

Digestibility and Immunoreactivity of Shrimp Extracts Using an *In Vitro* Digestibility Model with Pepsin and Pancreatin

Ondulla T. Toomer, Andrew B. Do, Tong J. Fu, and Kristina M. Williams

Abstract: Shellfish allergy affects 2% of the adult population in the United States. Identification of allergenic shrimp proteins is needed for improved management and assessment of shrimp allergy. We determined the temporal pepsin and pancreatin stability of total shrimp proteins using simulated physiological digestive conditions *in vitro*. Gel electrophoresis was used to determine protein stability, whereas immunoreactivity of protease stable proteins was determined using rabbit antigen-specific antibodies. Potential allergenicity of protease stable proteins was determined utilizing human sera from shrimp allergic patients. Total shrimp myofibrillar proteins were pepsin- and pancreatin-stable for up to 1 h after initiating digestion, whereas only pancreatin-stable total shrimp proteins were Immunoglobulin G (IgG) immunoreactive. However, shrimp proteins of 32 and 25 kDa were pepsin and/or pancreatin stable and Immunoglobulin E (IgE) reactive, denoting the stability and potential allergenicity. These findings suggest that this *in vitro* digestibility model may be useful for the identification of shrimp allergenic proteins that are more resistant to physiologic digestive conditions and may elicit an immunologic response *in vivo*.

Keywords: allergenicity, digestibility, food allergens, shrimp, tropomyosin

Practical Application: Unlike other food allergies, shellfish allergy is typically life-long and predominantly affects the adult population. A major difficulty in managing shellfish allergy is the lack of reliable diagnostic assays due to limited knowledge of clinically relevant shellfish allergens. Therefore, the identification and characterization of digestive-stable and immunoreactive food proteins is fundamental to the development of new polyclonal antibodies for improved food allergen detection methods within the food industry.

Introduction

With improved aquacultural farming, seafood consumption within the United States has peaked to an average of approximately 16 pounds per year per person (<http://seafoodhealthfacts.org/pdf/seafood-choices-overview.pdf>). IgE mediated allergic reactions to shellfish, such as shrimp and crab, are frequent occurrences within the United States (Ross and others 2008; Boyce and others 2010; Lopata and others 2010). Tropomyosin is a muscle myofibrillar protein and major food allergen found in shrimp and other shellfish. It is composed of 2 identical subunits with molecular masses of approximately 36 kDa arranged in helical formation.⁵ Tropomyosin is heat stable and retains IgE reactivity after thermal processing (Shanti and others 1993). Various studies have shown that >70% of shrimp-allergic patients have IgE reactivity against shrimp tropomyosin (Reese and others 2006; Albrecht and others 2008; Yange and others 2010; Gámez and others 2011). Shrimp myofibrillar proteins consist mainly of tropomyosin approximately 36 kDa (Lopata and others 2010), myosin heavy chain (MHC) approximately 200 kDa (Liu and others 2011), actin approximately 42 kDa (Liu and others 2011), α -actinins approximately 100 kDa (Liu and others 2011), troponin approximately 52 kDa (European

Academy of Allergy and Clinical Immunology 1993), and myosin light chain (MLC) approximately 20 kDa (Lopata and others 2010).

Unlike other food allergies, shellfish allergy is typically life-long and predominately affects the adult population (Crespo and Rodriguez 2003; Skypala 2011). A major difficulty in managing shellfish allergy is the lack of reliable diagnostic assays due to limited knowledge of clinically relevant shellfish allergens. Identification and characterization of digestive stable and immunoreactive food proteins is fundamental to the development of new polyclonal antibodies for improved food allergen detection methods. Pepsin stability has been the primary criterion for food allergen safety assessment. However, this approach is limited and does not parallel *in vivo* digestive conditions (acid denaturation, pepsin, pancreatin), resulting in stable digestive products with allergenic potential. Thus, in this study, we aimed to determine the digestive stability and immunoreactivity of total myofibrillar shrimp proteins using simulated gastric and intestinal physiological conditions *in vitro* to identify the major and minor allergenic food proteins produced with digestion.

Materials and Methods

Purified total shrimp proteins (catalog#XPF34D3A2.5) were purchased from Greer Source Materials (Lenoir, N.C., U.S.A.) and reconstituted in ultrapure distilled water per manufacturer's instructions. Pepsin, 2546 U/mg activity (catalog no. P7012), and pancreatin, 26296 U/mg activity (catalog no. P1750), were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) in the highest available purity. The gels and running buffer

MS 20150166 Submitted 1/30/2015, Accepted 4/26/2015. Authors Toomer, Do, and Williams are with U.S. Food and Drug Administration, Laurel, MD, 20708, U.S.A. Authors Toomer and Fu are with U.S. Food and Drug Administration, Natl. Center for Food Safety and Technology, Moffett Campus, Bedford Park, IL, 60501, U.S.A. Direct inquiries to author Toomer (E-mail: Ondulla.Toomer@fda.hhs.gov)

for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Mini-PROTEAN TGX (catalog no. 456 to 1034) were purchased from Bio-Rad (Hercules, Calif., U.S.A.) and reducing sample buffer (catalog no. 39000) was purchased from Thermo Fisher Scientific (Waltham, Mass., U.S.A.). All polyacrylamide gels were stained using Coomassie Brilliant Blue R-250 Staining Solution purchased from Bio-Rad per the manufacturer's instructions. Western blots were performed using the Trans-Blot Turbo Mini System (catalog no. 170 to 4155) and Nitrocellulose Transfer Packs (catalog no. 170 to 4158) purchased from Bio-Rad. Immunoreactive digestive protein fragments and control undigested proteins were detected using rabbit IgG antishrimp total protein primary antibodies, or rabbit IgG antishrimp tropomyosin primary antibodies. Donkey anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibodies (catalog no. sc-2313) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., U.S.A.), and chromogenic substrates chloronaphthol and diaminobenzidine (CN/DAB, catalog no. 34000) from Thermo Fisher Scientific. Allergen specific rabbit primary antibodies were produced in-house using a protocol that was approved by the Center for Food Safety and Applied Nutrition Institutional Animal Care and Use Committee (Laurel, Md., U.S.A.). Immunoreactive digested shrimp proteins and control undigested proteins were detected using sera from 2 shrimp-allergic human donors (Patient 1 ImmunoCAP 45.4 kU/L; Patient 2 ImmunoCAP 86.2 kU/L) purchased from Plasma Lab Intl. (Everett, Wash., U.S.A.), goat antihuman IgE-HRP secondary antibodies (catalog no. 074 to 1004) from KPL (Gaithersburg, Md., U.S.A.), and CN/DAB from Thermo Fisher Scientific (catalog no. 34000).

