Effect of Food Thermal Processing on Allergenicity Proteins in Bombay Locust (Patanga Succincta)

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***Abstract*—Food security is importance for people all the world. Bombay locust (*Patanga succincta*) is a major agricultural pest in Thailand and most popular for consumption as human food. Arthropods include crustacean (shrimp) and insect which can induce allergic reactions in sensitive humans. The aims of this study is to identified the cross-reactive allergens in *P. succincta* with sera who allergic to shrimp by IgE-immunoblotting and tandem mass spectrometry (LC-MS/MS) and investigated the effect of thermal processing (fried) on allergenicity of this edible insect. The electrophoresis profiles between raw and fried *P. succincta* is clearly differentiated. Hexamerin (HEX), enolase and arginine kinase (AK) were identified as raw *P. succincta* allergens whereas fried *P. succincta* can identified four proteins as HEX, pyruvate kinase, enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as allergen. In this result, thermal processing can alter the allergenicity of food allergens.**

***Index Terms*—edible insects, *patanga succincta*, Bombay locust, food processing, fried, allergen, IgE**

I. Introduction

Edible insects have always been a part of human diets. Many animals, such as spiders, lizards and birds, are entomophagous, as are many insects. People throughout the world have been eating insects as a regular part of their diets for millennia. Food and Agriculture Organization of the United Nations (FAO) suggested that edible insects can be the future sources of food and feed [1]. The nutritional values of edible insects are highly variable, not least because of the wide variety of species. Even within the same group of edible insect species, values may differ depending on the metamorphic stage of the insect, and their habitat and diet. The main components of insects are protein, fat and fibre [2]. Traditional processing methods, such as boiling, roasting and frying, are often applied to improve the taste and palatability of edible insects and have the added advantage of ensuring a safe food product. In Thailand have many species of insects but the popular species that can usually found as edible insects are; for example, Bombay locust (*Patanga succincta*) and the field cricket (*Gryllus bimaculatus*) [3]. Nowadays, locusts are an important essentially of the human diet. *Patanga succincta* (Bombay locust) is a major agricultural pest in Thailand. The deep fried locust is one of the best-known and most popular insects and important for commercial food. Hanboonsong (2013) [4] reported that Bombay locusts are also sold in frozen packages at all 51 Makro wholesale supermarkets throughout Thailand. Since the organism is a famous alternative food in South East, the study of allergenicity of insect protein is necessary for food safety policy. Moreover, insects are including in Phylum Arthropoda as same as shrimp (crustacean). Arginine kinase (AK) has been reported as minor allergen [5] and the major allergen is tropomyosin (TM), are also in shellfish and known as pan-allergen in invertebrate [6], [7]. Due to the close Phylum relation between crustacean and insect, Ayuso *et al*. (2002) are interesting about cross-reactivity between shrimp, cockroach and house

