Troponin from Smooth Adductor Muscle of Ezo-Giant Scallop

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Troponin which can confer Ca^{2+}-sensitivity upon rabbit actomyosin Mg-ATPase activity has been prepared from the smooth adductor muscle of Ezo-giant scallop (*Patinopecten yessoensis*). The troponin comprises 40-, 20-, and 19-kDa components. In order to characterize the components, they were separated from each other by CM-Toyopearl column chromatography in the presence of 6 M urea. Consequently, the 20-kDa component was identified as troponin C, based on the Ca^{2+}-binding ability. The amount of Ca^{2+} bound to the troponin C was estimated to be 0.75 mol/mol at 10^{-4} M Ca^{2+} by the equilibrium dialysis method. The 19-kDa component was identified as troponin I on the basis of not only its inhibitory effect on rabbit actomyosin Mg-ATPase activity along with the smooth adductor tropomyosin, but also the releasing effect of the smooth adductor troponin C on the inhibition. On the other hand, the 40-kDa component was regarded as troponin T on the basis that it bound to F-actin-tropomyosin filament and was indispensable for conferring Ca^{2+}-sensitivity upon rabbit actomyosin Mg-ATPase activity, along with troponin C and troponin I. The above assignments were confirmed by both amino acid analysis and immunoblotting using rabbit antisera raised against counterparts of scallop striated muscle troponin.

Key words: Ca^{2+}-regulation, scallop, smooth muscle, tropomyosin, troponin.

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Tn is an actin-linked regulatory protein for striated muscles (*1-3*), consisting of three distinct components, TnC, TnI, and TnT (*4*). Tn has also been isolated from dual-regulated muscles, such as scallop striated adductor muscles, which possess two regulatory systems through myosin-linked regulatory light chains (*5-7*) and actin-linked Tm-Tn (*8-10*). The striated adductor Tns of Akazara scallop (*Chlamys nipponensis akazara*) and Ezo-giant scallop show some differences in properties from vertebrate Tns, *e.g.*, the TnIs have a considerably larger molecular weight (52,000) than rabbit TnI (23,000) on SDS-PAGE and the TnCs bind only one Ca^{2+} in contrast to four Ca^{2+} in the case of rabbit TnC (*9-12*). Recently, the amino acid sequences of the scallop TnCs were determined and shown to have low homology to the vertebrate TnCs (*12, 13*).

Bivalve smooth adductor muscles as well as anterior byssus retractor muscle of *Mytilus* are known to show an energy-saving prolonged state of contraction, so-called "catch" (*14-16*). In our previous study, we isolated and characterized Tn from the catch muscle, that is, smooth adductor muscle of Akazara scallop (*17*). Later, Bennet and Marston (*18*) investigated actin-linked regulatory proteins in mussel and oyster catch muscles and reported the isolation of caldesmon-like protein. In addition, they suggested that some of the Tn-like proteins which have been isolated from molluscan muscles are breakdown products of a larger caldesmon-like protein. Thus, it seems still unsettled whether or not Tn exists in molluscan catch muscles other than Akazara scallop smooth adductor muscle.

In the present paper, we describe the isolation and characterization of Tn and its components of the smooth adductor muscle of Ezo-giant scallop.

MATERIALS AND METHODS

Crude Tn and Tm were prepared from Ezo-giant scallop smooth adductor muscle essentially by the method reported previously (*9, 10, 17*). Yields of crude Tn and Tm were approx. 30 and 200 mg, respectively, from 100 g of muscle. The crude Tn was purified by DEAE-Toyopearl column chromatography as described in the "RESULTS AND DISCUSSION." Crude Tm was purified by repeating the ammonium sulfate fractionation (35-40% saturation) as described previously (*17*). Scallop striated adductor Tn and its components were prepared by the methods described previously (*9, 10*).

Rabbit skeletal myosin and actin were prepared by the methods of Perry (*19*) and Spudich and Watt (*20*), respectively.

Mg-ATPase activity of reconstituted actomyosin was measured at 15°C in a reaction medium containing 50 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM $MgCl_2$, 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 1 mM ATP, either 0.2 mM EGTA (in the absence of Ca^{2+}) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (in the presence of Ca^{2+}), unless otherwise stated. Inorganic phosphate liberated was determined by the method of Youngburg and Youngburg (*21*).

