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The Impact of Salt Boiled and Acid Boiled Treatments on Allergenicity of Giant River Prawn (*Macrobrachium rosenbergii*)

KOMATHI SOCKALINGAM, ROSMILAH MISNAN*, ZAILATUL HANI MOHD YADZIR, NOORMALIN ABDULLAH, FAIZAL BAKHTIAR

ABSTRACT

The aim of this study was to determine the allergenicity of salt boiled and acid boiled treated prawn extracts. Raw and treated prawn extracts were prepared. The extracts were then analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine their protein profiling. Allergenic proteins were detected by immunoblotting tests using sera from 20 prawn-allergic patients. The raw prawn contains 27 protein fractions between 6 to 207 kDa. Meanwhile, in salt boiled and acid boiled treated extracts, most of the bands were seen to be disappeared. Salt boiled extract showed some prominent bands, while in acid boiled extract, no prominent band was observed. The immunoblotting of raw prawn identified six major allergens at 72, 65, 48, 38, 36, and 30 kDa. Overall, compared to the raw prawn, the treated prawns induced lesser allergenic bands. The immunoblotting results of acid boiled extract clearly show that almost all of the sera did not react to the prawn proteins except for the 30 and 36 kDa bands. However, immunoblotting of salt boiled extract demonstrated more IgE-binding bands including the 18, 20, 30, 36, 48, 52 and 65 kDa. As a conclusion, this study indicated that both treated *M. rosenbergii* prawns could triggered IgE binding reactions but at minimum capacities than the raw prawn. The degree of allergenicity was revealed in the order of: raw > salt boiled > acid boiled treatments. These results would facilitate for developing of effective diagnosis and management strategies of prawn allergy in this country.

Keywords: *Macrobrachium rosenbergii*, prawn, allergy, SDS-PAGE, immunoblotting

INTRODUCTION

Seafood constitutes a significant part in human diet. The worldwide consumption and demand on seafood has increased, that around the world, the increment on consuming various seafood products is rising rapidly (EFSA, 2006). Consistent health problems, particularly allergies have been associated by consumption of seafood products. Allergic reactions can be identified if a person having symptoms such as difficulty in breathing, skin-related signs, abdominal problems or anaphylactic shock after consuming certain food items (Lopata & Lehrer., 2009). An allergic reaction to seafood products in particular is on the rise, by affecting a huge number of patients that are sensitive to particular seafood types around the world. There are three most notable seafood groups which causes allergic reactions. There are fish, crustaceans and molluscs (Leung et al., 2012). The crustaceans and molluscs are known as "shellfish". Tropomyosin, an abundant shellfish muscle protein is known as the major shellfish allergen (Ayuso et al., 2008).

In Malaysia, *Macrobrachium rosenbergii* is well known for its popularity as a delicious dish and commonly consumed by local people. In Malaysia, it is known as '*udang galah*' and is an important species in aquaculture. These prawns are mainly to be found and caught in the coastal waters of the Indo-West Pacific, Southeast Asia, and South China Sea (Rosmilah et al., 2012). Amongst local patients with atopic diseases, this prawn is deliberated as one of the most common allergenic prawn. Previous local study has identified several major allergens of *M. rosenbergii* including proteins of 36 and 42 kDa, identical as tropomyosin and arginine kinase, respectively. The 36 and 42 kDa major allergens were identified as the heat-resistant and heat-sensitive protein, respectively, based on the results of boiling process in their study (Yadzir et al., 2012).

Prior consumption, seafood including prawns are commonly processed by thermal or non-thermal treatments. Thermal-treated prawn

commonly involved several heat treatments such as boiling, frying and roasting or non-thermal treatments such as salting and pickling. It was reported that processing methods could modify the allergenicity of shellfish such as decreasing, increasing or having no effect on the allerginicity (Abramovitch et al., 2013; Nowak-Wegrzyn et al., 2009). Reports on the impact of combination of thermal treatments and non-thermal treatments on allergenicity of this prawn are currently not available. Thus, this study was conducted to investigate the effects of acid-boiling and salt-boiling treatments on allergenicity of *M. rosenbergii* among local patients with prawn allergy.

