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Impacts of Thermal Treatments on Major and Minor Allergens of Sea Snail, *Cerithidea obtusa* (Obtuse Horn Shell)

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ABSTRACT

Snail is one of the worst causes of food allergy. Thus, the aim of this study was to identify the major and minor allergens of the local marine snail (*Cerithidea obtusa*) and subsequently to investigate the impacts of heat treatment on the IgE-binding activity of snail allergens.

Proteins from raw and heat-treated snails (boiled, roasted and fried) were extracted and then resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting of all extracts were then performed using sera from patients with snail allergy.

The results showed that the raw extract contains numerous protein bands between 12 to>250 kDa. Some thermostable proteins, predominantly the 33 and 42 kDa bands, remained detected in all cooked extracts with decreasing intensities from boiled to roasted to fried extracts, while the majority of thermolabile bands denatured after heating. Boiled snail had more protein bands compared to roasted and fried snails. Immunoblotting of raw extract demostrated 19 IgE-binding bands ranging from 15 to 240 kDa. The thermostable bands of 33 and 42 kDa and a thermolabile of 30 kDa band were identified as the major allergens of this snail. The cooked extracts yielded less allergenic bands. The boiled extract yielded approximately 14 IgE-binding bands with some smeared bands at high molecular weight regions. The roasted extract had lesser IgE-binding bands and the majority appeared as smears, while the IgE-reactivity in the fried extract was less visible and appeared as weak smears.

This study indicated that both raw and cooked snails played a crucial role in snail allergenicity, as this species of snail contains both thermostable and thermolabile major allergens. The degree of snail allergenicity was revealed in the order: raw> boiled > roasted> fried. Thus, the results would facilitate in the development of effective diagnosis and management strategies of snail allergy in this country.

Keywords: Allergen; *Cerithidea obtusa*; Immunoblotting; SDS-PAGE; Snail; Thermolabile; Thermostable

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INTRODUCTION

Snails are comprised of 80% water, 15% protein and 2.4% of fat, which is an ideal diet.[1] They are also rich sources of vitamins and minerals.[1] Snail is a very popular delicacy in many countries including Japan, China, Europe and other Asian countries.[1] However, snail is also considered as one of the worst causes of food allergy. In fact, some people may develop severe episodes of asthma after the ingestion of snails.[2,3] Mild symptoms such as oral allergy syndrome, urticaria (hives) and severe life threatening symptoms such as anaphylactic shock can also occur after snail consumption.[2,4] Allergic reactions have been reported to several species of snails including the brown garden snail (*Helix aspersa*), burgundy snail (*Helix pomatia*), vineyard snail (*Cernuella virgata*), snail *Helix terrestre*, white garden snail (*Theba pisana*) and the sea snail (*Bolinus brandaris*).[2-4]

In molluscs including snails, tropomyosin, an abundant muscle protein at 34 to 38 kDa is recognized as the major allergen responsible for food allergy.[2,5-8] Allergenic snail tropomyosins have been established in several species of snail including *Turbo cornutus* (turban shell)[7] and *Helix aspersa* (brown garden snail).[8] Tropomyosin is a major protein in eukaryotic cells which constitutes up to 20% of the total protein content[5,9] and plays a structural and functional role in contractile activity and the regulation of cell morphology and motility.[9]

Besides, other allergens such as actin also play a vital role in mollusc allergenicity.[9] Actin contributes the most abundant protein in all eukaryotic cells.[10-13] Although it is found in every living cell, actin is mostly discovered in muscle cells. This protein is a highly conserved protein of approximately 42 kDa with 375 amino acids in its polypeptide chain.[12,13]

Snail is frequently consumed after certain heat treatments such as boiling, roasting or frying.[1] It was reported that the heat denaturation of proteins can either minimize or maximize food allergenicity as the result of epitope alterations which include destruction, modification, masking or unmasking of epitope.[6,9,14-21]

Cerithidea obtusa (obtuse horn shell), locally known as "siput sedut" or "Belitung" is a species of sea snail, marine gastropod molluscs in Potamididae Family.[1] This snail is one of the fishery commodities which is commonly consumed and traditionally used for therapeutic purposes.[1] Thus, the aim of this study was to identify the major and minor allergens of this species of snail and subsequently evaluate how heat treatment processes (boiling, roasting and frying) may affect the allergenicity of *C. obtusa*.

