Journal of
Food and Pharmaceutical Sciences

Research Article

Intrinsic Factors Contributing to Shrimp and Blood Clamp Allergic Reaction and Allergic-Like Symptoms

Almasah Azzahra, Bramanti Nadya Kausara, Muhammad Novrizal Abdi Sahid*

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

*Corresponding author: Muhammad Novrizal Abdi Sahid | Email: m.novrizal.a@ugm.ac.id

Received: 04 January 2023; Revised: 07 May 2023; Accepted: 22 May 2023; Published: 23 June 2023

Abstract: Factors that contribute to food allergenicity is still unknown. To determine food intrinsic properties that contribute to *L. vannamei* and *A. granosa* allergenicity or allergy like symptoms this study identify total protein and metal profiles, histamine contents, and protein heat stability. Histamine content is measured colorimetrically. Protein heat-stability is observed with sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Allergy-related metal concentration are determined with Atomic Absorption Spectroscopy (AAS). Fresh *L. vannamei* and *A. granosa* contained ~100 and ~25 ppm histamine, respectively. Storage at -20 ^{o}C for 3 days relatively did not changed histamine content of both foods. Storage at 4-8 ^{o}C for 3 days increased histamine content in *L. vannamei*, but not in *A. granosa*. Protein sized <70 kDa of both samples are relatively heat-stable. *L. vannamei* and *A. granosa* contained Zn with concentration of 85 ppm and 92 ppm, subsequently. All samples did not contain nickel (Ni), chromium (Cr), and lead (Pb). Higher histamine content and heat-stable protein number of *L. vannamei* could contribute to higher prevalence of its allergy or allergic like symptoms than *A. granosa*. High Zn content might contribute to allergic like symptoms of both foods.

Keywords: allergy, histamine, protein, metal, seafood

1. INTRODUCTION

Around ten percent of American have food allergy and near 5% believe they have food allergy [1]. There are five most common food allergies among adults, i.e., shellfish (2.9%), peanut (1.8%), milk (1.9%), tree nut (1.2%), and finfish (0.9%) [1]. Shellfish is consisted of a wide type of edible marine animal covering crustacea and mollusks, which include i.e., shrimp, oyster, crab, and abalone [2]. Even though they have identic allergens that might cross-react each other [3], the severity that induced by the "same" allergen from different type of shellfish are different [3]. For example, tropomyosin from invertebrate i.e. dust mites, shrimp is allergenic compare to tropomyosins from cattle and chicken [3]. Within shellfish, shrimp is the most common cause of allergy, followed by crab, lobster, clam, and oyster, respectively [2].

Many allergens that are identified from seafood are proteins. Protein allergens are reported mostly sized below 70 kDa [4]. Protein with high stability is has a more potency to become an allergen [4]. Besides allergenic protein, metals (i.e., nickel, cobalt, zinc, chromium) [5], carbohydrate [6], and

lipid were reported to be able to induce allergic reaction [7]. Previous studies showed that the formation of IgE and allergic reaction could be increased by the consumption of food containing metals, carbohydrate, or lipid [6], [7].

During allergy process, antigen complex with IgE and its receptor, and will activate mast cell which further induce degranulation event that cause the release of histamine and other mediators (i.e., prostaglandins, leukotrienes) [8]. The release of histamine induced acute allergic inflammation condition such as vasodilatation, increase vascular permeability, contraction of bronchial smooth muscle and increase mucus secretion [8]. In present study we sough to determine the intrinsic factor that might contribute to allergic reaction of mollusks (represent by *Anadara granosa*) and crustacea (represent by *Litopenaeus vannamei*).

2. MATERIALS AND METHODS

2.1. Materials

Anadara granosa purchased from Pasar Sentul Yogyakarta (origin from Semarang, Indonesia). White leg shrimp (*L. vannamei)* was obtained by direct delivery from farmer in Yogyakarta, Indonesia. Histamine detection kit (Bioo Scientific HistaStrip™ Test Kit) was purchased from PerkinElmer (TX, USA). HEPES buffer, phosphate buffer saline (PBS) were obtained from Gibco (Gibco, Grand Island, NY, USA). Materials for SDS-PAGE preparation in protein stability observation were all purchased from Sigma-Aldrich (Singapore). Protein marker (PM 5100) for SDS-PAGE is the product of SMOBIO (Hsinchu, Taiwan).