MATERIALS AND METHODS

Extraction of Prawn Proteins

Live *M. rosenbergii* was obtained from a local seafood market. Prawn proteins were extracted from their flesh, following the methods described by Rosmilah et al. (2012). Briefly, the prawn flesh was homogenized in purified water, followed by an overnight extraction at 4ºC. The homogenates were then centrifuged, filtered, dialyzed, lyophilized and stored at -20ºC until use. Meanwhile for the salt boiled and acid boiled extracts, respective treatments of acetic acid (pH 2.5) hydrolysis and salting (20% NaCl) were applied followed by boiling processes.

Serum Samples

Sera from 20 patients with prawn allergy were used in this study. These sera were confirmed to have IgE antibodies specific to prawn proteins in immunoblotting experiments in previous study (Yadzir et al., 2012). Serum from a non-allergenic individual was used as a negative control. This research was approved by Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the protein profile in the prepared extracts by using the method described by Rosmilah et al. (2012). Briefly, the samples and prestained molecular markers (Bio-Rad, CA, USA) were incorporated into wells containing 12% of resolving gel and 5% of stacking gel using a Mini Protean 3 Apparatus (Bio-Rad, CA, USA), and separated at 120 mA for 50 minutes. After the electrophoresis, the gels were then stained using Coomassie Brilliant Blue R-250 and destained in destaining solution I and II. The molecular weights of the proteins were estimated using an imaging densitometer (Bio-Rad, CA).

Immunoblotting

Immunoblotting was conducted to identify the IgE-binding properties of the allergens using sera from 20 patients with prawn allergy. Immunoblotting was performed according to the methods by Rosmilah et al. (2012) with slight modifications. Proteins which were separated by SDS-PAGE process were first transferred to nitrocellulose membrane using Mini Transblot System (Bio-Rad, USA) at 100 V and 250 mA for 70 minutes. After Immunoblotting, the membrane was stained by Ponceau S (Sigma Diagnostic, USA) to ensure that the protein was transferred. Nitrocellulose blots were then cut into strips measuring 4 mm and washed with tris buffer solution (TBS, pH 7.2) containing 5% Tween 20 (TTBS) for three times. After that, the nitrocellulose strips were blocked with 5% low-fat milk in TBS solution. The strips were then incubated with serum of 20 patients for 14 to 16 hours at 4˚C. IgE binding proteins on nitrocellulose strips were identified after incubation in Biotinylated Goat antihuman IgE Antibody (KPL, UK), followed by incubation in Conjugated Streptavidin-Alkaline Phosphatase (BioRad, CA, USA) for 30 minutes at room temperature. Finally, Alkaline Phosphatase Conjugate Substrate Kit (BioRad, USA) was used to detect IgE-binding protein bands.

RESULTS AND DISCUSSION

SDS-PAGE

The protein components in raw, salt boiled and acid boiled treated extracts were separated by SDS-PAGE. Figure 1 displayed the comparison of protein profiles between the raw, salt boiled and acid boiled treated extracts of *M. rosenbergii*.

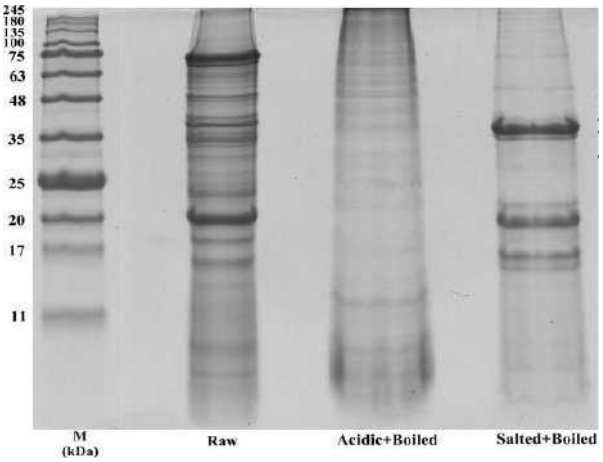

FIGURE 1. Protein profiles of raw, salt boiled and acid boiled treated extracts of *M. rosenbergii*. Lane M is molecular weight markers in kiloDalton (kDa).

