The Effects of Acid-Boiling Treatment on Allergenicity of *Cerithidea obtusa* (Obtusa Horn Snail)

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ABSTRACT

The aim of this study was to determine the effects of acid-boiling treatment on allergenicity of *Cerithidea obtusa*. Snails were treated in acid (white vinegar) and then boiled. The untreated and treated snails were then extracted and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine their protein profiles. Allergenic properties were then determined by immunoblotting using sera from snail-allergic patients. SDS-PAGE of the untreated snail extract exhibited 26 protein bands with molecular masses ranging from 8 to >250 kDa. Meanwhile, fewer protein bands were seen in the acid-boiled extract. Immunoblot of untreated extract yielded 23 IgE-binding proteins ranging from 25 to 245 kDa. Five major allergens at 35, 39, 48, 61 and 100 kDa were detected in untreated *C. obtusa*. Overall, compared to the untreated extract, the acid-boiled snails induced lesser allergenic bands. The immunoblotting results clearly show lesser number of bands and decreased band frequencies for most major allergens. As a conclusion, this study indicated that acid boiled snail has lesser degree of allergenicity than the raw snail. These results would help facilitate the development of an effective diagnosis and management strategies for snail allergy in this country.

Key words: *Cerithidea obtusa*, snail, allergy, SDS-PAGE, immunoblotting

INTRODUCTION

Obtusa horn snail (*Cerithidea obtusa*) locally known as 'siput sedut' is among the most widely consumed by society and traditionally used for therapeutic purposes (Purwaningsih, 2012). Snail is a marine gastropod mollusc in the family Potamididae (Noor Asyikin et al., 2016). Fleshy portion of sea snails utilized as food in Southeast Asia can cause allergy after its ingestion and tropomyosin has been identified as its allergen (Van et al., 1996). Snail allergy can be dangerous as most cases, report anaphylaxis resulting from inadvertent ingestion of snail in patients known to be allergic to this mollusc (Purwaningsih, 2012).

The food processing method may influence the food allergenicity (Wang, 2013). Acid ingredient especially vinegar has been used as daily food additives. Vinegar is made from raw materials containing starch or sugar through subsequent fermentation of ethanol and acetic acid and is utilized in a variety of food application (Budak et al., 2014). Acid processing by vinegar has been reported to be efficient in reducing the allergenicity of several different kinds of food. In one skin prick tests conducted amongst 18 paediatric and 26 adult patients, shrimp soaked in vinegar before cooking process produced a smaller wheal compared to shrimp prepared conventionally (Perez-Macalalag et al., 2007).

In spite of the high prevalence of snail allergy, there is limited information regarding snail allergens because it has not been well studied (Asturias et al., 2002). Limitation of information and diagnosis of snail allergy will make a snail's allergen more complicated to manage. The effect of thermal treatment on the snails' allergenicity was revealed by other studies (Rosmilah et al., 2016a; 2016b). However, studies on the effect of other treatments on allergenicity of local edible snails have not yet been identified. Thus, the aim of this study is to identify the effects of acid-boiled treatment on allergenicity of *C. obtusa*.

MATERIALS AND METHODS

Preparation of Snail Extracts

Live *C. obtusa* were purchased from a local market. 4 g of snail flesh were weighed into beakers. Vinegar was added to the samples in the ratio of 1:2 w/v, and the samples were soaked for 60 min. After treatment, the liquid was drained and the samples were rinsed with distilled water and patted dry on a clean paper towel. The beakers containing muscle samples were then covered with aluminium foil, sealed with adhesive tape, and cooked for 5 min in a boiling water bath. The allergen extracts were prepared

by homogenization of the snail (untreated and acid-boiled) in phosphate-buffered saline (PBS), pH 7.2 (1:10 weight/volume) using a blender, followed by overnight extraction at 4 °C. The homogenate was centrifuged at 14,000 rpm for 15 minutes at 4 °C. After centrifugation, the clear supernatant was filtered using a sterile 0.22 µm syringe filter. Extracts were then dried in freeze dryer. The lyophilized extracts were stored at -20 °C until use (Rosmilah et al., 2016a).

Serum Samples

Sera from 20 patients with history of snail allergy and have specific IgE to the snail proteins in immunoblotting in previous study (Rosmilah et al., 2016a) were used in this study. Sera from non-allergic individuals were used as negative controls.

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. (2016a). The snail proteins were treated in a denaturing Laemmli buffer and were heated at 97°C for 3 minutes. Then, protein samples (10 µg/ well) were run along with prestained molecular weight markers (BioRad, USA) in 12.5% separating gels with 5% stacking gels using a Mini Protean 3 System at 120 mA for 45 minutes (BioRad, USA). The separated proteins were visualized by staining with Coomassie brilliant blue R-250. Protein masses were estimated by comparing the snail protein bands with the molecular weight markers using an Imaging Densitometer GS800 and Quantity One Software (BioRad, USA).