Simulated gastric fluid digestion assay

The protocols of Astwood and others (1996) were followed with some modifications. Simulated gastric fluid (SGF) was prepared as described in the United States Pharmacopeia (2006): 0.25 mg/mL solution comprised 0.6365 U pepsin/ μ L in 0.03 mol/L NaCl (pH 1.2). SGF was aliquoted (500 μ L; 318.25 U pepsin) to a 5.0-mL glass tube for each time interval (0, 30, and 60 min) and incubated in a 37 °C water bath for 10 min. Test protein (400 μ g) was added to each tube to begin the SGF digestion assay at a 1:1.25 ratio (units of pepsin: μ g test protein), and 75 μ L of 1 N NaOH was added to each vial to stop the reaction. The 0-min time point was obtained by the addition of 500 μ L of SGF and 75 μ L NaOH (stop solution) simultaneously to the aliquot of test protein. Subsequently, 75 μ L of NaOH was added to the other aliquots of test proteins after 30 and 60 min of incubation at 37 °C, to obtain those respective time points of SGF digestion. SGF digestive products were subsequently stored at -20 °C until analyzed.

Simulated intestinal fluid digestion assay

Simulated intestinal fluid (SIF) was prepared as described in the United States Pharmacopeia (2006): 2.5 mg/mL pancreatin (chymotrypsin, trypsin, amylase, and lipase) in 0.05 mol/L KH_2PO_4 , (pH 7.5) containing 26296 U pancreatin/mg. SIF was aliquoted (500 μ L; 32870 U pancreatin) to a 5.0-mL glass tube for each time interval (0, 30, and 60 min) and incubated in a 37 °C water bath for 10 min. Test protein (400 μ g) was added to each tube to begin the SIF digestion assay at a 32:1 ratio (units of activity: μ g protein) at the respective time intervals. The 0-min time point was obtained by the addition of 500 μ L of SIF to the aliquot of test protein and immediately placing on ice. After the completion of the SIF digestive assay, all tubes were removed from ice and each

sample was boiled for 10 min (to inactivate pancreatic enzyme) and immediately placed on ice and stored long term at -20 °C.

An additional tube was prepared to determine the stability of test proteins to concurrent pepsin, trypsin, and chymotrypsin (SGF/SIF) digestion, in which 400 μ g of test protein was subjected to 60 min of pepsin digestion (reaction was stopped with the addition of 75 μ L NaOH after 60 min of *in vitro* pepsin digestion at 37 °C), followed by 30 min of pancreatin digestion at 37 °C. The pancreatin digestion was stopped by placement on ice and boiled in a water bath for 10 min before long-term storage at -20 °C.

Shrimp tropomyosin antigen purification and lyophilization

One hundred grams of shelled brown shrimp was boiled in deionized water for 15 min and blended with 4 $\times$ volumes of PBS (pH 7.4) for 2 min. The mixture was then shaken overnight in a shaking incubator at 4 °C. Total shrimp extract was obtained by centrifugation at 10000 g. Shrimp tropomyosin was purified using preparative SDS-PAGE procedures using apparatus, Model 491 Prep Cell from Bio-Rad using the modified protocols of Reese and others (2005). Fifty milliliters of preparative gel (40% Bis-acrylamide 13.75 mL, ddH₂O 23.75 mL, separating gel buffer 12.50 mL, 10% ammonium per-sulfate 125 μ L and TEMED 12.5 μ L) was poured in a gel tube casting assembly. The gel was allowed to solidify overnight at 4 °C, with a trace of butanol over it. Next day, a preparation of 30 mL of stacking gel (40% Bis-acrylamide 3 mL, ddH₂O 19.5 mL, stacking buffer 7.5 mL, 10% ammonium per-sulfate 150 μ L, TEMED 30 μ L) was poured above the preparative gel and allowed to solidify for 2 h, again with some butanol over it. The casted gel tube was assembled in the Model 491 Prep-Cell. A shrimp extract sample in PBS (pH 7.4) was mixed with 2 $\times$ Laemmli buffer in equal amounts and loaded on the top of the gel assembly for continuous elution electrophoresis. A flow rate of 1 mL/min was maintained and the elution was allowed to proceed for approximately 8 h until the dye front disappeared before sample collection for additional 3.5 h using a Gilson FC203 fraction collector (Middleton, Wis., U.S.A.). The collected samples were analyzed by SDS-PAGE gel electrophoresis to trace the eluted tropomyosin. The fractions that contained tropomyosin were pooled. The pooled samples were concentrated in Amicon 10000 MW cut off filter tubes (Millipore, Billerica, Mass., U.S.A.) by spinning it at 6500 relative centrifugal force (rcf) for 25 min. The concentrated samples were then desalted by micro-centrifuging the samples in protein desalting spin columns (Pierce, Ill., U.S.A.). The desalted tropomyosin sample was lyophilized overnight in a Labconco Freeze Dryer (Labconco Corp., Kansas City, Mo., U.S.A.).

Total shrimp allergen extract preparation

Red Shrimp was purchased from a local grocery store. Exactly 5 g of shrimp was aliquoted in each tray. The first tray was kept as raw shrimp. Shrimp in the 2nd tray was boiled with 5 mL of de-ionized water for 10 min. The raw and boiled samples were mixed with 50 mL PBS (pH 7.4) and separately blended for 30 s, using an osterizer blender (Sunbeam, Fla., U.S.A.) and then were mixed in a shaking incubator (Labline Orbit Environ Shaker, Labline Instruments Inc., Melrose Park, Ill., U.S.A.) for an hour before the homogenized mixtures were centrifuged at 10000 rcf for 10 min. The supernatant was collected for further analysis.