The raw prawn contains 27 protein fractions between 6 to 207 kDa. In acid boiled extract, bands that can be seen slightly are at the molecular weight of 30, 18, 14 and 10 kDa. Smears of bands can be seen at the molecular weight ranging in between 68 and 40 kDa. In general, the intensity of the bands of the acid boiled samples became lighter than all of the corresponding extracts. Compared to the control samples, the intensity of the 36 kDa bands in acid boiled samples was dramatically decreased. The fading of the bands indicates decreased protein content after vinegar treatment applied. This is because, the 36 kDa protein, predicted as tropomyosin is a heat stable protein and would have not been affected by boiling (Zailatul et al., 2015). Sletten and others (2009) also reported reduction in the intensity of the protein bands in acetic acid-salt brined herring products. Although the overall protein band intensity in acid boiled treated samples decreased, Figure 1 shows several novel bands appearing between 44 and 107 kDa in the respective extract. These minor novel bands may be caused by the acid hydrolyzation of larger proteins (Elsayed, 1971).

Meanwhile, in salt boiled extract, bands that can be seen clearly are at the molecular weight of 95, 41, 34, 30, 34, 26, 20, 18 and 14 kDa. Smears of bands can be seen at molecular weight of 92, 60, 41, 28, 20 and 16 to 14 kDa. Protein losses have been explained by the large uptake of salt (NaCl) by the muscle, resulting in competition with muscle protein for water molecules, and denaturation and aggregation of these proteins by a process of "salting out". In general, the number of bands decreases during salting processes owing to protein denaturation (Martínez-Alvarez & Gómez-Guillén, 2006). Martinez et al. (2001) investigated changes in protein patterns of shrimp muscle that was subjected at frozen state and in water, low salt and high-salt as well. The results showed that degradation of the myosin heavy chain occurred at all salt concentrations and one band of ranging between 67 kDa disappeared during storage. Similar to earlier reports and as expected, most of the bands after the salt-boiled process disappeared on the gels.

Immunoblotting

The IgE-binding protein components of raw, salt boiled and acid boiled extracts were detected by immunoblotting. Figure 2 displays the IgE-binding proteins of raw *M. rosenbergii* extract, while Figure 3 and 4 show the IgE-binding proteins of salt boiled and acid boiled of *M. rosenbergii* extracts, respectively.

This study demonstrated that all tested sera exhibited heterogeneous IgE-binding proteins towards raw *M. rosenbergii*, most probably due to the varieties of people's insusceptible reactions towards these allergens which be dependent on both hereditary and ecological factors (Granum & Levik, 2002). Allergenic proteins are defined as a major allergen if at least 50% of the tested sera have IgE reactions to the specific protein (Leung et al., 2014). Thus, in this study, six proteins at 30, 36, 38, 48, 65 and 72 kDa were recognized as the major allergens of *M. rosenbergii*, with the binding frequencies of 60, 90, 75, 55, 60 and 75%, respectively.

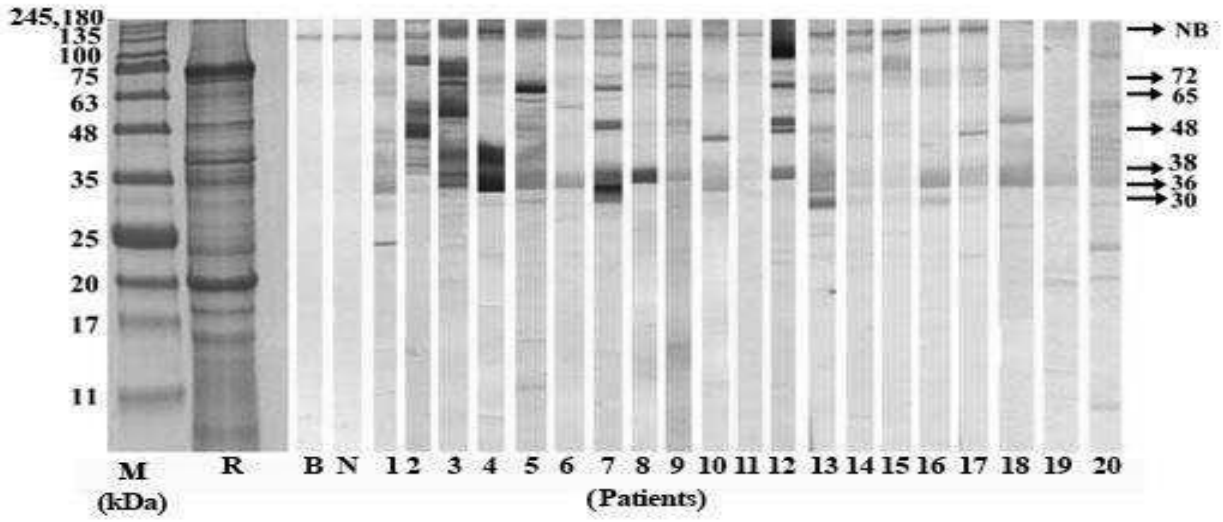

FIGURE 2. Immunoblotting results of raw *M. rosenbergii* using sera from 20 prawn-allergic patients (lane 1 to 20). Lane M is molecular mass markers in kiloDalton (kDa); lane R is raw; lane B is blank and lane N is immunoblot using a negative control serum. Arrows indicated the major allergens molecular weight in kDa.