2.2. Sample Preparation

One hundred g fresh *L. vannamei* (whole part) or *A. granosa* (meat part) is mashed, then placed into three 50 ml conical tubes (30 g each tube) and stored at 4-8^{o}C dan -20^{o}C for three days.

2.3. Histamine Content Measurement

Briefly, 4 g mashed sample was mixed with 16.0 mL seafood buffer. Mix by vortexing for 30 s then incubate for 1 min at room temperature. Repeat vortex step and incubate for 5 min. Centrifuge at 10000 rpm for 5 min. Supernatant was taken for histamine content measurement.

Mix neutralization solution with coloring solution (20 μL:20 μL) in 96-well plate. Add 200 μL of sample supernatant and mix well by pipetting. Histamine with concentration of 50 ppm was used as control. Dip HistaStrip for 1 min into the solution in the well, take out and incubate for 3 min at room temperature. The color on the HistaStrip then compared with standard. The darkest red color formed, the highest histamine concentration in the sample. This method can be used to measure histamine concentration 0 - 1000 ppm.

2.4. Protein Stability Measurement

Sample buffer containing 0.5 M Tris-HCl buffer pH 6.8, 70% glycerol, 10% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, and 1% blue bromphenol. Electrode buffer containing 25 mM Tris-base buffer, 250 mM glycine pH 8.3, and 0.1% SDS. Staining solution containing 0.25% Coomassie brilliant blue R250, 50% methanol, and 10% glacial acetic acid. De-staining solution containing 30% methanol and 10% glacial acetic acid. SDS-PAGE gel for electrophoresis was prepared with the

composition in Table 1. Iso-butanol was added to the upper part of separation gel before stacking buffer addition.

Table 1. Polyacrylamide gel (12%) preparation for SDS-PAGE

Materials	Separating Gel (mL)	Stacking Gel (mL)
30% Acrylamid/bisacrylamid (29:1)	6,0	1,7
1,5 M Tris-HCl pH 8,6	3,8	-
1 M Tris-HCl pH 6,8	-	1,25
Sterile aquades	4,9	6,8
SDS 10%	0,15	0,1
Ammonium Persulfat 10%	0,15	0,1
TEMED	0,006	0,01

Sample for electrophoresis is prepared by extracting protein from 4.0 g sampel with 16 mL HEPES buffer. The mixture then vortex for 30 sec, followed by incubation at room temperature for 1 min. Further, vortex for 30 sec and incubate for 5 min. Tissue and cell debris will precipitate, smaller particle i.e., protein will remain in the upper part of the solution. Collect 1 mL of the upper part that not contain the sediment and insert into 1.5 mL tube. Centrifuge for 10 min at 10.000 rpm and 4 ^{o}C. Supernatant obtained after this centrifugation process is considered as 1x protein extract. Ten times dilution of 1x protein extract is considered as 10x protein extract. Prior electrophoresis run, protein extracts were mixed with sample buffer according to protocol mentioned in Table 2.

Table 2. Sample solution preparation for SDS-PAGE

Sample	Volume (μL)	Sample buffer (μL)	Treatment
A. granosa (1x or 10x dilution)	10	5	Heated at 95-100^{o}C for 5 or 30 min
L. vannamei (1x or 10x dilution)	10	5	Heated at 95-100^{o}C for 5 or 30 min

Fifteen μL total sample and 5 μL marker proteins were run at 200 volt/cm for 45 min. The staining of protein attached on the gel was done with staining solution for 30-60 min. Stained gel then washed with de-staining solution until the background color is disappear, and the protein band clearly appear.

