SUPPLEMENTARY MATERIAL

Isolation and characterization of tropomyosin from shrimp (*Penaeus vannamei* Boone) and its association property at high ionic strength

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Abstract:

Shrimps are important and highly demanded seafood, but they have been reported as a cause of food hypersensitive reaction. The major allergen of shrimp is tropomyosin. However, so far, there has been few report on such purification procedure. In this study, we developed a strategy for purification of tropomyosin from shrimp (*Penaeus vannamei* Boone). Subsequently, we demonstrated that the apparent MW of this protein is about 66 kDa, and this protein naturally contains two subunits (38.5 kDa and 36.6 kDa) with a ratio of 1 to 1. Interestingly, different from other known tropomyosins from vertebrates, shrimp tropomyosin can self-assemble into nanofibers at high ionic strength induced by ATP. These findings help understand the structure and polymerization property of tropomyosin from shrimps.

Keywords: shrimp; tropomyosin; purification; self-assembly; nanofibers;

3. Experimental

3.1. Chemicals

3-(N-morpholino) propanesulfonic acid (MOPS) was obtained from Research Organics. N',N'-bis-methyleneacrylamide, sodium dodecyl sulfate (SDS), tris(hydroxymethyl)aminomethane (Tris), TEMED, β -Mercaptoethanol, Bovine Serum Albumin (BSA) and Coomassie Brilliant Blue R-250 were purchased from Sigma-Aldrich Co. (Beijing, PR China). ATP- Na_2 was obtained from Beijing BioDee BioTech Corporation Ltd. (Beijing, PR China). All other reagents used were of analytical grade or pure.

3.2. Protein isolation and purification

Penaeus vannamei Boone were obtained from local market. The species were identified and authenticated by Prof Y.K. Luo of the China Agricultural University. The identification number of the voucher specimen *Penaeus vannamei* Boone was ZLPV4001. Native tropomyosin from *Penaeus vannamei* Boone was prepared according to the reported methods with major modifications (Astbury et al. 1948; Rao et al. 2009). Briefly, 100 g of shrimp tissue was homogenised in a grinder at 4 °C along with 100 mL of buffer containing 5 mM Tris-HCl, pH 8.0. The homogenate was extracted at 4 °C for 30 min with stirring, followed by addition of five volumes of cold acetone and then centrifuged for 30 min at $12000 \times g$. Resulting precipitation (acetone powder) was then extracted in buffer A containing 0.2 mM ATP- Na_2 , 0.2 mM CaCl_2 , 5 mM Tris-HCl, pH 8.0 for two hours at 4 °C (30 mL/10 g), followed by centrifugation $12000 \times g$. Resultant supernatant was then filtered through pinhole filter of 0.45 μm , and the filtrate was then incubated in a boiling water bath for 5 min to eliminate proteases and other heat-sensitive proteins. The heat stable supernatants were further precipitated by ammonium sulfate fractionation (75% saturated fraction) overnight at 4 °C. Next the solution was centrifuged at $12,000 \times g$ for 15 min, and then resultant precipitation was dissolved in buffer (2 mM Tris-HCl, pH 8.0), and was dialysed against the same buffer three times. The protein was further purified by ion exchange chromatography (IEC) on a DEAE-Sepharose Fast Flow column previously equilibrated in 50 mM Tris-HCl buffer (pH 8.0).

3.3. Polyacrylamide gel electrophoresis

The molecular weight of native TM was estimated by Native-PAGE using a 4-20% polyacrylamide gradient gel run at 25V for 14 h at 4 °C employing Tris-HCl (25 mM, pH 8.3) as running buffer. Gels were stained with Coomassie brilliant blue R250. Gel electrophoresis under denaturing conditions was carried out with 15% polyacrylamide-SDS gel as reported by Laemmli (Laemmli 1970). 20 µL of SDS sample buffer containing 25% glycerol, 12.5% 0.5 M Tris-HCl, pH 6.8, 2% SDS, 1% bromphenol blue, and 5% β-mercaptoethanol were added to 20 µL TM aqua sample (~ 5 µM). After the solution was boiled for 5 min, the supernatant was isolated by centrifugation at $3,000 \times g$ for 5 min.

3.4. In-gel trypsin digestion

Purified TM subunits (38.5 and 36.6 kDa) gel bands, ~1 mm² were excised from a 15% SDS-polyacrylamide gel with a pipette tip and transferred to 1.5 mL microtubes, respectively. To each tube, 2 µL destaining solution containing 0.05 µg Trypsin, 0.1% n-dodecyl β-D-maltoside, 25 mM of NH₄HCO₃ was added and the mixture was incubated for 10 min, followed by addition of 10 µL solution containing 0.1% n-dodecyl β-D-maltoside, 25mM NH₄HCO₃. After incubation at 37 °C for 2 hours, peptides were desalted and concentrated using Ziptip C-18 columns prior to MS analysis (Deng et al. 2010).

