การวิเคราะห์สารก่อภูมิแพ้จากกุ้งกุลาดำ (*Penaeus monodon*) โดยเจลอิเล็กโทรฟอรีซิสแบบสองมิติร่วมกับการใช้เซลล์รีพอร์ตเตอร์ RS-ATL8

นางสาวธัญปัณย์ จารุปาลี

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

ANALYSIS OF ALLERGENS FROM BLACK TIGER SHRIMP (*Penaeus monodon*)
BY COMBINING TWO-DIMENSIONAL GEL ELECTROPHORESIS WITH
RS-ATL8 REPORTER CELL LINE

Miss Thanyapat Jarupalee

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	ANALYSIS OF ALLERGENS FROM BLACK TIGER SHRIMP (*Penaeus monodon*) BY COMBINING TWO-DIMENSIONAL GEL ELECTROPHORESIS WITH RS-ATL8 REPORTER CELL LINE
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ธัญปัถย์ จารุปาลี : การวิเคราะห์สารก่อภูมิแพ้จากกุ้งกุลาดำ (*Penaeus monodon*) โดยเจลอิเล็กโทรฟอรีซิสแบบสองมิติร่วมกับการใช้เซลล์รีพอร์ตเตอร์ RS-ATL8 (ANALYSIS OF ALLERGENS FROM BLACK TIGER SHRIMP (*Penaeus monodon*) BY COMBINING TWO-DIMENSIONAL GEL ELECTROPHORESIS WITH RS-ATL8 REPORTER CELL LINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ธนาภัทร ปาลกะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. พญ.พรรณทิพา ฉัตรชาตรี, 72 หน้า.

ภาวะภูมิแพ้อาหารเป็นภาวะที่ร่างกายตอบสนองทางภูมิคุ้มกันไวเกินต่อสารก่อภูมิแพ้ โดยอิมมูโนโกลบูลินชนิด อี (IgE) ที่มีความจำเพาะต่อสารก่อภูมิแพ้ที่ถูกผลิตขึ้นจะจับกับ IgE Fc-receptor ที่อยู่บนผิวของมาสต์เซลล์และเบโซฟิล สารก่อภูมิแพ้จะจับกับแอนติบอดี IgE แบบการต่อข้าม (cross-linking) และกระตุ้นให้มาสต์เซลล์ปล่อยสารเคมีออกฤทธิ์ เช่น ฮิสตามีน (histamine), พรอสตาแกลนดินส์ (prostaglandins) และลิวโคไตรอีนส์ (leukotrienes) ซึ่งทำให้เกิดอาการผิดปกติเพียงเล็กน้อยหรือมีอาการรุนแรงและถึงแก่เสียชีวิตได้ กุ้งกุลาดำ (*Penaeus monodon)* เป็นสัตว์น้ำที่มีความสำคัญทางเศรษฐกิจในแถบภูมิภาคเอเชียและพบว่าเป็นสาเหตุของการแพ้อาหารทะเลที่พบบ่อยในประเทศไทย ปัญหาที่สำคัญในการตรวจวินิจฉัยโรคภูมิแพ้อาหารทะเลนั้นยังขาดการวินิจฉัยที่ถูกต้องเนื่องจากข้อมูลทางชีวภาพและคุณสมบัติทางภูมิคุ้มกันของสารภูมิแพ้ในกุ้งกุลาดำนั้นยังไม่มีการศึกษามากนัก ในการวิจัยนี้จึงมีเป้าหมายเพื่อศึกษารูปแบบปฏิกิริยาของ แอนติบอดี IgE จากผู้ป่วยภูมิแพ้ที่จำเพาะต่อโปรตีนสกัดจากกุ้งกุลาดำดิบและกุ้งกุลาดำสุกและระบุชนิดของสารก่อภูมิแพ้จากกุ้งที่มีความสามารถในชักนำให้เกิด cross-linking ของ IgE โดยใช้เทคนิคเจลอิเล็กโทรฟอรีซิสแบบสองมิติร่วมกับการใช้เซลล์รีพอร์ตเตอร์ (RS-ATL8) โดยเทคนิค ELISA ผลจากการเปรียบเทียบความแตกต่างของปฏิกิริยากับแอนติบอดี IgE จากผู้ป่วยที่มีอาการแพ้กุ้งจำนวน 24 รายต่อโปรตีนสกัดจากกุ้งกุลาดำดิบและกุ้งกุลาดำสุก พบว่ามีความแตกต่างอย่างมีนัยสำคัญ (P= 0.0093) และนอกจากนี้โปรตีนสกัดจากกุ้งกุลาดำดิบยังมีปฏิกิริยากับแอนติบอดี IgE ที่สูงกว่าโปรตีนสกัดจากกุ้งกุลาดำสุก การทำ SDS-PAGE พบว่าโปรตีนสกัดจากกุ้งกุลาดำดิบมีแถบของโปรตีนจำนวนมากกว่าโปรตีนสกัดจากกุ้งกุลาดำสุก และการวิเคราะห์หาสารก่อภูมิแพ้ที่จำเพาะโดย Western blot พบว่าแอนติบอดี IgE ส่วนใหญ่แสดงการจับแบบจำเพาะอยู่ในช่วงขนาดน้ำหนักโมเลกุลที่ 32-39 kDa ทั้งในโปรตีนสกัดจากกุ้งกุลาดำดิบและกุ้งกุลาดำสุก และจากผู้ป่วยที่มีอาการแพ้กุ้ง 18 ราย จาก 24 ราย (75%) และ 24 ราย (100%) มีแอนติบอดี IgE จับแบบจำเพาะอยู่ในช่วงขนาดน้ำหนักโมเลกุลที่ 32-39 kDa ความเข้มข้นน้อยสุดของโปรตีนสกัดจากกุ้งกุลาดำดิบและกุ้งกุลาดำสุกที่สามารถชักนำให้เกิด cross-linking ของ IgE โดยใช้เซลล์รีพอร์ตเตอร์ (RS-ATL8) เท่ากับ 10 เฟมโตกรัม/มิลลิลิตร และ 100 เฟมโตกรัม/มิลลิลิตร ในโปรตีนสกัดจากกุ้งกุลาดำดิบและกุ้งกุลาดำสุก ตามลำดับ โปรตีน 10 จุดที่ได้ตัดมาจากการแยกโปรตีนด้วยเทคนิคเจลอิเล็กโทรฟอรีซิสแบบสองมิติ ไม่สามารถชักนำให้เกิด cross-linking ของ IgE โดยใช้เซลล์รีพอร์ตเตอร์ (RS-ATL8) โปรตีนจากกุ้งกุลาดำดิบที่ถูกชะจากการทำเจลอิเล็กโทรฟอรีซิสแบบมิติเดียวในช่วงขนาดน้ำหนักโมเลกุลที่ 115 และ 38 kDa พบว่าสามารถชักนำให้เกิด cross-linking ของ IgE และได้ทำการวิเคราะห์ชนิดของโปรตีนนั้นด้วยวิธี Mass spectrometry ซึ่งพบว่าชนิดของโปรตีนที่ได้นั้นอาจเป็นสารก่อภูมิแพ้ชนิดใหม่ซึ่งจะเป็นประโยชน์ต่อการพัฒนาชุดตรวจวินิจฉัยภาวะภูมิแพ้กุ้งต่อไป

