Isolation and Characterization of Troponin from Abdominal Muscle of Prawn *Penaeus japonicus*

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Troponin, which confers high Ca^{2+} -sensitivity to rabbit reconstituted actomyosin, was isolated from the abdominal muscle of prawn *Penaeus japonicus*. SDS-polyacrylamide gel electrophoresis showed that the prawn troponin consists of three components of M_r 17,000, 30,000–32,000 (doublet), and 42,000 in a molar ratio of 1:1:1, but M_r 52,000–59,000 component, which had been reported to be a troponin-T-like protein in arthropod troponin, was not found. The three components were separated from each other by DEAE-Toyopearl column chromatography in the presence of 6 M urea, and assigned as troponin-C, troponin-I, and troponin-T in increasing order of molecular weight. These features were basically identical with those of American lobster troponin of our previous study (Nishita and Ojima, *J. Biochem.*, **108**, 677–683 (1990)).

Key words: invertebrates, arthropod, prawn, troponin, muscular protein, Ca^{2+} -regulation

Troponin, a muscular regulatory protein complex located on actin filaments, consists of three components, TnC, TnI, and TnT, and regulates actin-myosin interaction responding to changes in intracellular Ca^{2+} concentration.^{1–5)} Troponin was at first isolated from rabbit fast skeletal muscle^{1,2)} and its regulatory mechanism has been studied mainly on vertebrate striated muscles.^{4,5)} Troponin and troponin components have also been isolated from various invertebrate striated and smooth muscles.^{6–18)} Further, their biochemical properties and primary structures^{19–27)} have been investigated mainly from the viewpoint of comparative biochemistry and molecular evolution of troponin.

Arthropod troponin possesses some characteristic features: the Ca^{2+} -binding number of TnC is rather small^{19–21,28)} (2 mol/mol in contrast with 4 mol/mol for rabbit skeletal TnC); some crustacea species show TnI isoforms of M_r 25,000–32,000 on SDS-polyacrylamide gel electrophoresis;^{9,16–18)} and TnT shows larger M_r than that of vertebrate muscle (M_r 52,000–59,000 for arthropod TnTs in contrast to M_r 30,000 for rabbit skeletal TnT).^{6,8,9,29)}

On the other hand, we previously showed that troponin from the abdominal muscle of American lobster *Homarus americanus* consists of M_r 17,000, 30,000–32,000 (doublet), and 42,000 components, but contains no M_r 52,000–59,000 component.¹⁷⁾ The M_r 17,000 and 42,000 components were identified as TnC and TnT, respectively, and the M_r 30,000–32,000 doublet component was identified as TnI-isoforms. On the other hand, Regenstein and Szent-Györgyi⁸⁾ have reported that lobster troponin contains M_r 52,000 TnT-like protein, which is inconsistent with our result. The reason for this inconsistency is unknown, however, Mykles³⁰⁾ has suggested that the M_r of lobster

troponin components vary depending on the muscle species. In addition, troponins from the other arthropod, insects³¹⁾ and a crayfish *Procambarus clarkii*¹⁶⁾ appeared to lack the M_r 52,000–59,000 component. Recently, Miegel *et al.*¹⁸⁾ have supported our results, showing lobster troponin components to be M_r 17,000 (TnC), 30,000–32,000 (TnI), and 42,000 (TnT). Judging from these circumstances, it is still unclear whether the M_r 52,000–59,000 protein is an obligatory component in arthropod troponin.

In the present paper, we describe isolation and characterization of troponin from the abdominal muscle of prawn and search for the M_r 52,000–59,000 component.

Materials and Methods

Living prawns *Penaeus japonicus* were purchased from a local supplier and the abdominal muscle was immediately cut off and used. Prawn troponin was extracted and purified essentially by the same methods for American lobster troponin.¹⁷⁾ All the solutions used for preparation of proteins contained 5 mM 2-mercaptoethanol and were maintained at 0–4°C. The abdominal muscles (100 g) were homogenized with 300 ml of 40 mM KCl, 10 mM potassium phosphate (pH 7.0) containing 0.2 mM phenylmethylsulfonyl fluoride, 0.2 $\mu\text{g}/\text{ml}$ pepstatin, and 0.2 $\mu\text{g}/\text{ml}$ leupeptin, in a Waring blender at 10,000 rpm for 15 s; homogenization was repeated three times at 1 min intervals. The homogenate was then added to 700 ml of the same solution, mixed thoroughly, and centrifuged at $10,000 \times g$ for 15 min. The precipitate obtained was homogenized as above. This washing procedure was repeated twice more.