Production of allergen-specific rabbit polyclonal antibodies

Rabbit antibodies were prepared by subcutaneous inoculation of adult New Zealand White rabbits (>2.0 kg). Antigens were shrimp tropomyosin and total shrimp proteins prepared in-house. One hundred micrograms of antigen was injected into multiple sites on the shaved back of the rabbit. The initial injection was antigen suspended 1:1 in normal saline. Complete Freund's adjuvant (Thermo Fisher Scientific, catalog no. 77140). Rabbits were inoculated 2 to 3 wk later with antigen suspended 1:1 in normal saline: Incomplete Freund's adjuvant (Thermo Fisher Scientific, catalog no. 77145). Two subsequent injections consisted of 100 µg protein suspended in normal saline. For blood collection, animals were anesthetized with an intramuscular injection of ketamine/xylazine/acepromazine. Rabbits were euthanized using intravenous injection of potassium chloride. All procedures were approved by the Center for Food Safety and Applied Nutrition Institutional Animal Care and Use Committee and rabbits were housed in an Assn. for Assessment and Accreditation of Laboratory Animal Care accredited facility.

Reducing SDS-PAGE analysis and immunoblotting

Digested/undigested protein samples (20 µg of protein digestion mixture was utilized for immunoblots with rabbit primary antibodies or 40 µg of protein digestion mixture was utilized for immunoblots with human primary antibodies) were allowed to thaw on ice and reducing/tracking dye (Bio-Rad) was added at a 1× final concentration. Each sample was boiled for 10 min to denature digested proteins and immediately placed on ice before loading on a 10% polyacrylamide Tris-glycine gel. Prepared protein samples were separated under reducing conditions with constant voltage with Tris/glycine/SDS buffer, according to the manufacturer's instructions.

Proteins were visualized by Coomassie Brilliant Blue staining and digitally imaged using the Gel Doc EZ System and The

Intuitive Image Lab™ software by Bio-Rad for the detection and determination of molecular weights of each protein band per the manufacturer's instructions. In addition, SDS-PAGE resolved proteins were transferred to nitrocellulose membranes electrophoretically for immunoblotting procedures according to the manufacturer's instructions. The membranes were washed 3 times in Tris-buffered saline with 0.1% Tween (TBST) and subsequently blocked in 5% nonfat dry milk (NFDM) in TBST for 2 h at room temperature. The membranes were incubated at 4 °C overnight in 1:1000 dilution of rabbit allergen-specific antibody in 5% NFDM in TBST or 1:2 dilution of human plasma from shrimp allergic and nonallergic (control) donors in 5% NFDM in TBST. For detection of tropomyosin and total shrimp IgG-binding proteins, immunoblot analysis was performed using primary antibodies generated in-house. The membranes were washed 3 times in TBST and incubated in secondary antibodies (donkey anti-rabbit IgG-HRP dilution 1:1000 in 5% NFDM in TBST or goat antihuman IgE-HRP 1:10 dilution in 5% NFDM in TBST) at room temperature for 1 h. Bio-detection was determined utilizing chromogenic peroxidase substrate CN/DAB-based detection of HRP activity using the Gel Doc EZ System and The Intuitive Image Lab software by Bio-Rad for the detection and determination of molecular weights of each protein band per the manufacturer's instructions.

Results and Discussion

SDS-PAGE analysis of simulated gastric and intestinal digestion of total shrimp proteins

Reducing electrophoretic analysis of total shrimp proteins revealed 9 protein bands: (1) 200, (2) 100, (3) 90, (4) 75, (5) 52, (6) 42, (7) 36, (8) 25, and (9) 20 kDa, and multiple small minor bands from approximately 20 to 8 kDa, creating a smear in the control undigested total shrimp protein sample (Figure 1).