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KEYWORDS: HYPERSENSITIVITY REACTION / BLACK TIGER SHRIMP / SHELLFISH ALLERGY / RS-ATL8 / LUCIFERASE

THANYAPAT JARUPALEE: ANALYSIS OF ALLERGENS FROM BLACK TIGER SHRIMP (*Penaeus monodon*) BY COMBINING TWO-DIMENSIONAL GEL ELECTROPHORESIS WITH RS-ATL8 REPORTER CELL LINE. ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., CO-ADVISOR: ASSOC. PROF. PANTIPA CHATCHATEE, M.D., 72 pp.

Food allergy is an immediate hypersensitivity reaction. Specific IgE is generated against allergens, and binds to the specific IgE receptor on the surface of basophils and mast cells. Allergen binding induces IgE cross-linking that triggers these cells to release chemical mediators such as histamine, prostaglandins and leukotrienes. Symptoms of allergic reactions vary from mild irritation to anaphylaxis and life-threatening. Black tiger shrimp (*Penaeus monodon*) is an important aquaculture species in Asia and is the common cause of food allergy in Thailand. One of the important problems in management of shellfish allergy is the lack of accurate diagnostic assay because the biological and immunological properties of allergens in the black tiger shrimps have not been well characterized. This study aims to investigate the reactive pattern of serum IgE from shrimp allergic patients to raw and cooked protein extract from black tiger shrimps and to identify shrimp allergens that can trigger IgE crosslinking by combining two-dimensional gel electrophoresis (2-DE) and RS-ATL8 reporter cell line. ELISA using sera from 24 shrimp allergic subjects, indicated that there were significant differences in reactivity to the raw and cooked shrimp extracts (P= 0.0093). Allergic serum IgE reacted stronger to raw shrimp extract than cooked shrimp extract. Consistent with these results, in SDS-PAGE, raw shrimp extract contained more protein bands than in the cooked extract. Western blot demonstrated that there was the major IgE reactivity area at 32-39 kDa in both raw and cooked shrimp extract. Eighteen of 24 patients (75%) and all patients (100%) had specific IgE to proteins in the range of 32-39 kDa in cooked and raw shrimp, respectively. The minimum concentration of crude shrimp extract to induce IgE cross-linking as measured by RS-ATL8 cell line were 10 fg/ml and 100 fg/ml in raw and cooked shrimp extract, respectively. The ten spots excised from 2-DE did not induce IgE cross-linking in RS-ATL8 cell line. The eluted protein from one-dimensional gel electrophoresis at the 115 and 38 kDa bands from raw shrimp extract induced an IgE cross-linking and the proteins were analysed by mass spectrometry. Some novel proteins were identified with the possibility of novel allergen. These results may be useful for shrimp allergy diagnostic test in the future.