MATERIALS AND METHODS

Snail Extracts

The raw snail (*C. obtusa*) extract was prepared, following the method by Rosmilah et al.[18] In brief, the flesh of the snail was homogenized in phosphate-buffered saline (PBS), pH 7.2 (1:10 weight/volume) using a blender, followed by an overnight extraction at 4˚C. The homogenate was centrifuged, sterile-filtered using a syringe filter and dried in a freeze dryer. The lyophilized extract was stored at -20˚C until use.

The three types of cooked extracts (boiled, roasted and fried) were prepared according to the procedure by Zailatul et al.[9] with a slight modification. For the boiled extract, the snail flesh was boiled in distilled water at 100˚C for 20 minutes, while for the roasted extract, the flesh was roasted at 180˚C for 20 minutes in an oven. The fried extract was carried out by frying the flesh for 15 minutes in vegetable oil. Each cooked snail was then extracted using the similar method as detailed above. The total protein content in the extracts was estimated by Bio-Rad Protein Assay (Biorad, Hercules, CA, USA).

Human Sera

Serum samples were obtained from 25 snail-allergic patients referred to the Allergy Clinic, Kuala Lumpur Hospital. The allergic responses were confirmed by the clinical history of snail allergy and positive skin prick test (SPT) to raw snail extract and/or in vitro test using ImmunoCAP test against sea snail (Phadia AB, Uppsala, Sweden). Serum from a non-allergic individual was used as a negative control. The ethics approval for this research was gained from the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (NMRR-08-697-1852).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the effects of thermal treatments on the snail protein profiles by using the previously described method.[17,18] Briefly, the snail proteins were treated in a Laemmli buffer and heated at 97°C for 3 minutes. Protein samples (10 µg/

well) were run along with pre-stained molecular weight markers (Biorad, Hercules, CA, USA) in 12.5% separating gels with 5% stacking gels using a Mini Protean 3 System at 120 mA for 45 min (Biorad, Hercules, CA, USA). The separated proteins were stained with Coomassie brilliant blue R-250. Protein masses were estimated by comparing the snail protein bands with the molecular weight markers using Imaging Densitometer GS800 and Quantity One Software (Biorad, Hercules, CA, USA).

Immunoblotting

Allergenic proteins were analyzed by immunoblotting, as described previously.[17,18] Briefly, the unstained snail proteins in the SDS-PAGE gel were transferred to 0.45 μm nitrocellulose membrane using Mini Transblot System (Biorad, Hercules, CA, USA). The membrane was then cut, washed with tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS) and blocked for 1 hours in a blocking buffer (10% non-fat milk in TBS). The strips were then probed overnight at 4°C with the diluted individual patient's sera (1:5 in blocking buffer), followed by a further incubation with 0.001% biotinylated goat-antihuman IgE as the secondary antibody (KPL, Gaithersburg, Maryland, USA) and 0.0002% streptavidin-conjugated alkaline phosphatase (Biorad, Hercules, CA, USA). The bound IgE was then discovered by incubating the strips in a colorimetric alkaline phosphatase substrate (BioRad, USA). A strip without the serum sample and a strip incubated with the serum from a non-allergenic individual were included in immunoblotting as a blank and negative controls, respectively. The immunoblotting of raw extract was conducted using 25 sera, while only six selected sera were further analyzed in immunoblotting of cooked extracts.

RESULTS

Protein Profiles Analysis

Figure 1 exhibits the SDS-PAGE protein profiles of raw and all cooked extracts of *C. obtusa*. Fractionation of complex protein mixtures in the raw extract expressed at least 38 protein bands between the molecular weights of 12 to 250 kDa. The majority of the prominent bands were detected between 30 to 250 kDa. In contrast, the cooked snail extracts revealed less protein components compared to the raw extract. Several thermolabile protein bands in the range of 10 to 17 kDa, 25 to 30 kDa, 40 to 74 kDa and some high molecular weight bands (124-250 kDa) in the raw extract had disappeared in all the cooked extracts. However, some higher molecular weight bands in the range of 74 to 124 kDa were also missing in the roasted extract, whereas almost all protein fractions between 25 to 30 and 40 to 100 kDa in the fried extract had completely disappeared. Therefore, the boiled snail contained more protein fractions than the roasted and fried snails.