Difference UV-absorption spectra of TnC were measured

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Abbreviations: Tn, troponin; TnT, troponin T; TnC, troponin C; TnI, troponin I; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

at 20°C in a medium containing 0.1 M KCl, 20 mM MOPS-KOH (pH 6.8), 0.5 mg/ml TnC, and 0.2 mM EGTA (reference cell) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (sample cell). Free Ca^{2+} concentration was varied by using Ca-EGTA buffer, assuming an apparent binding constant of 10.01×10^5 M^{-1} (*22*) for Ca-EGTA complex. The amount of Ca^{2+} bound to TnC was measured at 20°C by the equilibrium dialysis method using a microdialysis cell and $^{45}CaCl_2$ (*9*).

SDS-PAGE was performed in Tris-glycine buffer system by the method of Porzio and Pearson (*23*).

Amino acid composition was determined as follows; protein was hydrolyzed with 6 N HCl at 110°C for 24 h *in vacuo* and the resultant amino acids were reacted with phenyl isothiocyanate using a PICO-TAG work station (Waters). The phenylthiocarbamylated amino acids were analyzed by HPLC on a PICO-TAG column (3.9 × 150 mm, Waters).

Antisera against Tn components of scallop striated adductor muscle prepared from the immunized rabbits using SDS-PAGE-purified proteins. Peroxidase-conjugated anti-rabbit IgG goat IgG was purchased from Sigma. Immunoblotting of Tn components was carried out by using the method of Towbin *et al.* (*24*). Cross-reaction of the antibody was detected by color development with 4-chloro-1-naphthol (*25*).

RESULTS AND DISCUSSION

Preparation of Troponin—Crude Tn contained 40-, 35-, 20-, and 19-kDa components in addition to several trace components, so it was further purified by DEAE-Toyopearl column chromatography as follows: crude Tn (80-100 mg) was dialyzed against 1 M urea, 30 mM KCl, 10 mM Tris-HCl (pH 7.6) and then subjected to DEAE-Toyopearl column chromatography (Tosoh, 1.4 × 32 cm) using a linear KCl gradient. As shown in Fig. 1, five peaks were obtained and fractions d-f out of a-g were found to confer Ca^{2+}-sensitivity upon rabbit actomyosin Mg-ATPase activity, together with scallop smooth adductor Tm. Further, the fractions were shown to contain three components of 40-, 20-, and 19-kDa. The molar ratio of the three components was estimated to be approx. 1 : 1 : 1 by SDS-PAGE and densitometry. The amount of the 19-kDa component, however, appeared to be slightly smaller than the stoichiometric amount. Further, the ratio of the 19-kDa component to the 20-kDa component varied from the earlier to the later fractions of the elution peak. In addition, the 40-kDa component showed a doublet band on SDS-PAGE. These results may indicate the presence of multiple forms of Tn in the smooth muscle. Although the possibility of coexistence of small amounts of other species of troponin isoform can not be excluded, the major form seems to be eluted at around the peak fraction. Therefore, we pooled the fractions d-f and used this as the smooth adductor Tn for further characterization.

As shown in Fig. 2, the smooth adductor Tn strongly activated the rabbit actomyosin-smooth adductor Tm Mg-ATPase activity in the presence of Ca^{2+}, but had practically no effect in the absence of Ca^{2+}, thus conferring Ca^{2+}-sensitivity (see also Table I). This mode of conferring Ca^{2+}-sensitivity was similar to the case of striated adductor Tn (*9, 10*) and ascidian Tn (*26*), in which Tm showed marked inhibition of the Mg-ATPase activity so that Tn along with Tm might hardly be more inhibitory in the absence of Ca^{2+}.

