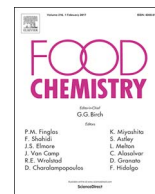




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Conformation stability, *in vitro* digestibility and allergenicity of tropomyosin from shrimp (*Exopalaemon modestus*) as affected by high intensity ultrasound

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ABSTRACT

The effects of high intensity ultrasound (HIU) modification (power: 100–800 W, 15 min) on the conformation stability, *in vitro* digestibility and allergenicity of tropomyosin (TM) from *Exopalaemon modestus* were investigated. HIU treatment oxidized the Cys, Met and Lys in TM and degraded TM to generate protein fragments by HIU-induced free radicals. The α -helix content decreased, while β -sheet, β -turn and random coil increased. The immunoblotting and ELISA data suggested HIU treatment reduced the allergenicity and contributed to higher digestibility of TM. TM had resistance to gastric digestion, while intestinal digestion totally hydrolyzed TM, and the sequential HIU-gastric-intestinal digestion most efficiently hydrolyzed TM and reduced the allergenicity of digestion products. Stronger HIU could loosen TM structure, strengthen the gastrointestinal digestibility of TM and reduce the allergenicity of TM and digestion products to low level.

1. Introduction

Shrimp allergy is a common allergic reaction worldwide and has become more serious in recent years, it may trigger severe allergic reactions and usually persist throughout life. The major allergen in shrimp was identified as tropomyosin (TM), which composed of two identical α -helical chains arranged in a coiled-coil secondary structure, with 284 amino acids and molecular weight of 35–40 kDa (Gómez et al., 2015; Liu et al., 2010; Lu, 2016). Shrimp allergic patients had immune response to tropomyosin, which was bound to shrimp-specific IgE from shrimp allergic patients (Lu, 2016).

Exopalaemon modestus (Taihu white shrimp) is a famous freshwater shrimp native to Taihu Lake, and has long been a fashionable delicacy with high nutritional value and culinary properties in Jiangsu and Zhejiang Province, China. This kind of shrimp constitutes a substantial freshwater fishery market with large production. While due to its increasing consumption in recent years, the occurrence of allergic reactions was also increasing. Reducing its major allergen would be beneficial to shrimp allergic individuals. However, up to now, there were no available data on the antigenicity and how to effectively reduce the antigenicity of tropomyosin from *Exopalaemon modestus*.

Among the measures to decrease the antigenicity of shrimp, high intensity ultrasound (HIU, 16–100 kHz, power 10–1000 W/cm²) is a

promising technology in food processing for its acoustic cavitation. During sonication, cavitation bubbles are formed, which grow over a few cycles and then violently collapse, resulting in high temperatures (5000 K), pressures (1000 atm), high shear energy waves and turbulence in the cavitation zone (Hu, Cheung, Pan, & Li-Chan, 2015), which modifies the conformation of proteins, loosen the protein structure and even break peptide chains of proteins, therefore altering antigenicity by changing its IgE reactive conformational or linear epitopes. Several studies have investigated the effect of sonication on the conformation of different proteins. Jiang et al. (2014) found that HIU increased the β -sheet of black bean protein isolates and Gülseren, Güzey, Bruce, and Weiss (2007) noticed that HIU reduced the β -turn of bovine serum albumin. Stathopoulos et al. (2004) pointed out that HIU of protein increased the β -structure while decreased the α -helix structure in the protein aggregates. Hu et al. (2015) found that HIU treatment could disrupt the tertiary and quaternary structure of soybean protein. The muscle protein tropomyosin is the major allergen identified in shrimp (Gómez et al., 2015; Liu et al., 2010), while limited reports focused on the effects of HIU on the conformation stability of tropomyosin and thereby antigenicity.

Resistance to gastrointestinal digestibility is one common characteristic for food allergens, which enables them to pass intact through the gastrointestinal tract, thereby result in retention of conformational

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or linear epitopes to bind with antibodies and therefore triggering allergic responses (Astwood, Leach, & Fuchs, 1996). Several studies report that tropomyosin is an extremely stable protein (Leung et al., 1994). However, the stability and allergenicity variation of shrimp tropomyosin to simulated gastrointestinal digestion are not well known. For this reason, it is necessary to gain a thorough understanding how tropomyosin digested during gastrointestinal tract and what impacted the allergenic potential in order to manage shrimp allergenicity.

Reducing the allergenicity of tropomyosin would be beneficial to shrimp allergic individuals. In this work, the objectives were to investigate the effects of HIU treatment on the TM conformation stability, allergenicity, gastrointestinal digestibility, and the allergenicity of gastrointestinal digestion products of TM from *Exopalaemon modestus*. The results of this work would enhance our understanding of the mechanism of allergenicity reduction of tropomyosin by HIU treatment, as well as indicating that HIU treatment may be an alternative efficient method for tropomyosin hyposensitization.

2. Materials and methods

2.1. Materials

Live *Exopalaemon modestus* (47.76 ± 3.31 mm in length, 1.59 ± 0.27 g in weight) were purchased in Nanquan fishery market of Taihu Lake, Wuxi, China. ANS (8-anilino-1-naphthalenesulphonic acid, CAS: 82-76-8), DTNB (5, 5-dithiobis [2-nitrobenzoic acid], CAS: 69-78-3), EDTA (ethylenediaminetetraacetic acid, CAS: 60-00-4), pepsin (3411 U/mg protein, CAS: 9001-75-6), trypsin (13165 U/mg protein, CAS: 9002-07-7) and urea (CAS: 57-13-6) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). ECL luminous liquid and TMB (Tetrazolium benzidine) were purchased from Beyotime Technology Co., Ltd. (Beijing, China), peroxidase conjugated goat anti-human IgE were purchased from KPL Inc. (Gaithersburg, MD, USA). BSA (bovine serum albumin, CAS: 9048-46-8) was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All other chemicals were analytical grade or better.

2.2. Patients' sera

Patients' sera from shrimp-allergic patients were obtained from the Department of Clinical Laboratory of Wuxi People's Hospital (Wuxi, China) and stored in aliquots at -80°C until analysis. The allergic response was confirmed by clinical diagnosis. A pool of sera from selected patients was used for IgE immunodetection. Ten sera from healthy volunteers (non-allergic and non-atopic individuals) were employed as negative controls.

2.3. Extraction and purification of tropomyosin (TM)

Raw shrimp muscles were minced and homogenized with 4-fold of 0.01 M PBS buffer (pH 7.4) in a homogenizer (DS-1, Shanghai Specimen Model Factory, Shanghai, China). After centrifugation at $10,000 \times g$ for 15 min, the supernatant was discarded and the pellet was resuspended in the 0.01 M PBS buffer, followed by homogenization and centrifugation. The washing, homogenization and centrifugation were repeated four times, to remove sarcoplasmic proteins effectively. The final resulting precipitate was then washed three times with cold absolute acetone (-20°C). After the final wash, the residues were spread on a filter paper and dried overnight.

The dried powder was suspended in 8-fold (w/v) of another buffer (1 M KCl, 0.5 mM DTT, 20 mM Tris-HCl, pH 7.5), and extraction was carried out with stirring at 4°C overnight. The solution was centrifuged at $15,000 \times g$ for 15 min. The supernatant passed through filter papers and then was subjected to isoelectric precipitation at pH 4.5 adjusted with 1 M HCl, kept at 4°C for 2 h, then centrifuged at $15,000 \times g$ for 15 min. The resulting precipitate was dissolved in PBS and subjected to

isoelectric precipitation at pH 4.5 with 1 M HCl once more. The resulting isoelectric precipitate was dissolved in PBS and brought to pH 7.6 with 1 M NaOH, then subjected to ammonium sulfate fractionation of 41% saturation, kept at 4°C for 4 h, centrifuged at $15,000 \times g$ for 15 min. The resulting precipitate was dissolved in PBS and brought to pH 7.6, then subjected to ammonium sulfate fractionation of 41% saturation once more. The precipitate was dialyzed (14 kDa, Shanghai Green Bird Science & Technology Development Co., Ltd, Shanghai, China) in ultrapure water (NW15VF, Heal Force® water purification system, Shanghai Canrex Analytic Instrument Co., Ltd, Shanghai, China) at 4°C for 48 h, then heated in a boiling water bath for 10 min. After extensive cooling in ice water, the boiled solution was centrifuged at $15,000 \times g$ for 15 min, the supernatant containing purified tropomyosin was passed through filter papers, then lyophilized using a vacuum freeze drier (BenchTop Pro, SP Scientific, Warminster, PA, USA) and stored at -20°C . The protein content was determined using the Biuret method with BSA as standard.