Field of Study: Biotechnology Student's Signature ....

Academic Year: 2016 Advisor's Signature ....

Co-Advisor's Signature ....

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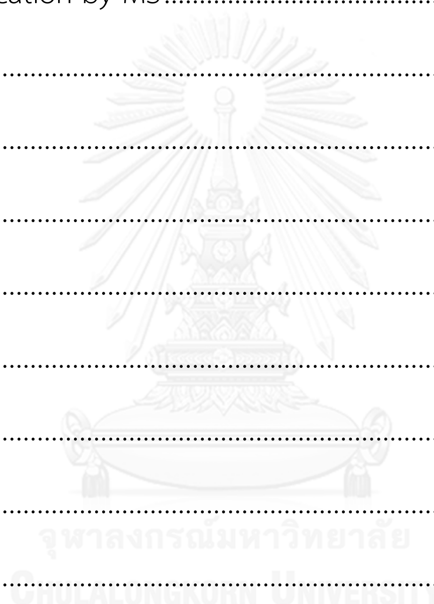
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LIST OF ABBREVIATIONS

Ab	Antibody
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CHAPs	(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)
cm	Centimeter
2D-GE	Two-dimensional gel electrophoresis
Da	Dalton
DMSO	Dimethyl sulfoxide
DTT	Dithioyhreitol
ECL	Enhanced chemiluminescence
ELISA	Enzyme-like immunosorbance assay
FBS	Fetal bovine serum
g	Gram
g (centrifugation speed)	Centrifugal accleration
hr	Hour
HRP	Horseradish peroxidase

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IL-4	Interleukin-4
IPG	Immobilized pH gradient
IgE	Immunoglobulin E
k	Kilo
M	Molar
MEM	Minimum essential medium
MW	Molecular weight marker
mA	Milliampere
mg	Milligram
mL	Milliliter
mM	Millimolar
min	minute
nM	Nano metre
N.D.	Not detectable
ng	Nanogram
O.D.	Optical Density
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline- Tween
PVDF	Polyvinylidene fluoride membrane

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sec	second
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
TEMED	Tetramethyllethylenediamine
TMB	3,3’,5,5’-Tetramethylbezidine
U	Unit
V	Volt
v/v	volume by volume
w/v	weight by volume
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
%	Percentage
/	Per
:	Ratio
x	Fold

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CHAPTER I

BACKGROUND

Food allergy is an immediate hypersensitivity reaction. IgE is generated against allergic causing allergens, and binds to the specific IgE receptor on the surface of basophils and mast cells. Allergen binding triggers mast cells to release chemical mediators such as histamine, prostaglandin and leukotriene. These mediators cause vasodilation, increased vascular permeability, mucus hypersecretion. Symptoms of allergic reaction may vary from mild irritation to anaphylaxis and life-threatening. Black tiger shrimp (*Penaeus monodon*) is an important aquaculture species in Asia and it is the common cause of food allergy in Thailand. One of the important problems in management of shellfish allergy is the lack of accurate diagnostic assay because the biological and immunological properties of allergens in the black tiger shrimps have not been well characterized. This study aimed to investigate the reactivity pattern of IgE from shrimp allergic patients to protein extracts from the black tiger shrimps. In addition, shrimp allergens that can trigger IgE crosslinking will be identified by a combination of two-dimensional gel electrophoresis and RS-ATL8 reporter cell line. The results from this study may provide a novel allergen (s) from black tiger shrimps that may be used for further diagnostic and therapeutic purposes.

Objectives

1. To analyse allergic serum IgE reactivity patterns to black tiger shrimp protein extract

2. To identify shrimp allergens that can trigger IgE crosslinking by combining two-dimensional gel electrophoresis and RS-ATL8 reporter cell line

Hypothesis

Novel shrimp allergen(s) can be detected by combining two-dimensional gel electrophoresis with the use RS-ATL8 reporter cell line

CHAPTER II

LITERATURE REVIEWS

2.1 Food allergy

Food allergy is an IgE immediate hypersensitivity reaction. When the food allergens are digested, they enter through the mucosal membrane via the microfold cells (M cells) in the digestive tract [1]. These allergens provoke Th2 cells to release interleukine (IL-4). IL-4 promotes B cells production of antigen-specific IgE antibodies that binds to the specific IgE receptor (FcεRI), a high-affinity receptor for the Fc region of IgE, on the surface of basophils and mast cells. The next time a person is re-exposed to the same food allergen, the allergen binds with the specific IgE on the surface of these cells and leading to cross-linking of the receptors. The granules of mast cells contain chemical mediators such as histamine, prostaglandin, leukotriene and cytokines. These mediators trigger various allergic symptoms including vasodilation, increased vascular permeability, and mucus hypersecretion. Symptoms of allergic reaction may vary from mild irritation to anaphylaxis, a life-threatening condition [2, 3]. The mucosal immune system in the gut during allergic reaction is shown in Figure 1.