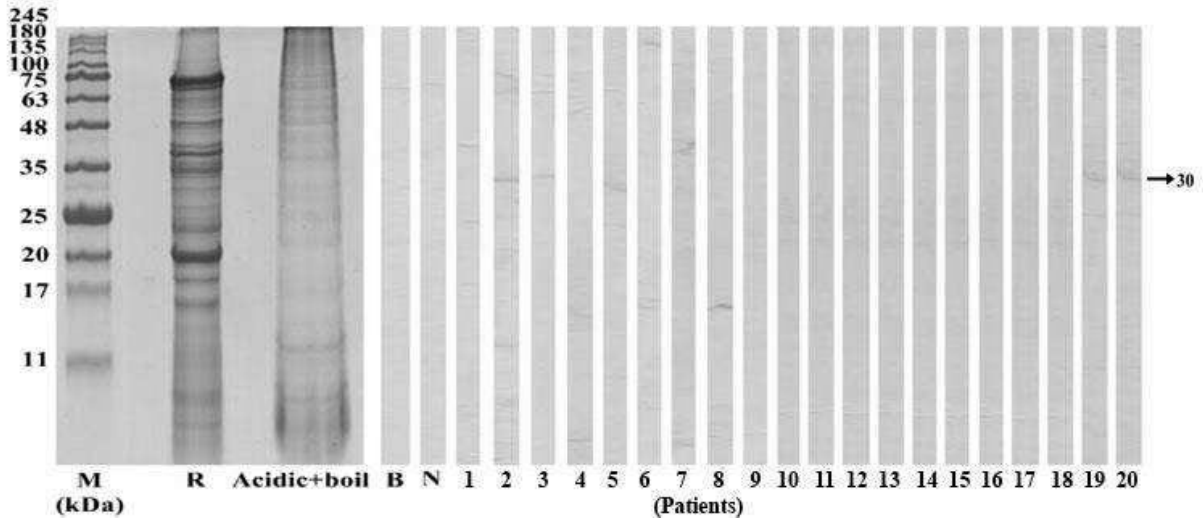

FIGURE 3. Immunoblotting results of acid boiled *M. rosenbergii* using sera from 20 prawn-allergic patients (lane 1 to 20). Lane M is molecular mass markers in kiloDalton (kDa); lane R is raw; lane B is blank and lane N is immunoblot using a negative control serum. Arrow indicated major allergen in kDa.