2.5. Metal Content Measurement

Five g samples digest with the combination of concentrate acid (HNO_3, H_2SO_4), heat, and peroxide. Metal (Ni, Cr, Zn, dan Pb) contents were measure in deionized water with atomic absoption spectrometry (AAS). In brief, add 5 mL HNO_3 and 5 mL H_2SO_4 into 5 g sample. Put tube in hot-block digestion apparatus and heated at 60 ^{o}C for 30 min. Let the temperature decrease to room temperature then add 10 ml of HNO_3. Tube then heated to 120 ^{o}C, the temperature was increase to 150 ^{o}C. After the sample becoming black, take out tube and let it reach room temperature by incubation. Then add 1 mL of H_2O_2. Put the tube on the digestion tube, then and H_2O_2 repetitively until the sample become clear. Take tube and ad 50 mL de-ionized water. Most elements can directly determine.

2.6. Proximate Analysis

Total protein content was determined using Kjeldahl method. Total lipid and rough fiber content were determined with Gravimetri method. Proximate analysis is performed in Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada, following their protocol.

2.7. Data Analysis

Histamine and metal concentration data were presented as mean ± SD of number of experiment state in figure or table legend. Statistics significance was measured with Student t-test (Prism 8, GraphPad Software, CA, USA) considering $p<0.05$ as statistically significant.

3. RESULTS AND DISCUSSIONS

3.1. Histamine Content

There are many factors that might directly trigger allergy or increasing food allergy severity. Thus, it is important to determine global factor that contribute to food allergic reaction or its severity. Histamine is the main mediator in allergic condition. However, consumption of histamine alone is not causing allergy. Histamine-rich foods may cause the condition known as food intolerance in sensitive individuals [9], [10]. The clinical symptoms of histamine intolerance, and allergy are difficult to differentiated [11]. Histamine was released by allergen-activated mast cells and contributed to allergy pathogenesis by binding to their H1 or H4 receptors [8]. Most of histamine receptor is expressed in human intestinal tract (H1R, H2R, H4R) [12], thus digested histamine-containing food can directly exert its biological activity.

Histamine concentration in the samples are summarized in Figure 1. Fresh shrimp (*L. vannamei*) contained 75 ppm of histamine, whereas *A. granosa* only containing around 25 ppm of histamine. Three measurement replications are results in three identic histamine concentration values (Figure 1).

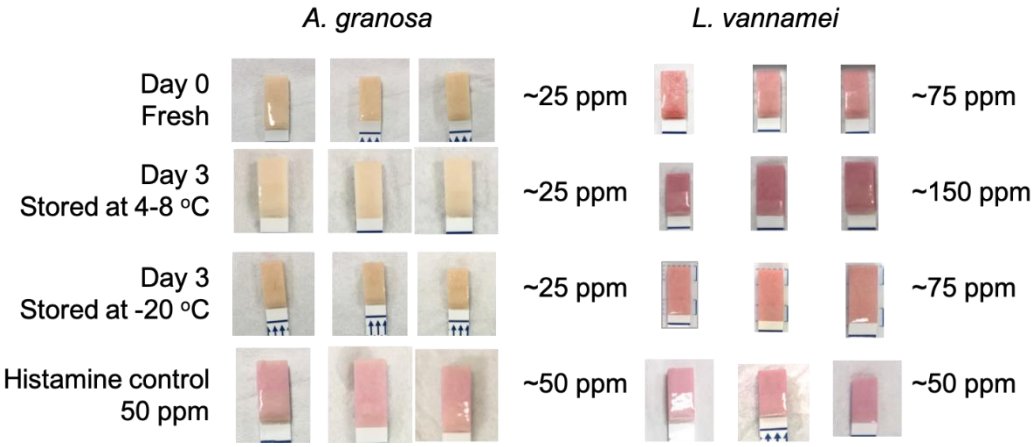

Figure 1. The effect of sample storage condition on the histamine concentration. Storage at 4-8 °C for 3 days increased histamine concentration in *L. vannamei* sample, but not in *A. granosa*. Storage at -20 °C did not affect histamine concentration in both samples. Experiments were performed in triplicate.