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dust mites and they identifying TM as cross-reactivity allergen protein by immunoblot with allergic serum [8]. Several studies proved the heat stability of the allergenic potential of food and mention that decreased of allergenicity is dependent on process such as boiled, heated and enzymatic hydrolysis [9]. The purpose of food processing are to ensure microbiological and chemical safety of foods, adequate nutrient content and bioavailability, and acceptability to the consumer and caregiver with regard to sensory properties and ease of preparation [10]. Edible insects can be processed and consumed in three ways: whole insects, in ground or paste form and as an extract of protein, fat or chitin for fortifying food and feed products. Heating of protein can increasing surface hydrophobicity due to unfolding of the molecule upon heating. Heating and technological food processing might lead to changes in target-protein structure affecting final detection, especially when immunoassay methods are used [11]. Bukkens (1996) [12] suggested that the type of food processing have an influence to protein content of insects, the mopane caterpillar had lower protein content when dry-roasted than when dried. Processing can have a disruptive effect on the native protein structure of an allergen, which can result in disruption of the IgE binding epitopes and consequently modify the ability of an allergen to elicit a reaction [13]. Maillard reaction and enzymatic browning (polyphenol oxidase catalyzed oxidation of 3, 4 diols) have been reported to irreversibly destroy conformational epitopes of cherry allergen *Pru av 1* [14]. Hansen *et al.* (2003) [15] reported that roasting decreased hazelnut allergenicity, particularly in patients sensitized to the birch pollenrelated hazelnut allergens Cor a 1.04 and Cor a 2. However, these investigators also found that 5/17 birch pollen allergic patients were positive to the roasted hazelnuts when double blind placebo controlled food challenge trials were conducted suggesting the roasted hazelnuts may not be safe for many hazelnut allergic patients. Traditional processing methods, such as boiling, roasting and frying, are often applied to improve the taste and palatability of edible insects and have the added advantage of ensuring a safe food product. In this study will describe the use of a proteomics approach; Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting techniques and liquid chromatography–two-dimensional mass spectrometry (LC-MS/MS) can be used to detect and identification of IgE binding protein respectively. The aims of the current study is compare how food processing may alter the allergenicity in *P. succincta* and study about cross-reactivity between shrimp and insects by IgE-binding proteins with serum from atopic and non-atopic subjects had a known allergic reaction to shrimp.

II. Materials and Method

A. Bombay Locust Preparation

Live Bombay locust, pooled weighing approximately 10 to 20g were purchased in Bangkok, Thailand. Whole body of Bombay locust were pooled and immediately frozen in liquid nitrogen. For food thermal processing prepared by frying raw Bombay locust without oil at 108.80±5.78 ℃ for 3min and raw insects were used as the control. The edible insects from food processing treatments were frozen at -80 ℃ until use.

B. Human Serum

The sera used in this study were allergen-specific to prawn and were obtained from 16 prawn-allergic patients at Phramongkutklao Hospital and Phyathai Hospital (Bangkok, Thailand). Control sera were obtained from 30 healthy subjects without any prawn allergy and no reported adverse reactions to any food stuff at Phramongkutklao Hospital. The uses of sera were approved by the Institutional Review Board at Phramongkutklao Hospital (R101h/2009). The level of the antibody IgE in the sera from allergic patients was verified to be greater than 0.35kU/L using modern EUROLINE inhalation Southeast Asian test strips (EUROIMMUN AG; Schleswig Holstein, Lübeck, Germany). After incubation of the test strip with patient serum and chromogen substrate, each test strip was scanned with a EUROBlotScanner and the results appeared on a EUROLineScan result sheet. This processing was done at Phyathai Hospital (Bangkok, Thailand). After verification of the IgE antibody, the sera from 16 prawn-allergic patients were pooled for 1D-immunoblot analysis. Every healthy subject's sera were pooled for 1D-immunoblot analysis as well. The sera were frozen at -80 ℃ until use.

C. Bombay Locust Extracts

Separate samples of frozen Bombay locust from each treatment were ground to a powder in a mortar and pestle using liquid nitrogen. The samples were solubilized in phosphate buffer saline (PBS; 9mM Na_2HPO_4, 1.13mM $NaH_2PO_4 \cdot H_2O$ and 0.152M NaCl, pH 7.4) for SDS-PAGE. A sample of 300 μg of locust powder protein was incubated for a while in 600 μL of extraction buffer with a protease inhibitor cocktail (#P8340; Sigma-Aldrich; St. Louis City, MO, USA). The mixture was centrifuged at 16,215 ×g for 30min at 4 ℃ and the supernatant was collected. Protein concentration was determined by means of UV-VIS spectrophotometry (UV-1700; Shimadzu; Kyoto, Japan) with the Bio-Rad protein assay (Bio-Rad; Hercules, CA, USA). Protein concentration was calculated from a standard curve is known concentrations of bovine serum albumin (BSA).