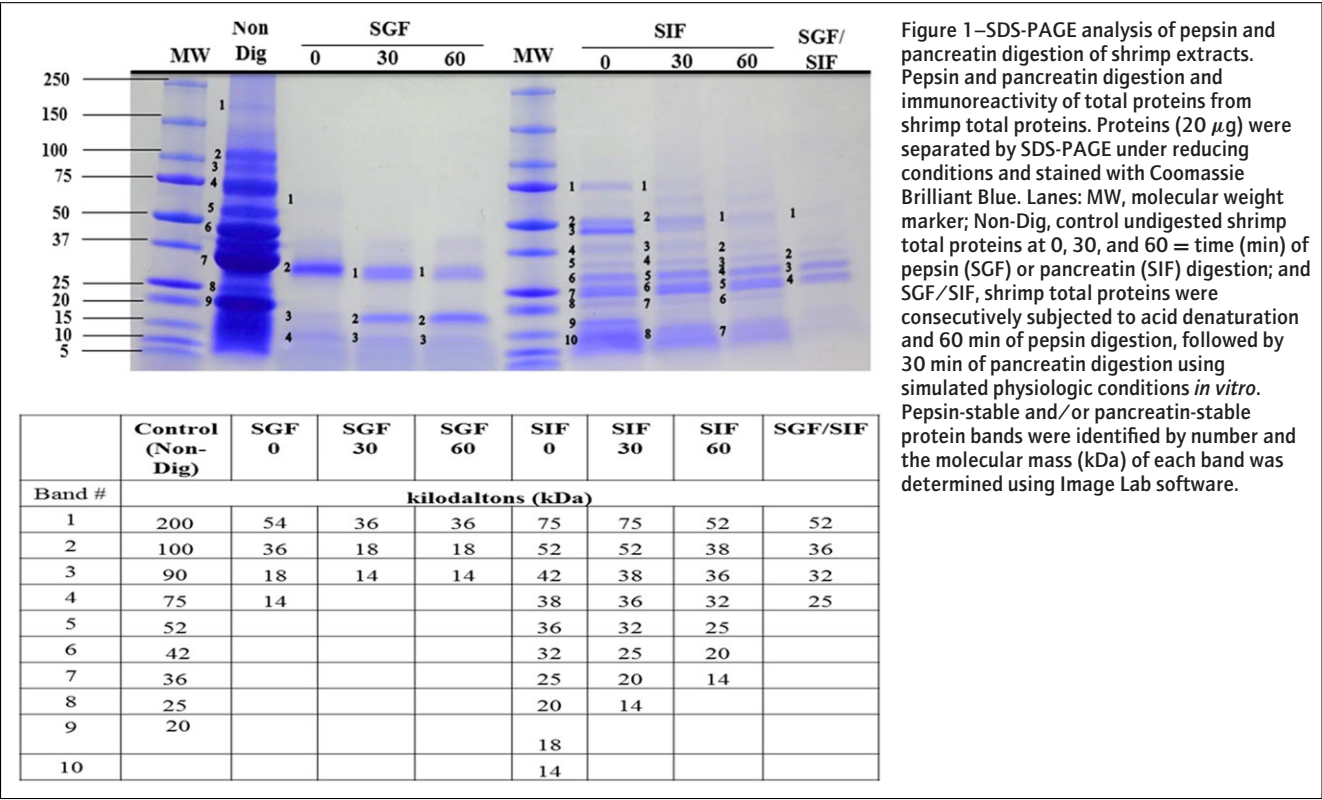


Figure 1—SDS-PAGE analysis of pepsin and pancreatin digestion of shrimp extracts. Pepsin and pancreatin digestion and immunoreactivity of total proteins from shrimp total proteins. Proteins (20 µg) were separated by SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue. Lanes: MW, molecular weight marker; Non-Dig, control undigested shrimp total proteins at 0, 30, and 60 = time (min) of pepsin (SGF) or pancreatin (SIF) digestion; and SGF/SIF, shrimp total proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion, followed by 30 min of pancreatin digestion using simulated physiologic conditions *in vitro*. Pepsin-stable and/or pancreatin-stable protein bands were identified by number and the molecular mass (kDa) of each band was determined using Image Lab software.

The molecular weight of known myofibrillar proteins are: MHC proteins (200 kDa), actinin (100 kDa), troponin (52 kDa), actin (42 kDa), tropomyosin (36 kDa), arginine kinase (40 kDa), and MLC (20 kDa), with similar molecular weights to bands 1 (200 kDa), 2 (100 kDa), 5 (52 kDa), 6 (42 kDa), 7 (36 kDa), and 9 (20 kDa) in lane 1 of Figure 1. Protein bands of 36, 18, and 14 kDa were stable at 0, 30, and 60 min using simulated gastric conditions *in vitro*, whereas proteins of 200, 100, 90, 75, 52, 42, 25, and 20 kDa were degraded (Figure 1). A very faint band of approximately 54 kDa and prominent protein bands of 18 and 14 kDa only appear after initial digestion with pepsin (0 min) with a quantitative increase in intensity of bands at 18 and 14 kDa (30 and 60 min), suggesting that these polypeptides are proteolytic fragments of total shrimp proteins (Figure 1). The band at 36 kDa demonstrated a reduction in banding intensity after 30 and 60 min of pepsin digestion *in vitro* (Figure 1).

Of the shrimp proteins, protein bands of 75, 52, 42, 36, 25, and 20 kDa were stable to pancreatin *in vitro* digestion at 0 min, with protein bands of 38, 32, 18, and 14 kDa appearing as potential proteolytic fragments (Figure 1). Proteins bands of 75, 52, 38, 36, 32, 25, 20, and 14 kDa were stable to 30 min of pancreatin digestion *in vitro*, whereas the proteins of 42 and 18 kDa were degraded with 30 min of pancreatin digestion. The protein band of 75 kDa was degraded with an hour of pancreatin *in vitro* digestion (Figure 1). Protein bands of 75 and 52 kDa show a reduction (0, 30, and 60 min) in the intensity of each band, suggesting degradation of these proteins by pancreatin digestion (Figure 1). Moreover, very faint protein bands of 52 and 36 kDa along with more prominent SIF-resistant fragments of 32 and 25 kDa were

stable to subsequent simulated gastric digestion with pepsin at 60 min (Figure 1).

Western blot analysis of digested total shrimp proteins

Tropomyosin (36 kDa) is a major shellfish allergen showing IgE reactivity in >70% of shellfish-allergic patients (Lehrer and others 2003). Thus, we aimed to specifically determine IgG tropomyosin reactivity with digestive stable total shrimp proteins by Western blot analysis using rabbit antishrimp tropomyosin IgG antibodies. Of the undigested control total shrimp proteins, protein bands between 200 and 50 kDa (bracketed area, Figure 2) demonstrated weak reactivity with rabbit antishrimp tropomyosin IgG antibody using Western blot analysis, whereas protein band of 36 kDa demonstrated strong reactivity (band 2, Figure 2). Also, band 2 at approximately 36 kDa remained immunoreactive with rabbit antishrimp IgG tropomyosin after *in vitro* pepsin digestion at 0, 30, and 60 min (Figure 2). Moreover, pepsin digestive products of 54 kDa observed at 0 min time point were immunoreactive with rabbit antishrimp tropomyosin, suggesting the formation of new proteolytic products that retain epitopes reactive with this antibody (Figure 2). Protein bands of 18 kDa appearing only after initial pepsin digestion (0 min), demonstrate increase in the banding intensity (Figure 1) and immunoreactivity with the antibodies (Figure 2). Protein bands of 75, 36, and, approximately, 20 kDa demonstrated weak reactivity to the antibodies after 0 min of pancreatin digestion (Figure 2), with only protein bands of 36 and 20 kDa remaining slightly reactive with the rabbit antitropomyosin IgG antibody after 30 and 60 min of *in vitro* pancreatin digestion (Figure 2). However, shrimp proteins that were