When the sample is stored at 4-8°C for 3 days the histamine content is increased in shrimp (*L. vannamei*) sample, but not in *A. granosa* sample. Storage in -20°C for the same period of time did not affect histamine concentration in shrimp (*L. vannamei*). Interestingly, histamine concentration of *A. granosa* is decreased. Together these results showed that histamine concentration is affected by the storage-temperature.

The higher histamine content in fresh *L. vannamei* might contribute to the higher allergy prevalence of *L. vannamei* compare to *A. granosa*. Histamine content of *L. vannamei* is highly probable to induce allergic or histamine intolerance symptoms as it is higher than 50 ppm. In contrast, histamine content of *A. granosa* were lower than than 50 ppm, thus might be not resulting any clinical symptoms. According to FAO/WHO, 50 ppm histamine are expected not causing a toxic symptom to healthy individuals [9]. However, the acceptable limit of histamine content are varied between country, from 50 – 200 ppm [13].

Storage at -20 °C as expected inhibited histamine production in *L. vannamei*. *A. granosa* sample have relatively unchanged histamine content during storage in different condition. Histamine generation during storage is affected by bacterial content, free histidine concentration, Histidine decarboxylase (HDC) activity, pH, salt, and oxygen availability, which are varied between animals [9], [13]. From all these factors, the increased in histamine content in *L. vannamei* after storage at 4-8 °C could be the contribution of HDC activity of bacterial in the sample. Histamine generating enzyme (HDC) of some bacterial is still active at 5 °C [14]. Food processing i.e., fried, boiling, grilled, and also fermentation cause the change in histamine content **[15]**. However, we did not investigate the effect of food processing as the histamine content changes appear mostly because of the reduction of water content of the sample.

3.2. Protein Stability

Proteins that are able to trigger an allergic reaction have a wide variation of molecular weight, commonly sized between 5-100 kDa. However allergenic protein that frequently caused allergy is sized between 10-70 kDa [4], [16]. In addition, allergenic proteins are usually highly stable, high water solubility, and an acidic isoelectric point [4], [16]**.** Heating process is commonly performed during food processing and was known to affect allergenicity of protein **[17].** Where heat-stable proteins are tent to be more allergenic **[17].** Thus, this study determined the heat-stability of protein with molecular weight between 10-70 kDa. Figure 2 depicting the 12% acrylamide gel after electrophoresis, stained, and de-staining process finished. In non-heated samples, four protein bands were appeared in the protein extract from shrimp sample (Fig. 2H and I), with molecular weight of ~75, ~60, ~36 and ~17 kDa. Band of protein with MW ~75, ~60, and ~17 kDa is thinner after dilution 10x (Figure 2H and I, box 1, 2, 4). Protein band around 7-8 (MW ~36 kDa) seems was not affected by dilution. For *A. granosa* protein extract, two protein bands were appeared (Figure 2C-D), with molecular weight of ~60 and ~36 kDa. Dilution seems did not affect the protein band thickness (Figure 2C-D, box 1, 2). In heated samples, the number of protein band is relatively unchanged. Two protein bands are appearing from *A. granosa* protein extract (Figure 2A-B), whereas shrimp protein-extract showing 4 protein bands (Figure 2F-G). Protein extract dilution only affecting band in box 1 and 4 of shrimp

sample (Figure 2F-G). Sample dilution only changed the thickness of protein band, but did not affect the number of band appeared. However, we can see the different band thickness after heating process. Together these results showed that protein ranged 10-70 kDa obtained from the extraction of *A. granosa* and *L. vannamei* are relatively heat-stable.