D. SDS-PAGE and Immunoblotting with Antibody IgE

The crude extract of Bombay locust was separated in triplicate by SDS-PAGE according to the method of Laemmli, 1970 [16]. Samples with a concentration of 30 μg were loaded onto 12.5% separating gel and 4% stacking gel of SDS polyacrylamide tris/glycine gel at 15mL/lane and electrically separated by the Mini-PROTEAN Tetra system (Bio-Rad; Hercules, CA, USA). Protein standard for SDS-PAGE was a PageRuler Prestained Protein Ladder (#26616: Thermo Fisher Scientific; Waltham, MA, USA). The gels were stained with Coomassie Brilliant Blue (CBB) R-250. For immunodetection of IgE-binding proteins, the separated proteins were electroblotted onto an Immobilon-P transfer

membrane (Millipore Corporation; Billerica, MA, USA) by applying a constant voltage of 100V for 1.5h at 4 ℃, according to the method of Towbin *et al*. [17], which was then blocked with Block Ace (Dainippon Sumitomo Pharma; Fukushima-Ku, Osaka, Japan) and incubated for 16h at 4 ℃ with a 1:25 dilution in 10% skim milk of the individual prawn-allergic serum. Bound IgE antibodies (Abs) were detected using horseradish peroxidase (HRP) conjugated goat anti-human IgE (Immunology Consultants Laboratory; Portland, OR, USA) with a dilution of 1:5,000 in 3% skim milk. The membrane was incubated with Amersham ECL Plus™ western blotting detection reagents (GE Healthcare Bio-Sciences AB; Little Chalfont, Buckinghamshire, UK). This process use enhanced chemiluminescence (ECL) as a substrate and used HPR as an enzyme. The reaction occurred under low-intensity light at 428nm. Finally, immunoreactive bands were visualized using exposure with Amersham® Hyperfilm® ECL™ (GE Healthcare Bio-Sciences AB; Little Chalfont, Buckinghamshire, UK) in a dark room.

E. Glycoprotein Staining of SDS-PAGE

Bombay locust protein in SDS-PAGE gel was transfer to an Immobilon-P transfer membrane. Protein membrane was staining by GelCode® Glycoprotein Staining Kit (Thermo Fisher Scientific; Waltham, MA, USA). Wash membrane with 20ml of 3% acetic acid for 10 minutes. Transfer membrane to 10ml of oxidizing solution for 15min. Wash membrane with 10ml of 3% acetic acid for 5min and transfer membrane to 10ml of GelCode® Glycoprotein Staining Reagent for 15min. After that, transfer membrane to 10ml of reducing solution for 5min and wash membrane extensively with 3% acetic acid and then with ultrapure water. Glycoproteins appear as magenta bands and store membrane in 3% acetic acid. Glycoprotein staining membranes were produced in triplicate.

F. Protein Image Analysis and Statistical Analysis

Stained gels and immunoreactive bands from the films were scanned using by image scanner and image scanner program. Image of protein gels and films were analyzed by ImageQuant TL Plus 7.0 Software for 1D-gel. The band intensity values of each 1D-immunoreactive band from the films were detected. Significant differences in intensity values of each allergen bands were determined by statistical analysis using SPSS software version 15.0 (SPSS Inc.: Chicago, IL, USA) and shown as interleaved bars graph with mean value by GraphPad Prism 5 to compare the allergenicity of IgE binding protein between treatments of Bombay locust. Paired-samples T-test was used and P-values less than 0.05 were considered statistically significant.

