNP-amino acid	Bull frog	Squid
	(mol)	(mol)
Asp or Glu	0.02	——
Ala	trace	0.03
Di-Lys	trace	——
Leu or ILeu	0.06	0.05

Values obtained from 16 hours hydrolysate of DNP-protein.

Water soluble fraction: Water soluble DNP-amino acid is detected in 16 hours hydrolysate of DNP-protein by use of paper chromatography, solvent system n-BuOH: CH_3COOH: H_2O = 250: 60: 250[16] and acetone: 3M CH_3COOH = 92: 8,[17] and with silica gel column chromatography, solvent system 66% methylethylketone-ether.[8] The spot or band corresponding to ε-DNP-lysine is merely detected in each tropomyosin.

From these results, no N-terminal residue exists in haddock tropomyosin and the same facts are found in bull frog and squid tropomycsin.

Assay for C-terminal residue

C-terminal residue is detected by the method of Akabori and Ohno[9)-12)] on 20-30

mg. of haddock tropomyosin which is dried over P_2O_5 at 100°C for 4 hours.

Prelimiary test: The protein is hydrazinolyzed with about 1 g. of anhydrous hydrazine in sealed glass tube at 100°C for 10 hours. After the excess of hydrazine is removed in vacuo over conc. H_2SO_4, the residue is dissolved in 5 cc. of water. For the removal of amino acid hydrazide which is formed in resionous precipitate with aldehyde, 0.3 cc. of isovaleraldehyde is added to the solution. Solid $NaHCO_3$ is added to the solution, maintaining pH 7 and stirring occasionaly for 20 min. The precipitate formed is filtered off and washed twice with 1 cc. of water. The removal of amino acid hydrazide is repeated again, and the solution is extracted with 5 and 3 cc. of ethyl acetate. The combined ethyl acetate layer is washed with 3 cc. of water and is rejected. The water phase obtained, about 10 cc. is subjected to dinitrophenylation with 0.3 cc. of DNFB in ethylalcohol 20 cc. and 0.3 g. $NaHCO_3$. After shaking for 2 hours in dark at room temperature, the reaction mexture is diluted with 50 cc. of water. The solution acidified with 2 N HCl is extracted with 30, 20 and 10 cc. of ethyl acetate. The ethyl acetate combined is extracted with 30,30, 20 and 10 cc. of 2 per cent $NaHCO_3$, and the $NaHCO_3$ solution combined is washed with 30 and 20 cc. of ethyl acetate. Most of DNP-amino acid and a part of isovaleral derivatives of DNP-aspartic and DNP-glutamic monohydazides are transferred into the $NaHCO_3$ solution, while all of di-DNP-tyrosine and a part of di-DNP-histidine remain in ethyl acetate layer. Fraction A: the $NaHCO_3$ solution is acidified with 2 N HCl and extracted with 30, 20 and 10 cc. of ethyl acetate. After evaporating ethyl acetat, dinitrophenol in residue is sublimated three times and chromatographed by Levy's method like as in the assay for N-terminal residue. Fraction B: after ethyl acetate layer is evaporated in vacuo, the residue dissolved in 50 cc. of ether is extracted with 30, 20 and 10 cc. of 2 per cent $NaHCO_3$ solution. The $NaHCO_3$ solution combined is acidified with 2 N HCl and extracted with ether. The residue, obtained from ether fraction, is chromatographed by Levy's method.

To ascertain spot separated, the eluted solution of spot is hydrolyzed with 6 N HCl for 4 hours, and rechromatogrpahed. The strong spot of DNP-leucine or DNP-isoleucine and the trace of DNP-alanine, DNP-glutamic acid or DNP-aspartic acid are obtained from only fraction A.

Identification of C-terminal amino acid by Dowex-50 chromatography: Hydrazinolysate of 52.1 mg. haddock tropomyosin is treated with isovaleradehyde as described above, except for the use of pyridine instead of $NaHCO_3$ to adjust pH. Aqueous solution washed with 5 and 3 cc. of ethyl acetate is dried up and the residue is chromatographed by the method of Moore and Stein[18] as shown in Fig. 1. Isoleucine, most positive free amino acid is obtained 0.69 mol per molar protein and for glutamic acid, 0.06 mol, and alanine, 0.04 mol. The strong spot DNP-leucine or DNP-isoleucine found in preliminary test, is due to isoleucine which is in one C-terminal residue of the protein. Other amino

acid, glutamic acid and alanine seem to be caused by impurities.

