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Thermostability of striated and smooth adductor muscle tropomyosins from Yesso scallop *Mizuhopecten yessoensis*

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The striated and smooth adductor muscle tropomyosins (TMs) of Yesso scallop *Mizuhopecten yessoensis* have been known to express different isoforms, but have not been characterized in detail to date. In the present study, TMs from both muscles of Yesso scallop were purified and their stabilities were compared by circular dichroism (CD) spectrometry and differential scanning calorimetry (DSC). From the CD data, the apparent melting temperature and the apparent free energy of folding at 20°C were calculated to be 30.5°C and −13.4 kJ/mol, and 36.0°C and −31.9 kJ/mol for the striated and smooth muscle TMs, respectively. From the DSC data, ΔH values were calculated to be 1.87×10^3 and 2.19×10^3 kJ/mol for striated and smooth muscle TMs. These results suggest that smooth muscle TM has higher thermostability than striated muscle TM. The amino acid residues responsible for such stability difference were considered to be the six amino acid substitutions in the middle region of the TM molecules.

Keywords: adductor muscle/α-helix/scallop/thermostability/tropomyosin.

Abbreviations: CD, circular dichroism; CNBr, cyanogen bromide; DSC, differential scanning calorimetry; DTT, dithiothreitol; TM, tropomyosin.

Most of the tropomyosins (TMs) so far studied consist of two parallel α-helical polypeptide chains of 284 residues, with a molecular mass of ~33 kDa. Skeletal muscle TM is involved in the regulation of muscle contraction, interacting with one troponin complex and with seven actin molecules (*1*–*5*). TM polymerizes by head-to-tail interaction (*6*, *7*), and the filament formed resides in the groove of actin filament.

TM is characterized by α-helical coiled-coil structure formed by the packing of two α-helices against one another (*8*). In this structure, characteristic heptad repeats (designated *abcdefg* from the N-terminal side) are recognized. The *a* and *d* positions are frequently occupied by hydrophobic amino acids and the *e* and *g* positions by ionic amino acids, while the *b*, *c* and *f* positions by hydrophilic amino acids. The relationship between residues at the *a*, *d*, *e* and *g* positions is considered to be responsible for the stabilization of coiled-coil structure. It has been established that there is a high correlation of thermostability with the sum of the hydrophobic moments of the residues at the *a* and *d* positions in TMs (*9*). It was also reported that at the *a* and *d* positions, more than three consecutive residues (consisting of Leu, Met, Ile, Val, Phe and Tyr) tend to stabilize the coiled-coil (*10*) and the stability would affect the function of TM (*11*).

TMs have been purified from many eukaryotic species, from yeast to mammals (*12*–*18*). The deduced amino acid sequences of vertebrate TMs (*19*–*22*) and invertebrate TMs (*23*–*25*) are now available for not a few species. The crystal structures of full-length TM have been determined, but the resolution is not high enough (*26*, *27*). However, the several regional structures (such as N- and C-termini) of high resolution are available to date (*28*–*32*).

In the striated muscles of vertebrates, only the actin-linked regulation functions, but not in a few organisms, both of actin- and myosin-linked regulations were found (*33*). Molluskan myosin directly binds Ca^{2+} via the regulatory light chains and the ATPase activity in the presence of actin requires Ca^{2+} (*34*, *35*). It has been believed for a long time that molluskan muscles do not possess thin filament-linked regulatory systems. However, by biochemical (*36*) and immunological (*37*) studies, the occurrence of troponin was demonstrated, and when the concentration of Mg^{2+} was high enough, molluskan thin filaments showed Ca^{2+}-sensitivity (*38*, *39*).

TMs from scallops show unique biochemical and biophysical properties, *i.e.* the TMs show stronger inhibition of Mg-ATPase and higher viscosity compared to the vertebrate TMs. The high viscosity of scallop TMs suggest that these TMs tend to take a highly polymerized form compared with vertebrate TMs (*40*). However, the mechanism for such a unique behavior has not yet been elucidated. Fujinoki *et al.* (*41*) examined the heterogeneity and tissue specificity of TM isoforms obtained from posterior adductor and cardiac muscles of four bivalves, namely, ark shell *Scapharca broughtonii*, mussel *Mytilus galloprovincialis*, surf clam *Atrina pectinata* and Pacific oyster *Crassostrea gigas*. The mussel has only one TM, while the others have two isoforms (*41*). Yesso scallop possesses striated and smooth adductor muscles, the latter containing translucent and opaque portions. The striated muscle facilitates jet water propulsion for quick locomotion, while the smooth muscle can keep the shell tightly closed by

maintaining large tension with little energy expenditure for a long time (so-called catch mechanism). Therefore, the protein components are considered to have different profiles between the two muscles.

It was reported that each of the striated muscle and the translucent portion in the smooth muscle has only one TM species, though the opaque portion in the smooth muscle has two TM species (*42*–*44*). The cDNA sequence of the Yesso scallop striated muscle TM has been determined, while three cDNA sequences encoding smooth muscle TMs, *e.g.* TM-1, TM-2 and TM-3, are available to date. The deduced amino acid sequence of TM-1 was completely identical to those of TM-2 and TM-3 in the range of 1st–125th and 214th–284th residues (*25*), and TM-1 and striated muscle TM have the same sequence except for the 84th residue. However, no information is so far available for their physiological and physico-chemical properties, although it is suggested that the flexibility of TM is critical to finely tune the regulatory dynamics (*11*). As mentioned above, the striated and smooth adductor muscles have different TM isoforms, which could represent the functional differences of the two muscles. It is expected that the stability difference of scallop TM isoforms, if any, could be discussed in view of characteristics difference of the two muscles. Above all, the key amino acid residues for stabilization of TMs are of great interest to understand the formation mechanism of coiled-coil structure, and the proteins of great stability difference but with high sequence identity could be excellent targets to pinpoint the residues responsible for stabilization. From this viewpoint, the TM isoforms from scallop adductor muscle are considered to be the ideal materials.