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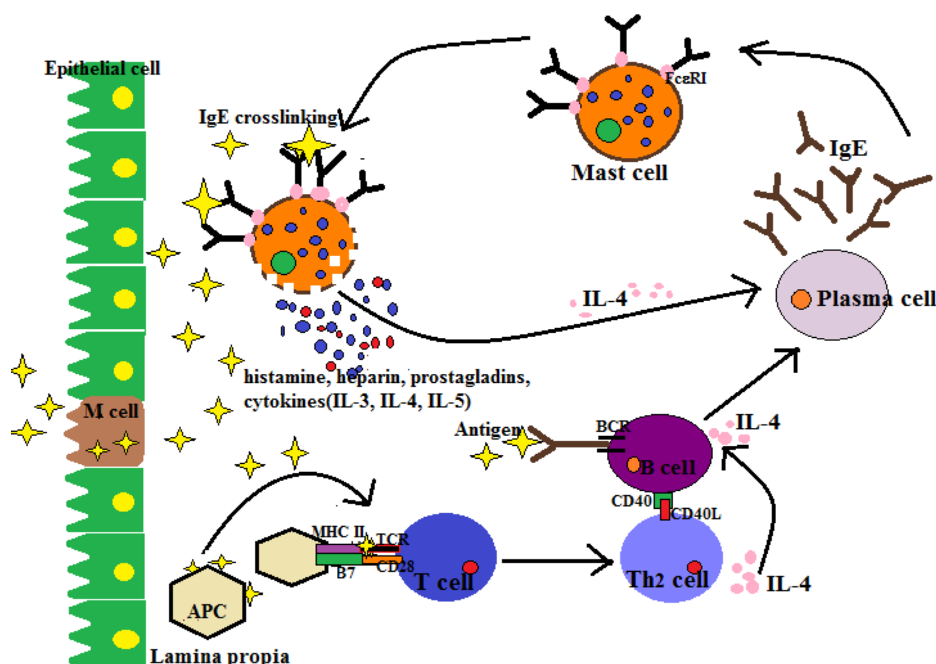

Figure 1. The mucosal immune system in the gut during allergic reaction. (Modified and adapted from Sabban, 2011 [4]).

2.2 Diagnosis of food allergies

A correct diagnosis of food allergy is essential for proper treatment of the allergic symptoms. To date, there is no specific treatment for food allergy. The best treatment is simply to avoid the foods that are the causative of allergy. Patients with severe allergic response should carry injectable epinephrine for anaphylaxis [5]. The development of diagnosis have not changed much in the past decade. Clinical history is an important step in the diagnosis of food allergy. It can provide information that related to the presenting symptoms. Also, this history is used for guidelines to select the appropriate treatment or allergy tests [6]. Skin prick test (SPT) is recommended as the first method for diagnosis of allergic reaction by used along with the clinical history. The size of the wheal-produced at the injection site (3mm or greater in diameter) shows that the individual is allergic to that allergen [7].

However, SPT has a low positive predictive value and often give false positive result. Furthermore, it can induce systemic allergic reaction [8]. Oral food challenge (OFC) is done by feeding moderately increasing suspected allergens food under mornitoring of doctor. It is the gold standard for diagnosis of a food allergy [9]. However, this test also has a great risk of severe reaction in allergy patients and expensive [10]. Nonetheless, several patients with positive SPT still had a negative OFC [9]. The ImmunoCAP test, a registered trademark of Pharmacia Diagnostics AB, is a commercial automated test kit that has been used to detect food specific IgE for the diagnosis of allergic reactions. The allergen of interest are bound on to a solid matrix and incubate with the patient’s serum. IgE antibodies that sepcific to the allergen can detected by use of a secondary fluorescence labels anti-human IgE. The results are determined as classes (class 0-4). This test has been selected as the standard quantification of specific IgE because of its accuracy and cost effectiveness. Nevertheless, false positive results may be obtained from this test due to cross-reactivity [11] and clinical history is not relevance in some case.The results of allergen-specific IgE measured by these methods cannot be interpreted for a clear diagnosis, particularly in the cases of food allergy [11].

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2.3 Prevalence of shellfish allergy

The consumption of seafood increases worldwide and one of food allergies is caused by shellfish [12-16]. In the United States, it is reported that 1 in 50 individuals has been diagnosed to be allergic to shrimps [17]. This high prevalence of food allergy greatly affects the economy because approximately US $ 18 billion was spent annually as the costs of allergy treatment [18]. This trend is also found in many Asian countries [3, 19]. The most shrimps consumed in Asia are the black tiger shrimps (*Penaeus monodon*), the banana shrimp (*Fenneropenaeus merguiensis*) and the

white leg pacific shrimp (*Litopenaeus vannamei*) [3, 20, 21]. In Thailand, black tiger shrimps are the most frequently consumed shrimp species (69%) [22]. The risk of shrimp allergy is triggered by various routes, such as through inhaling of the shrimp particles, touching, working and ingestion of meals containing shrimps. The prevalence of seafood allergy in Thai adults and children population from all food allergic cases, as reported by Allergy Clinic at Siriraj Hospital in Bangkok, Thailand, is 65% and 66%, respectively [23]. Shrimp allergy has symptoms expanding in several organs such as skin (52-90%), respiratory (42%), gastrointestinal (35%), and the cardiovascular system (anaphylaxis 10%) [24].

