

In Vitro Gastric and Intestinal Digestions of Pulsed Light-Treated Shrimp Extracts

Wade W. Yang · Sandra K. Shriver · Si-yin Chung ·
Susan Percival · Melanie J. Correll · Taha M. Rababah

Received: 1 August 2011 / Accepted: 29 December 2011
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Abstract Pulsed ultraviolet light (PUV), a novel technology most commonly used for microbial inactivation, has recently been employed to effectively mitigate food allergens in peanuts, soybean, shrimp, and almond. Putative mechanisms for the efficacy of PUV in reducing allergen reactivity include photothermal, photochemical, and photophysical effects. To date, there are no published data highlighting the effects of in vitro simulated gastric and intestinal digestion on the stability of PUV reduced allergen reactivity of food. In this study, PUV-treated shrimp extracts were subjected to simulated gastric fluid containing pepsin and simulated intestinal fluid containing trypsin and chymotrypsin, and then tested for changes in allergen potency. SDS-PAGE showed no major band deviation between undigested and digested PUV-treated shrimp extracts. IgE binding to tropomyosin remained markedly decreased as seen in Western blot analysis. Total shrimp allergen reactivity remained unchanged following in vitro peptic digestion and was markedly reduced following in vitro intestinal digestion as illustrated in indirect ELISA. The PUV reduced shrimp allergens remained at a low level under the in vitro simulated digestive conditions. The

W. W. Yang (✉) · S. K. Shriver · S. Percival
Department of Food Science & Human Nutrition, University of Florida, 359 FSHN Bldg. Newell Drive,
Gainesville, FL 32611, USA
e-mail: wade.yang@ufl.edu

S.-y. Chung
USDA-ARS, Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70124,
USA

M. J. Correll
Department of Agricultural and Biological Engineering, University of Florida, 207 Frazier Rogers Hall,
Gainesville, FL 32611, USA

T. M. Rababah
Department of Nutrition and Food Technology, Jordan University of Science and Technology, Irbid
22110, Jordan

results inferred that PUV could be a potential method to create less allergenic shrimp products that would remain at a low allergen level under human gastric and intestinal digestive conditions.

Keywords Shrimp · Allergen · Pulsed ultraviolet light · PUV · IgE binding · SGF · SIF

Introduction

Shrimp (*Litopenaeus setiferus*) is among the kinds of shellfish that can cause allergic reactions such as itching, hives, swelling of lips and tongue, pulmonary symptoms, gastrointestinal symptoms, and anaphylactic shock [1]. Although two other shrimp allergens, namely myosin light chain (20 kDa) [2, 3] and arginine kinase (40 kDa) [4, 5], have been recognized, the majority of shrimp allergen reactivity comes from tropomyosin, a heat-stable allergen of 34 to 38 kDa molecular weight, also known as Sa-II or Pen a 1 [6–8]. To date, the only completely effective method to prevent shrimp-induced allergies is total avoidance. However, avoidance is often difficult due to many uncontrollable factors such as unintentional cross-contamination or the inclusion of allergenic foods as ingredients during food processing. This underlies the importance of developing methods to mitigate the allergen reactivity of food products like shrimp extracts. Among the thermal and nonthermal methods that have been reported to reduce food allergens, pulsed ultraviolet light (PUV) is the most recent one reported to be able to reduce the antigenicity of allergenic foods [9].

Owing to its ability to store and emit pulses that have greater intensities than continuous ultraviolet light, PUV technology has been successfully used for surface disinfection of food and nonfood products, such as sterilization of packaging materials and pasteurization of milk products [10, 11]. Its ability to destroy microbes has been notable even with a single pulse [12]. Recently, PUV has been employed to reduce peanut [13, 14], soybean [15], and shrimp [16] allergen reactivity. A marked decrease in the potency of the major shrimp allergen, tropomyosin, by PUV radiation was noted, while boiling alone actually causes an increase in shrimp immunoreactivity [16].

Putative mechanisms for the interactions of PUV with foods include photothermal, photophysical, and photochemical effects [12, 16]. Previous research shows that for a short duration of exposure (e.g., seconds), PUV may be regarded as nonthermal [14, 16, 17]; however, extended exposure (e.g., minutes) triggered PUV's photothermal effect and could cause a pronounced temperature rise and moisture evaporation on foods, as well as modification of protein allergen reactivity via protein cross-linking [13–17], protein solubility reduction [13, 14], or protein hydrolysis in the high instantaneous temperature environment induced by PUV's photothermal effect [16, 17].

Previous study [16] has shown that the allergenic potency of a shrimp extract can be reduced by PUV. However, whether this reduced potency remains the same, is reversed to its original level, or may further be reduced in the digestive tract is not known. Some studies [9, 18] have indicated that digestion by digestive enzymes in the stomach can lead to a change in the potency of food allergens. In view of this, it is important to determine the allergenic potency of PUV-treated extracts digested by simulated gastric and intestinal enzymes versus the potency of undigested PUV-treated extracts and digested non-PUV-treated extracts. The objective of this study was to determine whether shrimp allergens subjected to PUV radiation retain their reduced potency or undergo more changes in potency after in vitro simulated gastric and intestinal digestions.