In the present study, Yesso scallop TMs were purified from the striated and smooth adductor muscles, and their thermostabilities were examined in detail by the aid of circular dichroism (CD) spectrometry and differential scanning calorimetry (DSC) analyses. The amino acid residues assumed to be responsible for the stability difference were also specified.

Materials and Methods

Isolation of TM

Live specimens of Yesso scallop (average body weight 198 g) were purchased at the Tokyo Central Wholesale Market and immediately transported to the lab on ice. All the procedures were carried out at 0–4°C, unless otherwise stated. The striated adductor muscle and the opaque portion of the smooth adductor muscle were carefully excised and washed with 10 volumes of 50 mM KCl and 2 mM $NaHCO_3$ and left for 30 min. Ten volumes of water was added to the sediment, and left for 30 min. This procedure was repeated once more. The precipitate was centrifuged at 600*g* for 5 min, and washed with 10 volumes of acetone. Immediately, it was filtered through layers of gauze, added 10 volumes of acetone and left for 30 min. The sediment was further soaked in 10 volumes of acetone, left for 30 min, and filtered through a sheet of gauze. For the control, TM was also purified from the fast skeletal muscle of fish (white croaker *Pennahia argentatus*) (*18*).

The acetone dried powder of striated muscle was extracted with 10 volumes of 20 mM Tris–HCl (pH 7.5) containing 1 M KCl and 5 mM 2-mercaptoethanol overnight. The mixture was centrifuged at 10,000*g* for 10 min. The supernatant was subjected to isoelectric precipitation at pH 4.5 with 1 N HCl. The pellet after centrifugation at 10,000*g* for 10 min was collected and dissolved in water. pH was adjusted to 7.6 with 1 N NaOH. Ammonium sulphate fractionation was carried out, and TM was obtained in 40–45% saturation fraction. Anion exchange chromatography using a Mono Q 5/50 GL column (0.5 × 5 cm) (GE Healthcare UK Ltd., Little Chalfont, England) was carried out at a flow rate of 1 ml/min. The proteins were eluted by a linear gradient from 200 to 520 mM KCl in the presence of 10 mM potassium phosphate buffer (pH 7.0) and 1 mM dithiothreitol (DTT) over 20 column volumes.

Smooth muscle TM was extracted as in the case of striated muscle TM, except that KCl concentration of the extract was decreased to 0.1 M in order to precipitate contaminating paramyosin. In addition, TM was obtained in 35–50% ammonium sulphate saturation. Hydrophobic interaction chromatography, using a TSKgel BioAssist Phenyl column (0.78 × 5 cm) (Tosoh, Tokyo, Japan), was carried out at a flow rate of 1 ml/min. The column was equilibrated with 40% ammonium sulphate, 20 mM potassium phosphate buffer (pH 7.0). Protein was eluted by linearly decreasing the concentration of ammonium sulphate from 40 to 0% saturation over 20 column volumes and washed with 20 mM potassium phosphate buffer (pH 7.0) of five column volumes.

SDS–PAGE

SDS–PAGE was performed, using 15% polyacrylamide slab gels (*45*). After the run, gels were stained with 0.05% Coomassie Brilliant Blue R-250 (Wako). Protein molecular weight markers SDS-7 were purchased from Sigma Chemicals (St. Louis, MO, USA).

Two-dimensional PAGE

Two-dimensional PAGE consisted of isoelectric focusing for the first dimension and SDS–PAGE for the second dimension. Immobiline DryStrip gel (pH range 3–10, 11 cm long, GE Healthcare, Uppsala, Sweden) was used for the first-dimensional isoelectric focusing. Second-dimensional electrophoresis (SDS–PAGE) was performed as described above.

Identification of isoforms

The smooth muscle TM was fragmented by endoproteinase Arg-C (Roche, Nonnenwald, Penzberg, Germany), to determine the isoform type of smooth muscle TM preparation. TM, at a concentration of 2 mg/ml, was dissolved in 8.5 mM $CaCl_2$, 5 mM DTT, 0.5 mM EDTA and 90 mM Tris–HCl (pH 7.6) with 5 μg/ml endoproteinase Arg-C and digested at 30°C. Digestion time was 1.5 h. Digestion was stopped by addition of 100 mM Tris–HCl (pH 6.8) containing 8% SDS, 2% 2-mercaptoethanol, 24% glycerol, 0.02% bromophenol blue and heating at 95°C for 5 min. Tris–Tricine PAGE was performed, using 16% gel for separating gel and 10% for spacer gel (*46*). After the run, blotting to a polyvinylidene difluoride membrane was performed.

All three smooth muscle TMs have Met at the 1st, 8th, 11th and 25th positions, but, only TM-2 has Met at the 204th position. In order to confirm the isoform composition, smooth muscle TM was fragmented by cyanogen bromide (CNBr), which cleaves the peptide bond at N-terminus of Met residues. Namely, TM was dissolved in 7 M urea and 0.1 M HCl at a concentration of 1 mg/ml (31 μM). CNBr, 4.7 M concentrate in acetonitrile, was added at 500-fold molar excess over the TM, and the mixture was left in the darkness at room temperature for 16 h (*47*). Fish (white croaker) TM was used as a control.