The resulting muscle residue was then homogenized with 900 ml of 0.4 M LiCl (pH 5.2) to extract troponin-tropomyosin. After centrifugation at $10,000 \times g$ for 15

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Abbreviations: TnC, Ca^{2+} -binding component of troponin; TnI, ATPase inhibitory component of troponin; TnT, tropomyosin-binding component of troponin; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis(β -amino ethylether)- N,N' -tetraacetic acid.

min, the supernatant was adjusted to pH 7.2 and fractionated with ammonium sulfate. Precipitates formed between 40–60% saturation were collected by centrifugation at $10,000 \times g$ for 15 min, and dissolved in and dialyzed against 0.4 M LiCl containing 1 mM sodium bicarbonate. The dialysate was then centrifuged at $100,000 \times g$ for 40 min, and the supernatant was subjected to isoelectric precipitation at pH 4.7. The precipitated tropomyosin was purified by repeating ammonium sulfate fractionation (between 45–55% saturation) and isoelectric precipitation (at pH 4.7). The troponin in the supernatant was precipitated with ammonium sulfate between 40–55% saturation and collected by centrifugation at $10,000 \times g$ for 15 min, and purified by DEAE-Toyopearl column chromatography (details are described under "Results and Discussion"). Rabbit myosin and actin were prepared by the methods of Perry,³²⁾ and Spudich and Watt,³³⁾ respectively. Mg-ATPase activity of actomyosin was measured in a medium containing 50 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, either 0.2 mM EGTA (in the absence of Ca^{2+} , $-Ca^{2+}$) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (in the presence of Ca^{2+} , $+Ca^{2+}$), 0.1 mg/ml rabbit myosin, 0.05 mg/ml F-actin, and when added, 0.025 mg/ml tropomyosin and various concentrations of troponin. Ca^{2+} -sensitivity was calculated from the following equation:

$$Ca^{2+}\text{-sensitivity} = \left(1 - \frac{\text{activity}(-Ca^{2+})}{\text{activity}(+Ca^{2+})} \right) \times 100 (\%)$$

Free Ca^{2+} concentrations were varied with Ca-EGTA buffer, using the stability constant for Ca-EGTA chelate of $8.45 \times 10^5 M^{-1}$.³⁴⁾ SDS-polyacrylamide gel electrophoresis was performed by the method of Porzio and Pearson³⁵⁾ using a 10% polyacrylamide slab gel. Proteins in the gel were stained with Coomassie Brilliant Blue R-250, and the relative amount of the protein was estimated by scanning the gel at 600 nm with a Shimadzu CS-9000 Chromatoscanner. Protein concentrations were determined by the biuret method³⁶⁾ using bovine serum albumin (Fraction V) as a standard protein. Amino acid composition was analyzed with an auto-analyzer (Hitachi 835) after hydrolysis of protein with 6 N HCl *in vacuo* in a sealed tube at 110°C for 24 h.

Results and Discussion

DEAE-Toyopearl Column Chromatography of Prawn Troponin

Crude troponin fraction obtained by the ammonium sulfate fractionation was dialyzed against 1 M urea, 30 mM KCl, 10 mM Tris-HCl (pH 7.6), and applied to a DEAE-Toyopearl 650 M (Tosoh) column (2.2×20 cm). Elution was performed with a linear gradient of 30–300 mM KCl and two major peaks were obtained (Fig. 1). Protein eluted in the first peak (fraction numbers, 42–47) showed no effect on Mg-ATPase activity of rabbit actomyosin-prawn tropomyosin, while protein in the second peak (fraction numbers, 55–59) conferred high Ca^{2+} -sensitivity. SDS-polyacrylamide gel electrophoresis (*inset* of Fig. 1) showed that the first peak contains *Mr* 65,000 protein, and the second peak contains *Mr* 17,000, 30,000–32,000 (doublet) and 42,000 proteins. Considering the previous results that

Fig. 1. Purification of prawn troponin by DEAE-Toyopearl column chromatography.

The crude troponin (120 mg) was dialyzed against 1 M urea, 30 mM KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol and subjected to a column of DEAE-Toyopearl 650 M (2.0×20 cm). Elution of proteins was performed with a linear KCl gradient from 30 to 300 mM. Each fraction contains 5.0 ml. The SDS-gel electrophoretic patterns of the sample before chromatography (P) and fractions indicated by the arrows (a–j) are shown in the inset. 65 K, *Mr* 65,000 protein; 42 K, *Mr* 42,000 component; 30 K, *Mr* 30,000–32,000 component; 17 K, *Mr* 17,000 component.