2.4 Studies of shrimp allergens

The studies of shrimp allergens have been conducted for many years but only a few allergens in shrimp have been identified. The tropomyosin (34-39 kDa) is the major allergen first identified in several shrimp species [25-31], other crustaceans [32], squid [33], cockroaches [34] and house dust mites [35]. In 1994, Dual et al. have identified the allergic proteins that present in brown shrimp (*Penaeus aztecus*) by using Western blot analysis in cook and uncooked extracts.They confirmed that the protein with molecular weight (MW) of 36 kDa, a group of muscle protein called tropomyosin, was major allergen [30]. In 2010, Rahman et al. have identified the allergic proteins that present in black tiger shrimp by using Western blot in a combination with peptide mass fingerprinting (PMF). There are shrimp allergens that were reported including tropomyosin (34-39 kDa), arginine kinase (40 kDa), myosin light chain (20 kDa) [20]. In several studies, beside tropomyosin as a major allergen, other allergens have been reported. Yu et al. have identified a novel allergen from the black tiger shrimp by two-dimentional immunoblotting. The molecular weight of the allergen was 39.9 kDa and it was identified as arginine kinase [36]. Moreover,

other shrimp allergens have been also identified by LC-MS/MS, such as myosin light chain [21] and sarcoplasmic calcium-binding protein [37] with molecular weight of 20 kDa. Recently, the study by Piboonpocanun el al. have reported that the hemocyanin, a protein with 75 kDa as allergen from the giant freshwater shrimp (*Macrobrachium rosenbergii*) [38]. Although several studies of shrimp allergens, the knowledge have not been applied for the treatment of shrimp allergic patients.

2.5 Reporter cell line (RS-ATL8)

One of the important problems in management of shellfish allergy is the lack of accurate diagnostic assay because the biological and immunological properties of allergens in the black tiger shrimps have not been well characterized. The diagnosis of shellfish allergy are often based on the quantification of allergen-specifics IgE or total IgE in serum. This technique does not measure the potential of an allergen to induce allergic reaction and the quantity of specific IgE may not correlate with the severity of the symptoms [39]. Recently, a new detection assay for IgE binding to specific allergens based on the NFAT (Nuclear factor of activated T-cells) related luciferase expression in a humanized rat basophilic leukaemia cell line (RS-ATL8) was developed (Figure 2). RS-ATL8, derived from RBL-SX38 cell stably transfected with NFAT-Luciferase clone 8, was improved by Nakamura et al. The reporter cell lines were used as a reporter system for mornitoring the activation of NFAT. NFAT is important in transcription of IL-4 when crosslinking of FcεRI by allergens. The report cellular activation by the interesting allergen are measured by luciferase activity. The system can be used for evaluation of allergenicity of various antigens (mite, cat dander and cedar pollen) including food allergens (milk, peanut, wheat, crab, shrimp) by evaluating the cross-linking capacity of allergens. This system showed a good correlation with oral food challenge test (OFC test) in patients with egg allergy

and also with the egg white specific serum IgE test (ImmunoCAP test) [11, 40]. Moreover, the specific IgE levels to allergen can be measured by sensitizing reporter cell lines with diluted serum and measuring the activation of cells after challenge with the interesting allergens [41]. In 2014, Wan et al. have used RS-ATL8 cell lines for assessment of potential allergenicity of *Schistosoma mansoni* antigens. They concluded that this cell line is suitable to study of vaccine producing from helminthic proteins [42].

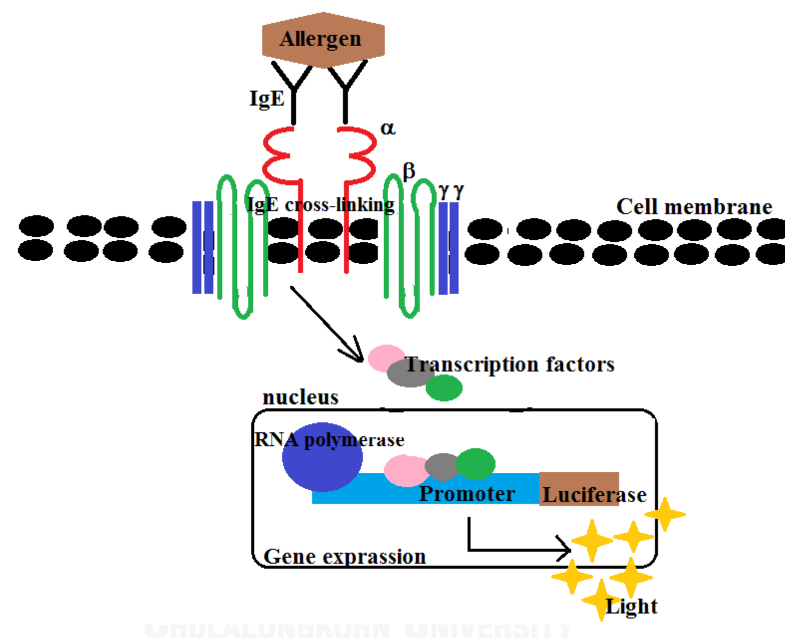

Figure 2. Schematic diagram of the mechanism of IgE cross linking reporter cell line (RS-ATL8). (Modified and adapted from Abbas & Lichtman, 2008).