Separation of Troponin Components—In order to sepa-

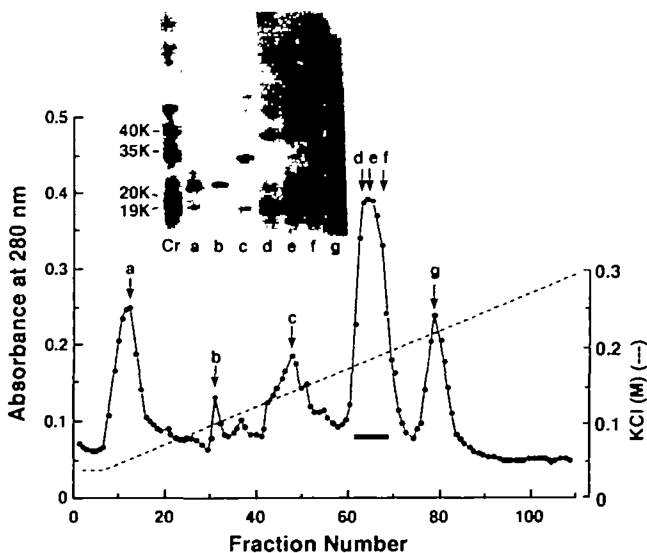

Fig. 1. **DEAE-Toyopearl column chromatography of crude troponin of scallop smooth adductor muscle.** Crude smooth adductor Tn (approx. 100 mg) was dialyzed against 1 M urea, 30 mM KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol, and then applied to a DEAE-Toyopearl column (1.4 × 32 cm). The proteins were eluted with a linear KCl gradient of 30-300 mM (total volume, 600 ml). Each fraction was 5.0 ml. The SDS-PAGE patterns of myofibrils, crude Tn and the fractions indicated by arrows a-g are shown in the inset of the figure. Electrophoresis was performed in 0.1% SDS, 50 mM Tris-150 mM glycine buffer using 10% polyacrylamide gel according to Porzio and Pearson (*23*). Cr, crude Tn. The fractions indicated by a solid bar were pooled.

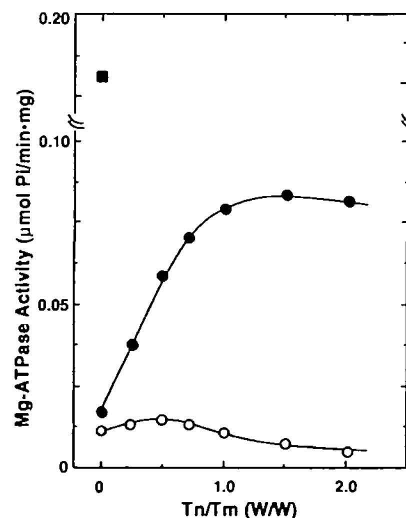

Fig. 2. **Effect of smooth adductor tropomyosin and troponin on actomyosin Mg-ATPase activity.** Mg-ATPase activity was measured at 15°C in a solution containing 50 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, and either 0.2 mM EGTA (○) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (●), 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 0.025 mg/ml scallop smooth adductor Tm, and various concentrations of scallop smooth adductor Tn. The activity of actomyosin alone is also shown (■).

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rate the three Tn-components, CM-Toyopearl 650M column chromatography was carried out in the presence of 6 M urea (*27*); smooth adductor Tn (approx. 30 mg) was dialyzed against 6 M urea, 30 mM KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol, and then applied to a CM-Toyopearl 650M column (2.2×21 cm). As shown in Fig. 3, the 20-kDa component was passed through, but the 40- and 19-kDa components were adsorbed and eluted separately.

It should be noted that the 19-kDa component was separated into two peaks and their components showed identical mobility on SDS-PAGE. Therefore, the component in the former peak was designated as 19-kDa-a and in the latter peak as 19-kDa-b.

The Tn-components were dialyzed separately against 0.6 M KCl, 20 mM imidazole-HCl (pH 7.0), and 5 mM 2-mer-