CD spectrometry

TMs were dissolved in 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M KCl, 0.1 mM DTT and 0.001% NaN_3. CD spectra were measured at 0.1°C intervals ranging from 5 to 80°C in the above medium with a J-720 spectropolarimeter (JASCO, Tokyo, Japan). A cuvette cell of 10 mm optical path length was used with constant N_2 flux. Wavelength and protein concentration for measurement were 222 nm and 0.025 mg/ml, respectively.

α-Helical content was estimated assuming that the mean residue ellipticity at 222 nm, $[\theta]_{222}$, of poly L-glutamic acid is $-36{,}000\ \mathrm{deg\,cm^2/dmole}$ when the substance is of completely helical structure (*48*, *49*). To compare the overall stability of TM, two criteria were used; the apparent melting temperature (T_{Mapp}) and the apparent free energy of folding at 20°C (ΔG_{app}). To evaluate ΔG_{app}, it was assumed that the unfolding could be fit by a helix–coil transition and folding and unfolding are irreversible. The CD data normalized to a scale of 0–1, were fit by

the following equation (*9*):

$$\theta = \varepsilon_1\alpha_1 + \varepsilon_2\alpha_2 \quad (1)$$

where

$$\alpha_1 = \frac{K_1}{(1+K_1)} \text{ and } \alpha_2 = \frac{K_2}{(1+K_2)}. \quad (2)$$

ε_1 and ε_2 are the extinction coefficients for the CD associated with each transition.

$$K_1 = \exp\left(\left(\frac{\Delta H_1}{RT}\right)\left(\left(\frac{T}{T_{M1}}\right)-1\right)\right), K_2 = \exp\left(\left(\frac{\Delta H_2}{RT}\right)\left(\left(\frac{T}{T_{M2}}\right)-1\right)\right). \quad (3)$$

ΔH_1 is the enthalpy of folding for the first transition at the lowest temperature and ΔH_2 is the enthalpy of folding for the second transition. T_{M1} and T_{M2} are the observed midpoints of each transition. To calculate these values that best describe the unfolding behaviour, the initial values of these parameters were estimated, and the unfolding equations were fit using Microsoft Excel 2007. The ΔS_1 and ΔS_2 values were determined at the T_{M1} and T_{M2} of each transition using the relationships:

$$\Delta G = -RT \ln K \text{ and } \Delta S = (\Delta H - \Delta G)/T.$$

The obtained values were then used to estimate ΔG_{app} using the following relationship:

$$\Delta G_{app} = \varepsilon_1(\Delta H_1 - 293.15\Delta S_1) + \varepsilon_2(\Delta H_2 - 293.15\Delta S_2). \quad (4)$$

DSC

TM was analysed with a differential scanning microcalorimeter (MicroCal model VP-DSC, Northampton, MA, USA) with the raising speed of temperature at 1°C/min from 5 to 90°C. Progress baseline mode was adopted, assuming that each point reflects the extent of progress of the reaction. DSC data were analysed for determination of transition temperature (T_m) and enthalpy (ΔH) using a software package Origin developed by MicroCal. The measurement was performed three times successively for each sample.

Bioinformatic analysis

Propensity for α-helix formation was used to estimate α-helix formation ability based on the amino-acid sequence (*50*). Hydrophobicity at the *a* and *d* positions on heptad repeat was estimated according to Kyte and Doolittle (*51*). The secondary-structure prediction was performed using the GOR IV program (*52*). Coiled-coil formation prediction was performed using the COILS program (*53*), with a window width of 14, MTIDK as a matrix, and no weighting of position.

Other analytical methods

Protein concentration was determined by bicinchoninic acid (BCA) method (*54*) using bovine serum albumin as a standard.

Results

Isolation of TM

Yesso scallop striated muscle TM was eluted in the range of 240–330 mM KCl by anion exchange chromatography (Fig. 1A). Contamination by paramyosin was recognized in the applied sample, but it was eluted in the 17–19 fractions. For the subsequent experiments, the 5th–16th fractions were pooled and used (Fig. 1B). The smooth muscle TM was dissolved in 40% saturation ammonium sulphate after ammonium sulphate fractionation. The fraction was slightly turbid, but the composition of protein did not essentially change by filtration. Smooth muscle TM was eluted in the range of 33–28% saturation ammonium sulphate by hydrophobic interaction chromatography (Fig. 1C). For the subsequent experiment, the 10th–12th fractions were pooled and used (Fig. 1D). The purity was more than 98% for both muscle TMs as checked by SDS–PAGE. Presence of any isoform was not recognized for both TMs by two-dimensional PAGE (Fig. 2). Although two isoforms have been reported for smooth muscle TM based on mRNA level (*25*), the procedure seemed to have resulted in loss of the minor isoform. However, in order to demonstrate the homogeneity of the purified proteins, the following experiments were performed.

First, smooth muscle TM was digested by endoproteinase Arg-C, separated by Tris–Tricine PAGE, blotted to a PVDF membrane, and the N-terminal sequences were determined. The sequences obtained were SLADDERIDA, which corresponded to the 134th–143rd residues of TM-2 and TM-3, and LEAADAKVHELEEEL, which corresponded to the 183rd–197th residues of TM-3. These results strongly suggested that the major translated TM was TM-3. Smooth muscle TM was then cleaved by CNBr to further verify the isoform composition. The cleavage position did not exist in the middle region of the molecule (20 and 10 kDa fragments would be obtained if cleaved), but seemed to exist only in the N-terminal region (Fig. 3). Only one TM isoform (TM-2) contains Met at the 204 position and is expected to produce the 20 and 10 kDa fragments by CNBr degradation. However, no such fragment was obtained. This result supported the view that the smooth muscle TM preparation solely consists of TM-3. In this experiment, white croaker TM was used as a control of this reaction. This TM is homogenous unlike mammalian counterparts and has seven Met residues at the 1st, 8th, 10th, 127th, 135th, 141st and 281st positions (*18*, *19*), and the fragments of expected molecular sizes were obtained, indicating that the fragmentation was successful.