Materials and Methods

Preparation of Shrimp Extracts

Twenty-five grams of shelled Atlantic white shrimp (*L. setiferus*) purchased frozen from Publix Super Markets, Inc. (Lakeland, FL) were ground in a KitchenAid food processor (St. Joseph, MI) at high speed. Two hundred milliliters of 0.1 M KCl in 0.07 M phosphate buffer (pH 7) was added to the processed tissue, and the mixture was homogenized using a BioSpec (Bartlesville, OK) homogenizer at high speed for 1 min. The samples were centrifuged for 30 min at 20,000×g at 4 °C to remove debris that might cause error in pipetting. Protein concentration was measured with Coomassie Plus (Bradford) protein assay (Pierce, Rockford, IL) using bovine serum albumin protein standards (Pierce, Rockford, IL). The homogenate was diluted to a concentration of 5 mg/mL with 0.1 M KCl in 0.07 M phosphate buffer (pH 7). Samples were used immediately or stored on ice at 4 °C for no longer than 1 week.

PUV Treatment of Shrimp Samples

For each run, 10 mL of raw or boiled shrimp extract (5 mg/mL) in an aluminum dish of 7.2 cm in diameter was treated in the PUV sterilization system (Model # LHS40 LMP HSG, Xenon Corp., Wilmington, MA) for 4 min. Pulses were emitted at a rate of 3/s with a width of 360 µs, positioned at a distance of 10 cm from the light source. At the chosen distance, the maximum energy level of the emitted radiation was 0.27 J/cm² per pulse as per factory calibration. Temperature was monitored using an Omega OS423-LS non-contact infrared thermometer (Omega Engineering, Inc., Stamford, CT), and protein concentration was measured in all samples following treatment. To correct for moisture loss during treatment, the sample volume was brought back to its original level by adding the buffer to maintain the protein concentration on the same level as before the PUV treatment.

Simulated Peptic Digestion

For simulated gastric digestion, pepsin was dissolved in simulated gastric fluid (SGF) as described by the United States Pharmacopoeia (USP) [19]. Briefly, 3.2 mg of porcine pepsin (Sigma) with an activity of 272 U/mg was dissolved in 1 mL of 0.03 M NaCl titrated to a pH of 1.2 with HCl. Following the methods of Liu et al. [20], digestion was performed using a ratio of 0.33 U of pepsin activity per µg of protein (1:50 w/w). A volume of 30 µL of SGF was added to 970 µL of shrimp protein extract (5 mg/mL; untreated and PUV treated) that had been adjusted to a pH of 1.2 with 13.5 µL of 8 N HCl. Digestion was carried out in a 1.5-mL microcentrifuge tube at 37 °C with continuous shaking. At 0, 5, 15, 30, 60, and 120 min, aliquots of 200 µL were terminated using 22 µL of 1 N NaOH, as described by Fu et al. [21]. Two 1-mL (control and PUV treated) samples were digested for 120 min for subsequent tryptic digestion; these samples were terminated with 110 µL of 1 N NaOH. Three independent trials were conducted ($n=3$). The samples (4.39 mg/mL) were stored on ice and under refrigeration (4 °C) for no longer than 1 week and analyzed by SDS-PAGE, immunoblot, and ELISA.

Simulated Intestinal Digestion

Simulated intestinal fluid (SIF) was prepared according to the USP [19] and consists of 0.05 M KH₂PO₄ at a pH of 6.8. Trypsin and α -chymotrypsin (Sigma) with enzyme activities

of 325 U/mg of protein and 62 U/mg of protein, respectively, were added to the SIF at concentrations of 3.2 mg/mL. Digestion conditions followed the procedure of Liu et al. [20], in which 0.20 U of trypsin activity per μg of shrimp protein (1:50 w/w) and 0.04 U of α -chymotrypsin activity per μg of shrimp protein (1:50 w/w) was used to digest the all the shrimp proteins present in the sample. Digestion was performed in volumes of 1 mL. Aliquots of 200 μL were removed and terminated at 0, 5, 15, 60, and 120 min with trypsin inhibitor (Sigma) at a ratio of 1:1 (w/w, trypsin inhibitor to trypsin and chymotrypsin) as described by Bublin et al. [22]. Three independent trials were conducted ($n=3$). The samples were stored on ice and under refrigeration (4 °C) for no longer than 1 week and analyzed by SDS-PAGE, immunoblot, ELISA, and dot blot.

SDS-PAGE Analysis of Digested PUV-Treated Shrimp Extracts

Digested and undigested PUV-treated extracts along with the digested non-PUV-treated samples were subjected to SDS-PAGE to analyze changes in protein bands due to simulated in vitro digestion. Samples were analyzed under denaturing conditions as described by Laemmli [23]. Twelve micrograms of each sample was combined with sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 0.05% β -mercaptoethanol; BioRad) at a 1:1 ratio and were boiled for 5 min. Proteins were separated with electrophoresis using BioRad precast Tris–glycine minigels (4–15%) in BioRad Mini-Protean tanks for 1.5 h at 150 V per the manufacturer's recommendations. Proteins were stained using GelCode Blue reagent (Pierce, Rockford, IL), and the gel was destained with deionized water for 2 h to reduce background.