2.4. High intensity ultrasound (HIU) treatment

The HIU treatment of TM was according to Zhang, Regenstien, Zhou, and Yang (2017) with some modifications. TM solution (1 mg/mL) was treated using an ultrasound processor (JY99-IIDN, 20 kHz, Scientz Biotechnology Co., Ltd, Ningbo, China) equipped with a 2.0 cm (diameter) titanium probe, which was used to sonicate 25 mL of TM solutions in 50 mL centrifuge tubes immersed in an ice-water bath. Samples were treated at different power outputs (100, 200, 300, 400, 500, 600, 700 and 800 W) for 15 min (pulse durations of 5 s on and 1 s off) and the samples without ultrasound treatment served as the control.

2.5. Conformation stability

2.5.1. Amino acid analysis

The determination of amino acid content was according to Zhang, Yang, Zhou, Zhang, and Wang (2017) with some modifications. The lyophilized HIU-treated TM samples (0.1 g) were mixed with 8 mL of 6 M HCl in 10 mL capped glass tubes, filled the tubes with pure nitrogen gas, then hydrolyzed at 110°C for 22 h; after cooled to room temperature (25°C), transferred the sample from the tube to the 25 mL volumetric flask, and added 4.8 mL 10 M NaOH to neutralize for acid hydrolysis. Washed the inner-wall of the flask by small amount of water, then diluted with water to 25 mL; part of the solution was filtered using two slices of filter paper and transferred to a centrifuge tube, centrifuged at $10,000 \times g$ for 10 min, then took 400 μL of the supernatant into HPLC sample bottles. The samples were analyzed using an automatic amino acid analyzer (Agilent 1260, Agilent Technology, Santa Clara, CA, USA) for amino acid analysis. The content of amino acid was expressed as the weight percentage (%) in total amount of amino acid.

2.5.2. High performance liquid chromatograph (HPLC) analysis

The HPLC analysis of HIU-treated TM were performed using an Alliance HPLC (Waters e2695, Waters, Corp., Milford, MA, USA) with a BioSuite 125, 5 μm , HR SEC column (7.8 mm $\times$ 300 mm, Waters Corp., Milford, MA, USA). The elution system was 0.01 M PBS (pH 7.0) containing 0.15 M NaCl. The flow rate was 1.0 mL/min and the analysis was conducted at 20°C . Protein was monitored at 220 nm with a Waters 2489 UV detector (Waters Corp., USA). HIU-treated TM was prepared by dissolving 1 mg protein in 1 mL 0.01 M PBS (pH 7.0) and injected on to the column (15 μL). Data acquisition was implemented using Waters Empower 3 software (Waters Corp., USA).

2.5.3. Circular dichroism (CD) spectra

Far-UV CD spectra were recorded by a MOS-450 spectrometer (Bio-Logic Science Instruments, Claix, France) at 25°C , using a quartz

cuvette with an optical path-length of 1 mm. Protein samples (0.1 mg/mL) were scanned from 190 to 250 nm at a scan rate of 100 nm/min with a wavelength step of 1 nm. The DichroWeb (<http://dichroweb.cryst.bbk.ac.uk>) was used for the analysis of protein secondary structures.

2.5.4. Surface hydrophobicity

The surface hydrophobicity of TM sample was determined using ANS as a fluorescence probe. TM solution (1 mg/mL) was diluted to a series of solutions ranged from 0.1 to 0.5 mg/mL. A 20 μ L aliquot of ANS solution (8 mM ANS in 0.1 M KH_2PO_4 buffer, pH 7.0) was added to 4 L of TM solution and mixed well. Samples were kept in the dark for 20 min. The fluorescence intensity was determined using a fluorescence spectrophotometer (F-7000, Hitachi Corp., Tokyo, Japan). The surface hydrophobicity (S_0 -ANS) of the samples was defined as the initial slope of the fluorescence intensity versus protein concentration plot, which was calculated by linear regression analysis.

2.5.5. Sulfhydryl (SH) group content

The total SH group content were determined as follows: 4.5 mL of a buffer containing 8 M urea, 0.1 M KH_2PO_4 and 10 mM EDTA (pH 7.0), and 100 μ L Ellman's reagent (10 mM DTNB in 0.1 M KH_2PO_4 buffer, pH 7.0) were added to 0.5 mL TM solutions (1 mg/mL). Samples were kept in the dark for 25 min. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer (UV-2700, Shimadzu Corp., Kyoto, Japan). Reactive SH groups were calculated as the measurement of total SH groups with the absence of urea. A molar extinction coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate the SH group content.

2.5.6. Particle size and zeta potential

One mL of TM solutions (1 mg/mL) were injected in clear zeta cells for particle size and zeta potential measurement, and analyzed using a Particle Sizer and Zeta Potential Analyzer (NanoBrook Omni, Brookhaven Instruments Corp., Holtsville, NY, USA) in triplicate, the particle size (nm) and zeta potential (mV) data were reported automatically by the Particle Solution 3.0 software (Brookhaven Instruments Corp., Holtsville, NY, USA).

2.6. Allergenicity analysis

2.6.1. Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

TM solutions (1 mg/mL) were diluted 1:1 in loading buffer (25 mM Tris-HCl, 10% glycerol (v/v), 2% SDS (w/v), 1.54% DTT (w/v), and 0.1% bromophenol blue (w/v)) and heated (95 °C, 3 min) prior to gel running. As to SDS-PAGE, a separating gel of 12% acrylamide and a stacking gel of 4% acrylamide were used. The amount of sample loaded per lane was 10 μ L for each. Molecular weight standards (10–250 kDa) (Bio-Rad, Philadelphia, PA, USA) were used in every gel. Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) with running buffer (50 mM Tris-HCl, 384 mM glycine and 0.1% SDS, pH 8.3). Electrophoresis was conducted at 80 V for stacking gel and 120 V for separating gel. Protein bands were fixed and stained with Coomassie Brilliant Blue (R-250) for 1 h, then destained with a solution containing 50 mL/L methanol and 75 mL/L acetic acid.

2.6.2. Immunoblotting

The proteins from electrophoretic gels were transferred to a PVDF membrane (0.45 μ m, Merk Millipore Ltd., Darmstadt, Germany) by a Mini Trans-Blot® cell (Bio-Rad, Philadelphia, PA, USA) using Tris-glycine transfer buffer system. The membrane was first washed with a Tris-buffered saline solution and then incubated in a blocking solution (5% defatted milk) for 1 h at 25 °C. The membrane was then transferred to a diluted pooled sera (1:10 dilution) and then incubated overnight at 4 °C. The blot was washed five times with TBST buffer (0.1 M Tris,

0.15 M NaCl, 0.05% Tween-20, pH 7.5) and then incubated with peroxidase conjugated goat anti-human IgE (KPL Inc., Gaithersburg, MD, USA) diluted in TBST (1:5000) for another hour. The membrane was then washed five times with TBST and the immobilized protein-antibody complex in the membrane was detected (BeyECL Plus, Beyotime Biotechnology Co., Ltd, Beijing, China) and recorded using a ChemiDoc XRS⁺ imager (Bio-Rad, Hercules, CA, USA).

2.6.3. Enzyme linked immune sorbent assay (ELISA)

ELISA was carried out as follows: the Polystyrene 96-well ELISA plates (Costar 2592, Corning Inc., NY, USA) were coated with TM samples (5 μ g/mL, 100 μ L per well) in the buffer containing 0.29% (w/w) NaOH and 0.16% (w/w) Na_2CO_3 , and incubated at 37 °C for 2 h. After washing the plates with TBST for 5 times, the nonspecific sites were blocked by 5% defatted milk (200 μ L per well) at 37 °C for 2 h. The plates were washed and incubated with 100 μ L of human sera (1:10 diluted with TBST, v/v). After incubation at 37 °C for 1 h, the plates were washed again and incubated with peroxidase conjugated goat anti-human IgE (1:5000, v/v, KPL Inc., Gaithersburg, MD, USA) at 37 °C for 1.5 h, 100 μ L per well. Finally, color was developed with TMB (Beyotime Biotechnology Co., Ltd., Beijing, China). After terminating the reaction by addition of 2 M H_2SO_4 , the absorbance was monitored at 450 nm using an automated ELISA plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

2.7. Simulated gastrointestinal digestibility

Simulated gastric fluid (SGF) digestion of the TM was conducted using the method by Astwood et al. (1996) with some modifications. Briefly, the SGF was prepared following the United States Pharmacopoeia Standard (Anonymous, 1995). The total volume of the reaction solution was 2 mL, with the ratio of pepsin to test protein at 1:50 (w/w). Digestion was carried out at 37 °C, and the SGF was incubated in a water bath at 37 °C for 10 min before adding the TM samples. At 0, 5, 15, 30, 40, 50 and 60 min, 100 μ L of the reaction solution was transferred to a 1.5 mL centrifuge tube, and 30 μ L of 0.2 M Na_2CO_3 was added to stop the digestion.