Immunoblotting

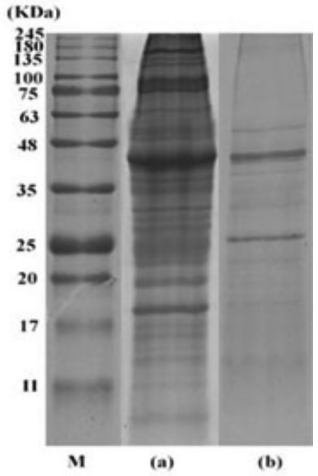
Allergenic proteins were analyzed by immunoblotting (Rosmilah et al., 2016a). The IgE-binding proteins of *C. obtus*a were performed by IgE-immunoblotting test using sera from snail-allergic patients as mentioned above. Briefly, the separated proteins of *C. obtusa* were electrophoretically transferred from unstained SDS-PAGE gel to a 0.45 µm nitrocellulose membrane using a Mini Transblot System (BioRad, USA). After this process had completed, the membrane was cut, washed with tris-buffered saline (TBS) containing 0.05 % Tween 20 (TTBS) and blocked for 1 hour in a solution of 10 % non-fat milk in TBS. The strips were then incubated overnight at 4 °C with the individual patient's sera (diluted 1:5 in blocking buffer). For detection of IgE-binding protein, the strips were probed in biotinylated goat-antihuman IgE (Kirkergaard and Perry Laboratories, UK), followed by incubation in streptavidin-conjugated alkaline phosphatase (BioRad, USA) and then in Alkaline Phosphatase Conjugate Substrate Kit (BioRad, USA). A strip without a serum sample was used as a blank, while serum from a non-allergic individual was used as a negative control in this experiment. The molecular weight of the protein fractions on the strips were estimated by comparing the bands with the molecular weight markers using a calibrated imaging densitometer scanning and Quantity One Software (BioRad, USA).

RESULTS AND DISCUSSION

SDS-PAGE

The protein components in untreated and acid-boiled extract of *C. obtusa* were separated by SDS-PAGE. Figure 1 displays the comparison of protein profiles among the untreated and the acid-boiled extracts of *C. obtusa.*

FIGURE 1. Protein profiles of chemical-treated extracts of *Cerithidea obtusa.*



(a) Untreated, (b) acid-boiled *C. obtusa*. Line M is the molecular weight marker in kiloDalton (kDa)

Fractionation of complex protein mixtures in the untreated *C. obtusa* extract resulted in at least 26 protein bands, between molecular weights of 8 to >250 kDa. Meanwhile, the acid-boiled extract produced lesser protein bands compared with the untreated extract. The

acid-boiled *C. obtusa* had only 9 protein bands. Protein bands above 46 kDa and several protein bands between 8 to 42 kDa disappeared in the acid-boiled extract of *C. obtusa* compared with SDS-PAGE of the untreated *C. obtusa* extract. Based on the SDS-PAGE gel results, the intensity of the visible protein bands in acid boiled extract was reduced than the untreated extract.

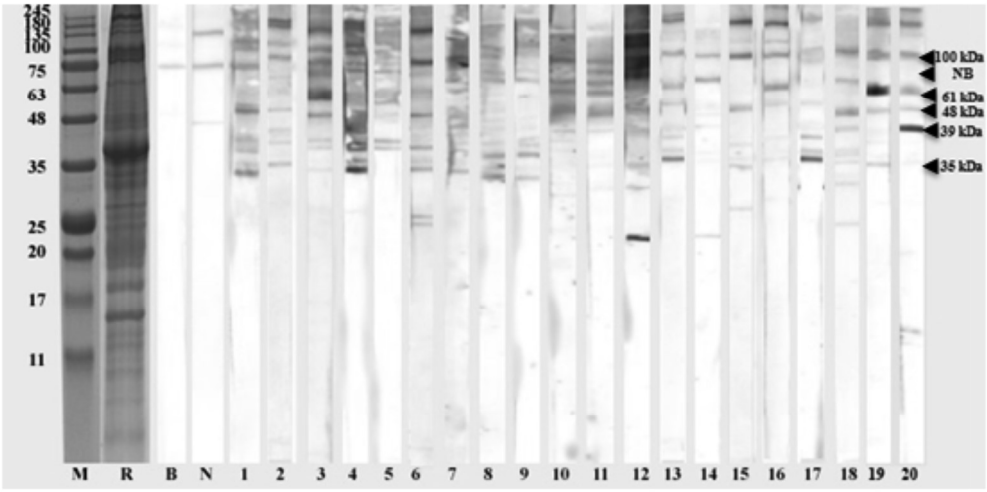
The proteins loss in the acid-boiled extract might be due to rearrangement of protein structures after treatment with acid, or aggregation of non-allergenic proteins (Kim et al., 2012). Therefore, these findings showed that treatment of *C. obtusa* with vinegar could significantly denatured the protein bands of this snail, which might also decreased the allergenicity of *C. obtusa.* This finding might provide an evidence for a decrease in the amount of snail allergens in vinegar-treated snail extracts, supporting the findings of studies that have reported that vinegar treatment reduced the allergenicity of shrimp, herring and peanuts (Kim et al., 2012; Sletten et al., 2010; Perez-Macalalag et al., 2007).

