The Structure of the Junction between Short Subfragment-2 and the Hinge from Adult Chicken Smooth Muscle Myosin.

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Abstract In the preceding papers [Watanabe B (1989), Biol. Chem. Hoppe-Seyler 370(9): 1027-1034 and Watanabe B, Tanigawa M (1993), Biol. Chem. Hoppe-Seyler 374(1): 27-35] we reported primary structures of subfragment-2 (S-2) from adult chicken skeletal and cardiac muscle myosins. This paper describes the structure of the junctional region between the short subfragment -2(short S-2) and the hinge from adult chicken gizzard muscle myosin.

The rod obtained by digesting myosin with papain was further subdivided into S-2 and light meromyosin (LMM) by limited digestion with α-chymotrypsin. A 134 amino-acid-residue peptide covered with carboxy-terminal portion of the short S-2 and amino-terminal portion of the hinge was isolated by conventional method from cyanogen bromide (CNBr) digests of S-2 and sequenced. To analyse the structural differences, the sequence of this junction thus determined was compared with those of corresponding portions of embryonic chicken gizzard, chicken brain, drosophila, chicken skeletal and chicken cardiac muscle myosin.

Interestingly, results show the higher sequence identities of 100%, 81.3% and 59.7% with embryonic chicken gizzard,chicken brain and drosophila nonmuscle myosin,respectively, and lower degrees, 38.8% and 40.3% with chicken skeletal and chicken cardiac ventricular muscle myosin respectively.

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Key words : amino-acid sequence, primary structure, smooth muscle myosin, isoform, the short subfragment-2, the hinge,

Introduction

Myosin is an important component of the contractile system that is present in all muscle and nonmuscle cells. It is composed of two heavy chains and two pairs of light chains and shares the same basic molecular structure. The myosin heavy chains (MHC) form a dimer consisting of two globular amino(NH_2)-terminal heads (subfragment-1, S-1) and carboxy(COOH)-terminal α-helical coiled-coil rods.

The rod are involved in the assembly of myosin molecules into filaments, whereas the heads contain an actin-activated Mg^{2+}-ATPase activity.[1]

Thus MHCs have been extensively studied the structure, function and regulation at the molecular level in skeletal and cardiac muscle. The role of the rod has been mainly investigated in self-assembling ability, however, many recent studies have revealed the important properties of the short S-2, the hinge,the short S-2/the hinge and the hinge/LMM junction for the contractile force generation and speed or for the isoform-specific function of the myosin.[2)-6)]

In striated muscle, MHC isoforms are encoded by a multigene family,[7)8)] however, smooth muscle MHC isoforms are the products of alternative mRNA processing from a single gene.[9)~13)] The characterization of the tissue specific and developmental isoform of the nonmuscle and smooth muscle MHC is not fully explored.

Furthermore, sequence analysis at the protein level is necessary for revealing the post translational processing of proteins. The amino-acid differences between sequences obtained by protein analysis and by cDNA sequencing have been reported.[14)]

The structural differences of the developmental isoforms of smooth muscle MHC between embryonic and adult chicken were suggested.[15)16)]

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From these reasons, the sequence of the junctional region of the short S-2 and the hinge from adult chicken gizzard muscle MHC was determined by direct protein sequencing.

Materials and Methods

preparation of S-2

Myosin was prepared from adult chicken gizzard muscle[17)-18)] and was digested with papain in 200mM CH_3COONH_4 and 5mM $MgCl_2$, pH 7.2 at 20℃ for 15min, according to the method as described.[19)] The generated rod was separated from S-1 by centrifugation and purified by ethanol precipitation. S-2 was obtained by digesting the rod with α-chymotrypsin at a substrate to enzyme ratio of 100 : 1(w/w) in 0.5M KCl, 100mM EDTA, and 20mM Tris-phosphate, pH 7.0 at 25℃ for 10min. S-2 was separated from LMM according to its solubility in lower ionic strength buffer, and was reduced and S-carboxymethylated.[20)] S-carboxymethylated S-2 (CM-S-2) was separated from LMM by DEAE-cellulose (DE-52, 2×18cm, Whatman Biochemical Co.) ion-exchange column chromatography in the presence of 8M urea. SDS polyacrylamide gel electrophoresis (SDS-page) was performed to examine the purity of myosin, the rod and CM-S-2.[21)]