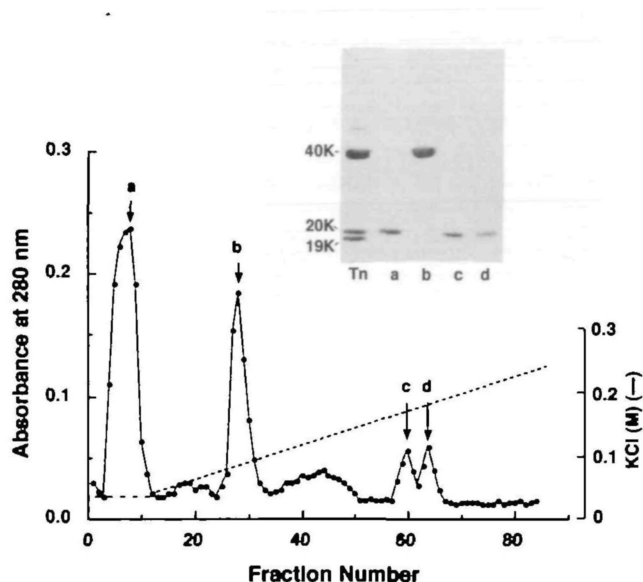

Fig. 3. **CM-Toyopearl column chromatography of smooth adductor troponin.** Scallop smooth adductor Tn (approx. 30 mg) was dialyzed against a solution containing 6 M urea, 1 mM EDTA, 30 mM KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol, and then applied to a CM-Toyopearl 650M column (2.2×21 cm). The protein was eluted with a linear gradient of 30-250 mM KCl (total volume, 600 ml). Each fraction was 5.0 ml. SDS-PAGE patterns of Tn before separation (Tn) and fractions eluted in each peak are shown in the inset.

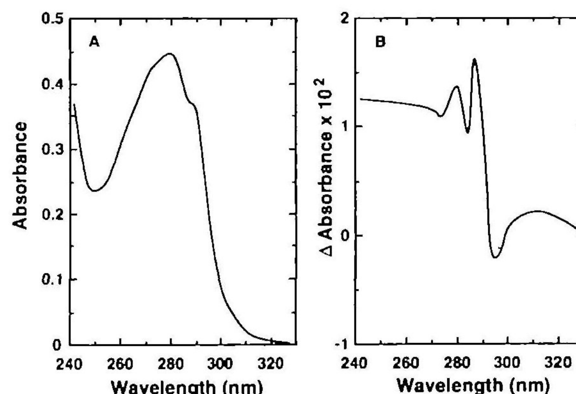

Fig. 4. **UV absorption and difference UV absorption spectra of smooth adductor TnC.** A, UV absorption spectrum of scallop smooth adductor TnC (0.5 mg/ml) in 0.1 M KCl, 20 mM MOPS-KOH (pH 6.8). B, Ca^{2+}-induced difference absorption spectrum of scallop smooth adductor TnC. The sample cell and reference cell contained 0.2 mM EGTA and 0.2 mM EGTA plus 0.3 mM $CaCl_2$, respectively. Other conditions were the same as in "A."

TABLE I. **Effects of scallop smooth adductor troponin and its components on Mg-ATPase activity of actomyosin.** Mg-ATPase activity was measured at 15°C in the presence of 0.2 mM EGTA ($-Ca^{2+}$) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ ($+Ca^{2+}$), 0.1 mg/ml rabbit myosin, and 0.05 mg/ml rabbit F-actin, and when added, 0.025 mg/ml scallop smooth adductor Tm, 0.025 mg/ml scallop smooth adductor Tn, 0.018 mg/ml 40-kDa component, and 0.01 mg/ml 20-kDa component, and 0.009 mg/ml 19-kDa component. Tn and its component were added in the combinations indicated in the table. Other experimental conditions were the same as in Fig. 2. AM, actomyosin; 40K, 40-kDa component, for example; Tm, tropomyosin; Tn, troponin.

Samples	Mg-ATPase activity (μmol P_i/min·mg)		
	$-Ca^{2+}$	$+Ca^{2+}$	Ca^{2+}-sensitivity (%)[a]
AM	0.188	0.164	–
AM+Tm	0.032	0.035	8.6
AM+Tm+Tn	0.035	0.096	63.5
AM+Tm+40K	0.047	0.042	–
AM+Tm+20K	0.034	0.040	15.0
AM+Tm+19K-a	0.027	0.022	–
AM+Tm+19K-b	0.027	0.028	3.6
AM+Tm+40K+20K	0.044	0.043	–
AM+Tm+40K+19K-a	0.020	0.024	16.7
AM+Tm+40K+19K-b	0.015	0.018	16.7
AM+Tm+20K+19K-a	0.025	0.033	24.2
AM+Tm+20K+19K-b	0.021	0.028	25.0
AM+Tm+40K+20K+19K-a	0.039	0.089	56.2
AM+Tm+40K+20K+19K-b	0.024	0.072	66.7

[a] Ca^{2+}-sensitivity $= (1 - \frac{\text{activity }(-Ca^{2+})}{\text{activity }(+Ca^{2+})}) \times 100$.