American lobster troponin consists of *Mr* 17,000, 32,000–30,000 (doublet), and 42,000,^{17,18)} the proteins in the second peak appeared to construct prawn troponin. In addition, the molar ratio of the three components was estimated to be 1:1:1 by SDS-polyacrylamide gel electrophoresis followed by densitometry if the calibration factors (or staining factors) for *Mr* 17,000, 30,000, and 42,000 components are 0.5, 1.05, and 1.0, respectively, as in the case of American lobster troponin.¹⁷⁾ Accordingly, we regarded these proteins as the prawn troponin subunits and characterized then further.

Effects of Prawn Troponin on Mg-ATPase Activity of Actomyosin

At first, various amounts of prawn troponin were added to rabbit reconstituted actomyosin together with prawn tropomyosin, and then Mg-ATPase activity was assayed in the presence and absence of Ca^{2+} . As shown in Fig. 2, the activity in the absence of Ca^{2+} was strongly inhibited by an increasing amount of prawn troponin, while that in the presence of Ca^{2+} showed a slight increase and subsequent decrease. However, Ca^{2+} -sensitivity was found to increase monophasically with increasing weight ratio of troponin:tropomyosin up to 1.2:1. This value corresponds to a molar ratio of 1:1 if the *Mr* of prawn troponin and tropomyosin are assumed to be 90,000 and 70,000, respectively. This profile of activity change was very similar to the case with American lobster troponin.¹⁷⁾

Next, the Mg-ATPase activity was measured as a function of Ca^{2+} concentration. As shown in Fig. 3, the activity increased by raising Ca^{2+} concentration from 10^{-6} to 10^{-5} M. The half maximal activation was observed at around 3–5 μM , being very close to that of American lobster troponin-tropomyosin system (5–8 μM).¹⁷⁾

Fig. 2. Effect of prawn troponin on Mg-ATPase activity of rabbit actomyosin.

Mg-ATPase activity was measured at 15°C in a solution containing 50 mM KCl, 2 mM $MgCl_2$, 20 mM Tris maleate (pH 6.8), 1 mM ATP, 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 prawn tropomyosin, and various concentrations of prawn troponin. Ca^{2+} -sensitivity (Δ) was calculated using the equation described in the text.

Fig. 3. Ca^{2+} -dependent Mg-ATPase activity of rabbit actomyosin with prawn troponin-tropomyosin.

The Mg-ATPase activity was measured in the presence of prawn tropomyosin (0.025 mg/ml)-troponin (0.04 mg/ml) under the same conditions as in Fig. 2, except that the Ca^{2+} concentration was varied with Ca-EGTA buffer consisting of 0.2 mM EGTA and various concentrations of $CaCl_2$.

Separation and Characterization of Troponin Components

Since prawn troponin consists of three components with practically the same *Mr* as those of American lobster troponin, we attempted to separate and characterize the components.

Components were separated by column chromatography on DEAE-Toyopearl 650 M (Tosoh) in the presence of 6 M urea. As shown in Fig. 4, the three components of prawn troponin were separately eluted in the ord-

Fig. 4. Separation of prawn troponin components by DEAE-Toyopearl column chromatography.

The prawn troponin (40 mg) was dialyzed against 6 M urea, 1 mM EDTA, 10 mM KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol to dissociate the components. Then, the components were applied to a column of DEAE-Toyopearl 650 M (2.2 × 15 cm) pre-equilibrated with the same buffer and eluted with a linear gradient of 10–250 mM KCl. Each fraction contains 5.0 ml. The SDS-gel electrophoretic patterns of the sample before chromatography (Tn) and of the fractions indicated by arrows (a–j) are shown in the inset. 42 K, *Mr* 42,000 component; 30 K, *Mr* 30,000–32,000 component; 17 K, *Mr* 17,000 component.

Fig. 5. UV absorption and difference UV absorption spectra of the prawn *Mr* 17,000 component.

A, UV absorption spectrum of *Mr* 17,000 component in 50 mM KCl and 20 mM MOPS-KOH (pH 6.8). B, Ca^{2+} -induced difference absorption spectrum of *Mr* 17,000 component. The sample and reference cells contained 0.1 mM EGTA and 0.1 mM EGTA plus 0.3 mM $CaCl_2$, respectively. Other conditions were the same as in A.

er of *Mr* 30,000–32,000 (doublet), 42,000, and 17,000. The *Mr* 30,000–32,000 doublet-component was unable to divide into two constituents, however, both of them were considered as TnI isoforms, since several arthropod TnIs show two protein bands on SDS-polyacrylamide gel electrophoresis.^{9,16–18)}

The *Mr* 17,000, 30,000–32,000 (doublet), and 42,000 components were dialyzed against 0.5 M KCl containing 1 mM sodium bicarbonate and 5 mM 2-mercaptoethanol, and then subjected to biochemical characterization.