Chemical cleavage of CM-S-2

CNBr cleavage was carried out in 70% formic acid at 30℃ for 24h with 200-fold excess of CNBr per Met.[22)] Partial hydrolysis of a peptide was performed with 0.4M formic acid at 110℃ for 2h in evacuated sealed tube.[23)]

Enzymatic cleavage of CM-S-2

CM-S-2 was digested with lysyl endopeptidase (S/E=100/1, w/w, Wako Pure Chemical Co.) in 0.1M Tris-HCl buffer, pH 8.0 at 37℃ for 6h.

Separation of peptides

CNBr fragments and lysyl endopeptidase peptides were applied to columns of Sephadex G-100 (1.8×90 cm, Pharmacia Co.) and Sephadex G-50 (1.8×180 cm, Pharmacia Co.) respectively, and were eluted with 50mM NH_4HCO_3, pH 8.5.

Peptides in each pool of above columns and hydrolysed peptides of a long CNBr fragment with formic acid were purified by reverse-phase HPLC

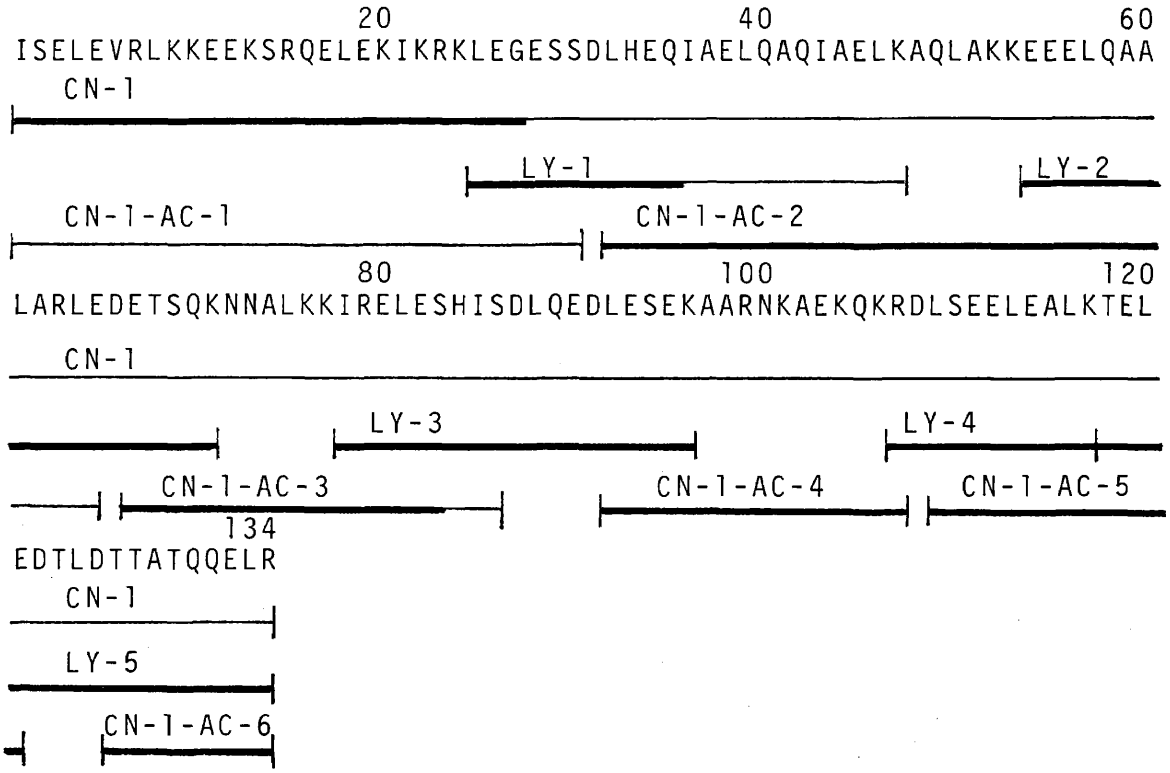

Fig. 1 Summary of sequence studies of the junctional region between the short S-2 and the hinge from adult chicken gizzard muscle myosin heavy chain. Thick lines show the amino-acids determined by automated Edman degradation. CN, AC and LY stand for peptides cleaved with CNBr, formic acid and lysyl endopeptidase, respectively.