Some thermostable protein bands were detected in all snail extracts, as they were preserved even after being heat-treated with boiling, roasting or frying. The most prominent thermostable protein bands were visualized at 33 and 42 kDa. However, both bands showed a significant reduction of band intensity in the boiled extract, followed by the roasted and fried extracts. In addition, several thermostable bands in the range of 17 to 20 kDa and 124 to 150 kDa were also detected in all snail extracts.

IgE-binding Proteins Analysis

Based on the immunoblotting results of the raw snail extract in Figure 2(a) and Table 1, 19 IgE binding bands from 15 to 240 kDa were detected. Three major allergens in raw snail were detected at 30, 33 and 42 kDa. The 33 kDa protein had the highest IgE-binding frequencies, as detected by all 25 sera (100%), whereas the 42 and 30 kDa bands were recognized by 23 (92%) and 21 (84%) of the sera, respectively.

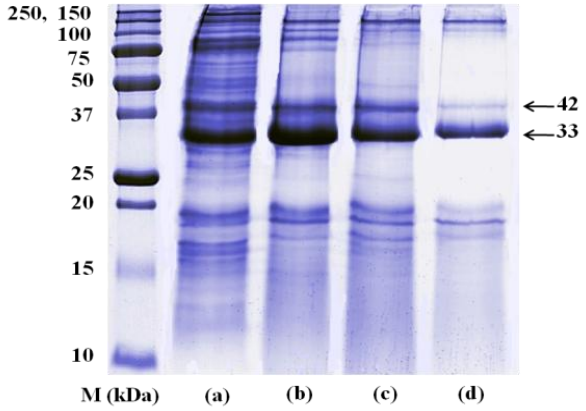

Figure 1. Protein profiles of *Cerithidea obtusa* extracts in SDS-PAGE gel. Lane (a), raw extract; lane (b), boiled extract; lane (c) roasted extract; lane (d) fried extract. Lane M, molecular weight markers.

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All patients in the immunoblotting of the raw extract were positive not only to major allergens, but also to several minor allergens. Several higher molecular weight bands from 58 to 240 and two low molecular weight bands at 26 and 29 kDa were recognized by 8 to 12 (32 to 48%) of the sera. Furthermore, proteins of 32, 40 and 49 kDa had reacted with 5 to 7 (20 to 28%) of the sera, while proteins at 17 and 25 kDa were detected by 4 (12%) and 3 (16%) of the sera, respectively. Only one serum (4%) was identified at the 15 kDa band.

The thermal property of the allergenic proteins was further evaluated by immunoblotting of the cooked extracts using six selected sera. The cooked extracts demonstrated a decreased number of IgE-binding bands, as presented in Figure 2 (b, c and d) and Table 1. The boiled extract yielded approximately 14 thermostable IgE-binding bands between 17 to 218 kDa with some smeared bands at the high molecular weight regions. The roasted extract had lesser IgE-binding bands between 33 to 218 kDa and the majority appeared as smears, while the IgE-reactivity in the fried extract was less visible and appeared as weak smears. It was shown that only one serum (no. 4) retained the IgE-binding to both thermostable major allergens (33 and 42 kDa proteins) in all extracts (notably smeared in the fried extract). Only two sera (no. 3 and 4) retained the IgE-binding to 42 kDa band in all cooked extracts, whereas rest of the sera had notably failed to detect the 42 kDa and 33 kDa bands in both roasted and fried extracts. Overall, all sera showed a reduction of thermostable band intensities, except for serum number 4 which had a slight enhancement in the IgE-binding intensities from regions 27 to 74 kDa. It should be noted that none of the sera (except for serum no. 4) bound to the 30 kDa thermolabile major allergens in all cooked extracts. No IgE-binding was observed in the blank and negative control serum, except for a non-specific binding at 70 kDa seen in all blots of the raw extract.