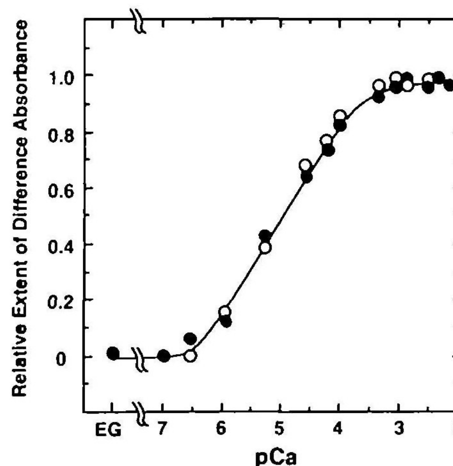

Fig. 5. **Difference spectra of smooth and striated adductor TnCs as a function of Ca^{2+}-concentration.** The relative extent of difference absorbance was estimated from the difference between positive peak-signal at 289 nm and negative peak-signal at 292 nm. The value 1.0 on the ordinate corresponds to the signal difference of 0.024. The Ca^{2+} concentrations were varied with Ca^{2+}-EGTA buffer consisting of 0.1 mM EGTA and various concentrations of $CaCl_2$ (*22*). EG, in the presence of 0.1 mM EGTA. Other conditions were the same as in Fig. 4B. ●, scallop smooth adductor TnC; ○, scallop striated adductor TnC.

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captoethanol and concentrated by ultrafiltration with a PM-10 membrane (Amicon).

Characterization of Troponin Components—The effects of the Tm, Tn, and Tn components on the rabbit actomyosin Mg-ATPase activity were examined. As shown in Table I, smooth adductor Tm inhibited the activity irrespective of the presence or absence of Ca^{2+}, and recovery of Ca^{2+}-sensitivity by Tn was a result of the activity increase in the presence of Ca^{2+}. The 19-kDa-a and -b components lowered the activity of actomyosin-Tm, but the 40- and 20-kDa components did not. It is recognized that all three components, including either the 19-kDa-a or -b component, are required for recovery of Ca^{2+}-sensitivity. It is, therefore, considered that the 20-kDa component is TnC, the 40-kDa component is TnT, and both the 19-kDa-a and -b components are TnI. Next, more detailed characterization was carried out.

As shown in Fig. 4, the 20-kDa component showed a UV-spectrum and a difference spectrum with three positive peaks at 280, 288, and 295 nm due to the perturbation of Tyr and Trp upon addition of Ca^{2+}, similarly to scallop striated adductor TnC (*9, 10*). In addition, the difference between the absorbances at 289 and 292 nm was plotted against free Ca^{2+} concentration and the half-maximal change was estimated to occur at 30 μM Ca^{2+} (Fig. 5). In general, TnC shows approximately ten times lower affinity for Ca^{2+} when isolated from Tn complex (*28*). Indeed, the Ca^{2+} concentration for half-maximal change in the difference spectrum was estimated to be a physiological concentration, 3 μM, in the case of scallop smooth adductor intact Tn (data not shown). Further, the smooth adductor TnC was found to bind approximately 0.75 mol of Ca^{2+} per mol irrespective of the presence or absence of 2 mM $MgCl_2$ (Table II). This indicates that the smooth adductor TnC possesses one Ca^{2+}-specific site, like the striated adductor TnC.

TABLE II. **Calcium binding of scallop smooth adductor TnC.** The amount of Ca^{2+} bound to the 20-kDa component was measured by the equilibrium dialysis method in the medium of 0.1 M KC, 20 mM MOPS-KOH (pH 6.8), and 1 mg/ml of the 20-kDa component. EGTA, in the presence of 1 mM EGTA; Ca^{2+}, in the presence of 0.1 mM $CaCl_2$; $Ca^{2+}+Mg^{2+}$, in the presence of 0.1 mM $CaCl_2$ and 2 mM $MgCl_2$. The average values of four experiments are shown with the standard deviation.

	EGTA	Ca^{2+}	$Ca^{2+}+Mg^{2+}$
Number of Ca^{2+} bound	0.08±0.13	0.73±0.04	0.75±0.01