(RP-HPLC) on columns of μBondasphere C18 (0.4 × 15cm, Millipore Co.), Puresil C18 (0.4 × 15cm, Millipore Co.) or TSK 120T (0.4 × 25cm, Toyosoda Co.) with acetonitrile gradient elution in 0.1% trifluoroacetic acid (TFA).

Amino-acid analysis and sequence determination

Amino-acid compositions of isolated peptides were analysed using a JLC-300 automatic amino-acid analyser (JEOL Co.) by the method described in literature after hydrolysis with 5.7M HCl at 110℃ for 22h in evacuated sealed tubes.[24]

Sequence determination was carried out in a 476A

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                                                                    40
(1)chicken gizzard      ISELEVRLKKEEKSRQELEKIKRKLEGESSDLHEQIAELQ
(2)chicken brain        +TD++E+++++++T++++++A++++D++TT++QD++++++
(3)drosophila           +T+++E++H+DQQQ+++SDRS+++I+T+VA++K++LN+RR
(4)chicken skeletal     VDD++GS+EQ+K+L+MD++RA++++++DLKLA+DS+MD+E
(5)chicken cardiac      VDD++GS+EQ+K+V+MD++RA++++++DLKLTQ+SVMD+E

                                                                   100
(1)AQIAELKAQLAKKEEELQAALARLEDETSQKNNALKKIRELESHISDLQEDLESEKAARN
(2)+++E+++I+++++++++++++++GDE+AV+++++++V++++QAQ+AE++++++++++S++
(3)V+VD+MQ+++++R++++TQT+L+IDE+SAT+AT+Q+AQ+++++QLAEI+++++A+++++A
(4)NDKQQ+DEK+K++DF+ISQIQSKI+++QALGMQLQ+++K++QAR+EE+E+EI+A+RTS+A
(5)NDKLQMEEK+K+++F+MSQLNSKI+++QAIVMQLQ+++K++QAR+EE+E+E++A+R+++A

                                                                   134
(1)KAEKQKRDLSEELEALKTELEDTLDTTATQQELR
(2)++++++++++++++++++++++++++++++A+++++
(3)++++VR++++++++++N++L+S+++++A+++++
(4)++++HRA+++R+++EISER++EAGGA++A+I+MN
(5)+V+++RS++AR+++E+SER++EAGGA++A+L+MN
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Fig. 2 Comparison of the junctional regions of known myosins. From the top, (1) adult chicken gizzard muscle (present study) and embryonic chicken gizzard muscle,[29] (2) chicken brain,[28] (3) drosophila nonmuscle,[30] (4) adult chicken skeletal muscle,[31] (5) adult chicken cardiac muscle,[32] myosin heavy chain, respectively. Identical residues among chicken gizzard (1) and others are indicated by '+'.

Table. 1 Amino-acid compositions of CN-1, hydrolysed peptides (AC) of CN-1 with dilute formic acid and lysyl endopeptidase peptides (LY) of CN-1.