Simulated intestinal fluid (SIF) digestion was also prepared as described in the United States Pharmacopoeia (Anonymous, 1995). The total volume of the reaction solution was 2 mL, and the ratio of trypsin to test protein was 1:100 (w/w). Digestion was performed at 37 °C with SIF preheated. At 0, 1, 5, 7, 15, 30, 60, 120 and 180 min, 100 μ L of the reaction solution was transferred to a 1.5 mL centrifuge tube, and the reaction was immediately terminated by heating in a boiling water bath for 10 min.

The sequential enzymatic treatments of SGF and SIF digestion was carried out as follows: after SGF digestion for 1 h, the pH of SGF solution was adjusted to 7.5, then adding the SIF to further the SIF digestion as the procedures described above. The digestibility of TM and allergenicity of simulated digestion products was analyzed using SDS-PAGE and indirect ELISA, respectively.

2.8. Statistical analysis

Data were presented as means $\pm$ standard deviation (SD). Statistical analysis was performed using SPSS software (SPSS Inc., Ver. 19, Chicago, IL, USA). The Duncan's multiple range test ($p < 0.05$) was used to determine the significant differences between means.

3. Results and discussion

3.1. Conformational stability of TM

3.1.1. Amino acid content

The amino acid sequence was the primary structure of a protein. Changes in amino acid content of a protein can be used to reflect the

Table 1
Amino acid analysis of non-HIU and HIU treated TM.

Amino acid	Ultrasonic power (W)									
	0	100	200	300	400	500	600	700	800	
Asp	5.97 ± 0.03 ^a	6.01 ± 0.05 ^a	5.98 ± 0.02 ^a	5.98 ± 0.03 ^a	5.98 ± 0.04 ^a	5.97 ± 0.03 ^a	5.97 ± 0.02 ^a	6.01 ± 0.03 ^a	5.98 ± 0.04 ^a	
Glu	21.71 ± 0.02 ^a	21.68 ± 0.01 ^a	21.68 ± 0.02 ^a	21.72 ± 0.04 ^a	21.69 ± 0.03 ^a	21.69 ± 0.02 ^a	21.70 ± 0.02 ^a	21.69 ± 0.03 ^a	21.69 ± 0.01 ^a	
Ser	4.26 ± 0.05 ^a	4.25 ± 0.02 ^a	4.27 ± 0.03 ^a	4.25 ± 0.04 ^a	4.25 ± 0.02 ^a	4.26 ± 0.05 ^a	4.25 ± 0.03 ^a	4.25 ± 0.04 ^a	4.26 ± 0.03 ^a	
His	0.42 ± 0.03 ^a	0.43 ± 0.02 ^a	0.42 ± 0.04 ^a	0.42 ± 0.03 ^a	0.42 ± 0.01 ^a	0.42 ± 0.04 ^a	0.43 ± 0.03 ^a	0.43 ± 0.02 ^a	0.43 ± 0.04 ^a	
Gly	0.72 ± 0.04 ^a	0.71 ± 0.03 ^a	0.71 ± 0.02 ^a	0.72 ± 0.01 ^a	0.72 ± 0.04 ^a	0.72 ± 0.03 ^a	0.71 ± 0.01 ^a	0.72 ± 0.02 ^a	0.71 ± 0.03 ^a	
Thr	2.79 ± 0.01 ^a	2.78 ± 0.03 ^a	2.78 ± 0.02 ^a	2.78 ± 0.04 ^a	2.78 ± 0.02 ^a	2.79 ± 0.05 ^a	2.79 ± 0.06 ^a	2.78 ± 0.04 ^a	2.79 ± 0.05 ^a	
Arg	10.03 ± 0.06 ^a	10.05 ± 0.04 ^a	10.03 ± 0.02 ^a	10.02 ± 0.03 ^a	10.03 ± 0.05 ^a	10.04 ± 0.04 ^a	10.05 ± 0.02 ^a	10.05 ± 0.05 ^a	10.06 ± 0.03 ^a	
Ala	7.39 ± 0.03 ^a	7.40 ± 0.02 ^a	7.40 ± 0.04 ^a	7.39 ± 0.01 ^a	7.39 ± 0.02 ^a	7.39 ± 0.03 ^a	7.41 ± 0.05 ^a	7.40 ± 0.03 ^a	7.40 ± 0.04 ^a	
Tyr	2.01 ± 0.04 ^a	1.99 ± 0.03 ^a	1.99 ± 0.05 ^a	2.00 ± 0.06 ^a	2.00 ± 0.04 ^a	2.01 ± 0.03 ^a	2.00 ± 0.02 ^a	2.01 ± 0.04 ^a	2.01 ± 0.05 ^a	
Cys	0.32 ± 0.05 ^a	0.31 ± 0.01 ^a	0.30 ± 0.02 ^a	0.30 ± 0.04 ^a	0.29 ± 0.02 ^a	0.27 ± 0.03 ^{ab}	0.21 ± 0.02 ^b	0.16 ± 0.02 ^c	0.11 ± 0.01 ^d	
Val	3.95 ± 0.03 ^a	3.94 ± 0.05 ^a	3.94 ± 0.02 ^a	3.95 ± 0.02 ^a	3.93 ± 0.04 ^a	3.93 ± 0.04 ^a	3.95 ± 0.04 ^a	3.94 ± 0.03 ^a	3.95 ± 0.02 ^a	
Met	3.21 ± 0.02 ^a	3.21 ± 0.04 ^a	3.21 ± 0.01 ^a	3.21 ± 0.03 ^a	3.21 ± 0.05 ^a	3.21 ± 0.02 ^a	3.15 ± 0.01 ^b	3.06 ± 0.02 ^c	2.97 ± 0.03 ^d	
Phe	1.82 ± 0.04 ^a	1.81 ± 0.02 ^a	1.81 ± 0.03 ^a	1.82 ± 0.05 ^a	1.81 ± 0.04 ^a	1.83 ± 0.02 ^a	1.82 ± 0.03 ^a	1.82 ± 0.01 ^a	1.81 ± 0.04 ^a	
Ile	1.75 ± 0.01 ^a	1.75 ± 0.03 ^a	1.73 ± 0.02 ^a	1.75 ± 0.04 ^a	1.74 ± 0.06 ^a	1.73 ± 0.04 ^a	1.74 ± 0.01 ^a	1.74 ± 0.02 ^a	1.44 ± 0.05 ^a	
Leu	11.45 ± 0.03 ^a	11.43 ± 0.06 ^a	11.43 ± 0.02 ^a	11.45 ± 0.01 ^a	11.42 ± 0.03 ^a	11.43 ± 0.05 ^a	11.45 ± 0.02 ^a	11.44 ± 0.03 ^a	11.45 ± 0.03 ^a	
Lys	10.97 ± 0.04 ^a	10.98 ± 0.05 ^a	10.97 ± 0.04 ^a	10.95 ± 0.06 ^a	10.95 ± 0.05 ^a	10.92 ± 0.03 ^a	10.83 ± 0.02 ^b	10.71 ± 0.03 ^c	10.52 ± 0.05 ^d	

Note: Different superscripted letters in the same line means significant differences ($p < 0.05$).

alteration on its conformation. Table 1 showed the weight percentage (%) of amino acids in non-HIU and HIU treated tropomyosin (TM) samples. Compared with the control (0 W), the content of most amino acids of HIU-treated TM had no significant changes ($p > 0.05$), while the content of Cys, Met and Lys decreased at higher HIU power ($p < 0.05$). This might be due to that ultrasonication of water leads to electronically excited water molecules, resulting in the formation of H and OH radicals, then reactive oxygen species (ROS) such as H_2O_2 , as follows: $\text{H}_2\text{O} \rightarrow \text{H}^\cdot + \text{HO}^\cdot$, $\text{H}^\cdot + \text{O}_2 \rightarrow \text{HO}_2^\cdot$, $2\text{HO}^\cdot \rightarrow \text{H}_2\text{O}_2$, $2\text{HO}_2^\cdot \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ (Makino, Mossoba, & Riesz, 1983; Mason & Peters, 2002; Riesz & Kondo, 1992; Wang & Xu, 2012). These HIU-induced free radicals could attack the amino acid residues, leading to their loss. Specifically, the sulfur amino acids (Cys and Met) were the prone to oxidative modifications in radical oxidation. Oxidation of Cys is known to lead to the formation of disulfide bonds, mixed disulfides (e.g., with glutathione), and thiyl radicals (Kalyanaraman, 1995). As to Met residues, the major oxidation product is methionine sulfoxide (Vogt, 1995). The loss of Lys might be attributed to that, as HIU became stronger, HIU-induced free radical breakdown products bond covalently to Lys (Requena et al., 1997), thereby resulting in the loss.