The allergens bind with the specific IgE on the surface of these cells. The crosslinking of the FcεRI receptors (α, β and γ_2 chains) on the surface of cell leads to the activation of NFAT. NFAT induced reporter gene expression that was monitored by the enzymatic activity of luciferase [43].

2.6 Two-dimensional gel electrophoresis (2-DE) for detection of allergens

In recent years, proteomic techniques was used for allergen analysis in foods and plants [44, 45]. 2-DE is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique was used to detect allergens by identifying proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights [46]. Each spot from the two-dimensional gel electrophoresis corresponds to a single protein species in the sample. To identify allergenic proteins were used by MALDI-TOF MS or LC-MS [47-49]. A patient's IgE can bind to allergens, but not all IgE-binding proteins cause allergenic reactions. The role of allergenic reaction is IgE-binding proteins that cross-link the FcεRI on mast cells [44]. By combining 2-DE with RS-ATL8 cell line, it is now possible to identify novel allergen that can trigger allergic reaction.

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CHAPTER III

MATERIAL AND METHODS

3.1 Preparation of serum and shrimp extracts

3.1.1. Patient sera

Twenty-four sera were obtained from shrimp allergic patients from the Allergy Clinic of King Chulalongkorn Memorial Hospital. The inclusion criteria of this study were:

1. All patients had history of shrimp allergy

2. The allergic response was confirmed by the positive skin prick test (wheal $\geq$3 mm) and Immuno CAP test.

Serum from non-allergic healthy donor with no history of shrimp allergy and skin prick test negative was used as a negative control. All procedure involved human subject was reviewed and approved by the Institutional Review Board of Chulalongkorn University (Project number 469/58).

3.1.2. Preparation of shrimp extracts

Fresh black tiger shrimp (*Penaeus monodon*) was purchased from a local market. The shrimp extracts were prepared as previously described [50]. Briefly, the outer shell of raw shrimp was removed, cut into pieces, and resuspended in phosphate buffered saline (PBS), pH 7.4. The homogenate was centrifuged at 8,600 x g for 10 minutes at 4 °C. The supernatant was collected and the centrifuge steps were repeated. After the end of centrifuge steps, supernatant was sterilized by syringe filtration (Pall, USA, 0.22 μm) and stored at -80 °C until used as raw shrimp extracts. For the cooked shrimp extracts, shrimp was boiled in PBS at 100 °C for 5

minutes before extraction, using the method previously described for raw extract. The bicinchoninic acid (BCA Assay Protein Assay kit, Pierce, USA) protein assay was used to determine total protein concentration of each extract, using bovine serum albumin as a standard.

3.2 Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was performed as described previously [51] with some modifications. ELISA plates were coated with raw or cooked shrimp extract; 250 µg per ml in PBS (100 µl per well). The plates were kept overnight at 4 °C. On the next day, wells were washed with 200 µl per well of PBS-Tween20 (PBS-T) 3 times. Each well was incubated with blocking solution (200 µl per well) for 1 hour at room temperature. The patient sera (100 µl of a dilution 1:50 in 5% skim milk powder/PBS-T) were added to each well and incubated at room temperature for 1 hour. Secondary antibody, goat anti-human IgE (KPL, USA), conjugated with horse radish peroxidase (100 µl of 1:5000 in 5% skim milk powder/PBS-T) were added to each well and then incubated for 1 hour at room temperature. Signals were detected using 3, 3', 5, 5'- tetramethylbezidine (TMB). After sufficient color development for 5-10 minutes.The reaction was stopped by using 1 M H_2SO_4 (100 µl per well). The OD was measured at 450 nm by microplate reader (Biochrom, Anthos 2010, USA)

3.3 Western blot

3.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Fifteen micrograms of black tiger shrimp extract solution and equal volume of 2x loading buffer were mixed and heated at 99 °C for 5 minutes. The prepared samples were loaded into gels and resolved at 100 V for 90 minutes. Prestained color plus protein was used as a molecular weight (MW) marker (New England Biolab,

USA). The resulting bands were stained by Coomassie brilliant blue R-250 (GE Healthcare, UK).

3.3.2 Staining Gels with Coomassie Blue R-250

After electrophoresis, gels were placed in plastic tray and washed 2-3 times in deionized water to remove SDS present in the gel. The Coomassie solution was poured over the gels. The gel was gentle shaked for 30 minutes untill the gel was in a uniform blue color. The stained gel were washed with distilled water for 10 minutes and excess stain was eluted with destain solution for 4 - 24 hours. This treatment allowed the visualization of proteins as blue bands on a clear background.