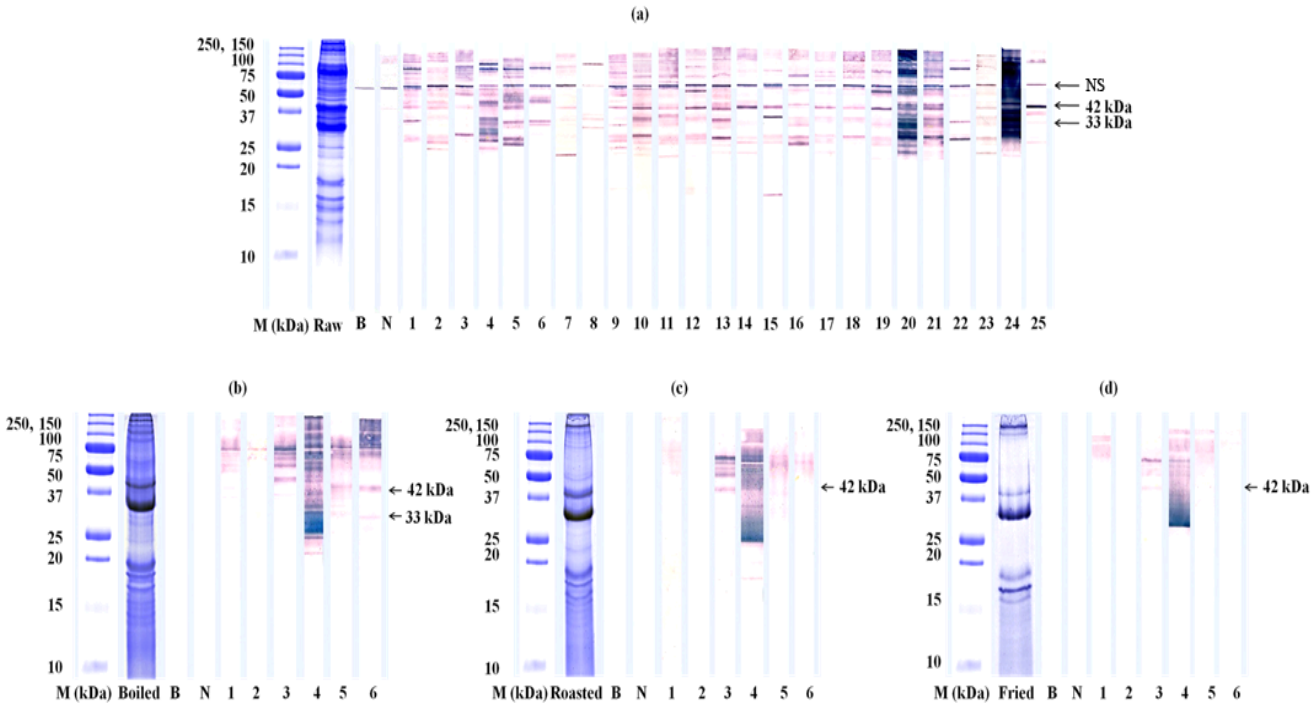

Figure 2. Immunoblotting results of raw (a), boiled (b), roasted (c) and fried (d) extracts of *Cerithidea obtusa*. Lane M, molecular mass markers; lanes 1-25, immunoblots showing binding of IgE from different serum samples; lane N, control serum; and lane B, blank. NS shows non-specific binding.

Table 1. The frequency of the specific-IgE binding proteins in 25 patients sensitized to *Cerithidea obtusa*. Raw (a), boiled (b), roasted (c) and fried (d) extracts of *Cerithidea obtusa*.

(a)

Subject

Protein Marker (kDa) \ M(kDa)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	%
240			√				√				√	√	√	√		√		√	√	√	√			√		12(48)
218	√		√	√					√	√	√		√							√	√		√	√	√	12(48)
124	√		√	√	√	√		√	√											√	√	√		√		11(44)
92	√											√		√	√		√	√	√	√	√			√		10(40)
82	√				√				√							√	√	√		√	√			√		9(36)
80	√					√				√	√	√	√		√		√			√				√		10(40)
74	√		√	√	√	√			√		√				√					√	√			√		11(44)
58			√	√	√				√			√							√	√				√		8(32)
49					√				√								√			√	√			√		6(24)
42	√	√	√	√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√		√	√	√	**23(92)***
40					√															√	√			√	√	5(20)
33	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	**25(100)***
32				√		√		√								√				√	√			√		7(28)
30	√	√	√	√	√				√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	**21(84%)***
29		√		√	√				√	√			√	√	√	√				√						10(40)
26		√		√					√	√			√	√			√			√	√		√	√		11(44)
25							√				√								√							3(12)
17					√				√	√		√														4(16)
15															√											1(4)