3.1.2. Degradation of TM by HIU treatment

Since HIU-induced free radicals could oxidize amino acids, could they attack protein primary structure and thereby degrade TM? As shown in SDS-PAGE (Fig. 1A), the intensity of the band of TM tended to decrease gradually with higher HIU power, which suggested the degradation effects of HIU on TM molecules. While there were no protein fragment bands, this might be due to the small amount and molecular weight of protein fragments, which could not be detected by SDS-PAGE. To further pinpoint the protein fragments induced by HIU, the HPLC was used to analyze TM samples (Fig. 1B–J, Table 2). In control (0 W), there was only one peak referred to TM, which indicated the high purity of TM samples. With the strength of HIU power (100–800 W), there were two main peaks in 100–200 W, three peaks in 300–500 W, and five peaks in 600–800 W, which suggested that stronger HIU could destroy TM molecules to generate various protein fragments. Specifically, as HIU strengthened (0–800 W), the peak of TM (at about 5.7 min) decreased, the peak at about 10.25, 10.52 and 13.06 min increased, while the peaks at about 11.22 min increased in 0–600 W, then decreased at 600–800 W. This indicated that stronger HIU induced the greater degradation of TM molecules, the generation of more protein fragments, and the generation of protein fragments at about 10.25 and 10.52 min might be related to the reduction of TM or the decompose of protein fragments at about 11.22 min.

Previous literatures suggested that ultrasound could degrade biomolecules by ultrasound-induced free radicals (Makino et al., 1983; Mason & Peters, 2002; Pingret, Fabiano-Tixier, & Chemat, 2013; Soria & Villamiel, 2010). The biodegradation of TM (Fig. 1) might be due to the cleavage of bonds by HIU-induced free radicals attack. Possible reasons as follows: Free radical species are atoms or molecules that are capable of independent existence and possess one or more unpaired electrons, such as hydroxyl radical ($\text{HO}^\cdot$) and hydroperoxyl radical ($\text{HO}_2^\cdot$) (Tai, Gu, Zou, & Guo, 2002). Among these various radicals, the hydroxyl radical is thought to play a central role in biomolecule degradation (Tai et al., 2002). Due to its high standard potentials of 2.8 V versus normal hydrogen electrode (NHE) in acidic environment and 1.55 V versus NHE in basic environment, hydroxyl radicals is highly reactive and nonselective that react with molecules in atom abstraction or addition reactions driven by bond strength energetics (García-Montaña et al., 2008; Pera-Titus, García-Molina, Baños, Giménez, & Esplugas, 2004), then disrupt or cleavage the primary structure of TM molecules, leading to protein fragments.

3.1.3. Secondary structure

The secondary structure compositions of tropomyosin were shown in Fig. 2A. Tropomyosin is not a uniform structure (Eckard & Cowgill, 1976), as shown in Fig. 2A, the secondary structure components in the non-HIU treated TM (control) consisted of 78.4% α -helix, 5.2% β -turn and 14.4% random coils, which were similar with the data reported by Jin et al. (2015). As HIU power increased, the content of α -helix decreased significantly from 78.4% (control) to 16.1% (800 W), while the contents of β -sheets, β -turns and random coils increased markedly from 0 to 20.5%, 5.2 to 22.4% and 14.4 to 40.6% ($p < 0.05$), respectively. These results suggested that HIU induced a conversion of α -helix to β -sheet, β -turn and random coils (most predominantly of the random coils type) in the TM molecules, and the degree of this transformation was positively correlated with the power of HIU from 100 W to 800 W. Similar results were reported by Stathopoulos et al. (2004), who found that proteins with high α -helical content (bovine serum albumin, myoglobin and lysozyme) underwent an increase of β -structure. Hu et al. (2015) also reported that HIU treated 7S proteins had higher levels of β -structure and lower levels of α -helical structure. Jin et al. (2015) compared the effects of sweeping frequency and pulsed ultrasound (SFPU) and sequential dual frequency ultrasound (SDFU) on glutenin, both SFPU and SDFU increased β -sheet and decreased α -helix of glutenin.

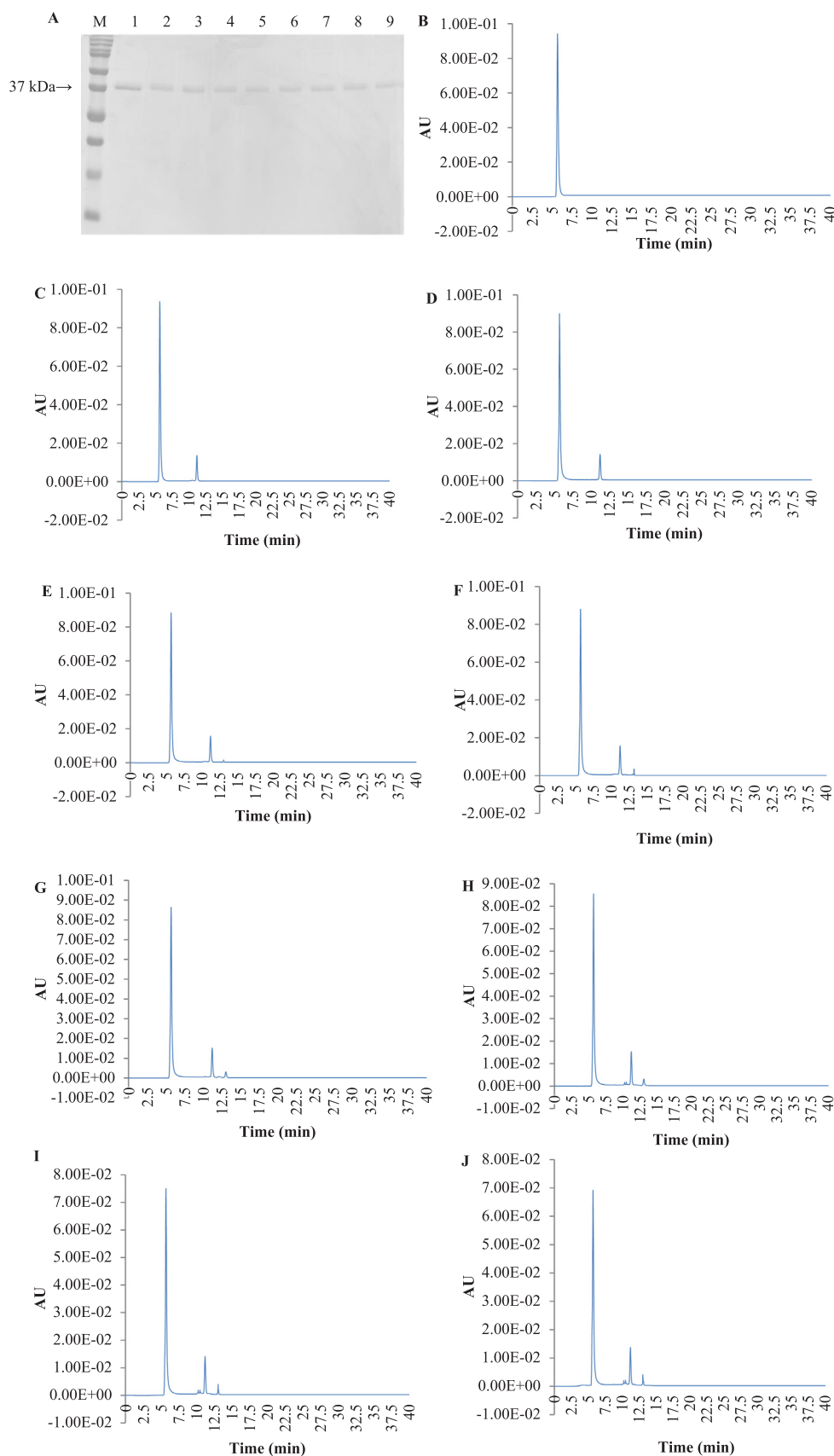


Fig. 1. SDS-PAGE (A) and HPLC analysis (B-J) on the degradation of non-HIU and HIU treated troponomyosin (Note: A: M, marker; lane 1, 0 W; lane 2, 100 W; lane 3, 200 W; lane 4, 300 W; lane 5, 400 W; lane 6, 500 W; lane 7, 600 W; lane 8, 700 W; lane 9, 800 W. B, control; C, 100 W; D, 200 W; E, 300 W; F, 400 W; G, 500 W; H, 600 W; I, 700 W; J, 800 W).

Table 2
The analysis of HIU-induced degradation of TM by HPLC.