3.3.3 Gel drying

After complete of staining and destaining steps, the cellophane sheet were immersed in the gel drying solution (Appendix C). The glass frame was placed on the table and covered with wet cellophane. The gel was layed on top of cellophane and placed another sheet of cellophane over the gel. The drying frame was clamped and transported into the hot air oven. Drying was taken between 2–3 hours depending on humidity and gel thickness. When the cellophane was dry to touch, removed the gel from the drying frame. The excess cellophane was cut for scanning, photography.

3.3.4 Western blot

After electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millopore Corporation, USA). The transfer was performed by using a constant voltage of 90 mA (for one gel) or 150 mA for 90 minutes. After the transfer, the membranes were blocked with 5% skim milk powder in PBS-T. The membrane were cut into 1-cm strips and incubated with patient sera (diluted at 1:100 in 5% skim milk in PBS-T) at room temperature for 1 hour. On the next day, the membrane strips were washed with 3 times of PBS-T for 5 minutes and

4 times of PBS-T for 15 minutes. The blots were further incubated with goat anti-human IgE (KPL, USA) labelled with horse radish peroxidase (at a dilution of 1:5000) at temperature with agitation for 1 hour and washed with three times of PBS-T for 5 minutes and 4 times of PBS-T for 15 minutes. After the last washing, the blots were incubated with chemiluminescent substrates for 1 minute with gentle shaking. The signals were detected Chemiluminescence by using X-ray film (Amersham Biosicences, UK).

3.4 Cell culture

3.4.1 Reporter cell line

RS-ATL8 reporter cell line (kind gift of Professor Ryosuke Nakamura, Division of Novel Foods and Immunochemistry, National Institute of Health Science, Japan) was maintained in minimum essential medium (MEM) (GIBCO, USA) with supplement of 10% FBS (GIBCO, USA), 100 U/ml penicillin (Hyclone, USA), 0.5 mg/ml geneticin, 0.2 mg/ml hygromycin B, Gluta MAX-I (GIBCO, USA) at 37°C in humidified 5% CO_2 incubator (Thermo Scientific, TC 230, USA).

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3.4.2 Cell preparation

RS-ATL8 cell was cultured in 25 cm^2 flask in 8 ml completed MEM. To prepare cells for experiment, cells were removed from 25 cm^2 flasks by using cell scrapers. Cell suspension were centrifuged at 300 x g for 5 minutes. The culture supernatant were discarded and cells were re-suspended in complete MEM to 1 x 10^6 cells per ml and added 50 μl per well onto a 96 well plate. The cells were incubated for 3 hours in humidified 5% CO_2 incubator at 37 °C. After incubation, 5μl of sera from shrimp allergic patients were diluted (final dilution at 1:100) in complete MEM and were added to cells. After sensitization, cells were washed once gently with sterile PBS. The dissolved allergens in complete MEM were added 50μl per well and were

incubated for 3 hours in humidified 5% CO_2 incubator at 37 ^{o}C. After stimulation, luciferase substrate buffer including cell lysis reagent (ONE-Glo, Promega, UK) was added to the cells at 50µl per well. Luciferase expression levels was measured on a microplate reader (Thermo Scientific, Varioskan Flash, USA). Measurements were done in triplicate, and the average were used for analysis. The positive control was stimulated with the affinity purified goat anti-human IgE (Bethyl, USA) and the negative control was medium alone or serum alone. The summary of cell preparation is shown below:

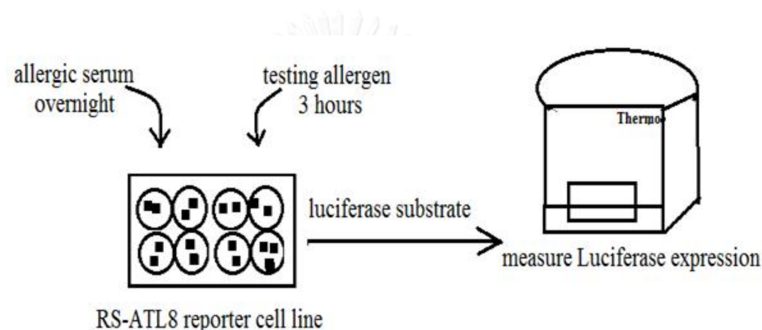

Figure 3. The summary of cell preparation.

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3.4.3 Cell preservation

After centrifugation, the supernatant was removed from the centrifuged cells and the cell pellet were resuspended in cold freezing medium (Appendix A). Aliquot the 1 ml of cells suspension in cryogenic vials. This method were done on ice. The cryogenic vials were immediately placed in the freezer (-80 ^{o}C) overnight. After 24 hours, cells were transferred to a liquid nitrogen for permanent storage.

3.4.4 Thawing cells for use

The frozen vial of RS-ATL8 cell line was thawed swiftly in a water bath (Memmert, Germany) at 37 ^{o}C. The cells suspension was washed in 4 ml of pre-

warmed complete MEM and centrifuged 5 minutes at 1000 x g. Supernatant was removed and 5 ml of MEM complete media was added. The suspended cells were transfered to 25 cm^2 flask and were incubated at 37°C in humidified 5% CO_2 incubator (Thermo Scientific, TC 230, USA).