Frequency (%)

(b)

M (kDa)	1	2	3	4	5	6	n(%)
218				■			1(17)
124				■			1(17)
92				■			1(17)
82				■			1(17)
80				■			1(17)
74	■		√	√	■	■	5(83)
58			■	■			2(33)
49			■	■			2(33)
42			√	√	■		3(50)
33				■	■	■	3(50)
26				■			1(17)
17				√			1(17)

(c)

M (kDa)	1	2	3	4	5	6	n(%)
218				√		■	2(33)
124				√		■	2(33)
92				√		■	2(33)
82				√		■	2(33)
80				√		■	2(33)
74	√	■	√	√	■	■	6(100)
58			√	■			2(33)
49			√	■			2(33)
42	■		√	■	√	√	5(83)
40				■			1(17)
33				■	√	√	3(50)
29				■			1(17)
26				■			1(17)
17				√			1(17)

(d)

M (kDa)	1	2	3	4	5	6	n(%)
218				■			1(17)
124				■			1(17)
92				■			1(17)
82				■			1(17)
80				■			1(17)
74	■		√	√	■		4(67)
58				■			1(17)
49				■			1(17)
42			√				2(33)
33				■	■	■	3(50)
26				■	■	■	3(50)

√=IgE-binding band, ■ = Smear-binding region, *=major allergen

DISCUSSION

Snail meat is a healthy alternative food as it has high protein and low fat.[1] In total, raw extract of *C. obtusa* has 38 protein bands between 12 to 250 kDa with either thermostable or thermolabile properties. The thermostable proteins can withstand high temperature and are resistant to changes in their protein structure, thus were found in both raw and cooked snail extracts. In contrast, heating the thermolabile proteins above 80˚C typically causes denaturation, due to the destruction of quaternary, tertiary and secondary protein structures.[19,20] These proteins might be aggregated and suffered loss of solubility upon heat treatment, hence were predominantly found in the raw snail extract and were not retained in the heated extracts, as reported in other studies.[17-19]

The rate of thermal denaturation and structural alteration of proteins not only depends on the type of proteins, but also on the thermal load.[9,19-21] In this study, we studied the effects of three types of common thermal treatments (boiling, roasting and frying) on SDS-PAGE profiles and the IgE-binding ability of *C. obtusa.* As seen from the results, minor effects of boiling of snails were detected in the SDS-PAGE profile, whereas in the roasted snails, lesser bands were detected in the gel. The fried snails showed the highest significant reduction of the number of bands as well as in their intensities, as more bands disappeared and smearing regions increased. This was not surprising as in an extreme thermal treatment like frying, protein denaturation will be irreversible.[9,19] Thus, in this study, thermolabile proteins found in all cooked extracts were proteins in the range of 10 to 17 kDa, 25 to 30 kDa, 40 to 74 kDa and some high molecular weight bands (124-250 kDa). In addition, some higher molecular weight bands in the range of 74 to 124 kDa were also detected as being sensitive to heat in the roasted extract, whereas in the fried extract, almost all protein fractions between 25 to 30 and 40 to 100 kDa were completely heat-denatured. Therefore, we found that the majority of snail proteins were sensitive to heat, except for a few bands at 17, 18, 20, 33, 42, 124 kDa which were resistant to heat denaturation. The findings were in accordance with numerous studies which reported that seafood proteins between 40 to 90 kDa where diminished after heating, while hardly any effects on 32-38 kDa proteins were seen, thus they were reported as highly resistant proteins.[9,17,18,21]

Immunoblotting revealed that half of the protein bands of raw *C. obtusa* were capable to bind IgE of the tested sera. 19 of the 38 bands (50%) from 15 to 240 kDa were recognized as IgE binding proteins with numerous IgE-binding frequencies. Major allergen was defined as the protein that elicits IgE binding in the sera of at least half of the patients with allergies to the specific source.[9,17,18,21] Thus, we had detected three major allergens in this species of snail at the molecular weight of 30, 33 and 42 kDa. Similar to other reports[17,18,21-23], the majority of allergenic proteins were identified as protein bands between the molecular weight of 10 to 70 kDa.