3.5. Peptide mass fingerprinting (PMF) by MALDI-TOF-MS

All mass spectra of *MALDI-TOF-MS* was obtained on a Bruker ultraflex III TOF/TOF (Bruker Daltonik GmbH, Germany) in positive ion mode at an accelerating voltage of 20 kV with a nitrogen laser (337 nm). Aliquots prepared using trypsin of 0.5 µL were applied onto the steel target plate and immediately mixed with an equal volume of α-cyano-hydroxy-cinnamic acid (CHCA) (10 mg/mL in 50%ACN/0.1%TFA) and allowed to air-dried before analysis. PMFs obtained was used to search through the SWISS-PROT and NCBI database by the MASCOT search engine (<http://www.matrixscience.co.uk>) with one missed cleavage site. The database search was finished with the Mascot search engine (<http://www.matrixscience.co.uk>) using the data processed through MaxEnt3 and MasSeq.

3.6. Stopped-flow light scattering measurement

ATP-induced TM polymerization was observed by stopped-flow apparatus in conjunction with the Cary Eclipse spectrofluorimeter (Varian Palo Alto, USA) at 25 °C with both excitation and emission wavelengths set at 450 nm, scattering light at 90° perpendicular to the beam and the mixing dead time was determined to be 6.8±0.5 ms as previously described (Deng et al. 2010). Time-dependent change in scattering intensity was measured after mixing different final concentrations of ATP with TM (5.0 µM) in different buffersystems and data were acquired every 12.5 ms.

3.7. Characterization of protein polymerization by TEM

Transmission electron microscope (TEM) samples were observed by negative staining after the preparation of the samples (Sousa et al. 2010). Briefly, samples were diluted into quoted concentrations, and a droplet of the diluted sample was put onto a carbon support film on a copper grid. The droplet was removed with a filter paper five minutes later. Then, a droplet of 2% uranyl acetate was put onto the grid and removed after 2 min. The micrographs were made using a Hitach H-7650 electron microscope operating at 40kV~120kV.

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Table S1. Mass peaks of gel bands 38.5 kDa and 36.6 kDa for MASCOT database search.

Gel Band 38.5 kDa		Gel Band 36.6 kDa	
PMF (m/z)		PMF (m/z)	
722.300	1878.113	838.980	1878.672
854.997	1915.211	854.944	1915.037
861.021	1933.226	860.969	1933.039
871.070	1940.211	876.926	1940.086
877.002	1956.240	892.880	1982.131
906.359	1971.212	906.310	2031.104
1066.044	2031.250	1050.006	2053.088
1129.667	2095.260	1066.010	2069.071
1325.796	2118.339	1081.983	2096.088
1376.782	2134.297	1129.557	2118.127
1391.791	2157.307	1277.104	2135.154
1414.850	2187.388	1325.684	2157.157
1496.970	2202.416	1376.656	2187.217
1570.988	2234.369	1414.750	2221.351
1606.983	2333.412	1570.863	2234.192
1648.022	2343.692	1631.870	2333.250
1663.972	2364.500	1663.829	2463.620
1692.984	2462.540	1735.820	2488.426
1735.971	2488.614	1759.909	2508.392
1759.086	2508.575	1767.938	2530.397
1812.095	2530.529	1799.158	2555.498
1827.036	2681.686	1812.644	2644.502
1845.148	2709.831	1827.641	2681.536
1854.256	2910.708	1854.113	2709.612

Figure S1. Native PAGE and SDS-PAGE analyses of purified native tropomyosin from *Penaeus vannamei* Boone. Sample was loaded after purification. (A) Native PAGE; (B) SDS-PAGE. Lane 1, Protein markers; Lane 2, TM from shrimp.

Figure S2. Tryptic PMF of the (A), 38.5 kDa and (B), 36.6 kDa gel bands from SDS-PAGE acquired by MALDI-TOF-MS.

Figure S3. Transmission electron microscopy images of ATP-induced native tropomyosin polymerization. (A) Scale bar corresponding to 1.0 μm ; (B) Scale bar corresponding to 50 nm. Samples were negatively stained by 2% uranyl acetate.

Figure S4. Reversible polymerization of native tropomyosin induced by ATP detected by light scattering measurement. 1, TM alone; 2, TM plus 12 mM of ATP; 3, TM was mixed with 12 mM of ATP, followed by dialysis. Conditions were as follows: $[\text{TM}] = 5 \mu\text{M}$, 25 $^{\circ}\text{C}$, and both ATP and TM are in the same buffer containing 100 mM KCl, 50 mM Tris-HCl, pH 8.0.