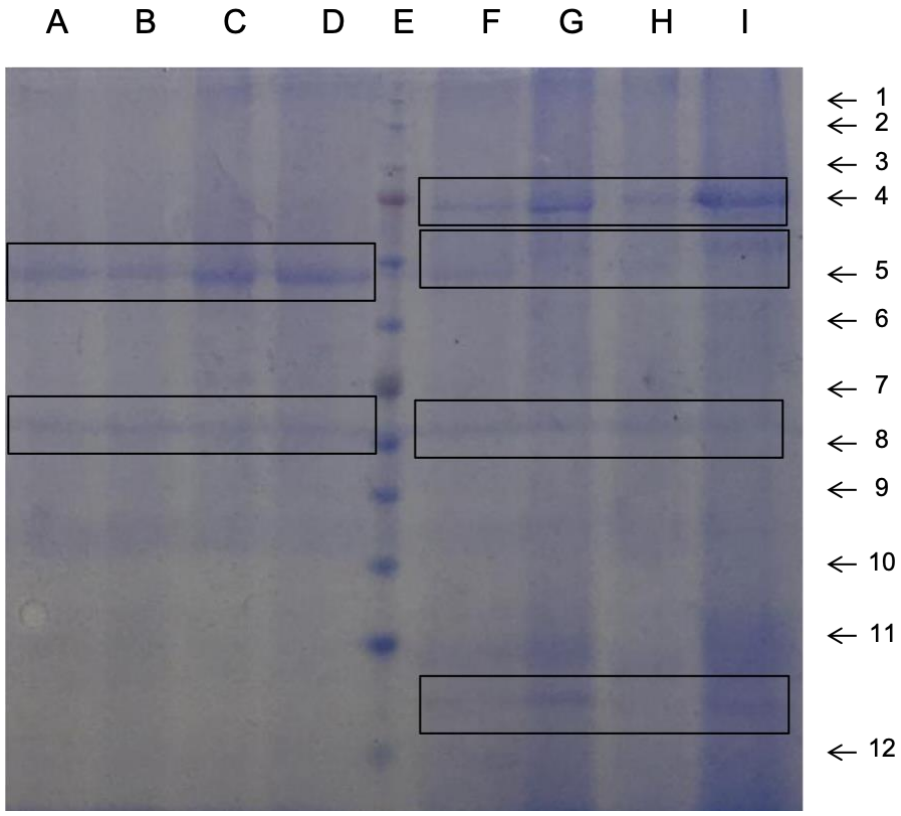

Figure 2. Protein bands after separation with SDS-PAGE. A-D is sample from *A. granosa* and F-I is sample from *L. vannamei*. Samples A, C, G and I are samples with 1 times dilution. Samples B, D, F and H are samples with 10 times dilution. Samples A, B, F and G are samples boiled for 30 min at 95 °C. Samples C, D, H and I are samples boiled only following SDS-PAGE protocol, for 5 min at 95 °C. Lane 1-12 are marker protein with size subsequently, 180, 140, 100, 75, 60, 50, 40, 35, 30, 25, 20, 15, and 10 kDa. In the box between lines 7-8 are predicted tropomyosin (36-41 kDa) or arginine kinase (40-45 kDa). In the box at line 4 are predicted sarcoplasmic calcium-binding protein (60 kDa), pyruvate kinase (60 kDa) or haemocyanine (72-75 kDa). In the box between lines 11-12 are predicted myosin light chain (17-20 kDa).

Electrophoresis of polyacrylamide gel shows that *L. vannamei* (4 proteins) contains more heat-stable protein sized <70 kDa compare to *A. granosa* (2 proteins). In addition, those proteins might also be protease stable as no protease inhibitor was used in the isolation buffer. Low protein band numbers in SDS-PAGE result is unexpected. HEPES buffer was used as protein extraction solution, which was also recommended for protein isolation [18]. However, protease inhibitors were not included in the recipe. Cell lysis is generally lead to the release of protease that could lower overall protein obtained. This is commonly controlled by the administration of protease inhibitor in the buffer used [18]. The absence of protease inhibitors in the protein extraction solution might contribute to low protein band obtained in this study. Protease inhibitor was not included in the protein

extraction solution to mimic food processing by digestive tract where there are many proteases involved [19]. PAGE concentration used here also suitable for separation of target protein (size 10-70 kDa). Ten percent of acrylamide was reported to be suitable for separation of protein with size 14-200 kDa (linear 14-66 kDa), whereas 15% acrylamide is suitable for separation protein with size 14-66 kDa (linear 14-36 kDa) **[20].** Thus, 12% is appropriate for separation of our target protein 10-70 kDa.