Western Blot Analysis of Digested PUV-Treated Shrimp Extracts

Treated samples were electrophoresed as described above. Following electrophoresis, the gel was removed from the cassette, equilibrated in Tris–glycine transfer buffer (BioRad) for 15–30 min and transferred onto Immobilon P blotting membrane (Millipore Corporation, Bedford, MA) using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad) at 15 V for 30 min. The membrane was blocked (StartingBlock TBST, Pierce) and incubated overnight at 4 °C with pooled human plasma containing antishrimp IgE diluted in blocking buffer (1:5). The secondary detection antibody was mouse antihuman IgE conjugated to horseradish peroxidase (HRP; Invitrogen, Carlsbad, CA) diluted in blocking buffer at 1:250 (1 h). To detect IgE and visualize allergen bands, SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used.

Determination of IgE Binding to Digested PUV-Treated Shrimp Extracts in Indirect ELISA and Dot Blot

ELISA was carried out according to the method of Shriver et al. [16]. Polystyrene 96-well plates (Corning, NY) were coated with proteins of digested and undigested PUV-treated extracts and also with proteins of digested non-PUV-treated shrimp samples (diluted in PBS to 20 $\mu\text{g}/\text{mL}$, 100 μL per well) at 37 °C for 2 h. Following blocking (StartingBlock TBST), pooled human plasma containing IgE antibody specific for shrimp allergens diluted in PBS was added in equal amounts to each well (1:20, 100 μL), for 1 h (RT). Secondary antibody incubation using monoclonal mouse antihuman IgE conjugated to HRP (1:100) was performed for 1 h (100 μL per well). After the plate was washed five times and patted dry, *o*-phenylenediamine dihydrochloride (Pierce) dissolved in stable peroxide substrate buffer

(Pierce) was added to each well (0.5 $\mu\text{g}/\mu\text{L}$, 100 μL per well). The reaction was stopped at 15 min with 2.5 N sulfuric acid (100 μL per well). Absorbance was read on Spectramax 340³⁸⁴ Spectrophotometer (Molecular Devices, Inc. Sunnyvale, CA) at 490 nm.

For dot blot, a nitrocellulose membrane was blotted with 1.5 μg of digested and undigested PUV-treated and digested non-PUV-treated shrimp samples and allowed to dry at 4 °C. Following blocking, the blot was incubated in human plasma containing antishrimp IgE diluted in blocking buffer (1:80) overnight at 4 °C. The blot was incubated in mouse antihuman IgE-HRP diluted in blocking buffer (1:1,000; 1 h; RT). A 4-chloro-1-naphthol substrate was added to the blot and incubated for 30 min to visualize protein spots. Spots appearing more pronounced after incubation indicated an increase in the amount of IgE bound to the shrimp proteins.

While SDS-PAGE and Western blot separate proteins based on their molecular weight in order to analyze individual protein bands, dot blot as well ELISA just described above test the sample for its allergen reactivity of all the proteins present in the solution. In other words, dot blot and ELISA can detect a pooled effect of allergenic proteins in a sample. This is important in the sense that SDS-PAGE and Western blot have resolution minimums and maximums, depending on the pore sizes of the gel or membrane. Dot blot partly overcomes this issue by removing the electrophoretic procedure, however the pore size of the membrane may still be an issue. The ELISA analysis does not require polyacrylamide gels or membranes, thus molecular weight is less of an issue and proteins that are too large (e.g., due to cross-linking [13, 14]) or small (e.g., due to hydrolysis [14, 16]) for SDS-PAGE and Western blot analysis can be studied.

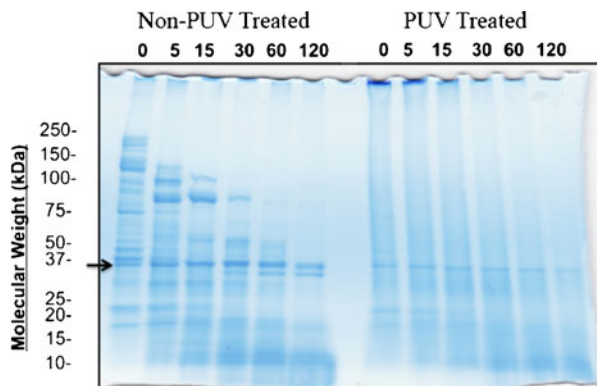
Results and Discussion

Simulated Gastric Digestion

SDS-PAGE

Non-PUV-treated and PUV-treated shrimp extracts were tested for their stability under gastric conditions (e.g., pepsin and low pH) for 2 h. The SDS-PAGE analysis was intended to determine whether the protein profiles of PUV-treated shrimp extracts remained unchanged after simulated gastric digestion. Figure 1 illustrates the SDS-PAGE result of

Fig. 1 SDS-PAGE analysis of non-PUV-treated and PUV-treated shrimp extracts subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min. The tropomyosin band (36 kDa) is highlighted with an arrow



PUV-treated and non-PUV-treated (control) samples after up to 120 min of simulated gastric digestion.