CD spectrometry

In the case of striated muscle TM, T_{Mapp} was estimated to be 30.5°C and ΔG_{app} was −13.4 kJ/mol from the CD data (Fig. 4, Table I). The value of $-d(\theta_{calculated})/dT$ had the maximum at 30.7°C, while the α-helical contents were 91 and 10% at 5 and 80°C, respectively. In the case of smooth muscle TM, T_{Mapp} was 36.0°C and ΔG_{app} was estimated to be −31.9 kJ/mol (Fig. 1, Table I). The value of $-d(\theta_{calculated})/dT$ showed the maximum at 36.5°C, while the α-helical contents were 100 and 11% at 5 and 80°C, respectively. The perfect α-helical structure may have been overestimated, because TMs do not show perfect heptad repeats (*4*) and some breakage of α-helix is present. On the other hand, the value of $-d(\theta_{calculated})/dT$ for smooth muscle TM showed sharper bell-shaped curve than that for striated muscle TM because of higher absolute figures of ΔH_2 ($|\Delta H_2|$) and ε_2 value, and this seems to have lead to the smaller ΔG_{app} value of this TM.

DSC

For the striated muscle TM, the values of total denaturation enthalpy (ΔH_{total}) from DSC were 1.87×10^3, 1.74×10^3, and 1.64×10^3 kJ/mol for the first, second and third scans, respectively (Table II).

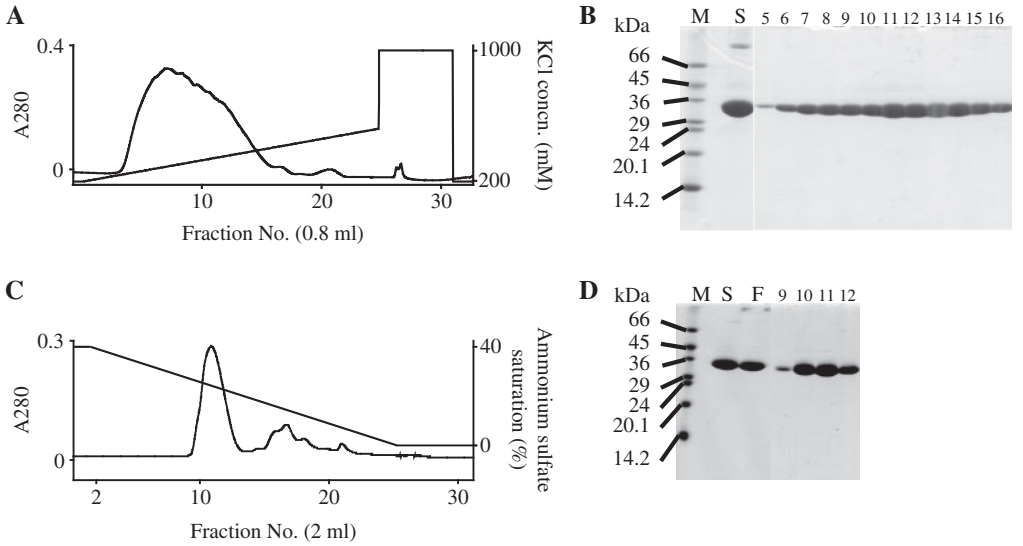

Fig. 1 Chromatograms and elution profiles from adductor striated and smooth muscles. (A and B) Elution pattern of striated muscle tropomyosin from a Mono Q 5/50 GL column (0.5 × 5 cm) and SDS–PAGE patterns of the fractions obtained by chromatography. The proteins were eluted by a linear gradient from 200 to 520 mM KCl (pH7.0) in the presence of 10 mM potassium phosphate buffer (pH 7.0) and 1 mM DTT. M, marker (SDS-7); S, the loaded sample; each number on the top of the gel corresponds to the fraction number in A. The fractions 1 and 2 are the flow through fractions. 15% gel. (C and D) Elution pattern of the opaque potion in the smooth muscle tropomyosin from a TSKgel BioAssist Phenyl column (0.78 × 5 cm) and SDS–PAGE patterns of the fractions obtained by chromatography. The proteins were eluted by the linear gradient from 40 to 0% saturation ammonium sulphate in the presence of 20 mM potassium phosphate buffer (pH 7.0). M, marker (SDS-7); S, the sample before filtration; F, the loaded sample. Each number on the top of gel corresponds to the fraction number in C and D. The fraction 1 is the flow through fraction. 15% gel.

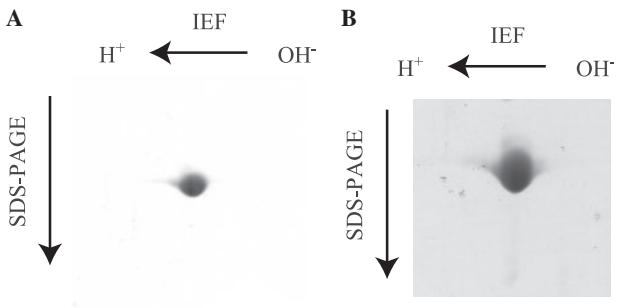

Fig. 2 Two-dimensional polyacrylamide gel electrophoresis of adductor striated and smooth muscle tropomyosins. (A) Striated muscle tropomyosin. (B) Smooth muscle tropomyosin. pH range 3–10 and 15% gel.