Immunoblotting

The IgE-binding protein components of untreated and acid-boiled extracts were detected by immunoblotting. Figure 2 displays the IgE-binding proteins of untreated *C. obtusa* extract, while Figure 3 shows the IgE-binding proteins of acid-boiled *C. obtusa* extract, respectively.

This study demonstrated that all sera also demonstrated different binding profiles against snail proteins. Interestingly, immunoblotting tests indicated that, more than half of the *C. obtusa* proteins were capable of binding to IgE antibodies from snail-allergic patients. Immunoblotting of untreated *C. obtusa* extract identified numerous IgE-binding proteins at various molecular weights between 25 to 245 kDa. In this study, a protein at 48 kDa (80%) showed the highest frequency of IgE-binding proteins in untreated extracts of *C. obtusa*. In addition, proteins of 35, 39, 61 and 100 kDa have also been detected as major allergens in the untreated *C. obtusa* extract by 70, 65, 50 and 60% subjects, respectively.

FIGURE 2. Immunoblotting results of untreated *C. obtusa using* sera from 20 snail-allergic patients (lane 1 to 20).

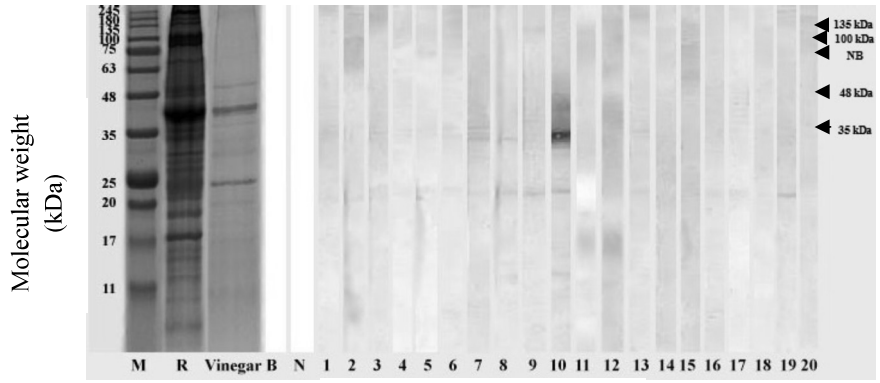


Lane M is molecular mass markers in kiloDalton (kDa); lane R is raw; lane B is blank and lane N is immunoblot using a negative control serum. Arrows indicated the major allergens molecular weight in kDa.

The 48 kDa were acknowledged as the most important major allergens in *C. obtusa*. Other than tropomyosin, there are other major allergens recognized in shellfish at numerous molecular weights (Khora, 2016). Arginine kinase with 40 kDa is identified as a novel shrimp allergen (Yu et al., 2003), crab species (Nurul Izzah et al., 2015) and in some other invertebrates, such as the House-dust mite (Taylor, 2008). However, this study was unable to verify whether the 48 kDa of major allergen in local sea snail were actually arginine kinase or other proteins. Besides that, half of the tested sera demonstrated IgE-binding to the 35 and 39

kDa band. This band was consistent with the size of tropomyosin, the well-known pan-allergens in crustaceans, molluscs, insects, mites, and other invertebrates (Zailatul et al., 2015a) which involved in the highly cross-reactivity reactions among these organisms (Lockey & Ledford, 2014).

FIGURE 3. Immunoblotting results of acid-boiled extract of *C. obtusa* 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.

The wet acid-boiled *C. obtusa* extract exhibited only 14 IgE-binding bands in the range of 20 to 180 kDa with some smeared bands. This result proved that acid-boiled treatment decreases the IgE-binding activity by either lowering solubility and thus the amount of antigenic protein in the extract, or destroying its epitope. Besides that, the exposure of hidden epitopes that slightly increased the IgE-binding could explain the enhanced intensity of bands of the lengthier vinegar treated snail samples as proposed by Wang (2013). The band intensity of major allergens (35, 48, 100 and 135 kDa) of *C. obtusa* become unclear and appeared as weak smearing bands, except for the 35 kDa band, corresponding to tropomyosin, which still remain apparent after acid-boiled treatment.