Amino acids	CN-1	CN-1 AC-1	CN-1 AC-2	CN-1 AC-3	CN-1 AC-4	CN-1 AC-5	CN-1 AC-6	LY-1	LY-2	LY-3	LY-4	LY-5
Asp	10.4(10)			2.2(2)	1.1(1)				1.0(1)	2.3(2)	0.9(1)	2.0(2)
Thr	5.9(6)			0.9(1)		0.9(1)	3.1(3)		0.9(1)			4.7(5)
Ser	8.1(9)	3.5(4)		2.5(3)	0.8(1)	0.8(1)		2.6(3)	0.8(1)	2.6(3)	0.9(1)	
Glu	40.2(39)	9.4(9)	12.4(12)	4.4(4)	4.5(4)	5.2(5)	3.2(3)	8.4(8)	7.4(7)	6.3(6)	3.2(3)	5.4(5)
Gly	1.0(1)	0.8(1)						1.1(1)				
Ala	13.5(14)		7.7(8)	1.0(1)	2.7(3)	0.9(1)	1.0(1)	2.9(3)	2.7(3)		1.0(1)	1.0(1)
Val	1.2(1)	0.7(1)										
Ile	5.6(6)	2.1(2)	1.8(2)	2.0(2)				1.9(2)		2.0(2)		
Leu	21.2(21)	4.2(4)	7.2(7)	2.2(2)	1.0(1)	4.2(4)	0.9(1)	4.2(4)	2.9(3)	3.3(3)	3.0(3)	2.9(3)
His	2.2(2)		0.8(1)	0.8(1)				0.8(1)		0.7(1)		
Lys	17.6(18)	6.2(6)	3.1(3)	3.1(3)	4.1(4)	1.0(1)		1.1(1)	1.2(1)	0.9(1)	0.9(1)	
Arg	7.1(7)	3.1(3)	1.0(1)	0.9(1)	1.8(2)		0.8(1)		1.1(1)	0.9(1)	1.1(1)	0.9(1)
Total	134	30	34	20	16	13	9	23	18	19	11	17
Position	1/134	1/30	32/65	67/86	92/107	109/121	126/134	25/47	54/71	78/96	107/117	118/134

Cysteine, methionine, tyrosine and phenylalanine were not detected in this region.
Values in parentheses were taken from the sequence data.

micro sequence analysis system (Parkin Elmer Co.) using a pulsed liquid method.[25]

Results

Typically aproximately 250mg of crude CM-S-2 was obtained from 2Kg of gizzard muscle. By ion-exchange column chromatography on DE-52 (Fig. 3), 80mg of purified CM-S-2 was prepared.

Purification and sequence analysis of the junctional region

CM-S-2 (40mg) was cleaved with CNBr in 70% formic acid under nitrogen at 30℃ for 24h. Resulting fragments were fractionated by Sephadex G-100 (1.8×90cm) column chromatography into five pools (Fig. 4). A large peptide of the junctional region (CN-1) between the short S-2 and the hinge was isolated from pool 1 in Fig. 4 by following RP-HPLC on a column of μBondasphere (0.4×15cm) (Fig. 5). The amino-acid composition of this CN-1 are analysed (Table. 1) and the partial sequence of the NH_2-terminal portion was determined up to 27th residue by automated Edman degradation (Fig. 1).

Subfragmentation, purification and sequence analysis of CNBr peptides, CN-1

CN-1 was subfragmented with 0.4M diluted formic acid at 110℃ for 2h, and generated peptides were directly subjected to RP-HPLC on a column of Puresil C18 (0.4×15cm) (Fig. 6).

Six hydrolysed peptides (CN-1-AC-1 to CN-1-AC-6) were purified and amino-acid composition of each peptide was analysed (Table. 1). These peptides were sequenced completely or partially except for CN-1-AC-1 (Fig. 1).

To obtain overlapping fragments of hydrolysed peptides, CM-S-2 (40mg) was digested with lysyl endopeptidase (S/E =100/1, w/w) at 37℃ for 6h and the digest was separated into 4 pools by Sephadex- G50 (1.8×180cm) column chromatography (Fig. 7). Five lysyl endopeptidase peptides were purified from pool 2 and 3 in Fig. 7 by following RP-HPLC on a column of Puresil (0.4×15cm).

LY-1, LY-2, LY-3 and LY-5 were isolated from pool 2 in Fig. 7 (Fig. 8) and LY-4 was from pool 3 in Fig. 7 (Fig. 9), respectively. Amino-acid compositions of these five peptides were summarized in Table.1 These lysyl endopeptidase peptides were sequenced completely (LY-2 to LY-5) or partially (LY-1) by automated Edman degradiation. From above results, the complete amino-acid sequence of the junctional portion between the short S-2 and the hinge from adult chicken gizzard muscle MHC was established (Fig. 1).