G. Trypsin In-Gel Digestion

Interested protein bands/spots were excised and transferred to 0.5mL microcentrifuge tubes. CBB were removed by using 0.1M NH_4HCO_3 in 50% (v/v) acetonitrile (ACN) until the gel pieces were colorless. After drying by speed vacuum (CentriVap Console: Labconco; Kansas City, MO, USA), reduction and alkylation were performed by swelling the gel pieces in 50 μL buffer solution (0.1M NH_4HCO_3, 10mM DTT and 1mM EDTA) and incubating at 60 ℃ for 45min by thermomixer (R Mixer: Eppendorf; Hamburg-Nord, Hamburg, Germany). The liquid was removed and then the gel slices were covered in 100mM IAA in 0.1M NH_4HCO_3 solution and incubated at room temperature in a dark room for 30min. The residual of IAA solution was removed and the gel pieces were washed with 50% (v/v) ACN in water, and dried in a speed vacuum. Aliquots of trypsin (Promega Corporation; Fitchburg, Madison WI, USA) (1 μg trypsin: 10 μL 1% acetic acid) were prepared and stored at -20 ℃. 50 μL of digestion buffer (0.05M tris HCl, 10% ACN, 1mM $CaCl_2$, pH 8.5) and 1 μL of trypsin were added. After incubating at 37 ℃ overnight, the digestion buffer was removed. Then, the gel pieces were extracted by adding 60 μL of 2% freshly prepared of trifluoroacetic acid and incubating for 30min at 60 ℃. The solutions were pooled and dried by speed vacuum and stored at -20 ℃ until analyze.

H. Protein Identification by LC-MS/MS

LC-MS/MS analysis was used to identify protein as previous described [18]. Briefly, 6μL of trypsin-digested peptides were concentrated and desalted using nanoflow LC (Waters, Milford, MA, USA) with 75mm id × 150mm C18 PepMap column (LC Packing, Amsterdam, Netherlands). Gradient separation was performed using eluents A and B, which were composed of 0.1% formic acid in 97% water, 3% ACN and 0.1% formic acid in 97% ACN, respectively. Sample was injected into the nanoLC system, and separation was performed using the following gradient: 0min 7% B, 35min 50% B, 45min 80% B, 49min 80% B, 50min 7% B, and 60min 7% B. Then, Nano ESI MS/MS (Q-TOF micro; Micromass, Manchester, UK) was used to generate MS/MS spectra. Parent mass peak with range from 400 to 1600m/z were selected for MS/MS analysis and collision energy was fixed at 38eV. MS/MS spectra were processed using MassLynx 4.0 software (Micromass) and convert to PKL files by the ProteinLynx 2.2 software (Waters). The files were used to identify proteins using MASCOT search engine (http://www.matrixscience.com) against, the Mascot search tool available on the Matrix Science site screening NCBInr was used. The search parameters were set as follows: peptide mass tolerance = 1.2 Da, MS/MS ion mass tolerance = 0.2 Da; enzyme set as trypsin and allowance was set up to 1 missed cleavages; peptide charges were limited to 1^+, 2^+ and 3^+. The proteins were identified at a probability level $P<0.05$ and Mascot scores >35 were considered as promising hits.

III. Results and Discussion

A. Total Protein Extraction from SDS-PAGE with Immunoblotting and 2D-PAGE Separations

The SDS-PAGE profiles of the sample extracts with each food processing condition showed multiple protein bands ranging from ~10 to ~100 kDa. The electrophoretic patterns are shown in lane 1A and 2A (Fig. 1) for raw sample, lane 1A and 2A for fried *P. succincta*. To identify

IgE-binding proteins, western blotting and immunoblotting with serum samples from 16 prawn-allergic patients were tested and shown IgE binding protein band in lane 1B and 2B (Fig. 1). No positive band was detected using serum from non-allergic individuals shown in lane 1C and 2C (Fig. 1). The identification of allergens was show in Table I.

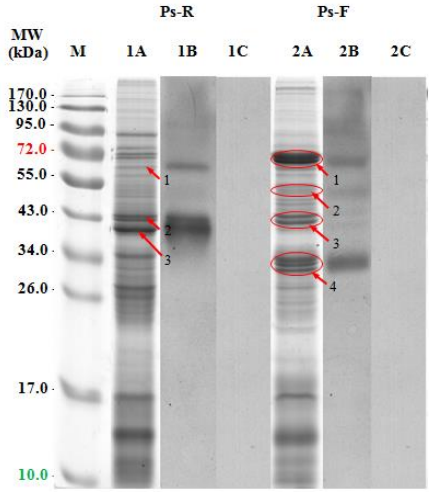

Figure 1. SDS-PAGE proteins pattern with CBB staining (lane 1A and 2A) compared with immunoblot analysis from 16 pooled prawn-allergic patients sera (lane 1B and 2B) and 30 non-allergic subject serums (lane 1C and 2C). Lane M, molecular weight. The arrow labels represented the IgE-binding protein that identified by LC-MS/MS as shown in Table I.