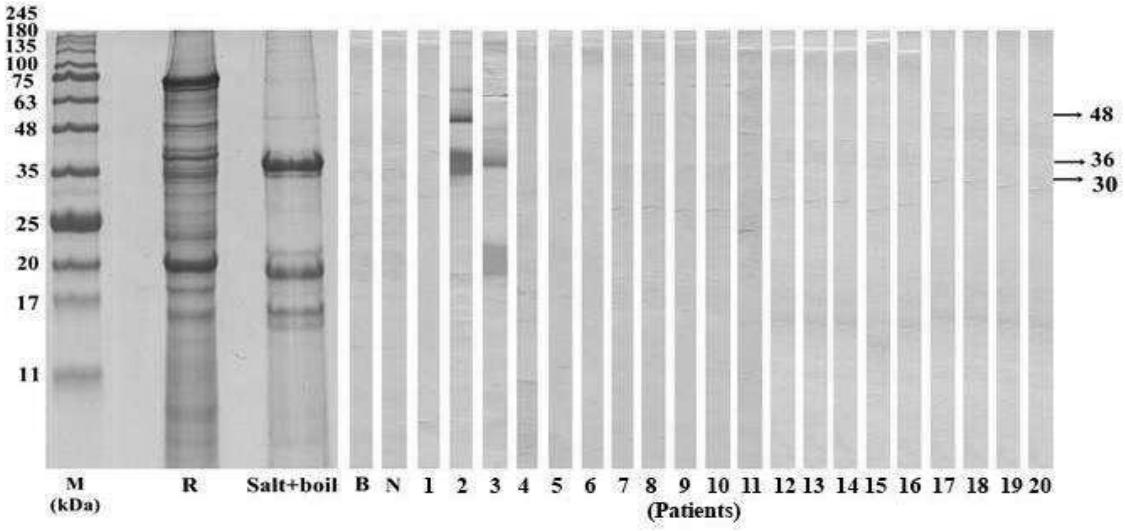

FIGURE 4. Immunoblotting results of salt boiled *M. rosenbergii* using sera from 20 prawn-allergic patients (lane 1 to 20). Lane M is molecular mass markers in kiloDalton (kDa); lane R is raw; lane B is blank and lane N is immunoblot using a negative control serum. Arrows indicated major allergen in kDa.

The immunoblotting results of acid boiled extract of *M. rosenbergii* clearly showed that almost all of the sera did not react to any protein bands (Figure 3). Only two sera (No. 2 and No. 3) demonstrated IgE-binding smear regions at molecular weight of 30 to 36 kDa in the acid boiled extract. This may due to all the protein bands have been destroyed by the boiling process and also chemical reaction in acidic pH (Hildebrandt et al., 2010). Boiling causes protein denaturation by heat while acidic proteases in prawn muscle which is activated by vinegar, triggered the proteolysis of the antigenic proteins in prawn muscles. So far, only one study reported on the influence of combining various heat treatments with acid hydrolysis on the structure and allergenicity of pasteurised liquid whole egg (Hildebrandt et al., 2010). This study found that the IgE-binding capacity of the end product, which underwent heating and acid treatments was more than 100-fold reduced compared to untreated liquid whole egg (Hildebrandt et al., 2010).

Meanwhile, in immunoblotting of salt boiled extracts, only sera No. 2 and No. 3 retain the IgE binding capabilities, but with stronger IgE reactivity to the 30 and 36 kDa bands compared to the other tested sera. However, compared to the immunoblotting of acid boiled extract, immunoblotting of salt boiled extract demonstrated more IgE-binding bands including the 18, 20, 48, 52 and 65 kDa (Figure 4).

It can be said that the patient No. 2 and No. 3 are highly allergic to prawn allergens of 30 and 36 kDa as they showed positive reactivity to IgE binding to both salt boiled and acid boiled prawn extracts. The 36 kDa major allergen might corresponding to be tropomyosin, myofibrillar protein which is the substantial allergen responsible in cross-reactivity reactions between different types of shrimps and prawns. Tropomyosin is well known to be a highly heat and pH stable in shellfish (Zailatul Hani et al., 2012). The ability of tropomyosin to withstand high temperature causes tropomyosin resistant to denaturation hence still appeared after heat treatment has been applied in both acid boiled and salt boiled prawn extracts.

It should be noted that, this study found the 30 kDa protein as one of the major allergens in *M. rosenbergii*, recognized by 60% of tested sera in raw *M. rosenbergii*. Therefore, this band has been identified as one of the important major allergens of *M. rosenbergii.* Unfortunately, protein band at this molecular weight is rarely described as the major allergen of shellfish in literatures. To date, there has been only one report on shellfish allergens that has identified a 28 kDa band (close to the molecular weight of 30 kDa) as triose phosphate isomerase, an important allergen in

shrimp *Crangon crangon* (Bauermeister et al., 2011). However, whether the 30 kDa major allergen found in this study is homologous to the 28 kDa is still not known and further research is needed to identify it.

CONCLUSION

This study indicated the loss of almost all IgE-binding capabilities of the tested sera against both combining thermal and non-thermal treatments. The degree of allergenicity based on the IgE-binding capacities was revealed in the order of: raw > salt boiled > acid boiled treatments. For future study, research on identification of the major allergens of *M. rosenbergii* using proteomics approach is needed to identify the proteins.

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Komathi Sockalingam
Rosmilah Misnan*
Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia

Zailatul Hani Mohd Yadzir
Noormalin Abdullah
Faizal Bakhtiar
Allergy and Immunology Research Centre, Institute for Medical Research, 50588 Kuala Lumpur, Malaysia

*Corresponding author:
rosmilah@fsmt.upsi.edu.my