For the non-PUV-treated samples (i.e., the left-hand side of Fig. 1), tropomyosin remained present in nearly equal amounts up to 120 min SGF digestion compared to the undigested sample (i.e., time 0). This suggested that tropomyosin in the non-PUV-treated sample remained stable and constant throughout the SGF digestion. These data are concurrent with SDS-PAGE profiles of SGF digested tropomyosin from grass prawn and Pacific white shrimp described by Liu et al. [20]. In their study, purified tropomyosin from the two species of shrimp was subjected to *in vitro* gastric digestion in SGF at a ratio of 1:50 (pepsin to protein, *w/w*) for up to 60 min. The tropomyosin band intensity remained unchanged in both species of shrimp under these conditions. For other protein bands, as the SFG digestion went on, the SDS-PAGE profile showed a succession of reduction with time for proteins of molecular weights around 12 kDa, but an increase for those below 12 kDa.

For the PUV-treated sample (i.e., right-hand side of Fig. 1), the initial bands at time 0 (i.e., undigested) consisted mostly of tropomyosin and two bands around 20 kDa, while the tropomyosin band was lighter than that of the non-PUV-treated samples, because part of tropomyosin and most other heat-labile proteins were cross-linked to form large molecules due to PUV treatment [16] and congested on the very top of the lane 0. As the digestion went on, the cross-linked proteins seemed to have been gradually digested into smaller molecules and partially lost in the buffer and partially appeared as low molecular (e.g., below 12 kDa) bands, since the congested protein bands on the top subsided as the digestion went on and almost disappeared at 120 min. The SFG digestion for 5–60 min did not seem to have changed the SDS-PAGE profile of tropomyosin, except for 120 min, when tropomyosin band intensity was slightly reduced. However, in general, tropomyosin in the PUV-treated sample seemed to remain stable during the SGF digestion. It should be noted that the existence of tropomyosin band on the SDS-PAGE after SGF digestion only indicated the presence of the tropomyosin molecules but not necessarily their allergen reactivity, which can be probed by Western blot and ELISA, as shown later.

Western Blot

Western blot analysis was used to detect the reactivity of the digested PUV-treated shrimp extracts following simulated gastric digestion, compared to non-PUV-treated and undigested samples (i.e., digestion time 0). Figure 2 shows that the tropomyosin reactivity of the

Fig. 2 Western blot analysis of non-PUV-treated and PUV-treated shrimp extracts digested with SGF containing pepsin for 0 (undigested), 5, 15, 30, 60, and 120 min. IgE was detected using a pooled plasma against tropomyosin. The tropomyosin band (36 kDa) is highlighted with an arrow

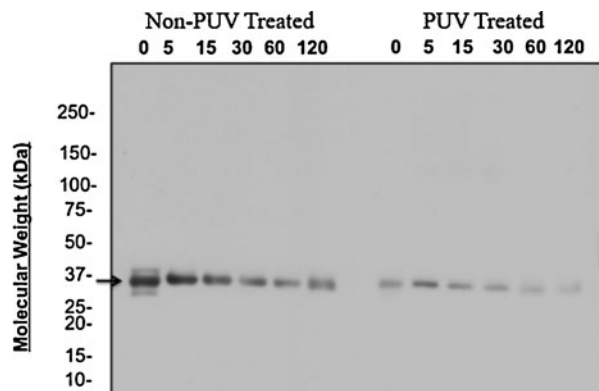
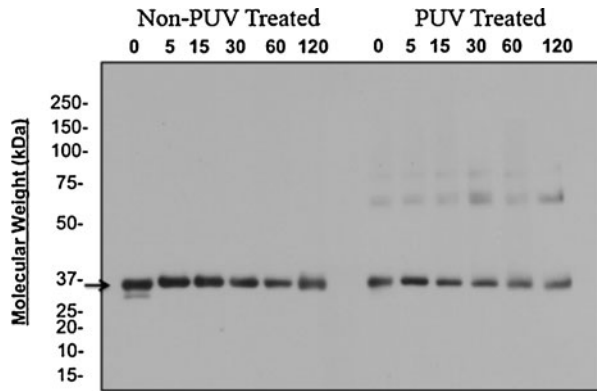


Fig. 3 Western blot analysis of non-PUV-treated and PUV-treated shrimp extracts that have been subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min. The detection antibody used was a monoclonal anti-tropomyosin (IgG). Band smearing is noted in the PUV-treated samples. The tropomyosin band (36 kDa) is highlighted with an arrow



undigested and the digested samples was only marginally reduced during the SGF digestion. Thus, pepsin digestion did not appear to significantly reduce tropomyosin reactivity, with no increase either on the tropomyosin activity.

Furthermore, tropomyosin levels, as detected by an anti-tropomyosin antibody (IgG), were basically unchanged as illustrated in Fig. 3. For the undigested samples (i.e., lane 0), PUV treatment caused the tropomyosin level to diminish. For the digested samples, the IgE binding was marginally reduced as the digestion time increased. It is also noted that in the PUV-treated sample (right-hand side, Fig. 3), the anti-tropomyosin antibody also detected two additional light bands on the 65–75-kDa range, which were also basically unchanged by the SGF digestion. Thus, peptic digestion did not appear to further alter the allergen after modification by the PUV treatment.