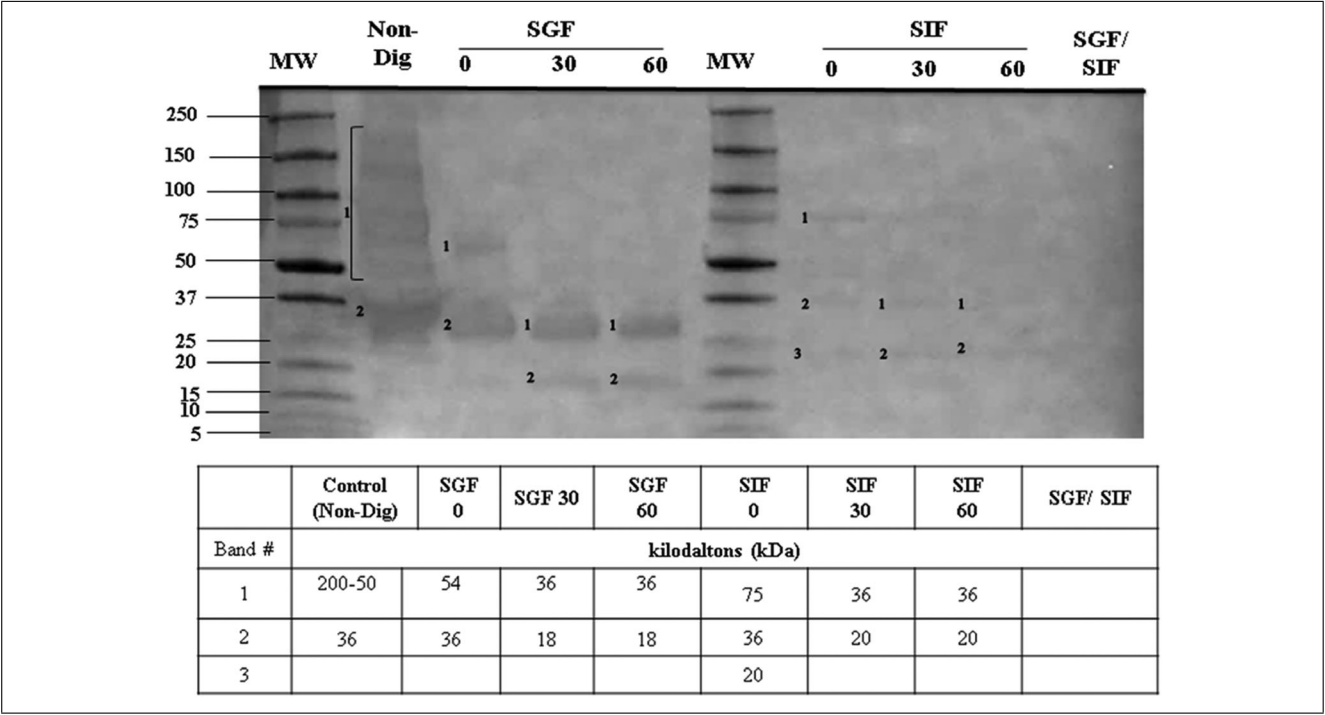


Figure 2–Rabbit antitropomyosin immunoreactivity of pepsin and pancreatin digested shrimp extracts. Electrophoretically separated shrimp total proteins were transferred to a nitrocellulose membrane and immunoblotted with rabbit antishrimp tropomyosin primary antibodies (1:1000) and donkey anti-rabbit secondary-HRP antibodies (1:1000) to determine the immunoreactivity. Biodetection was determined utilizing chromogenic peroxidase substrate CN/DAB-based detection of HRP activity. Lanes: MW, molecular weight marker; Non-Dig, control undigested shrimp total proteins at 0, 30, and 60 = time (min) of pepsin (SGF) or pancreatin (SIF) digestion; and SGF/SIF, shrimp total proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion, followed by 30 min of pancreatin digestion using simulated physiologic conditions *in vitro*. Tropomyosin immunoreactive bands were identified by number and the molecular mass (kDa) of each band was determined using Image Lab software. Bracketed area, proteins of 200 to 50 kDa.

stable to consecutive pepsin and pancreatin digestion (SGF/SIF) in Figure 1 were nonreactive with the rabbit antishrimp tropomyosin antibody, suggesting the loss of epitope binding with pancreatin protease treatment, possibly due to denaturation and loss of secondary and/or tertiary structures (Figure 2).

Next, we aimed to determine the immunoreactivity of pancreatin-stable total shrimp proteins of potential allergenicity using Western blot analysis with rabbit antishrimp total protein IgG antibodies. Similar to Figure 2, proteins of the control sample between approximately 200 to 50 kDa were reactive with the antibodies (Figure 3). More specifically, control total undigested shrimp proteins of 200, 90, 75, 36, and 20 kDa (seen in the Coomassie stained gel, Figure 1) were reactive with rabbit antishrimp total protein antibodies, whereas proteins of 100, 52, 42, and 25 kDa were nonreactive (Figure 3). Interestingly, a protein band of 47 kDa, not visualized as a prominent protein band with gel electrophoresis (Figure 1) of the control undigested sample, was also immunoreactive with rabbit antishrimp total protein IgG antibodies after 0 min of pepsin *in vitro* digestion (Figure 3). Moreover, protein bands of 90, 75, 47, 36, 20, and 18 kDa were reactive to the rabbit antishrimp total antibodies after 0 min of pepsin digestion, with protein bands of 75 and 36 kDa remaining only slightly reactive to the antibodies after 30 min of pepsin digestion (Figure 3). However, in Figure 1 (SDS-PAGE) of the pepsin digested samples (0 and 30 min), the 75 kDa protein band is not visualized and may be below the level of detection of Coomassie blue polyacrylamide gel staining. However, after 60 min of pepsin *in vitro* digestion, all stable shrimp proteins seen in Figure 1 (36, 18, and 14 kDa) were nonreactive with the rabbit antishrimp total protein antibodies (Figure 3), whereas these digestive stable

shrimp proteins were reactive with the rabbit antitropomyosin antibodies (Figure 2). The differences in reactivity of these pepsin stable proteins may be possibly due to variations in tropomyosin epitope binding affinity between these 2 different polyclonal antibodies.

Pancreatin-stable proteins (0, 30, and 60 min) of 75, 52, 36, 22, and 20 kDa were immunoreactive with rabbit antishrimp total protein antibodies, whereas the protein band of 36 kDa lost immunoreactivity after 30 min of pancreatin *in vitro* digestion (Figure 3). Interestingly, a 22 kDa protein band was reactive with the antibodies after 0, 30, and 60 min of pancreatin *in vitro* digestion and was also reactive after consecutive protease digestion with pepsin and pancreatin (SGF/SIF, Figure 3). These findings parallel the results of Liu and others (2011), demonstrating the appearance of a 22 kDa protein band reactive to tropomyosin specific polyclonal antibodies after 15 min of *in vitro* digestion with trypsin. A control blot was performed in parallel using undigested and digested (pepsin and/or pancreatin) total shrimp proteins and immunoblotted with rabbit antipine nut IgG antibodies and all total shrimp proteins were nonreactive (data not shown).