Ultrasonic power (W)	HPLC peak parameters					
	Peak 1		Peak 2		Peak 3	
	Time (min)	Area (μV·s)	Time (min)	Area (μV·s)	Time (min)	Area (μV·s)
0	5.674 ± 0.01	1171798 ± 60.69				
100	5.675 ± 0.02	1167678 ± 52.37	11.226 ± 0.01	144215 ± 10.27		
200	5.707 ± 0.01	1154016 ± 50.18	11.225 ± 0.01	151025 ± 12.32		
300	5.708 ± 0.01	1142096 ± 48.53	11.227 ± 0.01	167657 ± 15.28	13.062 ± 0.01	5590 ± 2.15
400	5.709 ± 0.01	1138751 ± 45.21	11.227 ± 0.01	167850 ± 16.06	13.060 ± 0.01	9612 ± 3.27
500	5.710 ± 0.01	1133439 ± 42.86	11.226 ± 0.01	168759 ± 16.95	13.064 ± 0.02	16326 ± 3.91
600	5.710 ± 0.01	1126204 ± 41.34	10.255 ± 0.01	3370 ± 1.57	10.531 ± 0.01	5522 ± 1.68
700	5.713 ± 0.02	978935 ± 28.61	10.253 ± 0.02	5390 ± 1.86	10.524 ± 0.01	6655 ± 1.97
800	5.712 ± 0.01	949946 ± 22.59	10.257 ± 0.01	5742 ± 1.59	10.524 ± 0.01	8220 ± 2.26
					11.226 ± 0.01	165848 ± 15.39
					11.228 ± 0.01	148920 ± 13.28
					11.228 ± 0.01	142919 ± 12.61
					13.060 ± 0.01	17582 ± 4.25
					13.059 ± 0.02	18434 ± 5.18
					13.057 ± 0.01	18869 ± 5.51

3.1.4. Tertiary structure

Surface hydrophobicity (S_0 -ANS) is an index of the amount of hydrophobic groups on the surface of protein molecules contacting with the polar aqueous environment, therefore, it is closely related to the conformation of proteins. As shown in Fig. 2B, the S_0 -ANS of TM increased as HIU increased significantly during 0–800 W ($p < 0.05$). The above results indicated that the cavitation phenomenon could disrupt the conformation of TM, leading to more exposure of hydrophobic residues and thereby strength of surface hydrophobicity. Several previous studies also found that HIU could increase the S_0 -ANS of food proteins such as black bean protein (Jiang et al., 2014), bovine serum albumin (Gülseren et al., 2007), zein protein and glutelin (Jin et al., 2015).

The effects of HIU on the total and reactive SH contents of TM samples were presented in Fig. 2C. The total SH content decreased with the increase of HIU power, which might be due to the oxidation of SH groups to form disulfide bonds. Ultrasonic wave could generate highly reactive free radicals from water molecules, these free radicals could oxidize susceptible free-SH groups to form S–S bonds, resulting in the decrease of total SH groups. The reactive sulfhydryl content of the control (0 W) was $12.55 \pm 0.11 \mu\text{mol/g}$ protein (Fig. 2C), HIU treated TM at 100–600 W exhibited significant increase in comparison with the control, this might be due to the exposure of interior SH groups. However, when treated during 700–800 W, the reactive sulfhydryl contents were markedly decreased ($p < 0.05$, compared with 600 W), which might be due to the oxidation of more free radicals generated during stronger ultrasonic cavitation. At 800 W, the total and reactive SH content were almost the same amount, which suggested almost all sulfhydryl groups were exposed outside.

3.1.5. Particle size and zeta potential

As shown in Fig. 2D, the average particle size of TM decreased obviously from 322 nm (0 W) to 197 nm (800 W) with the strength of HIU power ($p < 0.05$), while tropomyosin was a 400 Å-long (40 nm) protein (Frank & George, 2000), therefore the TM molecules were in aggregates. As HIU power increased, the size reduction was probably due to protein dissociation during cavitation, which included the destruction of agglomerates and aggregates. Several studies have investigated the effect of sonication on particle size of different proteins and similar findings were reported. Jambak, Lelas, Mason, Krešić, and Badanjak (2009) found that sonication using a 20 kHz probe for 15 and 30 min reduced the size of particles in soy protein solutions. Hu et al. (2013) found that the particle size of HIU treated SPI dispersions decreased after treatment at 400 W, and Zhao et al. (2014) found that HIU pretreated milk samples were smaller and more uniform in size.

The zeta potentials of all TM samples were negative (Fig. 2E), suggesting that there were more negative amino acids than positive ones on the protein surface. The current results showed that the absolute value of the zeta potential of HIU treated TM samples increased with higher ultrasonic power (Fig. 2E). The stronger ultrasonication enhanced protein zeta potential by disassociating protein aggregates, changing protein conformation and structure in the way that charged amino acids or groups (COO^-) from inside were opened toward water (Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'h, 2000).

3.2. Simulated in vitro digestibility of TM

The digestibility of HIU treated TM were determined by SDS-PAGE. As shown in Fig. 3A1, the band corresponding to the molecular mass around 38 kDa referred to TM.

In SGF digestion, TM began to be digested during the first five minutes, fragments around 34, 28 and 17 kDa were detected (Fig. 3A2). The protein band around 34 kDa disappeared within the 30 min digestion (Fig. 3A3–4). The new band around 19 and 14 kDa proteins were detected within 40 min digestion (Fig. 3A5). As SGF went further (Fig. 3A6–7), the band of TM and 28 kDa became weaker, while the band of 17 kDa came to be denser. At SGF within 50–60 min

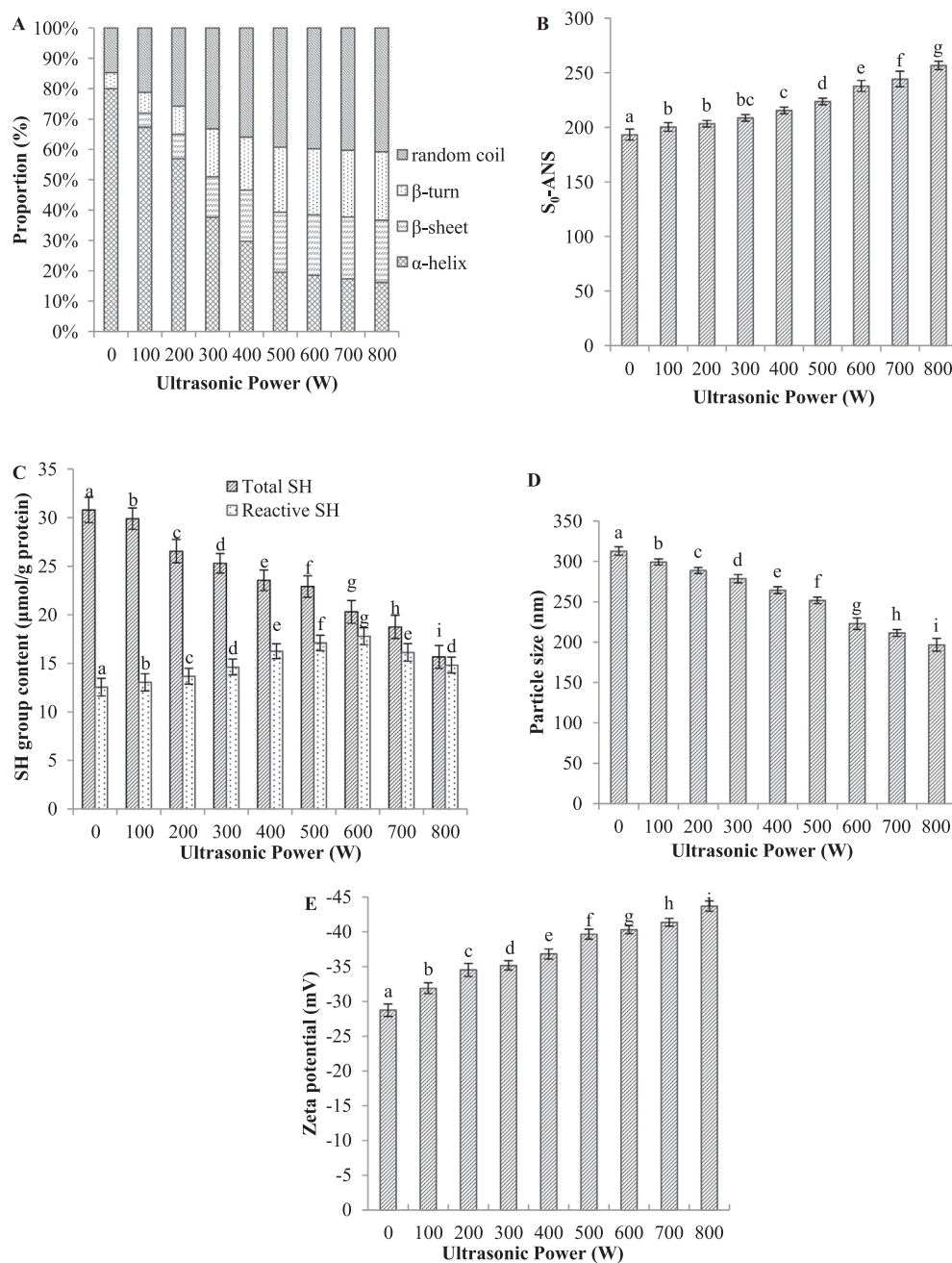


Fig. 2. Effects of HIU on the secondary structure proportion (A), surface hydrophobicity (B), sulfhydryl group content (C), particle size (D) and zeta potential (E) of non-HIU and HIU-treated tropomyosin (mean $\pm$ SD, $n = 3$). Different superscript letters means significant differences between values with different ultrasonic power ($p < 0.05$).