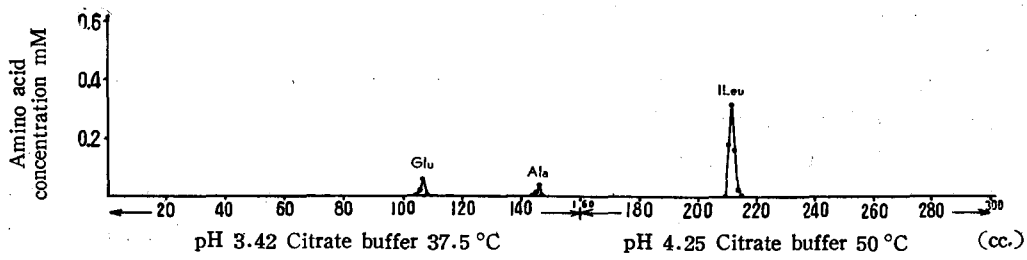

Fig. 1. Elution curves of free amino acids for a 10 hours hydrazinolysis of haddock tropomyosin on a 0.9×100 cm. Dowex-50 (X-8) column (52.1 mg. of protein hydrazinolysate is used, after isovaleraldehyde treatment)

Estimation of DNP-amino acid from C-terminal residue: Haddock tropomyosin is hydrazinolyzed for 5, 10, 15 and 20 hours, and treated as in preliminary test. Spot separated from fraction A is eluted and estimated at 360 mμ. The results obtained are shown in Table 3. Isoleucine yields about 1 mol per molar protein for every hydrazinolysis time, and seems to be correspond to C-terminal residue for haddock tropomyosin.

Table 3. DNP-amino acids in hydrazinolysate from molar haddock tropomyosin

DNP-amino acid	Hydrazinolysis time			
	5 hrs.	10 hrs.	15 hrs.	20 hrs.
	(mol)	(mol)	(mol)	(mol)
ILeu	0.72	1.00	0.75	0.65
Glu	0.05	0.05	0.04	0.01
Ala	0.02	0.03	0.01	trace

The values of isoleucine are corrected as follows: its recovery yields 65% for 5 hours hydrazinolysis, 65% 10 hours, 62% 15 hours and 55% 20 hours.

Isoleucine has been detected in about 25 per cent of the theoretical amount for C-terminal residue in rabbit tropomyosin,[3] and in the trace amount of glutamic acid, serine, glycine and alanire for impurities. But in this study, the yields of isoleucine are better on 10 hours hydrazination, about 0.65–0.52 mol per molar protein without correction.

In Table 4 is given the estimations of DNP-amino acid on 10 hours hydranization of bull frog and squid tropomyosin, after the performance of preliminary test and of identification by Dowex-50 chromatography. Isoleucine is also detected as C-terminal residue in both proteins.

Table 4. DNP-amino acids in hydrazinolysate from molar bull frog and squid tropomyosin

DNP-amino acid	Bull frog	Squid
	(mol)	(mol)
ILeu	0.81	0.81
Ser	0.03	——
Glu	——	0.04
Ala	trace	trace

Values obtained from 10 hours hydrazinolysates of proteins, and isolucine is corrected as in Table 3.

DISCUSSION

The tropomyosin of haddock, fish, have no N-terminal and one isoleucine for C-terminal residue. The same facts are found in tropomyosin of bull frog, amphibia, and of squid, cephalopoda.

On the amino acid composition of general protein, the specificity of animal origin is remarkable in the contents of neutral and aromatic amino acid, for example, serum albumin,[19] and hemoglobin.[20,21] The differences of terminal amino acid according to animal origin are also observed in these proteins: leucine or alanine corresponds to C-terminal residue for serum albumin[22] and valine or methionine to N-terminal residue for B chain of hemoglobin.[23]

Although there are some specificities to general amino acid composition of tropomyosin, high acidic amino acid, arginine and lysine contents, it is observed regularity that arginine replaces some of lysine[4-6] with alteration from high phyla to low phyla and this replacement is completed in annelid.[6] Fish tropomyosin is distinctly more close to mammalian tropomyosin in general property than to that of invertebrates.[7] But the quantity and the quality of terminal amino acid is the same in rabbit, haddock, bull frog and squid.

It may be considered respecting the molecular pattern of rabbit tropomyosin possibility of cyclopeptide structure having a branch point at glutamic γ- or aspartic β-linkage is caused by the fact of no N-terminal and one C-terminal residue, together with its axial ratio. The same fact, that does not agree in the number of each terminal, has also been observed in pepsin[24] and taka-amylase.[25] In the view of molecular shape, tropomyosin of fish, bull frog and squid may be the same as rabbit's from the results obtained.

SUMMARY

Terminal residue of fish tropomyosin is studies, using DNFB method for N-terminal and hydrazine method for C-terminal residue.

Fish tropomyosin has no N-terminal residue and one isoleucine for C-terminal residue the same as rabbit's. In addition to fish, the same results obtain in bull frog and squid tropomyosin.

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