Stability to digestive conditions is characteristic of some food allergens, which may actually contribute to their immunoreactivity [24, 25]. An undigested allergen may have a greater potential to cross the digestive membranes and elicit an immune response. Huang et al. [26] noted a similar stability of crab tropomyosin to SGF. They also found that increasing the pepsin to protein ratio from 1:50 to 1:1 (w/w) caused IgE binding to crab tropomyosin to noticeably decrease. However, it is estimated [25, 27] that humans have an average pepsin release of approximately 10 mg per day with a daily average intake of 75 g of protein,

Fig. 4 ELISA of non-PUV-treated (indicated as control) and PUV-treated shrimp extracts that have been subjected to simulated gastric digestion. The protease used was pepsin in SGF. Samples were digested for 0, 5, 15, 30, 60, and 120 min. Human IgE antibodies were used to determine shrimp allergen reactivity. Relative IgE binding is expressed as A/A_0 , where A is the absorbance of the sample divided by the absorbance of the untreated. Data are expressed as mean $\pm$ SD

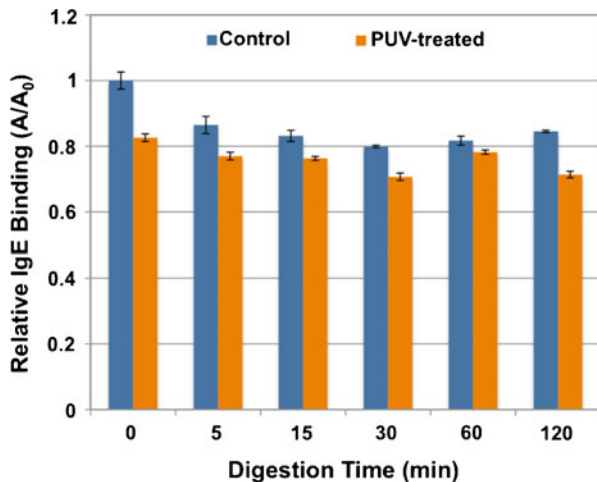
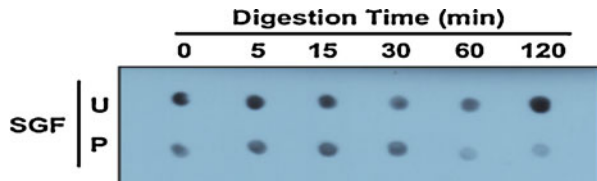


Fig. 5 Dot blot analysis of undigested vs. digested for non-PUV-treated (*U*) and PUV-treated (*P*) shrimp extracts. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min in SGF. Proteases used were pepsin



making the pepsin to protein ratio (w/w) approximately 1:7,500 for 24 h. This suggests that a ratio of 1:1 is highly unlikely. Furthermore, because optimal pepsin activity is related to pH, namely a low pH of 1.2 to 3.5 [25], the pepsin activity in individuals may vary due to physiological differences [27].

Indirect ELISA and Dot Blot

Indirect ELISA and dot blot were used to examine the reactivity of the shrimp extracts for the possible contributing factors following digestion in SGF. Figure 4 illustrates the ELISA results for the PUV-treated and non-PUV-treated (indicated as control in figure) samples after up to 120 min SGF digestion, which show only a mild reduction in allergen reactivity during the peptic digestions for both samples, and the reduction trend was not markedly different between the PUV-treated and non-PUV treated. It is noted that at time 0 (undigested), the PUV-treated sample had about 20% reduction in allergen level compared to non-PUV treated (control). The PUV treatment condition for this sample was so chosen as to minimize the sample moisture loss during the PUV treatment [16], and it was not the optimal condition for allergen reduction yet. With a longer PUV exposure, the IgE binding potential of the shrimp extract at time 0 could be further reduced.

Dot blot analysis (Fig. 5) shows that the IgE binding in the non-PUV-treated shrimp extracts basically remained unchanged; yet, the PUV-treated sample marginally decreased after 60 and 120 min digestion in SGF. These results agreed with the SDS-PAGE and Western blot data, and eluded to the postulation that peptic digestion alone may not increase the reactivity of PUV-treated samples.

The reason why tropomyosin is stable to pepsin has not yet been elucidated. However, research on one of the major peanut allergens (Ara h 2) has shown that disulfide bonds may enhance stability to digestive conditions [28]. When disulfide bonds were reduced before digestion, it was found that the allergen was no longer resistant. Circular dichroism further confirmed the importance of disulfide bonds in regards to resistance to digestion.