(Fig. 3A6–7), compared with the control (lane 1, Fig. 3A6–7), the 28 kDa band with digestion of 50–60 min became weaker, which might be due to that ultrasonic treatment loosen its structure, made enzyme easy to diffuse/penetrate into and contact the fragments to be cleaved (Yu et al., 2012). At SGF digestion of 60 min (Fig. 3A7), there were still TM existed in SGF digestion products, indicating a high digestion resistance of TM to gastric digestion. Compared with previously reported SGF digestibility of other crustacean tropomyosin allergens with 60 min SGF digestion, the TM from *Exopalaemon modestus* had higher SGF digestion resistance than other TM allergens from shrimp (*Solenocera melanoto*, Gámez et al. (2015)), from squid (*Todarodes pacificus*, Jin et al. (2015)) and from crab (*Scylla serrata*, Huang et al. (2010)).

In SIF digestion, after digestion for 1 min, the band of TM disappeared, and one fragment was seen at around 24 kDa (Fig. 3B2). Within 1–7 min digestion (Fig. 3B2–4), the 24 kDa became weaker, and disappeared after SIF digestion for 15 min (Fig. 3B5). As SIF digestion prolonged to 30–180 min (Fig. 3B6–7), there were no visible protein

bands in the SDS-PAGE. The different digestive patterns observed between pepsin (SGF) and trypsin (SIF) were due to different cleavage specificities for peptide bonds (Huang et al., 2010). Pepsin tended to cleave peptide bond next to phenylalanine or tyrosine residues, while trypsin was more likely to cleave next to lysine and arginine (Mikita & Padlan, 2007). TM was proved to be resistant to pepsin while relatively susceptible to trypsin digestion due to the low phenylalanine and tyrosine content (1.82% and 2.01% in Table 1), and the high lysine and arginine content (10.97% and 10.03% in Table 1) in TM (Huang et al., 2010).

The sequential SGF and SIF digestion on allergen reduction in HIU treated TM were carried out firstly treated using gastric digestion, then adjusted the pH to 7.5 and continually hydrolyzed by intestinal digestion. The results showed that, within 1 min SIG digestion (Fig. 3C2), the bands around 38 kDa (TM), 28 kDa, 19 and 17 kDa from pre-hydrolysis of pepsin digestion (Fig. 3C1) disappeared, the band around 14 kDa became weaker, and generating new weak bands around 24 and 12 kDa

A

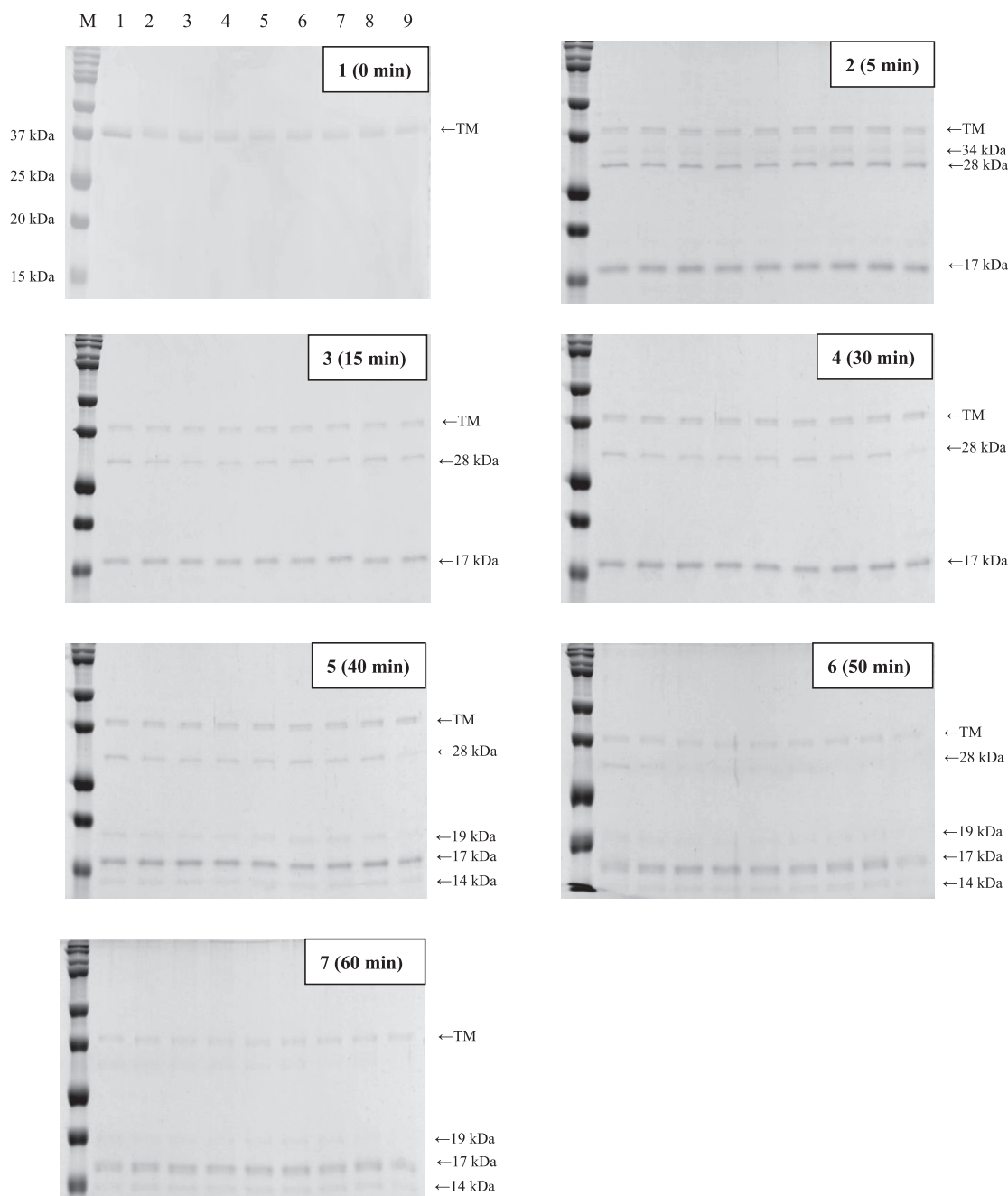


Fig. 3. The simulated gastric (A), intestinal (B) and sequential gastric and intestinal (C) digestibility of non-HIU and HIU-treated tropomyosin by SDS-PAGE (Note: M, marker; lane 1, control; lane 2, 100 W; lane 3, 200 W; lane 4, 300 W; lane 5, 400 W; lane 6, 500 W; lane 7, 600 W; lane 8, 700 W; lane 9, 800 W).

(Fig. 3C2). The band around 14 kDa disappeared with digestion of 7 min (Fig. 3C4), generated new intermediate weak bands around 10 kDa (Fig. 3C4) and then disappeared with digestion of 30 min (Fig. 3C6). The bands around 24 and 12 kDa disappeared when intestinal digestion went further to 60 min (Fig. 3C7). When SIF digestion prolonged to 60–180 min (Fig. 3C7–9), there were no visible protein bands existed. Compared with single SIF, the sequential SGF and SIF digestion took much more time (15 min for SIF digestion, 60 min for sequential SGF and SIF digestion) to see no visible bands of TM and protein fragments on SDS-PAGE, which suggested that pepsin digestion might pre-disrupt some spatial hydrolysis sites on TM for trypsin digestion, which delayed the following trypsin digestion.

3.3. Allergenicity

3.3.1. Allergenicity of HIU-treated TM

The changes in allergenicity of HIU-treated TM were confirmed by immunoblotting analysis using human sera of shrimp allergic patients. As shown in Fig. 4A, HIU-treated TM exhibited weaker immune response gradually as power increased, and the immune response disappeared after HIU treatment at 800 W, which might be due to the ultrasound destroy on conformational or linear epitopes of TM.