L. vannamei protein extract shows protein band at 75, 60, 36, and 15 kDa. *A. granosa* only two protein bands that appear at 60 and 36 kDa. These proteins molecular weight are identic with the molecular weight of in shellfish allergenic protein described previously, i.e., tropomyosin (38-41 kDa), arginine kinase (40-45 kDa), myosin light chain (17-20 kDa), sarcoplasmic calcium-binding protein, pyruvate kinase (~60 kDa) and hemocyanin (72-75 kDa) [16], [21], [22]. These allergens were also reported to be heat-stable [16], [21], [22]. Further characterization of each protein is needed as in this study the protein is only predicted based on its molecular weight.

3.3. Metal Content

Seafood organs and bodies of different invertebrates might accumulate trace metals [23]**.** Metals that exist in foodstuff are cadmium (Cd), lead (Pb), copper (Cu), chromium (Cr), nickel (Ni), zinc (Zn) **[24]**. Both *L. vannamei* and *A. granosa* samples not contain Ni, Cr, and Pb. Zinc with the concentration of 92.29 $\pm$ 0.71 ppm and 85.60 $\pm$ 0.71 ppm are detected in *A. granosa* and *L. vannamei*, respectively (Table 3). This Zn concentration is higher than limitation by several guidelines i.e., 40 ppm [23]. Zinc is needed for mast cell degranulation in antigen mediated pathway [25], and also affected basophil and T-cell function during allergic responses [25]. Zinc acute toxicity symptoms including nausea and vomiting, stomachache, diarrhea [26], which are also common in histamine-intolerant, food-hypersensitive, or food-allergic patients [11].

Table 3. Metal content of food samples. *Statistically significant between samples after t-test analysis.

Sample	Metal contents, n = 3 (ppm)			
	Cr	**Ni**	**Pb**	**Zn**
A. granosa	n.d.	n.d.	n.d.	92.29 $\pm$ 0.71*
L. vannamei	n.d.	n.d.	n.d.	85.60 $\pm$ 0.71

3.4. Proximate Analysis

Proximate compositions of the samples are performed to check the other possibilities that contribute to allergy, especially carbohydrate and lipid. Lipid and carbohydrates were also known to contribute to allergic reaction, even though most of the mechanism is not directly as immunological allergen. Lipid was shown to increase allergic response by increasing acting as hapten or indirectly activating molecular pathway that results in allergic reaction [7]. The investigation performed here is not specific to certain carbohydrate or lipid content as the lack of specified apparatus. The proximate analysis shows that *A. granosa* contain protein (15.49 $\pm$ 1.04%) and fiber (5.72 $\pm$ 0.82%) lower than *L. vannamei*. But the total lipid is higher (2.11 $\pm$ 0.75%). In contrast, *L. vannamei* contained more protein (18.26 $\pm$ 2.09%) and rough fiber (7.05 $\pm$ 0.33%) Table 4. Further detail experiment is needed to show

the contribution of proximate analysis results of *A. granosa* and *L. vannamei* to their overall allergenicity.

Table 4. Total lipid, protein, and rough fiber content of samples

Sample	Food Component Content, n = 2 (%)		
	Total lipid	Total Protein	Rough fiber
A. granosa	2.11 ± 0.75	15.49 ± 1.04	5.72 ± 0.82
L. vannamei	0.55 ± 0.40	18.26 ± 2.09	7.05 ± 0.33

4. CONCLUSION

Collectively, this study implied the possibility of heat-stable protein and high histamine content in *L. vannamei* could contribute to its higher allergy prevalence compare to *A. granosa*. Zinc content might also contribute to the high prevalence.