Fig. 6 SDS-PAGE analysis of non-PUV-treated and PUV-treated shrimp extracts subjected to simulated intestinal digestion. The proteases used were trypsin and α -chymotrypsin in SIF. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min. The tropomyosin band (36 kDa) is highlighted with an arrow

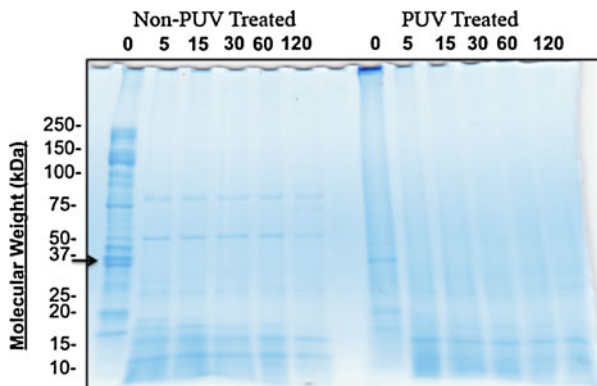
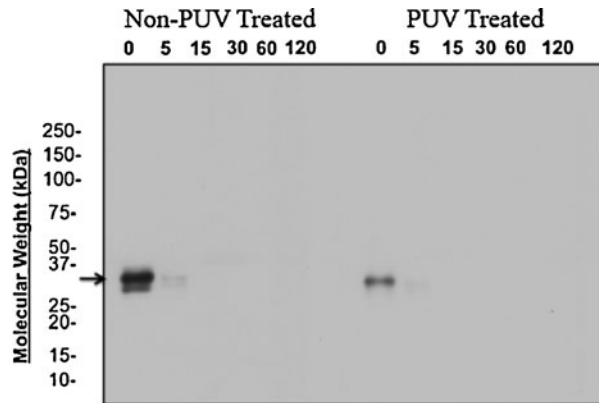


Fig. 7 Western blot analysis of non-PUV-treated and PUV-treated shrimp extracts subjected to simulated intestinal digestion. The proteases used were trypsin and α -chymotrypsin in SIF. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min. Human IgE antibodies were used to determine the reactivity of tropomyosin. The tropomyosin band (36 kDa) is highlighted with an arrow



Simulated Tryptic Digestion

SDS-PAGE

Non-PUV-treated and PUV-treated shrimp extracts were subjected to trypsin and α -chymotrypsin in SIF for up to 2 h to examine the stability of tropomyosin or any cross-linked or precipitated proteins related to it. Unlike in simulated gastric digestion, tropomyosin did not appear to be stable in SIF, as illustrated in SDS-PAGE profiles (Fig. 6). Compared to the undigested samples (i.e., lane 0), tropomyosin was not present in detectable levels in the non-PUV-treated or PUV-treated shrimp extracts. Compared to a study by Liu et al. [20], tropomyosin from Atlantic white shrimp was degraded much faster than tropomyosin from grass prawn and Pacific white shrimp when trypsin and chymotrypsin were combined. In the foregoing study, the effects of trypsin and chymotrypsin were separated; however, physiologically this is not the case.

Western Blot

According to Western blot analysis (Fig. 7), IgE binding to tropomyosin was not detectable in the non-PUV-treated or PUV-treated shrimp extracts compared to the undigested extracts (i.e., lane 0). Tropomyosin bands were also not detected with the tropomyosin-specific

Fig. 8 Western blot analysis of non-PUV-treated and PUV-treated shrimp extracts subjected to simulated intestinal digestion. The proteases used were trypsin and α -chymotrypsin in SIF. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min in SIF. The detection antibody used was a monoclonal anti-tropomyosin (IgG). The tropomyosin band (36 kDa) is highlighted with an arrow

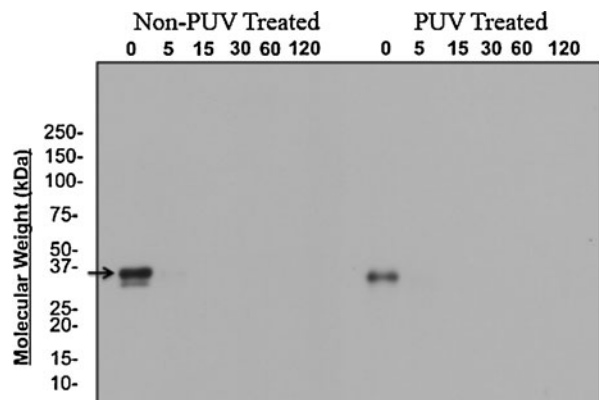
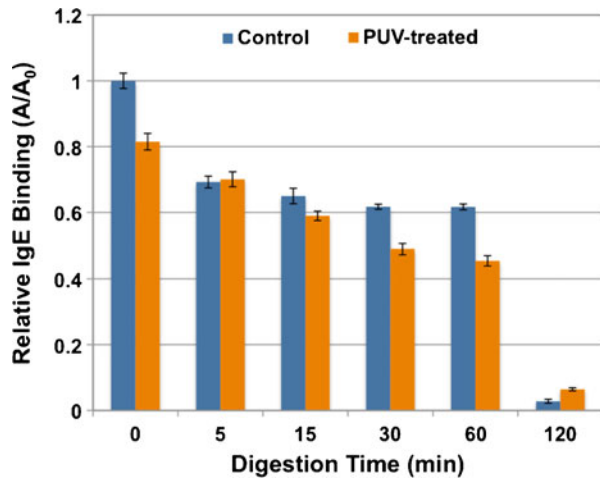