As shown in Fig. 4A and B, the immunoblotting and ELISA had similar changes. While the changes in allergenicity tested by the western blot was higher than the decrease obtained by ELISA, especially at 800 W, where the immunoblotting had no immune response, while the

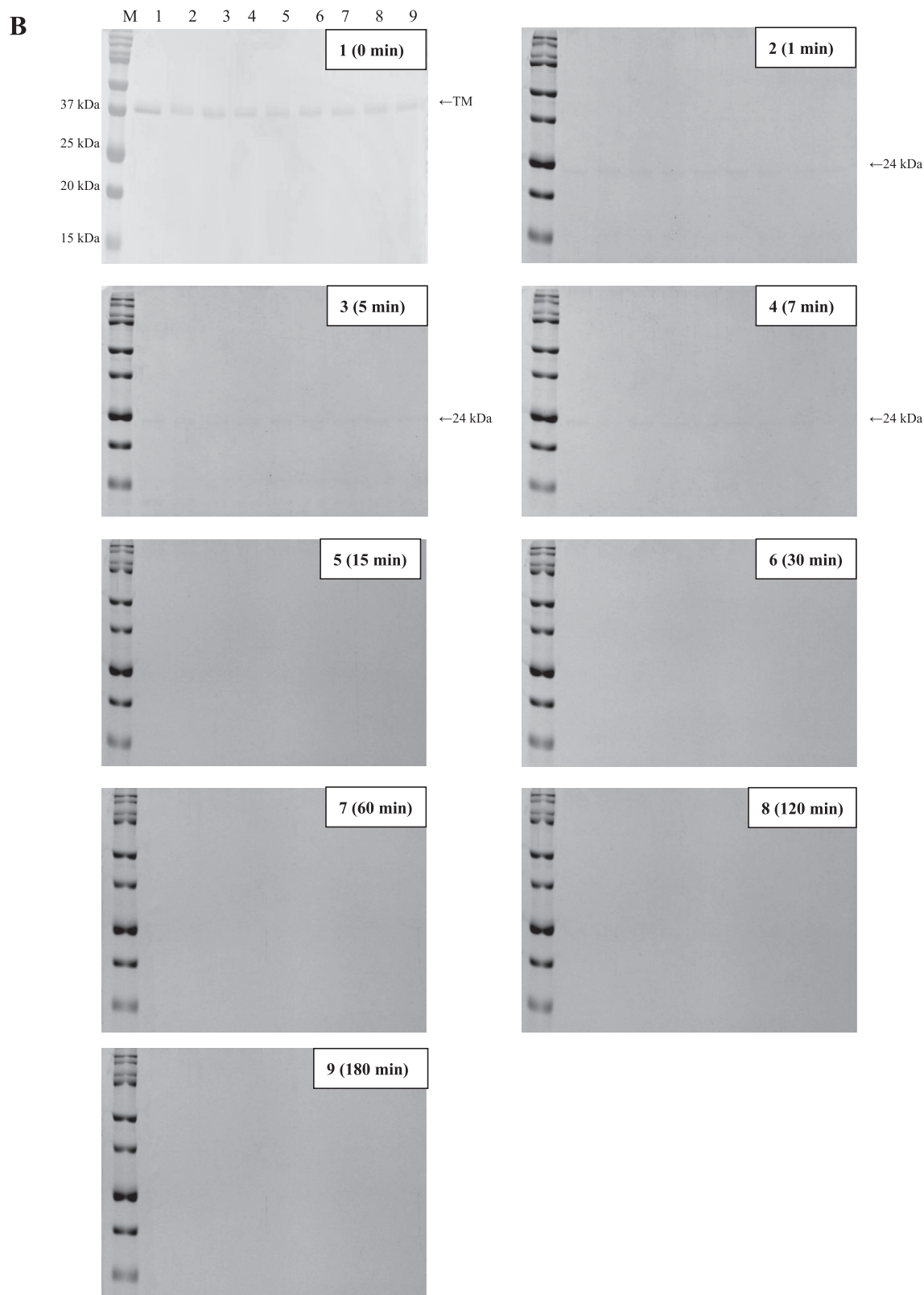


Fig. 3. (continued)

OD₄₅₀ still had a value of 0.95. It suggested that some protein fragments induced by HIU with allergenicity could not be detected by western blot, but could be tested by ELISA. The difference between western blot and ELISA results might be due to that, in immunoblotting, some smaller protein fragments with epitopes had not been transferred to PVDF membrane, inducing the loss of allergic components, therefore had no

corresponding immune response. Another reason was that some linear epitopes separated from the allergen when treated with HIU, these epitopes could not be detected by immunoblotting, but could be measured by ELISA (Lee et al., 2001). Therefore, compared the two methods, ELISA could better reflect the real allergenicity than western blot. Thereby, the ELISA was employed to further test the allergenicity

C

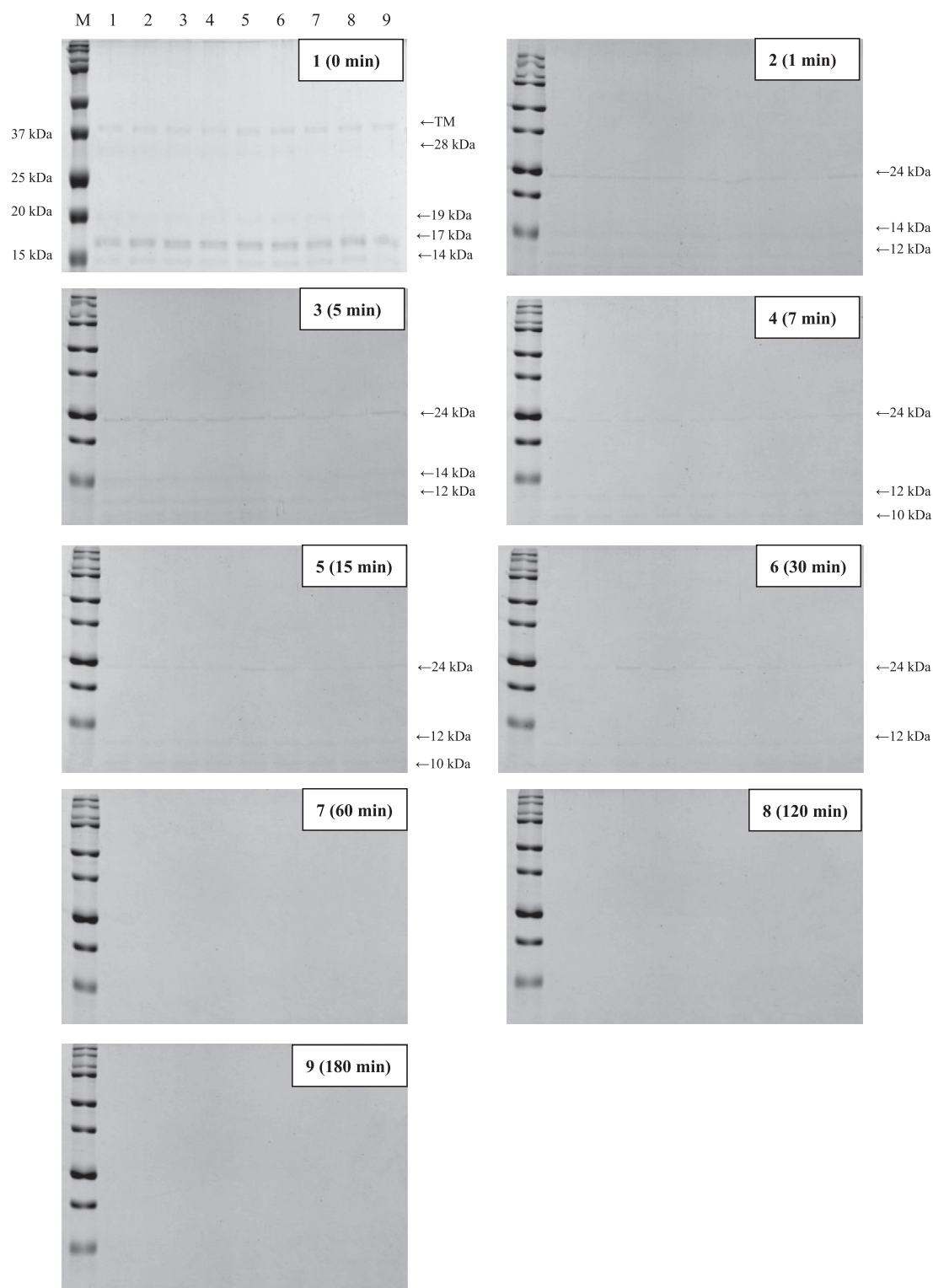


Fig. 3. (continued)

of digestion products.