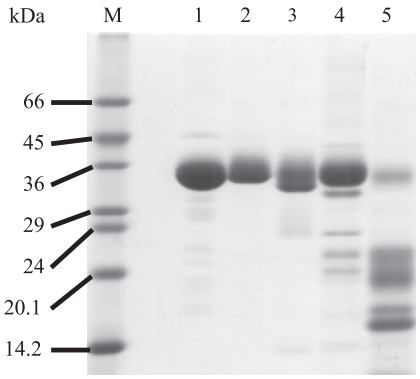

Fig. 3 SDS–PAGE patterns of CNBr-treated tropomyosins. M, marker (SDS-7); 1, smooth muscle tropomyosin before CNBr treatment; 2, smooth muscle tropomyosin treated with 0.1 M HCl and 7 M urea; 3, smooth muscle tropomyosin after cyanogen bromide treatment; 4, white croaker tropomyosin treated by 0.1 M HCl and 7 M urea; 5, white croaker tropomyosin after CNBr treatment. White croaker tropomyosin was used as a positive control. 15% gel.

Five transitions were observed for all the cases (Fig. 5). The value of denaturation heat capacity (C_p) showed the maximum at 28.5, 28.7 and 28.2°C for the first, second and third scans, respectively. The values of ΔH_{total} were reduced to 93 and 88% at the second and third scans, respectively, compared with the first scan because of incomplete refolding. The temperature of maximal C_p value changed slightly, but the maximal C_p values were reduced to 76 and 64% at the second and third scans, respectively.

For the smooth muscle TM, the values of ΔH_{total} were 2.19×10^3, 1.80×10^3 and 1.64×10^3 kJ/mol for the first, second and third scans, respectively (Table II). Four transitions were observed for all the cases (Fig. 5). The value of C_p gave the maximum at 35.4, 35.2 and 35.2°C for the first, second and third scans, respectively. The values of ΔH_{total} were reduced to 82 and 75% at the second and third scans, respectively, compared with the first scan. It is thus likely that smooth muscle TM has lower refolding ability than striated muscle TM. The temperature of the maximal C_p value changed slightly, but the maximal C_p values were reduced to 67 and 53% at the second and third scans, respectively.

Discussion

In the present study, Yesso scallop adductor muscle TMs were examined, because the adductor muscle consists of neighbouring striated and smooth muscles with

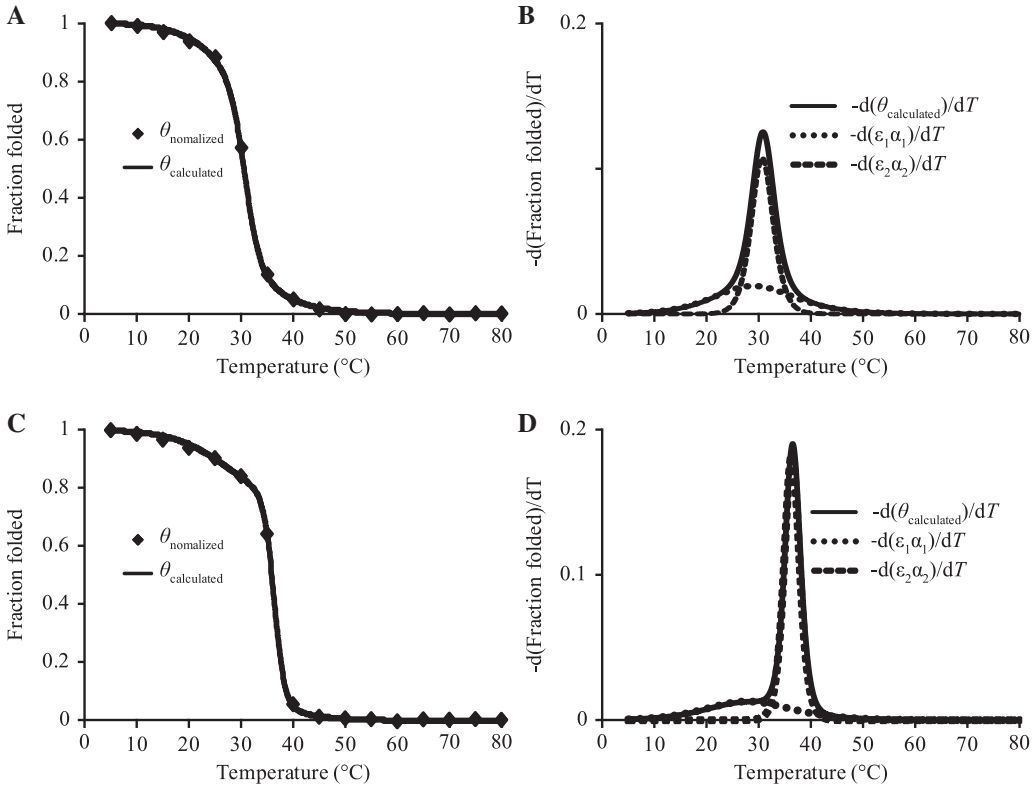

Fig. 4 CD analysis on the temperature-dependent unfolding of striated and smooth adductor muscle tropomyosins. (A and C) $[\theta]_{normalized}$ (dots) was obtained by normalizing CD data and $[\theta]_{calculated}$ (line) was based on the analysis. (B and D) $-d[\theta]_{calculated}/dT$ is shown.