To determine the potential allergenicity of shrimp total proteins, Western blot analysis was conducted with control undigested and digested (pepsin and/or pancreatin) shrimp proteins using commercially available plasma from 2 shrimp-allergic patients. Protein bands of 200, 150, 90, 75, 62, 42, 36, and 22 kDa of the control undigested sample were reactive with IgE human antishrimp antibodies from 2 different donors (Figure 4). Of these bands, proteins of 200, 90, 75, and 36 kDa were also reactive with rabbit antishrimp total protein IgG antibodies (Figure 3). Moreover, pepsin-stable protein bands (0, 30, and 60 min) of 36, 18,

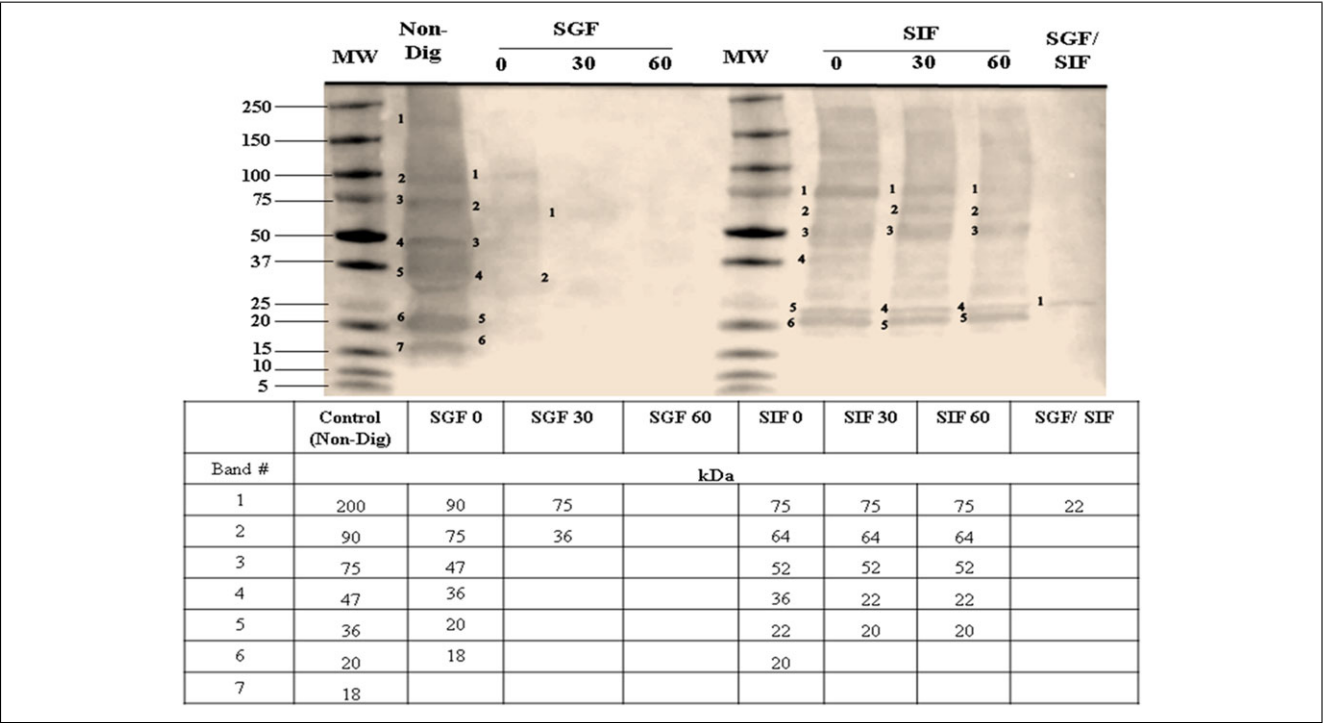


Figure 3–Rabbit antishrimp total protein immunoreactivity of pepsin and pancreatin digested shrimp extracts. Electrophoretically separated shrimp total proteins were transferred to a nitrocellulose membrane and immunoblotted with rabbit antishrimp total proteins primary antibodies (1:1000) and donkey anti-rabbit secondary-HRP antibodies (1:1000) to determine the immunoreactivity. Biotin detection was determined utilizing chromogenic peroxidase substrate CN/DAB-based detection of HRP activity. Lanes: MW, molecular weight marker; Non-Dig, control undigested shrimp total proteins at 0, 30, and 60 = time (min) of pepsin (SGF) or pancreatin (SIF) digestion; and SGF/SIF, shrimp total proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion, followed by 30 min of pancreatin digestion using simulated physiologic conditions *in vitro*. Immunoreactive shrimp total protein bands were identified by number and the molecular mass in kilodaltons (kDa) of each band was determined using Image Lab software.

and 14 kDa visualized with gel electrophoresis (Figure 1) were also IgE-reactive with the sera from both shrimp-allergic patients (Figure 4).

Of the pancreatin-stable (0 min) shrimp proteins visualized in Figure 1, protein bands of 75, 42, 36, 32, 25, and 20 kDa were immunoreactive with human sera of 2 different shrimp-allergic patients (Figure 4), whereas protein bands 75, 62, and 42 kDa were either hydrolyzed and/or loss epitope binding to IgE antibodies of shrimp-allergic patient 1 after 60 min of pancreatin *in vitro* digestion (Figure 4). However, shrimp proteins of 75, 42, 36, 34, and 20 kDa were either hydrolyzed and/or loss epitope binding to IgE antibodies of shrimp-allergic patient 2 after 30 and 60 min of pancreatin *in vitro* digestion (Figure 4). Shrimp proteins of 25 kDa and digestive fragment of 32 kDa were stable and immunoreactive with shrimp-allergic sera from both patients after consecutive pepsin and pancreatin *in vitro* digestion (Figure 4), suggesting potential *in vivo* allergenicity of these stable shrimp proteins. Control undigested and digested (pepsin and/or pancreatin) total shrimp proteins were immunoblotted with the serum from grass-allergic only donor patients and all shrimp proteins were nonreactive (data not shown).