The *Mr* 17,000 component was regarded as TnC, since its UV absorption spectrum resembled those of typical Ca^{2+} -binding proteins^{37–40)} and showed UV-difference ab-

Fig. 6. Inhibition of Mg-ATPase activity of rabbit actomyosin by *Mr* 30,000–32,000 component, and its neutralization by *Mr* 17,000 component.

Mg-ATPase activity was measured in the presence of 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 0.025 mg/ml prawn tropomyosin, and various concentrations of *Mr* 30,000–32,000 component (A) and 0.013 mg/ml *Mr* 30,000–32,000 component plus various concentrations of *Mr* 17,000 component (B). Other conditions are the same as in Fig. 2. ○, in the absence of Ca^{2+} ; ●, in the presence of Ca^{2+} . 30 K, *Mr* 30,000–32,000 component; Tm, tropomyosin; 17 K, *Mr* 17,000 component.

sorption around 250–280 nm upon addition of Ca^{2+} (Fig. 5A and B). On the other hand, the *Mr* 30,000–32,000 (doublet) components were regarded as TnIs, since they strongly inhibited the Mg-ATPase activity of rabbit reconstituted actomyosin in the presence of prawn tropomyosin (Fig. 6A), and this inhibition was quantitatively neutralized by the addition of the prawn *Mr* 17,000 component (TnC) (Fig. 6B). Finally, the *Mr* 42,000 component was regarded as TnT since this component was indispensable for recovery of Ca^{2+} -regulation together with the *Mr* 17,000 (TnC) and 30,000–32,000 (TnI) components (Table 1). However, the *Mr* 65,000 protein isolated in the first DEAE-Toyopearl column chromatography (Fig. 1), was incapable of substituting either the *Mr* 42,000 component or the *Mr* 30,000–32,000 doublet component (data not shown). Thus, this protein might be excluded from troponin components.

These assignments are consistent with those of American lobster counterparts. Further, the amino acid composi-

Table 2. Amino acid composition of the components of prawn troponin

Amino acid	17 K	30 K	42 K
Asx	12.0	9.1	7.7
Thr	5.7	2.2	3.4
Ser	5.5	3.4	10.0
Glx	19.7	18.5	17.8
Pro	2.1	2.6	2.9
Gly	8.2	9.5	18.2
Ala	8.2	11.9	7.1
Val	3.8	2.2	3.2
Met	3.8	1.3	1.8
Ile	6.3	3.9	2.6
Leu	8.9	6.9	5.8
Tyr	1.3	2.2	2.4
Phe	5.0	2.6	2.1
Lys	5.7	15.9	8.2
His	0.6	1.3	1.8
Arg	3.2	6.5	5.0
Total	100.0	100.0	100.0

Values are expressed as mol%. 17 K, *Mr* 17,000 component; 30 K, *Mr* 30,000–32,000-doublet component; 42 K, *Mr* 42,000 component; Asx, Asp and Asn; Glx, Glu, and Gln.

tions were very similar between the prawn troponin components and American lobster counterparts, except that the prawn *Mr* 42,000 component showed considerably higher Gly and lower Glx contents (Table 2).

Thus, we conclude that prawn troponin consists of *Mr* 17,000 TnC, *Mr* 30,000–32,000 (doublet) TnI, and *Mr* 42,000 TnT, and that *Mr* of arthropod TnT seems to vary depending on the animal species.

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Table 1. Effects of prawn troponin components on Mg-ATPase activity of reconstituted actomyosin

Samples	Mg-ATPase activity ($\mu\text{mol Pi/min} \cdot \text{mg}$)		Ca^{2+} -sensitivity (%)
	– Ca^{2+}	+ Ca^{2+}	
AM + Tm	0.048	0.050	4.0
AM + Tm + Tn	0.004	0.062	93.5
AM + Tm + 17 K	0.050	0.050	0.0
AM + Tm + 30 K	0.009	0.010	10.0
AM + Tm + 42 K	0.057	0.058	1.7
AM + Tm + 17 K + 30 K	0.036	0.037	2.7
AM + Tm + 17 K + 42 K	0.075	0.063	– 19.0
AM + Tm + 30 K + 42 K	0.004	0.004	0.0
AM + Tm + 17 K + 30 K + 42 K	0.005	0.108	95.4

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, 0.05 mg/ml rabbit F-actin, and 0.025 mg/ml prawn tropomyosin (Tm), and when added, 0.05 mg/ml prawn troponin (Tn), 0.015 mg/ml prawn *Mr* 17,000 component (17 K), 0.025 mg/ml prawn *Mr* 30,000–32,000-doublet component (30 K), and 0.03 mg/ml prawn *Mr* 42,000 component (42 K). AM, rabbit reconstituted actomyosin.

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