Table I. Estimation of the thermodynamic parameters for the folding of scallop adductor muscle tropomyosins based on the ellipticity at 222 nm as a function of temperature[a].

Source	ΔH_1	ΔH_2	T_{M1}	T_{M2}	ε_1	ε_2	ΔG_{app}	T_{Mapp}
Striated muscle	−146	−545	29.3	30.7	0.295	0.605	−13.4	30.5
Smooth muscle	−136	−808	28.3	36.4	0.281	0.719	−31.9	36.0

[a]ΔH_1 and ΔH_2 are the enthalpies in kJ/mol for folding of the helix–coil transitions. T_{M1} and T_{M2} are the observed midpoints of each transition. The symbols ε_1 and ε_2 are the fractions of the TM molecule folded in each transition, and T_{Mapp} is the temperature at which the ellipticity, normalized to a scale of 0–1, was equal to 0.5. ΔG_{app} is the apparent free energy of folding at 20°C.

Table II. Estimation of the thermodynamic parameters for the folding of scallop adductor muscle tropomyosins based on DSC data as a function of temperature.

	Striated muscle			Smooth muscle		
	First scan	Second scan	Third scan	First scan	Second scan	Third scan
ΔH (kJ/mol)						
Total	1,867	1,738	1,636	2,189	1,799	1,638
First transition	423	293	287	395	245	238
Second transition	462	475	430	562	434	387
Third transition	464	450	408	846	760	673
Fourth transition	206	216	216	386	360	340
Fifth transition	313	304	294	–	–	–
T_m (°C)						
First transition	27.4	24.0	22.1	29.4	30.5	28.9
Second transition	28.2	27.7	27.2	35.4	32.1	31.3
Third transition	31.1	30.8	30.3	35.4	35.4	35.2
Fourth transition	34.2	37.5	38.3	42.8	43.0	43.2
Fifth transition	42.7	43.0	43.0	–	–	–

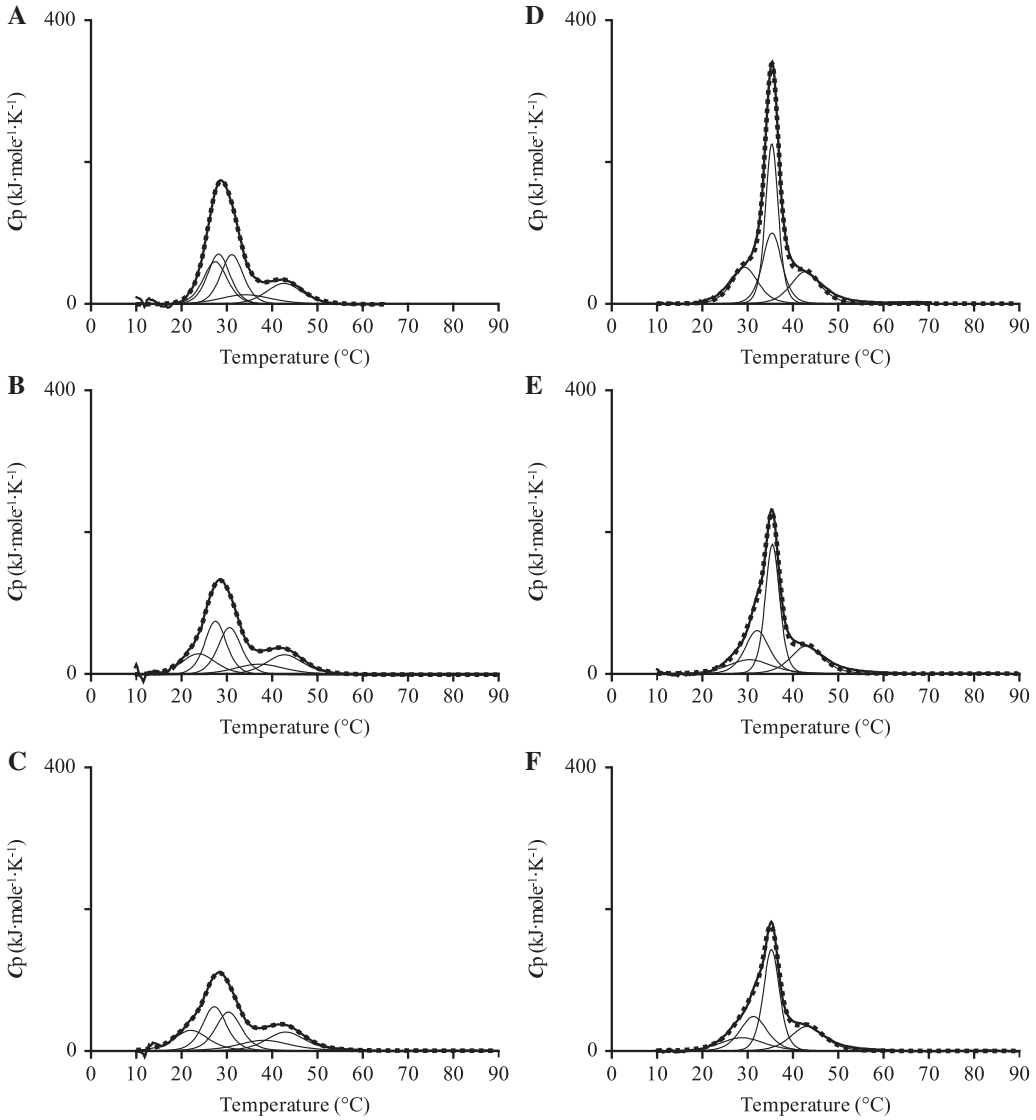

Fig. 5 DSC scans of striated and smooth adductor muscle tropomyosins. (A–C) Striated muscle tropomyosin. (D–F) Smooth muscle tropomyosin. The observed DSC patterns and the sum of subsequent deconvolution analysis are shown with solid and dotted lines, and the subsequent analysis is shown with thin lines. The DSC data were subtracted with a progress baseline, and analysed assuming tropomyosins form a dimer. A and D, first scan; B and E, second scan; C and F, third scan.

different biological functions, and TMs from these muscles have different isoforms with high sequence similarity (Fig. 6). The striated and smooth muscle TMs were successfully purified by column chromatography (Fig. 1). Hydrophobic chromatography was successful for purification of smooth muscle TM.