In summary, by comparing western blot analysis using rabbit antishrimp polyclonal antibodies and IgE antibodies from shrimp-allergic donors, we identified common immunoreactivity of protein bands ranging from approximately 200 to 20 kDa in the control undigested shrimp proteins. Moreover, shrimp proteins of 32 and 25 kDa were stable and immunoreactive to human IgE post consecutive pepsin and pancreatin treatment *in vitro*, implying the potential stability and intact transit of these proteins through the digestive tract and potential to trigger an immunological response *in vivo*.

In this study, tropomyosin (36 kDa) remains stable and immunoreactive after gastric digestion whereas predominately losing epitope binding capacity after exposure to pancreatin and the digestive conditions within the duodenum (Figure 2). In addition, although shrimp tropomyosin has been identified as a major shrimp allergen (Shanti and others 1993), these results suggest that other shrimp total proteins (32 and 25 kDa) are digestive-stable and may also be capable of eliciting type I hypersensitivity responses *in vivo*. Similarly, other studies have demonstrated shrimp proteins of approximately 75, 49, 42 (Sahabudin and others 2011), and 40 kDa (García-Orozco and others 2007) to be lesser known

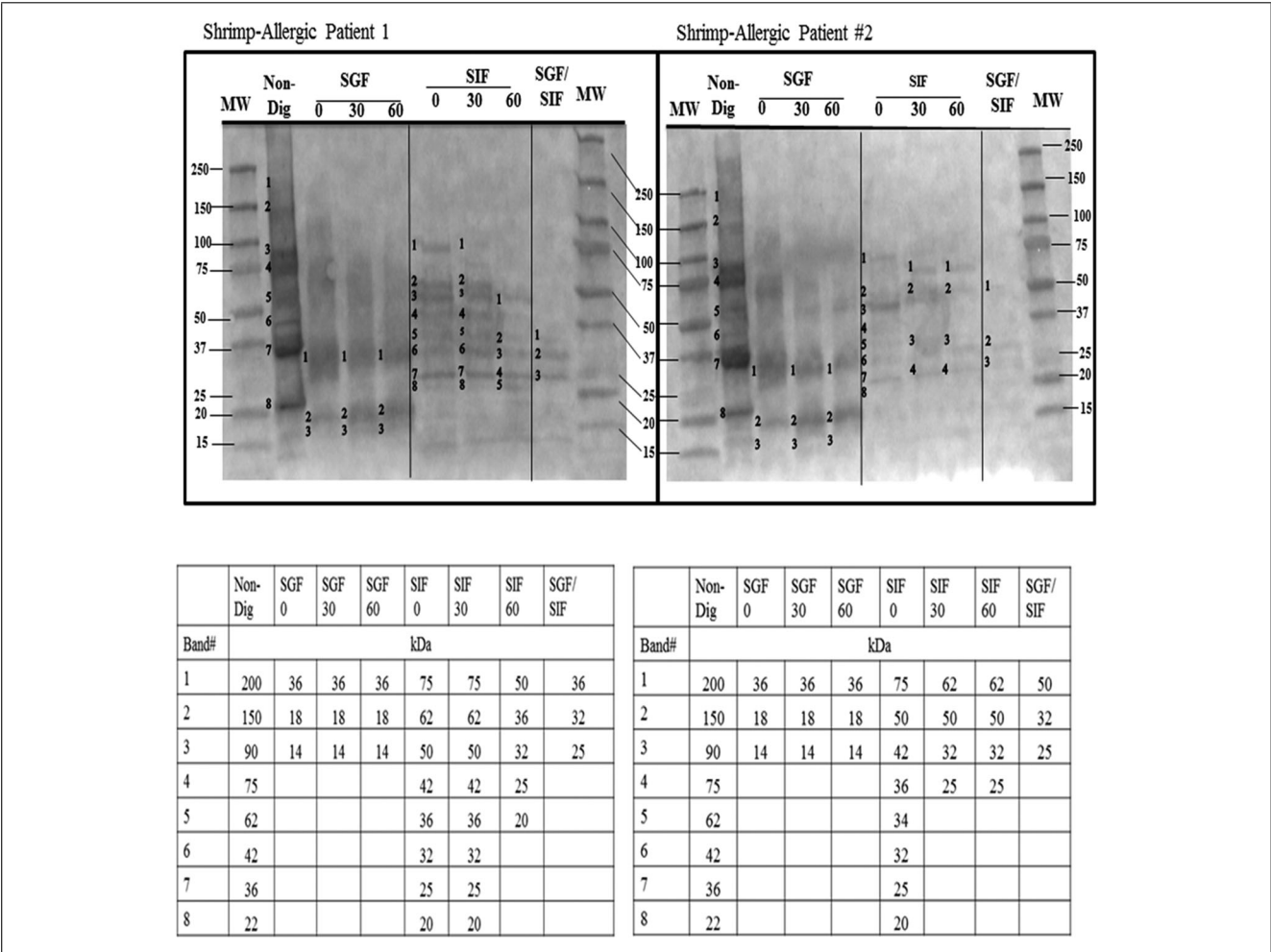


Figure 4—IgE immunoreactivity of pepsin and pancreatin digested shrimp extracts electrophoretically separated shrimp total proteins were transferred to a nitrocellulose membrane and immunoblotted with commercially available sera from shrimp-allergic donor patients (1:2) and goat antihuman IgE-HRP secondary antibodies (1:10) to determine allergenicity of total shrimp proteins. Biodetection was determined utilizing chromogenic peroxidase substrate CN/DAB-based detection of HRP activity. Lanes: MW, molecular weight marker; Non-Dig, control undigested shrimp total proteins at 0, 30, and 60 = time (min) of pepsin (SGF) or pancreatin (SIF) digestion; and SGF/SIF, shrimp total proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion, followed by 30 min of pancreatin digestion using simulated physiologic conditions *in vitro*. Allergenic shrimp protein bands were identified by number and the molecular mass in kilodaltons (kDa) of each band was determined using Image Lab software.

shellfish allergens. Although these shellfish allergens have been reported, their characterization has yet to be determined biochemically (Sahabudin and others 2011).