The 33 kDa protein was the most important major allergen in *C. obtusa*, as detected by all the tested sera. We believed that this band was tropomyosin, which was widely reported to have a molecular weight of 32 to 38 kDa.[2,4,23] Tropomyosin had been recognized as a major allergen responsible for allergic reactions to a wide range of shellfish, including snails.[4,5,23] Tropomyosin is water soluble and a highly thermostable protein which plays a crucial role in muscle contraction.[9,24] In accordance with these studies[21], we also found that this 33 kDa major allergen was very stable at a high temperature, and this may explain why this protein was still present in the SDS-PAGE gel even after the snails were boiled, roasted and fried. The ability of tropomyosin to resist heat-treatment and other food processing techniques is due to its extremely stable alpha helical coils in its secondary structure.[6,9]

The second important major allergen of *C. obtusa* was the heat stable 42 kDa band, recognized in the immunoblotting of 92% of the tested sera. In contrast to the 36 kDa protein, recognition of this band in immunoblotting decreased by increasing the heat, from raw > boiled > roasted > fried, indicating that this band was sensitive to high temperatures, but its epitope was still capable to bind IgE in some sera as weak smears. This band might be homologous to either actin or arginine kinase, as both proteins were recently identified as heat-sensitive major allergens at ~40-42 kDa in shellfish, including mollusks.[12,13] Actin is a crucial component of the cytoskeleton and is related with tropomyosin in muscle contraction[10-13,25], while arginine kinase plays a critical role in energy metabolism in invertebrates.[9,17,18,25]

The 30 kDa bands were the third major allergen in *C. obtusa*, recognized by 84% of the tested sera. In

contrary to the 33 and 42 kDa major allergens, this major allergen was very sensitive to heat and was not detected in the immunoblotting of all cooked extracts, with the exception of sample 4 which demonstrated smearing regions between 26 to 218 kDa. This band had been rarely recognized in other studies, therefore further characterization is crucial as it might induce allergic reactions via direct contact of raw snails which could be implicated in snail allergy.

The cooking process, either boiling, roasting or frying, might cause an alteration of protein conformation, which then could destroy or modify existing epitopes or generate neoallergens.[9,26] Our study found that the majority of IgE-binding proteins were heat-sensitive, including the major allergen of 30 kDa. The number of IgE-binding bands and their intensities were decreased in the order of boiled, roasted to fried snails. The explanation for this, could be that heat treatment resulted in the masking of allergenic epitopes, thus reducing allergen recognition and subsequently reducing allergenicity.[9,17,18,26]

In contrast, boiling at 100°C had no significant effect on the IgE-binding capacity of several thermostable protein bands, including the major allergens of 36 and 42 kDa. However, roasting at 180°C revealed a decrease in recognition of the proteins bands, while frying at higher temperature had abolished the bands but retained minimal IgE-binding as weak smears. Therefore, the order of the heat resistance was from boiled, roasted to fried snails, similar to other reports.[9,26]

Interestingly, we detected a slight enhancement in the IgE-binding intensities in the immunoblots of one serum (patient no. 4), probably as a result of the Maillard reaction. This reaction occurs due to the chemical interaction of amino acid residues with the sugar moieties at elevated temperatures. This phenomenon had been previously reported in shellfish and peanuts allergens.[20,27-29] In shellfish, this reaction was usually seen with tropomyosin, which is rich in lysine residues and might readily react with reducing sugars at elevated temperatures, causing the Maillard reaction.[24]

In summary, this species of snail has both thermostable and thermolabile major allergens, showing that both raw and cooked snails can cause allergic reactions. However, based on the number of allergenic proteins, the raw snail was more allergenic than the cooked snails in the order: boiled>roasted>fried. Therefore, thermal treatment approaches can be applied to diminish snail allergenicity by decreasing the number of IgE-binding proteins. However, further studies using larger number of samples are needed to confirm these findings. In addition, identification of the major allergens is crucial for a better understanding of snail allergy, allowing a better diagnosis and management for snail allergic patients in this country.

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