In the two-dimensional PAGE of striated and smooth muscle TMs, a single spot was obtained for each TM (Fig. 2). In Yesso scallop striated muscle, only one TM molecular species was detected by SDS–PAGE (*42–44*). Smooth muscle can be separated into two parts: opaque and translucent portions. In our experiment, only the opaque portion was used. In the translucent portion, only one TM species was detected by SDS–PAGE, although, in the opaque potion, two isoforms were detected (*42–44*). The major TM species in the opaque portion is the same as that in the translucent portion, and the other isoform is the same as that in the cardiac muscle (*43*, *44*). TMs from the striated and smooth muscles were clearly different in amino acid sequences from each other as revealed by tryptic digestion pattern (*44*). In our experiment, only one isoform was detected in the opaque potion of smooth muscle, and the minor TM species might have been lost through purification procedure.

By cDNA cloning, three types of smooth muscle TM have been detected and designated as TM-1, TM-2 and TM-3 (*25*). To confirm the major component of the purified TM, endoproteinase Arg-C digestion of TM preparation and subsequent N-terminal sequencing of the fragments were performed. The sequences obtained indicated that the major translated TM was TM-3. The result of CNBr fragmentation also supported the view (Fig. 3).

The helix propensity was predicted to be 1.14 for the striated muscle TM and 1.15 for smooth muscle TM-3 (Table III). Hydrophobicity per residue at the *a* and *d*

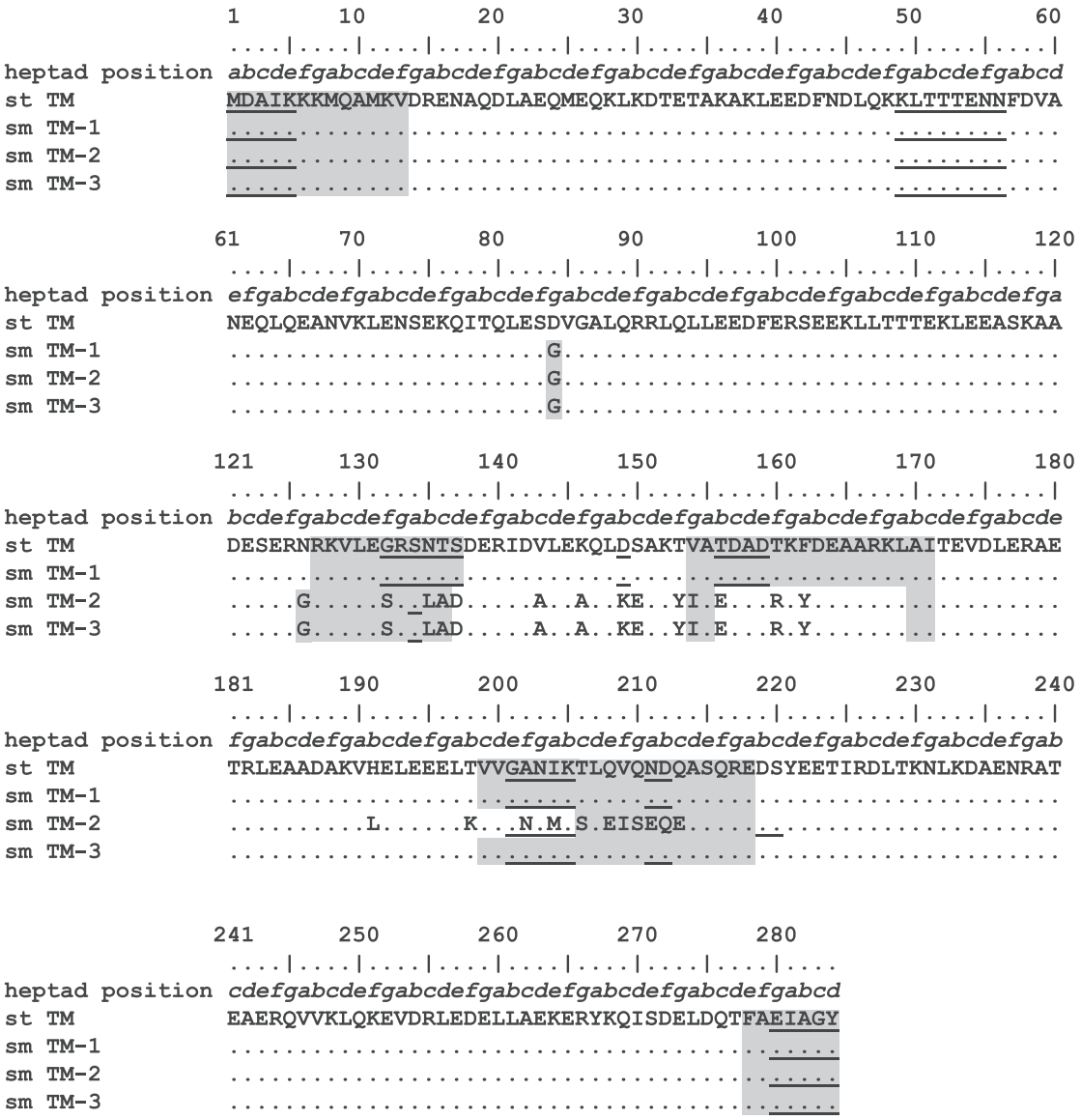

Fig. 6 Sequence alignment of striated and smooth muscle tropomyosins. The positions that are expected not to form α-helix by GOR IV are underlined. The positions where coiled-coil formation values by COILS are higher than 0.8 point are shaded. The st TM was reported as the GeneBank nucleotide sequence database with accession no. AB004636, and sm TMs were reported by Hasegawa (*25*). Abbreviations: st, striated muscle; sm, smooth muscle.