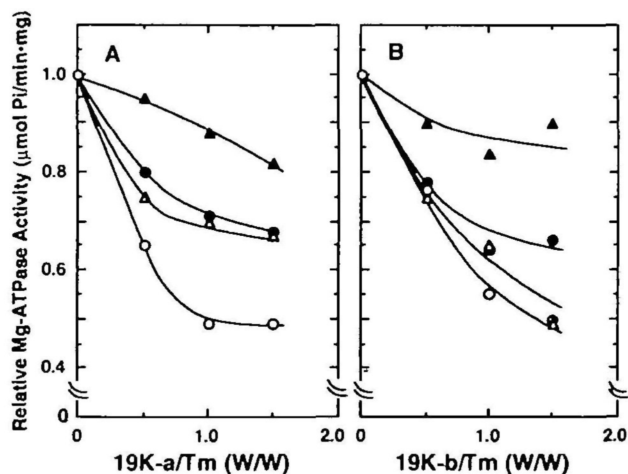

Fig. 6. **Effects of smooth adductor 19-kDa-a and 19-kDa-b components on rabbit actomyosin Mg-ATPase activity.** Mg-ATPase activity was measured at 15°C in a reaction medium containing 30 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, either 0.2 mM EGTA (○, △) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (●, ▲), 0.25 mg/ml rabbit myosin, 0.025 mg/ml rabbit F-actin, 0.013 mg/ml scallop smooth adductor Tm and various concentrations of the 19-kDa component with (△, ▲) and without (○, ●) an equimolar amount of scallop smooth adductor TnC. A, 19-kDa-a component; B, 19-kDa-b component. The relative ATPase activity 1.0 is 0.10 μmol P_i/min·mg myosin.

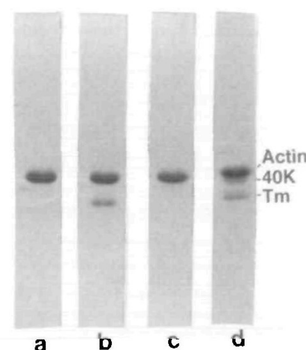

Fig. 7. **SDS-PAGE pattern of coprecipitates of the 40-kDa component and actin-tropomyosin filament.** To 0.2 mg/ml of rabbit F-actin, 0.1 mg/ml of the smooth adductor Tm and 0-0.1 mg/ml of the 40-kDa component (TnT), which was centrifuged at 100,000×*g* for 2 h prior to use, were added in a solution containing 30 mM KCl, 1 mM $MgCl_2$, and 20 mM Tris maleate (pH 6.8), and the mixture was centrifuged at 100,000×*g* for 1 h. The pellet obtained was dissolved in 8 M urea, 1% SDS, 0.05 M Tris-HCl (pH 8.9), and 0.6 M 2-mercaptoethanol, and then subjected to SDS-PAGE. a, F-actin alone; b, F-actin+Tm; c, F-actin+40-kDa component; d, F-actin+Tm+40-kDa component (0.1 mg/ml). 40K means 40-kDa component.

TABLE III. **Amino acid compositions of the troponin components.** Values are expressed as mol%. 20K, 40K, 19K-a, and 19K-b; 20-kDa, 40-kDa, 19-kDa-a, and 19-kDa-b components, respectively, of Ezo-giant scallop smooth adductor muscle; S-TnC, TnC of Ezo-giant scallop striated adductor muscle (*10*); S-TnT, TnT of Ezo-giant scallop striated adductor muscle (*10*); A-19K-b, 19-kDa-b TnI of Akazara scallop striated adductor muscle (*29*).

Amino acid	20K	S-TnC	40K	S-TnT	19K-a	19K-b	A-19K-b
Asx	13.1	15.2	9.4	9.4	12.1	12.0	11.7
Thr	5.1	4.1	3.2	2.9	2.4	2.7	2.4
Ser	4.9	4.9	3.8	3.8	4.1	4.2	4.7
Glx	17.6	17.8	28.2	28.0	16.4	16.4	16.5
Pro	0.0	0.0	3.9	3.9	2.0	2.1	2.3
Gly	7.2	6.2	2.7	2.8	7.6	6.1	5.8
Ala	7.2	6.9	10.8	10.9	9.9	10.1	9.7
Val	5.1	5.3	3.1	3.1	4.8	4.9	4.3
Met	2.1	2.1	3.7	3.5	1.1	1.1	1.2
Ile	5.3	5.5	4.1	4.0	3.0	3.1	3.2
Leu	12.3	10.5	7.7	6.9	8.8	9.4	9.0
Tyr	0.6	0.9	2.9	2.9	1.1	1.2	1.1
Phe	3.6	3.9	1.7	1.7	3.0	3.2	3.0
Lys	11.7	12.5	14.2	15.3	22.4	22.3	20.0
His	0.0	0.0	0.8	0.8	1.3	1.3	1.3
Arg	4.1	4.1	9.3	9.4	2.4	2.4	3.5
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