Fig. 9 Indirect ELISA of non-PUV-treated (indicated as control) and PUV-treated shrimp extracts that have been subjected to simulated intestinal digestion. The proteases used were trypsin and α -chymotrypsin in SIF. Samples were digested for 0, 5, 15, 30, 60, and 120 min in SIF. Human IgE antibodies were used to determine allergen reactivity. Relative IgE binding is expressed as A/A_0 , where A is the absorbance of the sample divided by the absorbance of the non-PUV treated. Data are expressed as mean $\pm$ SD



antibody (Fig. 8). A smaller reactive protein band also began to appear after 60 min at approximately 10 kDa in both the non-PUV-treated and PUV-treated Western blots (data not shown). To determine whether these bands were tropomyosin fragments, the Western blot was probed with the tropomyosin-specific antibody; however, these bands were not detected with this antibody. These results may either indicate that the bands were not tropomyosin fragments, or that the fragments did not contain the epitope to which the tropomyosin-specific antibody binds. Huang et al. [26] used trypsin and chymotrypsin and also noted that smaller molecular weight bands which disappeared after 3 h of digestion.

Indirect ELISA and Dot Blot

Indirect ELISA (Fig. 9) illustrates a decrease in shrimp reactivity as time progressed, with almost no detectable reactivity at 120 min for both the non-PUV-treated (indicated as control in figure) and PUV-treated samples. Unlike the Western blot and dot blot (Fig. 10), the reactivity did not decrease completely after just 5 min. The disparity between the Western blot data and the indirect ELISA was likely due to the fragmentation, or enzymatic breakdown, of tropomyosin. If the protein was cleaved by the proteases, it would no longer appear at the same molecular weight and would be too small to be resolved on a polyacrylamide gel (i.e., undetectable on Western blot); however, the tropomyosin fragments might still contain IgE-binding epitopes. As time progresses, these epitopes would begin to disappear, as well as the digestion reached completion.

Fig. 10 Dot blot analysis of digested, untreated (*U*) and PUV-treated (*P*) shrimp extracts. Samples were digested for 0 (undigested), 5, 15, 30, 60, and 120 min in SIF or SGF followed by SIF (i.e., *SGF*+*SIF*). Proteases used were pepsin (*SGF*) and trypsin and chymotrypsin (*SIF*)

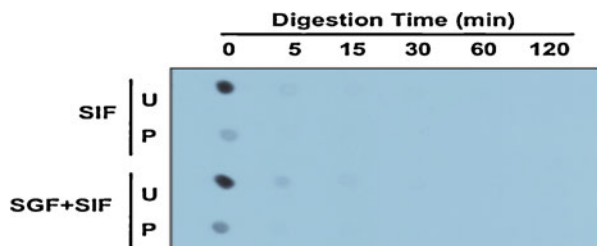
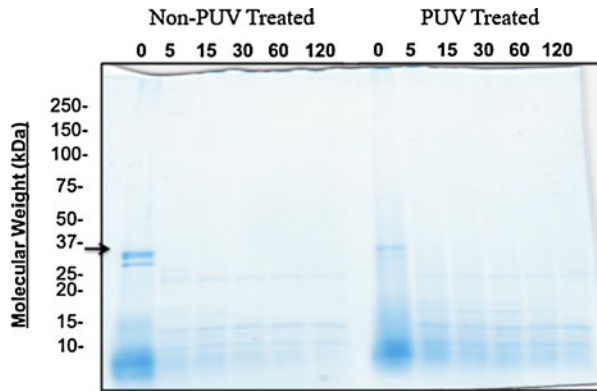


Fig. 11 SDS-PAGE analysis of non-PUV-treated and PUV-treated shrimp extracts subjected to simulated gastric digestion followed by simulated intestinal digestion. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α -chymotrypsin in SIF. Samples were digested for 120 min in SGF followed by 0 (undigested), 5, 15, 30, 60, and 120 min in SIF. The tropomyosin band (36 kDa) is highlighted with an arrow



Peptic and Tryptic Digestion

SDS-PAGE

Samples subjected to SGF for 2 h were treated with SIF to simulate the natural digestive succession. According to SDS-PAGE analysis, it was found that no detectable tropomyosin remained following 5 min or longer under every condition (Fig. 11). Furthermore, most proteins were digested under these conditions, which is predictable considering that intestinal digestion is the last step in human digestion.

Western Blot

In concurrence with SDS-PAGE, IgE binding to tropomyosin was undetectable following 5 min or longer under every condition tested (Fig. 12). However, a smaller molecular weight band (approximately 10 kDa) appeared after 5 min and became more intense as time lapsed (data not shown). This band appeared to be the same protein as seen in the SIF only trials and was not detected with the tropomyosin-specific antibody (Fig. 13). It is possible that this band represented a fragment of tropomyosin and contained an IgE-binding epitope or that

Fig. 12 Western blot analysis of non-PUV-treated and PUV-treated shrimp extracts subjected to simulated gastric digestion followed by simulated intestinal digestion. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α -chymotrypsin in SIF. Samples were digested for 120 min in SGF followed by 0 (undigested), 5, 15, 30, 60, and 120 min in SIF. Human IgE antibodies were used to determine the reactivity of tropomyosin. The tropomyosin band (36 kDa) is highlighted with an arrow