3.3.2. The allergenicity of SGF, SIF and sequential SGF and SIF digestion products

In SGF digestion (Fig. 4C), as digestion time stretched, the OD₄₅₀ of

digestion products decreased gradually, which indicated that part of conformational or linear epitopes of TM were hydrolyzed by pepsin. While there were still a relatively high value of OD₄₅₀ after SGF digestion of 60 min, which suggested pepsin could not thoroughly hydrolyzed TM due to the low content of its hydrolysis sites (Pepsin tends

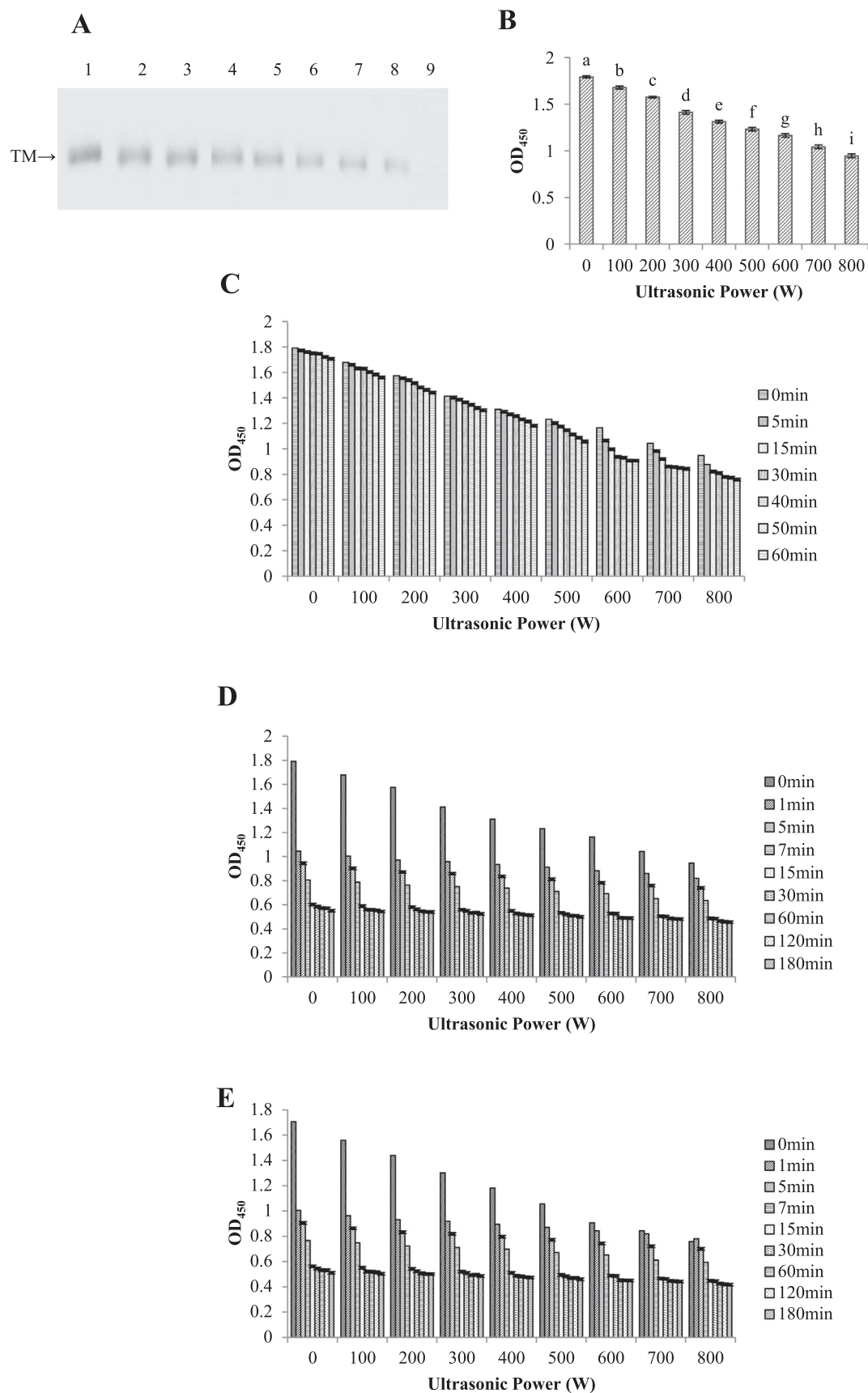


Fig. 4. The immunoblotting (A) and allergenicity (B) of TM, allergenicity of simulated gastric digestion product (C), simulated intestinal digestion product (D) and sequential simulated gastric and intestinal digestion product (E) of non-HIU and HIU-treated tropomyosin (mean $\pm$ SD, $n = 3$). Different superscript letters means significant differences between values with different ultrasonic power ($p < 0.05$) (Note: A: lane 1, control; lane 2, 100 W; lane 3, 200 W; lane 4, 300 W; lane 5, 400 W; lane 6, 500 W; lane 7, 600 W; lane 8, 700 W; lane 9, 800 W).

to cleave peptide bond next to phenylalanine or tyrosine residues (Mikita & Padlan, 2007), while 1.82% phenylalanine and 2.01% tyrosine content of TM in Table 1). As to HIU power, when HIU became stronger, the OD₄₅₀ of digestion products also decreased, and during 600–800 W, the OD₄₅₀ had relatively higher reduction speed during 0–15 min digestion, which might be due to that the HIU loosened the TM structure to make pepsin easy to diffuse/penetrate into and contact the TM to be cleaved (Yu et al., 2012).

In SIF digestion (Fig. 4D), as SIF digestion went further, the OD₄₅₀ of digestion products decreased rapidly to a low level, which indicated that most of conformational and linear epitopes of TM could be hydrolyzed by trypsin due to the multiple accessible sites of TM to trypsin (high arginine and lysine content in TM, Table 1). Also, as HIU strengthened, the OD₄₅₀ of digestion products also decreased, which might be due to that HIU induced conformational changes with exposure of cleavage sites to increase the susceptibility of protein to trypsin. While trypsin digestion fragments of TM still had IgE-binding ability even SIF digestion prolonged to 180 min.

Comparing to the OD₄₅₀ of SGF digestion (Fig. 4C) or SIF digestion (Fig. 4D), Fig. 4E showed that the allergenicity of TM digestion samples produced by sequential gastric digestion and intestinal digestion had the lowest OD₄₅₀, this might be due to that sequential hydrolysis of TM with two enzymes could cleave more peptide bonds than the single enzyme and produce peptides with fewer epitopes and thus more effectively reduced allergenicity. Besides, although pepsin prolonged the following trypsin digestion time (Fig. 3B and C), as to the SIF and sequential SGF and SIF digestion, the allergenicity results in Fig. 4E were lower than those in Fig. 4D and they were in the same decreasing tendency. This suggested trypsin played a more important role in gastrointestinal digestion than pepsin, and sequential SGF and SIF digestion was more effective than single SGF or SIF digestion in reducing TM allergenicity.

Low SGF digestibility and consequent persistence of intact TM in Fig. 3A with sufficient conformational and sequential epitopes resulted in a higher allergenicity in SGF digestion products. Subsequent hydrolysis of the samples with trypsin (SIF digestion) significantly hydrolyzed the remaining intact molecules of TM, as observed by electrophoresis and ELISA data, that resulted in reduced allergenicity ($p < 0.05$). Thus, although TM was difficultly digested by pepsin, the multiple proteolytic actions of trypsin were efficient in hydrolyzing it to an extent that lowered its allergenicity significantly (Chicón, Belloque, Alonso, & López-Fandiño, 2008). Besides, HIU treatment significantly affected both pepsin and trypsin digestion and thereby their allergenicity, due to that HIU induced conformational changes of TM and TM aggregates, leading to exposure of pepsin and trypsin cleavage sites resulting in enhanced digestibility and consequently reduced allergenicity.

4. Conclusions

HIU treatment oxidized the Cys, Met and Lys in TM, degraded TM to generate protein fragments. The α -helix decreased, while β -sheet, β -turn and random coil increased. HIU treatment reduced the allergenicity and promoted digestibility of TM. Gastric digestion partly hydrolyzed TM, and intestinal digestion totally hydrolyzed TM, while the sequential HIU-SGF-SIF digestion effectively hydrolyzed TM and reduced the allergenicity of digestion products. Stronger HIU could loosen TM structure, reduce TM allergenicity, strengthen the gastrointestinal digestibility of TM and obtain digestion products with low allergenicity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.11.072>.

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Information of shrimp allergy patients

No.	Gender	Age (year)	Clinical symptoms	IgE (kU _A /L)
1	Male	5	AS	22.4
2	Female	2	AS	23.2
3	Female	55	HI, EC	6.8
4	Male	4	AS,HI	65.5
5	Female	40	HI, CW	11.4
6	Female	20	HI, NW	19.3
7	Male	40	EC,RH	28.2
8	Male	2	AS, NW	35.9

Note: AS, asthma; HI, hives; EC, eczema; RH, rhinitis; NW, nose wheezing; CW, chest wheezing.