TABLE I. IDENTIFIED ALLERGENS FROM *P. SUCCINCTA* USING LC-MS/MS FROM SDS-PAGE

Band No.	Protein name/ Acc. No.	LC-MS/MS analysis		
		Score	Peptide match	% Coverage
Ps-R-1	Hexamerin /gi|60256959	88	2	1
Ps-R-2	Enolase/gi|359843244	92	8	11
Ps-R-3	Arginine kinase/gi|3183060	119	16	26
Ps-F-1	Hexamerin-like protein 3, partial/gi|256368120	50	2	5
	Hexamerin-like protein 2/ gi|256368118	111	3	4
Ps-F-2	Pyruvate kinase/gi|170043461	129	2	3
Ps-F-3	Enolase/gi|359843244	122	6	12
Ps-F-4	Glyceraldehyde-3-phosphate dehydrogenase/ gi|380447700	192	14	21
	Glyceraldehyde-3-phosphate dehydrogenase/gi|22450121	65	2	5

Raw *P. succincta* shows three IgE binding protein bands have molecular masses ranging ~38 to ~65kDa (Fig. 1, lane 1B) whereas fried *P. succincta* shows four group of IgE binding protein bands ranging ~30 to ~70kDa (Fig. 1, lane 2B). The remaining IgE-binding components with various molecular masses were detected at lower intensity. Iparraguirre *et al.* (2009) has identify allergens from lobster by using patient serum who allergic to house dust mite and the results show two IgE binding proteins around 198kDa and 65kDa as allergen, demonstrated that the patients who allergic to house dust mite could be allergic to lobster [19]. In this result shows IgE binding protein bands with atopic serum from shrimp but cannot show any band in non-allergic subject serum. So, we can conclude that its might has cross-reactivity with the patients who got the shrimp allergic.

In this study has identified many novel allergens from raw and fried Bombay locust. From 1D-immunobloting, there are three proteins as hexamerin (HEX) (~65kDa), enolase (~43kDa) and arginine kinase (AK) (~38kDa) can identified as allergen in raw *P. succincta* but in fried *P. succincta* can identified four proteins as HEX (~65 to ~70kDa), pyruvate kinase (~52 to ~55kDa), enolase (~40 to ~43kDa) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (~30 to ~34kDa) as allergen. Martins *et al.* (2011) [20] suggested that arylphorin hexamerin is likely used for adult cuticle construction and can be found in hemolymph of insects. GAPDH was considered a classical glycolytic protein examined for its pivotal role in energy production. GAPDH is found as minor allergen in bread wheat (*Triticum aestivum*) [21] and also been report as allergen in indoor mould (*Aspergillus versicolor*) [22]. Enolase is the new allergen found in fish; cod, tuna and salmon [23], [24]. GAPDH, enolase and pyruvate kinase (PK) are the glycolytic enzymes. There is evidence for the existence of glycolytic and pentose phosphate pathways in various species [25].

B. Glycoprotein Staining of SDS-PAGE

Glycoproteins are appeared on membrane as magenta bands shown in Fig. 2 have molecular masses ranging ~65 to ~72kDa. Glycoproteins bands with allergenic properties from fried Bombay locust can identified as hexamerin (HEX).