Acknowledgement: This work is financially supported by Faculty of Pharmacy, Universitas Gadjah Mada with Grant year 2018 (no: UGM/FA/1967.a/M/05/01) and 2019 (no: 18.05.04/UN1/FFA1/SET.PIM/PT/2019).

References

[1] R. S. Gupta *et al.*, "Prevalence and Severity of Food Allergies Among US Adults," *JAMA Netw. Open*, vol. 2, no. 1, p. e185630, Jan. 2019, doi: 10.1001/jamanetworkopen.2018.5630.

[2] C. K. Woo and S. L. Bahna, "Not all shellfish 'allergy' is allergy!," *Clin. Transl. Allergy*, vol. 1, no. 1, p. 3, Jan. 2011, doi: 10.1186/2045-7022-1-3.

[3] C. P. Mikita and E. A. Padlan, "Why is there a greater incidence of allergy to the tropomyosin of certain animals than to that of others?," *Med. Hypotheses*, vol. 69, no. 5, pp. 1070–1073, 2007, doi: 10.1016/j.mehy.2006.12.060.

[4] J. Pekar, D. Ret, and E. Untersmayr, "Stability of allergens," *Mol. Immunol.*, vol. 100, pp. 14–20, Aug. 2018, doi: 10.1016/j.molimm.2018.03.017.

[5] K. Roach and J. Roberts, "A comprehensive summary of disease variants implicated in metal allergy," *J. Toxicol. Environ. Health Part B*, vol. 25, no. 6, pp. 279–341, Aug. 2022, doi: 10.1080/10937404.2022.2104981.

[6] T. A. Platts-Mills *et al.*, "Carbohydrate epitopes currently recognized as targets for IgE antibodies," *Allergy*, vol. 76, no. 8, pp. 2383–2394, Aug. 2021, doi: 10.1111/all.14802.

[7] M. G. del Moral and E. Martínez-Naves, "The Role of Lipids in Development of Allergic Responses," *Immune Netw.*, vol. 17, no. 3, p. 133, 2017, doi: 10.4110/in.2017.17.3.133.

[8] E. B. Thangam *et al.*, "The Role of Histamine and Histamine Receptors in Mast Cell-Mediated Allergy and Inflammation: The Hunt for New Therapeutic Targets," *Front. Immunol.*, vol. 9, p. 1873, Aug. 2018, doi: 10.3389/fimmu.2018.01873.

[9] FAO, Ed., *Meeting report / Joint FAO WHO Expert Meeting on the Public Health Risks of Histamine and Other Biogenic Amines from Fish and Fishery Products: 23 - 27 July 2012, FAO headquarters, Rome Italy*. Rome: Food and Agriculture Organization of the United Nations [u.a.], 2013.

[10] C. J. Tuck, J. R. Biesiekierski, P. Schmid-Grendelmeier, and D. Pohl, "Food Intolerances," *Nutrients*, vol. 11, no. 7, p. 1684, Jul. 2019, doi: 10.3390/nu11071684.

[11] T. C. Pinzer, E. Tietz, E. Waldmann, M. Schink, M. F. Neurath, and Y. Zopf, "Circadian profiling reveals higher histamine plasma levels and lower diamine oxidase serum activities in 24% of patients with suspected histamine intolerance compared to food allergy and controls," *Allergy*, vol. 73, no. 4, pp. 949–957, Apr. 2018, doi: 10.1111/all.13361.

[12] S. Smolinska, E. Winiarska, A. Globinska, and M. Jutel, "Histamine: A Mediator of Intestinal Disorders—A Review," *Metabolites*, vol. 12, no. 10, p. 895, Sep. 2022, doi: 10.3390/metabo12100895.

[13] K. B. Biji, C. N. Ravishankar, R. Venkateswarlu, C. O. Mohan, and T. K. S. Gopal, "Biogenic amines in seafood: a review," *J. Food Sci. Technol.*, vol. 53, no. 5, pp. 2210–2218, May 2016, doi: 10.1007/s13197-016-2224-x.