CONCLUSION

This study indicated that the acid-boiled treatment approaches can be applied to diminish snail allergenicity by decreasing the number of IgE-binding proteins. For future study, more research on identification of several allergenic proteins must be evaluated thoroughly using proteomics approach since some of the proteins have yet to be identified.

ACKNOWLEDGEMENT

The authors thank Universiti Pendidikan Sultan Idris (UPSI) for funding this study through research grants UPSI 2016-0187-101-01.

REFERENCES

Asturias, J.A., Eraso, E., Arilla, M.C., Gomez-Bayon, N., Inacio, F. & Martinez, A. 2002. Cloning, isolation and IgE-binding properties of *Helix aspersa* (brown garden snail) tropomysin. International Archives of Allergy Immunology, 128, 90-96.

Budak, N. H., Aykin, E., Seydim, A. C., Greene, A. K., & Guzel-Seydim, Z. B. 2014. Functional properties of vinegar. Journal of food science, 79(5).

Khora, S. S. 2016. Seafood-associated shellfish allergy: acomprehensive review. Immunological Investigations, 45(6), 504-530.

Kim, J., Lee, J., Seo, W. H., Han, Y., Ahn, K., & Lee, S. I. 2012. Changes in major peanut allergens under different pH conditions. Allergy, asthma & immunology research, 4(3), 157-160.

Lockey, R. F., & Ledford, D. K. 2014. Allergens and Allergen Immunotherapy: Subcutaneous, Sublingual, and Oral. CRC Press.

Noor Asyikin, K., Rosmilah, M., & Zailatul, H.M.Y. 2016. Identification of major allergenic spots of *Cerithidea Obtusa* (obtusa horn shell) by two-dimensional electrophoresis (2-DE) and Immunoblotting. International Journal of Science, Environment and Technology, Vol. 5, No 1, 2016, 222 – 228

Nurul Izzah, A.R., Rosmilah, M., Zailatul Hani, M.Y., Noormalin, A., Faizal, B., & Shahnaz, M. 2015. Identification of major and minor allergens of mud crab (*Scylla Serrata*). Medicine and Health, 10(2), 90-97

Perez-Macalalag, E., Sumpaico, M., & Agbayani, B. 2007. Shrimp allergy: effect of vinegar soaking on allergenicity. The World Allergy Organization Journal, S317.

Purwaningsih, S. 2012. Aktivitas antioksidan dan komposisi kimia Keong Matah Merah (*Cerithidea obtusa*) (Antioxidant activity and nutrient composition of atah Merah Snail (*Cerithidea obtusa*). ILMU KELAUTAN: Indonesian Journal of Marine Sciences, 17(1), 39-48.

Rosmilah, M., Norazlin, S.A.A., Zailatul, H. M. Y., Faizal, B., Noormalin, A., & Shahnaz, M. 2016a. Impacts of thermal treatments on major and minor allergens of sea snail, *Cerithidea obtusa* (Obtuse Horn Shell). Iranian Journal of Allergy, Asthma and Immunology, 15(4), 309-316.

Rosmilah, M., Norazlin, S.A.A., Zailatul, H.M.Y., Noormalin, A., Faizal, B., & Shahnaz, M. 2016b. Comparison of allergenic proteins of sea snail (*Cerithidea Obtusa*) and freshwater snail (*Pomacea Canaliculata*). Jurnal Teknologi, 78(11), 113-119.

Sletten, G., Van Do, T., Lindvik, H., Egaas, E., & Florvaag, E. 2010. Effects of industrial processing on the immunogenicity of commonly ingested fish species. International Archives of Allergy and Immunology, 151(3), 223-236.

Taylor, S.L. 2008. Molluscan shellfish allergy. Advances in Food Nutrition Research, 54, 140-177.

Van, R.R., Antonicelli, L., Akkerdaas, J.H., Pajno, G.B., Barberio, G., Corbetta, L., Ferro, G., Zambito, M., Garritani, M.S., Aalberse, R.C. & Bonifazi, F. 1996. Asthma after consumption of snails in house-dust-mite-allergic patients: a case of IgE cross-reactivity. Allergy, 51, 387-393.

Wang, Y. 2013. Effects of vinegar treatment on detectability and allergenicity of finfish (Doctoral dissertation, The Florida State University).

Yu, C. J., Lin, Y. F., Chiang, B. L., & Chow, L. P. 2003. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. The Journal of Immunology, 170(1), 445-453.

Zailatul, H. M. Y., Rosmilah, M., Faizal, B., Noormalin, A., & Shahnaz, M. 2015. Malaysian cockle (*Anadara granosa*) allergy: Identification of IgE-binding proteins and effects of different cooking methods. Tropical Biomedicine, 32(2), 323-334.

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