Table III. Secondary-structure prediction of scallop adductor muscle tropomyosins.

	Helix propensity[a]	Hydrophobicity[b]	α-Helical content[c] (%)	Coiled-coil formation[d] (%)
Striated muscle	1.14	2.05	87	86
Smooth muscle	1.15	1.97	91	87

[a]Chou and Fasman (*50*). Per residue.
[b]Kyte and Doolittle (*51*) At the *a* and *d* residues and per residue.
[c]Predicted by GOR IV.
[d]Estimated by COILS.

positions of heptad repeat was 2.05 and 1.96 for the striated muscle TM and smooth muscle TM-3, respectively. α-Helical contents predicted by the GOR IV were 87 and 91% for the striated muscle TM and smooth muscle TM-3, respectively. The coiled-coil scores were estimated to be 86 and 87% for the striated muscle TM and smooth muscle TM-3, respectively.

The striated TM and TM-3 have 15 different amino acid residues (Fig. 6). Helix propensity per residue of these TMs was slightly different (Table III). It was reported that the thermostability is influenced by the hydrophobicity at the *a* and *d* positions (*9*). Only one amino acid replacement was recognized each at the *a* and *d* positions; namely, the 137th residue (Ser in the

striated muscle TM was substituted by Asp in the smooth muscle TM) and the 162nd residue (Phe in the striated muscle TM was substituted by Tyr in the smooth muscle TM). At the *a* and *d* positions, stabilizing residues were assumed to be Leu, Met, Ile, Val, Phe and Tyr, and the other amino acid residues would destabilize the coiled-coil (*10*). Thus, the substitutions of Ser by Asp and Phe by Tyr might have small effects on the stability of TM, and the difference in hydrophobicity would not explain the stability difference between the striated and smooth muscle TMs.

Prediction of α-helical contents suggested that the 132nd–137th residues in the striated muscle TM do not form α-helix, and that the 134th residue in the smooth muscle TM neither does so. In this region, substitutions at the 132nd, 135th and 136th residues could mainly affect the stability of TM, because Gly perturbs the α-helical structure, and Leu and Ala tend to exert much higher helix propensity than Asn and Thr. Prediction of coiled-coil propensity suggested that the 154th–171st residues in the striated muscle TM do not form a coiled-coil structure, and that the 154th–155th and 170th–171st residues in the smooth muscle TM behave in a similar manner. In these regions, substitutions at the 156th, 160th and 162nd residues would mainly affect the stability, although Arg shows slightly higher helix propensity than Thr. The different stability could mainly originate from the substitutions at the 132nd, 135th, 136th, 156th, 160th and 162nd residues.

The values of T_{Mapp} and ΔG_{app} based on the CD data indicated that smooth muscle TM was more stable than the striated muscle TM. White croaker TM was used as a control, because it is homogenous unlike mammalian counterparts and has been well characterized by our previous studies (*18*, *19*).

In case of DSC measurement, five transitions were observed for the striated muscle TM and four for smooth muscle TM (Table II). Striated and smooth muscles have high sequence similarity, and it was thus expected that some deconvoluted transitions of the striated muscle TM might correspond to those of the smooth muscle TM. Actually, the lowest transition temperature of striated muscle TM was very close to that of smooth muscle TM, and the highest transition temperature showed similar relationship, although the other transitions did not show any correspondence.

The values of $|\Delta H|$ obtained by DSC and CD showed a similar tendency, although those obtained by DSC were much larger than that by CD. The temperature with the maximum value of $-\mathrm{d}(\theta_{\mathrm{calculated}})/\mathrm{d}T$ by CD was in good correspondence to the temperature with the maximum C_{p} obtained by DSC, for both striated and smooth muscle TMs.

These values (T_{Mapp} and ΔG_{app} by CD, and ΔH and C_{p} by DSC) strongly suggested that the striated muscle TM is less stable than the smooth muscle counterpart. The result is in a good agreement with the fact that the striated muscle TM was more susceptible to tryptic digestion than the smooth muscle TM (*44*). Incidentally, rat skeletal muscle TM shows higher stability than its smooth muscle TM (*9*). The difference in stability might reflect the difference in the contractile properties between these two muscles. It has been shown, by using vertebrate proteins, that the higher stability around 130th amino acid residues increased the Ca^{2+}-regulated S1-thin filament ATPase activity and cooperativity (*11*). Optimization in structural flexibility of TM would be required to exert its best regulatory dynamics.

The ATPase activity of scallop smooth muscle myosin in the presence of rabbit skeletal muscle F-actin was enhanced two folds by the addition of scallop smooth adductor muscle TM compared to scallop striated adductor muscle TM (*42*). It has been reported that relationship of TM stability and the thin filament-myosin ATPase depends on the context of amino acid sequence (*11*, *55*).

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Conflict of interest
None declared.

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