Species variations between shrimp allergens and potency of major and minor shrimp allergens between study subjects and human study populations makes interpretation of identified shrimp allergens more complex (Reese and others 2005). Nevertheless, studies that parallel *in vivo* digestion of shrimp allergens are necessary for the identification of nonallergenic and allergenic food proteins and their epitope-binding and IgE reactivity. In future digestibility studies, we aim to sequence digestive stable and immunoreactive proteins identified in this study for identification and sequence homology, and biochemical characterization.

Conclusions

Current digestibility models of potential allergenic food proteins utilize pepsin stability as the criterion for safety assessment. However, this approach may be somewhat limited in identifying food protein stability *in vivo* in the mammalian gut. Protein digestion is dependent upon age, health, diet, stage of development, and varies between individuals. The findings suggest that an improved *in vitro* digestibility model utilizing simulated physiological conditions with pepsin and pancreatin may be more useful for identifying proteins that are more resistant to physiologic digestive conditions and most likely to elicit an immunologic response *in vivo*. Although these models are representative of human digestion (gastric and intestinal), they do not predict protein half-life or protein stability *in vivo*. An improved digestion model for food allergens, however, may enhance our understanding regarding the stability of the digestive products of food allergens in the human gut and facilitate the development of more sensitive and effective antibodies for use with food allergen detection and criteria used for food allergen safety assessment.

References

- Albrecht M, Alessandri S, Conti A, Reuter A, Lauer I, Vieths S, Reese G. 2008. High level expression, purification and physico- and immunochemical characterisation of recombinant Pen a 1: a major allergen of shrimp. *Mol Nutr Food Res* 52:S186–95.

- Astwood J, Leach J, Fuchs R. 1996. Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* 14:1269–73.
- Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, Plaut M, Cooper SF, Fenton MJ, Arshad SH, Bahna SL, Beck LA, Byrd-Bredbenner C, Camargo Jr CA, Eichenfield L, Furuta GT, Hanifin JM, Jones C, Kraft M, Levy BD, Lieberman P, Luccioli S, McCall KM, Schneider LC, Simon RA, Simons FE, Teach SJ, Yawn BP, Schwaninger JM. 2010. Guidelines for the diagnosis and management of food allergy in the united states: summary of the NIAID-sponsored expert panel report. *J Allergy Clin Immunol* 126:1105–18.
- Crespo JF, Rodriguez J. 2003. Food allergy in adulthood. *Allergy* 58:98–113.
- European Academy of Allergy and Clinical Immunology (EAACI) Subcommittee on Skin Tests. 1993. Allergen standardization and skin tests. *Allergy* 48:48–82.
- Gómez C, Sánchez-García S, Ibáñez MD, López R, Aguado E, López E, Sastre B, Sastre J, del Pozo V. 2011. Tropomyosin IgE-positive results are a good predictor of shrimp allergy. *Allergy* 66:1375–83.
- García-Orozco KD, Aispuro-Hernández E, Yepiz-Plascencia G, Calderón-de-la-Barca AM, Sotelo-Mundo RR. 2007. Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*. *Int Arch Allergy Immunol* 144:23–8.
- Lehrer SB, Ayuso R, Reese G. 2003. Seafood allergy and allergens: a review. *Mar Biotechnol* 5:339–48.
- Liu GM, Huang YY, Cai QF, Weng WY, Su WJ, Cao MJ. 2011. Comparative study of *in vitro* digestibility of major allergen, tropomyosin and other proteins between Grass prawn (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*). *J Sci Food Agric* 91: 163–70.
- Lopata AL, O'Hehir RE, Lehrer SB. 2010. Shellfish allergy. *Clin Exp Allergy* 40:850–8.
- Reese G, Schickentanz S, Lauer I, Randow S, Lüttkopf D, Vogel L, Lehrer SB, Vieths S. 2006. Structural, immunological and functional properties of natural recombinant Pen a 1, the major allergen of Brown Shrimp, *Penaeus aztecus*. *Clin Exp Allergy* 36:517–24.
- Reese G, Viebranz J, Leong-Kee SM, Plante M, Lauer I, Randow S, Moncin MS, Ayuso R, Lehrer SB, Vieths S. 2005. Reduced allergenic potency of VR9–1, a mutant of the major shrimp allergen Pen a 1 (tropomyosin). *J Immunol* 175:8354–64.
- Ross MP, Ferguson M, Street D, Klontz K, Schroeder T, Luccioli S. 2008. Analysis of food-allergic and anaphylactic events in the national electronic injury surveillance system. *J Allergy Clin Immunol* 121:166–71.
- Sahabudin S, Misnan R, Yazir ZH, Mohamad J, Abdullah N, Bakhtiar F, Murad S. 2011. Identification of major and minor allergens of black tiger prawn (*penaeus monodon*) and king prawn (*penaeus latisulcatus*). *Malays J Med Sci* 18:27–32.
- Seafood Health Facts: Making Smart Choices, Balancing the Benefits and Risk of Seafood Consumption, A joint project of Oregon State Univ., Cornell Univ., the Universities of Delaware, Rhode Island, Florida & California and the Community Seafood Initiative. 2004. Available from: <http://seafoodhealthfacts.org/pdf/seafood-choices-overview.pdf>.
- Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. 1993. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* 151:5354–63.
- Skypala I. 2011. Adverse food reactions—an emerging issue for adults. *J Am Diet Assoc* 111: 1877–91.
- United States Pharmacopeia 23, Simulated Gastric Fluid and Simulated Intestinal Fluid, The Natl. Formulary 18; The United States Pharmacopeial Convention, Inc., Rockville, MD, 2006, p. 3171.
- Yang AC, Arruda LK, Santos AB, Barbosa MC, Chapman MD, Galvão CE, Kalil J, Morato-Castro FF. 2010. Measurement of IgE antibodies to shrimp tropomyosin is superior to skin prick testing with commercial extract and measurement of IgE to shrimp for predicting clinically relevant allergic reactions after shrimp ingestion. *J Allergy Clin Immunol* 125: 872–8.