Discussion

In striated muscles including cardiac muscle, many myosin heavy chain (MHC) isoforms have been reported to be formed a large multigene family. Their expression is regulated by various physiological or pathophysiological states.

This isoform diversity is necessary to enable muscles to modulate its contractile properties in response to internal or external stimuli.

Although, the rod is involved in the assembly of myosin molecules into filaments ,recently many studies have been demonstrated the important roles of the short S-2, the short S-2/the hinge junction, the hinge and the hinge/LMM junctional portions on the modulation of ATPase activity and muscle contraction force and speed.[2)-6)]

Moreover, it has been known that isoform specific regions are located in S-2.[3)] Therefore, it may be possible to thought that S-2 affects the binding of Mg^{2+} -ATP and actin to S-1. In smooth muscles, MHC isoform diversity has been shown to be occured at NH_2-terminal 25KDa-50KDa junction and COOH-terminal portion in the myosin molecule by their cDNA analysis.[15), 26)-28)] These isoforms are likely to be encoded by a single gene, and above four isoforms are produced by alternative mRNA splicing.[29)] The structure, function, regulation of enzymatic activity and expression of distinct isoforms of smooth muscle MHC are relatively unexplored compared with striated muscle MHC.

Our interest has been in the structure-function relationship of S-2 in the myosin and tissue specific expression mechanism in the physiological or pathophysiological diversity of muscle tissues. The sequence determination by direct protein analysis is necessary to elucidate the molecular basis of functions or post translational modifications in distinct isoforms. In the present paper, the structure of the short S-2/the hinge junctional portion in S-2 from adult chicken gizzard muscle MHC is described.

The results demonstrated here(Fig.1) are completely identical with that of cDNA sequence from embryonic chicken gizzard muscle MHC,[30)] and any

amino-acid difference could not be observed in these portions. Any heterogeneity was not recognized. The periodicity of hydrophobic and charged residues in the 7- and 28-residue units and frequency values of hydrophobic residues at "a" plus "d" in the 7-residue repeat unit pattern[1] in this junctional portion are well conserved. This sequence was compared with corresponding regions of chicken brain MHC,[28] drosophila nonmuscle MHC,[30] chicken skeletal muscle MHC[31] and chicken cardiac muscle MHC[32] as presented in Fig. 2. The results showed 81.3%, 59.7%, 38.8%, and 40.3% sequence identities, respectively, suggesting that the degrees of sequence homology of MHC are depended on tissues rather than species.

At present, it is unclear whether the entire structure of two MHCs from embryonic and adult chicken gizzard muscle are completely identical or not. In this regard, further evidence is necessary to elucidate the developmental diversity of chicken smooth muscle MHC. It will be of interest to analyse gizzard MHC isoforms among various physiological changes. Studies such as these should lead to a more complete understanding of the role of each portion and isoforms of smooth muscle MHC in normal and pathological states.

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Supplemental Materials

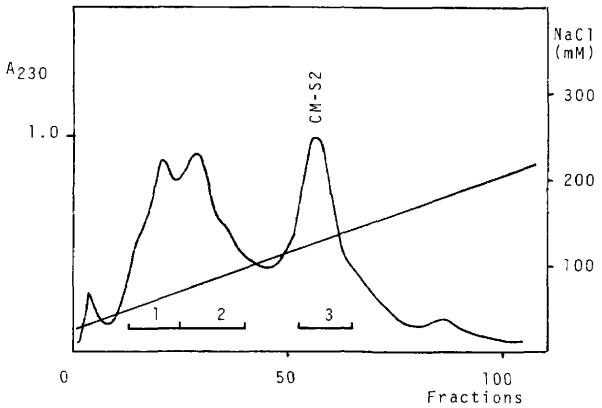

Fig. 3 Purification of CM-S-2 on DEAE-cellulose (DE-52, 2×18cm) equilibrated with 25mM Tris-HCl buffer, pH8.4 in 8M urea and eluted with a linear gradient of NaCl (25 →250mM, 500+500ml). Flow rate was 60 ml/h and 10ml fractions were collected. The effluent was pooled as indicated by the bars (pools 1-3).