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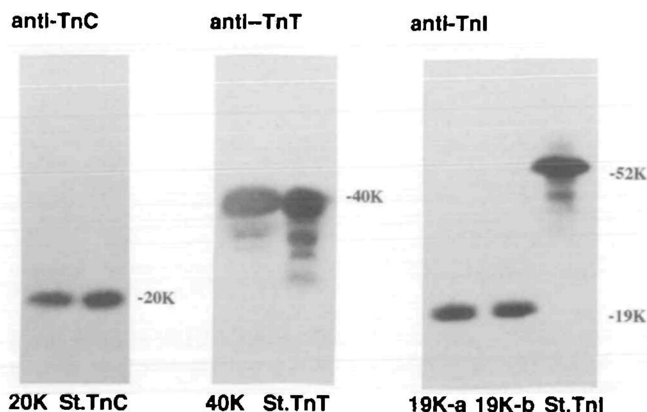

Fig. 8. **Cross-reactivity of smooth adductor troponin components with antisera against striated adductor troponin components.** Immunoblotting was carried out by separating the smooth adductor troponin components of Ezo-giant scallop on SDS-PAGE and then transferring them to nitrocellulose membrane to react with rabbit antibodies against troponin components of Akazara scallop striated adductor muscle. Ezo-giant scallop striated adductor TnC (St. TnC), TnT (St. TnT), and TnI (St. TnI) were used as control components. Color development was carried out using routine procedures (*24, 25*).

On the other hand, either the 19-kDa-a or 19-kDa-b component was shown to inhibit the actomyosin Mg-ATPase activity along with smooth adductor Tm, irrespective of the presence or absence of Ca^{2+} (Table I). Further, they repressed the activity with increase of the weight ratio of 19-kDa component to Tm up to 1.5 (Fig. 6). The inhibition was, however, significantly removed by the addition of an equimolar amount of the smooth adductor TnC, similarly to the case of scallop striated adductor TnI and TnC (*9, 10*). Consequently, both the 19-kDa-a and 19-kDa-b components are identified as TnI. Thus, the difference between 19-kDa-a and 19-kDa-b components was studied. The only difference that we have recognized so far is that the 19-kDa-a component yielded no PTH-amino acid, but the 19-kDa-b component gave a 24-amino acid sequence, AEQKKKKKKGLGGLSPEKKKMLKK, when subjected to amino acid sequencing.

Finally, the 40-kDa component is regarded as TnT based on the following facts. It activates the rabbit actomyosin-smooth adductor Tm Mg-ATPase activity, although only slightly, and is indispensable for conferring Ca^{2+}-sensitivity upon rabbit actomyosin, together with smooth adductor TnC and 19-kDa TnI (Table I). It was also confirmed by ultracentrifugal sedimentation to bind to F-actin-Tm (Fig. 7).

Amino Acid Composition of the Troponin Components—To confirm the above assignments of the Tn-components, their amino acid compositions were analyzed and compared with those of the striated adductor Tn-components (Table III). The 20-kDa and 40-kDa components showed closely similar compositions to those of the Ezo-giant scallop striated adductor TnC (20-kDa) and TnT (40-kDa), respectively. On the other hand, the smooth adductor 19-kDa-a and 19-kDa-b components revealed practically identical compositions to that of Akazara striated adductor 19-kDa-b (TnI), which was found as a complex with TnT (*29*).

Immunoblotting of the Components—We examined whether the smooth adductor Tn-components have cross-reactivity with rabbit antibodies against the Tn-components of Akazara scallop striated adductor muscle. As shown in Fig. 8, 20-kDa, 40-kDa, and 19-kDa-a and 19-kDa-b components of the smooth adductor muscle were found to cross-react with rabbit antibodies against TnC, TnT, and 52-kDa TnI of Akazara scallop striated adductor Tn, respectively.

In the present paper, we have succeeded in isolation and characterization of Tn and its components of Ezo-giant scallop smooth adductor muscle. It would be interesting to know why Tn exists in scallop smooth muscles but not in some other molluscan catch muscles and vertebrate smooth muscles, even though these muscles possess myosin-linked regulatory systems. It is therefore important to investigate the distribution of Tn in various smooth muscles and its physiological roles in Ca^{2+}-regulation.

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