[14] F. Gardini, Y. Özogul, G. Suzzi, G. Tabanelli, and F. Özogul, "Technological Factors Affecting Biogenic Amine Content in Foods: A Review," *Front. Microbiol.*, vol. 7, Aug. 2016, doi: 10.3389/fmicb.2016.01218.

[15] B. Y. Chung *et al.*, "Effect of Different Cooking Methods on Histamine Levels in Selected Foods," *Ann. Dermatol.*, vol. 29, no. 6, p. 706, 2017, doi: 10.5021/ad.2017.29.6.706.

[16] A. L. Lopata, J. Kleine-Tebbe, and S. D. Kamath, "Allergens and molecular diagnostics of shellfish allergy: Part 22 of the Series Molecular Allergology," *Allergo J. Int.*, vol. 25, no. 7, pp. 210–218, Nov. 2016, doi: 10.1007/s40629-016-0124-2.

[17] K. C. M. Verhoeckx *et al.*, "Food processing and allergenicity," *Food Chem. Toxicol.*, vol. 80, pp. 223–240, Jun. 2015, doi: 10.1016/j.fct.2015.03.005.

[18] EMBL, "Choice of lysis buffer – Protein Expression and Purification Core Facility." https://www.embl.org/groups/protein-expression-purification/services/protein-purification/choice-of-lysis-buffer/ (accessed May 12, 2023).

[19] A. Kårlund, I. Paukkonen, C. Gómez-Gallego, and M. Kolehmainen, "Intestinal Exposure to Food-Derived Protease Inhibitors: Digestion Physiology- and Gut Health-Related Effects," *Healthcare*, vol. 9, no. 8, p. 1002, Aug. 2021, doi: 10.3390/healthcare9081002.

[20] S. R. Gallagher, "SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)," in *Current Protocols Essential Laboratory Techniques*, S. Gallagher and E. Wiley, Eds., Hoboken, NJ, USA: John Wiley & Sons, Inc., 2012, p. et0703s06. doi: 10.1002/9780470089941.et0703s06.

[21] D. Guillen *et al.*, "Anaphylaxis caused by hemocyanin contained in shrimp cephalothorax," *Ann. Allergy. Asthma. Immunol.*, vol. 113, no. 6, pp. 674-675.e2, Dec. 2014, doi: 10.1016/j.anai.2014.09.011.

[22] C.-H. Lee, C.-C. Wu, Y.-C. Tyan, W.-T. Yu, E. S. Huang, and H.-S. Yu, "Identification of pyruvate kinase as a novel allergen in whiteleg shrimp (Litopenaeus vannamei) by specific-IgE present in patients with shrimp allergy," *Food Chem.*, vol. 258, pp. 359–365, Aug. 2018, doi: 10.1016/j.foodchem.2018.03.088.

[23] M. A. Baki *et al.*, "Concentration of heavy metals in seafood (fishes, shrimp, lobster and crabs) and human health assessment in Saint Martin Island, Bangladesh," *Ecotoxicol. Environ. Saf.*, vol. 159, pp. 153–163, Sep. 2018, doi: 10.1016/j.ecoenv.2018.04.035.

[24] P. K. Rai, S. S. Lee, M. Zhang, Y. F. Tsang, and K.-H. Kim, "Heavy metals in food crops: Health risks, fate, mechanisms, and management," *Environ. Int.*, vol. 125, pp. 365–385, Apr. 2019, doi: 10.1016/j.envint.2019.01.067.

[25] K. Nishida and R. Uchida, "Role of Zinc Signaling in the Regulation of Mast Cell-, Basophil-, and T Cell-Mediated Allergic Responses," *J. Immunol. Res.*, vol. 2018, pp. 1–9, Nov. 2018, doi: 10.1155/2018/5749120.

[26] N. Galan, "Too much zinc: Symptoms, causes, and treatments." https://www.medicalnewstoday.com/articles/326760#recommended-guidelines (accessed May 12, 2023).