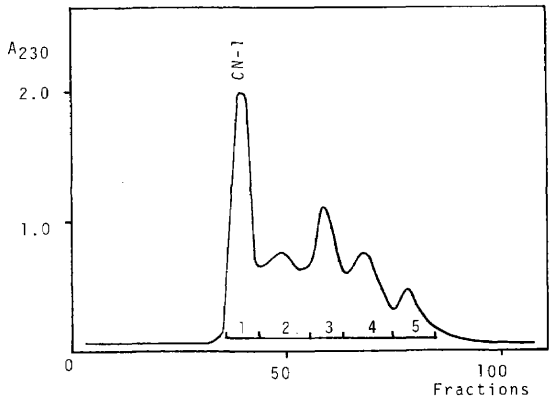

Fig. 4 Separation of CNBr degrated CM-S-2 on Sephadex G-100(1.8×90cm) equilibrated and eluted with 50mM NH_4HCO_3, pH8.5 at a flow rate of 20ml/h. 2.6ml fractions were collected. The effluent was pooled as indicated by the bars (pools 1-5).

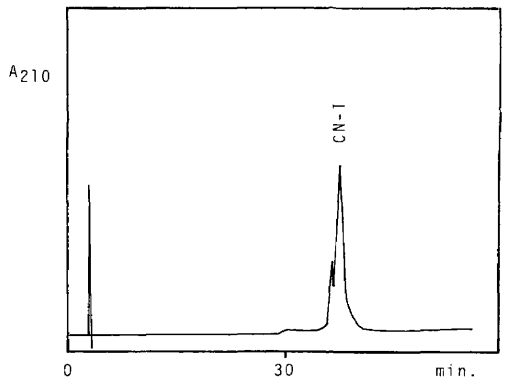

Fig. 5 Separation of peptides in pool 1 in Fig.4 by RP-HPLC on a column of μBondasphere (0.4×15cm). Elution was performed with a liner gradient of acetonitrile in 0.1% trifluoroacetic acid from 20% to 80% in 100min. at a flow rate of 0.7ml/min.

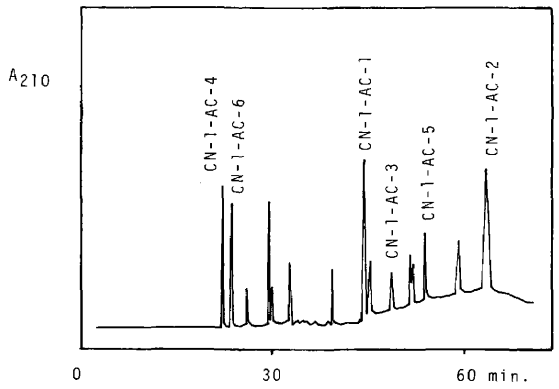

Fig. 6 Separation of hydrolysed peptides of CN-1 with formic acid by RP-HPLC on a column of Puresil (0.4 × 15cm). Elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid from 0 to 80% in 120min. at a flow rate of 0.7ml/min.

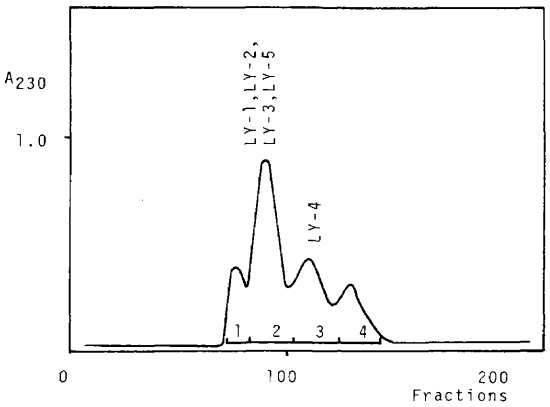

Fig. 7 Separation of lysyl endopeptidase peptides of CM-S-2 on Sephadex G-50 (1.8×180cm) equilibrated and eluted with 50mM NH_4HCO_3, pH8.5 at a flow rate of 18ml/h. 3ml of fractions were collected. The effluent was pooled as indicated by the bars (pools 1-4).