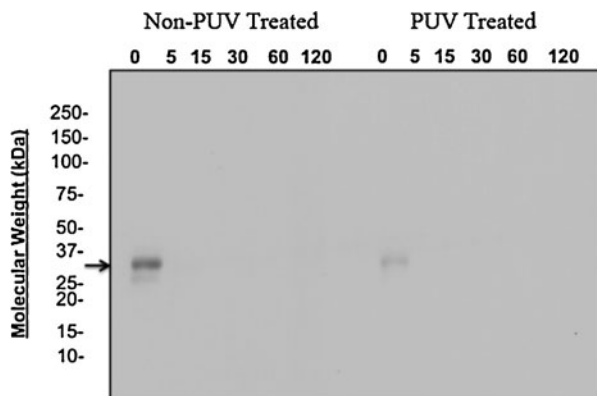
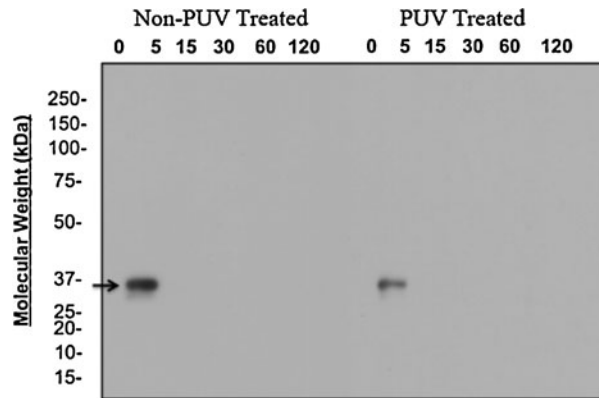


Fig. 13 Western blot analysis of non-PUV- versus PUV-treated shrimp extracts subjected to SGF digestion (120 min) followed by SIF digestion for 0 (undigested), 5, 15, 30, 60, and 120 min. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α -chymotrypsin in SIF. The detection antibody used was a monoclonal anti-tropomyosin (IgG). The tropomyosin band (36 kDa) is highlighted with an arrow

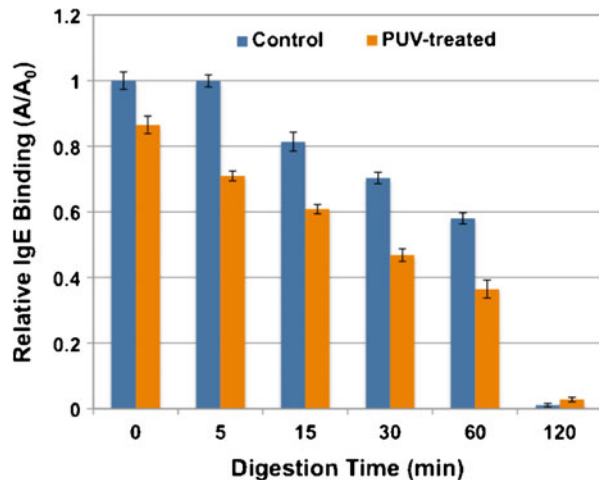


this was a fragment of another shrimp allergen, possibly myosin light chain or arginine kinase.

Indirect ELISA and Dot Blot

Indirect ELISA (Fig. 14) also determined a reduction in shrimp extract reactivity following SGF+SIF digestion. The decrease in reactivity was a stepwise progression with time and was nearly undetectable at 120 min. However, as shown by dot blot analysis (Fig. 10), IgE binding to PUV-treated samples was undetectable following 5 min treatment. The stepwise progression in digestion was not apparent in SDS-PAGE or immunoblot analysis but was apparent in ELISA. Thus, although the band corresponding to tropomyosin disappeared after just 5 min under conditions of simulated intestinal digestion, the tropomyosin fragments might not have been fully degraded and might have maintained IgE-binding epitopes. As described above, it appeared that these fragments were gradually broken down as time elapsed, because the IgE-binding epitopes were progressively destroyed. This would explain the disparity between SDS-PAGE, Western blot, and ELISA.

Fig. 14 Indirect ELISA of non-PUV vs. PUV-treated shrimp extracts subjected to SGF (120 min) followed by SIF digestions for 0 (undigested), 5, 15, 30, 60, and 120 min. The protease first used was pepsin in SGF, followed by a mixture of trypsin and α -chymotrypsin in SIF. Human IgE antibodies were used to determine allergen reactivity. Relative IgE binding is expressed as A/A_0 , where A is the absorbance of the sample divided by the absorbance of the non-PUV treated. Data are expressed as mean $\pm$ SD



Conclusions

Under the conditions of digestions described, SGF, SIF, and SGF+SIF, PUV-treated samples, which show pronouncedly reduced allergen reactivity under PUV treatments, did not return to their initial reactive state. Thus, PUV treatment may be suitable for allergen treatment and could be a potential method to create less allergenic shrimp products that would remain at a low allergen level under human gastric and intestinal digestive conditions; however, this postulation must first be tested and further verified with in vivo trials.

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