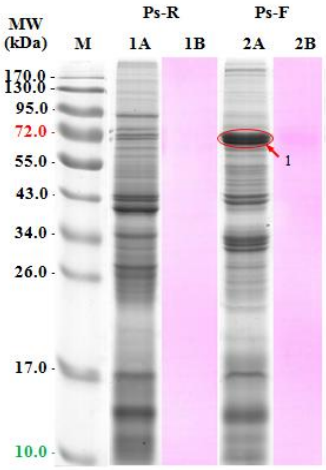

Figure 2. Glycoprotein staining on membrane protein bands label in lane 2A identified as hexamerin (HEX), lane 1A-2A: SDS-PAGE of Bombay locust from each treatment, lane 1B-2B: glycoprotein staining membrane. Lane M: molecular weight markers; Ps-R: raw Bombay locust; A: fried Bombay locust.

Hexamerin, abundant proteins found in the hemolymph of insects, high molecular weight molecules composed of six homologous or heterologous subunits. Hexamerin has been reported as hexameric glycoprotein with an apparent native molecular weight of 500kDa [26]. Moreover, Altmann (2006) [27] has review about structural of insect glycoprotein that discovery of core 1, 3 fucose in insect glycoproteins, it became apparent that this widely distributed residue is involved in cross-reactions between plant and insect glycoproteins.

C. Food Processing on Allergenicity

Food processing have affect to the allergenicity of Bombay locust allergen. Sen *et al*. (2002) [28] suggested that peanut allergen disulfide bonds reduction and their accessibility to IgE-binding epitopes can reduce overall allergenicity. Thus, the structure/epitope degradation of the allergen may reduce the allergenicity. The results from immunoblotting refer that allergic patient has difference immunological reactions with differ allergens as shown in the prevalence of IgE-binding (Table I) and intensity values of IgE binding (Fig. 3) from 1D-immunoblotting to compare the allergenicity of IgE binding protein between raw and fried Bombay locust. Enolase and HEX are still having allergenicity after heated. So, these allergen proteins might be heat stable protein. Two allergens, HEX and PK were not different between food processes, whereas three allergens; AK, GAPDH and enolase were differ with statistically significant (95%).

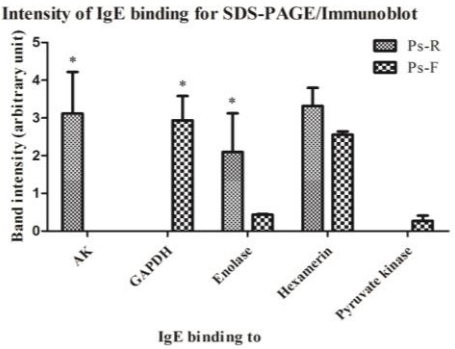

Figure 3. Band intensity of IgE binding of each allergen for 1D-immunoblot from pooled allergic human sera to Bombay locust allergens from raw (Ps-R) and fried (Ps-F). *P < 0.05 vs. raw Bombay locust.

The intensity value from these result assumed that some allergens proteins have decrease the allergenicity properties after food processed such as AK, enolase and HEX, whereas GAPDH and PK are increase. The effect of heating may appear in two different ways by unfolding of molecules and heat-induced aggregation. A consequence of unfolding and aggregation during cooking is destroys the conformational epitopes, IgE-reactivity and ability to trigger a reaction in sensitized individuals is generally reduced by food processing [11], [29].

IV. Conclusion

Allergens from Bombay locust were cross-reactivity to shrimp allergic serum and we can assume that the people who had allergic to shrimp might be had allergic reaction to insects. Moreover, thermal processing might be alteration structural and may change the allergenicity of Bombay locust by destroy existing protein epitopes to decreasing allergenicity or may generate new epitopes to increasing allergenicity. After fried, the allerginicity of arginin kinase and enolase in Bombay locust were decreased, while glyceraldehyde-3-phosphate dehydrogenase was increased. A study of epitope pattern for each type of allergen is necessary in further studies to even more understand in allergy. So, this experiment is the first step to understanding about safety processing for food consumption and can warning about allergic symptom from edible insect to shrimp allergic person.

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