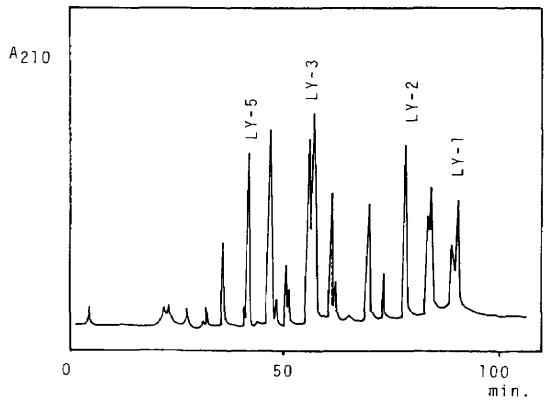

Fig. 8 Separation of peptides in pool 2 in Fig.7 by RP-HPLC on a column of Puresil (0.4×15 cm) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid from 0 to 60% in 100min, at a flow rate of 0.7ml/min.

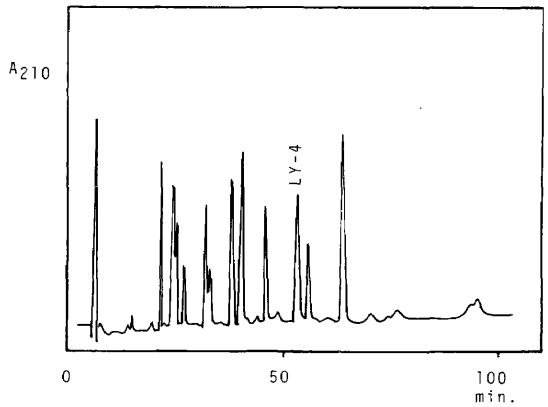

Fig. 9 Separation of peptides in pool 3 in Fig.7 by RP-HPLC on a column of Puresil (0.4×15 cm). conditions were as in Fig.8.

成鶏平滑筋ショート・サブフラグメント-2，ヒンジ連結領域の一次構造

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要　旨　われわれは［Watanabe B (1989), Biol. Chem. Hoppe-Seyler 370(9): 1027-1034 and Watanabe B, Tanigawa M (1993), Biol. Chem. Hoppe-Seyler 374(1): 27-35］において成鶏の骨格筋，心室筋ミオシンのサブフラグメント-2の構造と機能の関連性について発表したが，今回の論文は成鶏砂のう筋ミオシン（平滑筋）のショート・サブフラグメント-2とヒンジとの連結部領域の構造を明らかにしたものである。

ミオシンをパパインで消化して得られた尾部（ロッド）をさらにα-キモトリプシンで限定消化を行い，サブフラグメント-2（S-2）とライトメロミオシン（LMM）に分離した。次いでサブフラグメント-2のブロムシアン分解ペプチドの中から，ショート・サブフラグメント-2のカルボキシ末端領域とヒンジのアミノ末端領域を含む134個のアミノ酸からなるペプチドを精製単離し，一次構造を決定した.

さらに構造的相異を分析するために上記のようにして決定した連結部分の構造を胎鶏の砂のう筋（平滑筋），成鶏の脳，ドロソフィラ，成鶏骨格筋，成鶏心室筋ミオシンの相同部分の構造と比較した．その結果，興味あることに胎鶏の砂のう筋，成鶏の脳，ドロソフィラの非筋ミオシンとはそれぞれ100%，81.3%，59.7%の高いアミノ酸の同一性が認められ，これに対し成鶏骨格筋と成鶏心室筋ミオシンとはそれぞれ38.8%，40.3%と低いアミノ酸の同一性が認められた.

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