3.5 Two-dimensional gel electrophoresis (2-DE)

3.5.1 Protein clean up

The salts, detergents, lipids, nucleic acids in shrimp extract that interfere with analysis were removed by 2D cleanup kit (Bio-Rad, USA). The process was performed as described according to the manufacturer’s instruction. Briefly, two hundred micrograms from shrimp extract (final volume 100 µl) and 300 µl of precipitation agent 1 were transferred into a 1.5 ml microcentrifuge tube . The protein mixture was mixed by vortexing between each steps and incubated on ice for 15 minutes. Next, the precipitation agent 2 300 µl were added to the protein mixture and centrifuged at 12,000 x g for 5 minutes. Supernatant was carefully removed and centrifuge step was repeated (≈ 15-30 second) to remove the remaining supernatant. After this step, the wash reagent 1 was added on top of the pellet and centrifuged at 12,000 x g for 5 minutes . Supernatant was carefully removed. Twenty-five µl of ReadyPrep proteomic grade water, 1 ml of wash reagent 2 (prechilled at -20 °C for at least 1 hour) and 5 µl of wash 2 additive were added on top of the pellet. The protein mixture was incubated at at -20 °C for 30 minutes during the inculation period the mixture was mixed for 30 second every 10 minutes. After the incubation period, the microcentrifuge tubes were centrifuged at 12,000 x g for 5 minutes to remove the supernatant and centrifuge step was repeated (≈ 15-30 second) to remove the remaining supernatant. The pellets were air dried at room temperature for no more than 5 minutes, and resuspend in rehydration buffer

(Appendix E) containing 7 M urea (Bio-Rad, USA), 2M thiourea (Bio-Rad, USA), 4% (w/v) CHAPs (Bio-Rad, USA), 40 mM DTT (Bio-Rad, USA), IPG buffer pH 3-10 (Bio-Rad, USA), 0.002% (w/v) bromophenol blue. The proteins were incubated at room temperature for 5 minutes and mixed well by vortexing for dissolving the protein pellet. Finally, the proteins were centrifuged at 12,000 x g for 5 minutes. The supernatant were used for IEF in IPG strips or stored in a clean tube at -80 °C for analysis.

3.5.2 First-dimension isoelectric focusing (IEF)

One hundred twenty-five µl of rehydration solution containing the protein was pipetted into the channel of the rehydration tray (Bio-Rad, USA). The protective cover the IPG strip (7 cm of immobilized pH 3-10 non linear) (Bio-Rad, USA) was removed by using forcep and gently placed the IPG strip gel-side down in the channel of the rehydration tray. The IPG strip was incubated at room temperature for 1 hour. After this step, the IPG strip and the remaining solution were transferred to an iso-electric forcusing tray. Then each of the strips were overlaid with 2 ml of mineral oil and the rehydration period was programmed as 50 µA per IPG strip of electric current for 16 hours of 20 °C for complete rehydration. After rehydration was complete, the IPG strips gel side up on a piece of dry filter paper and placed wet a second piece of filter paper to remove unabsorbed protein from the surface of the gel and the oil. Next, the IPG strips were transfer to new chanel of the IEF tray and wet two paper wicks were inserted between the IPG strip and the electrodes. Two milliter of fresh mineral oil was overlaid again on the strip. The focusing conditions were applied to the strips as follow: step 1 applying linear current from 0 volt to 250 volt within 20 minutes, step 2 applying linear current from 250 volt to 4,000 volt within 2 hours and finally, step 3 applying rapidly current from 4,000 volt to 12,000 volt.

3.5.3 IPG strip equilibration

After the iso-electric forcusing step was complete, the IPG strips gel side up on a piece of dry filter paper and placed wet a second piece of filter paper to remove the oil from the surface of the gel. Next, the focused IPG strips were equilibrated in 2.5 ml equilibration buffer I (Appendix E) which containing 6 M urea (Bio-Rad), 0.375 M tris-HCL pH 8.8 (Bio-Rad, USA), 2% SDS (Bio-Rad, USA), 20% glycerol (Merck, Germany) and 2% (w/v) DTT (Bio-Rad, USA) was added fresh before used. The tray was gently shaked at room temperature for 10 minutes. At the end of 10 minutes incubation, the used equilibration buffer I was discarded and added equilibration buffer II (Appendix E) which containing 6M urea (Bio-Rad, USA) 0.375 M tris-HCL pH 8.8 (Bio-Rad, USA), 2% SDS (Bio-Rad, USA), 20% glycerol (Merck, Germany) and 2.5 %(w/v) iodoacetamide (Bio-Rad, USA) was added fresh before used. The tray was gently shaked at room temperature for 10 minutes.

3.5.4 SDS-PAGE for 2-DE

The IPG strip was ready for SDS-PAGE electrophoresis. The IPG strip was diped into SDS-running buffer ≈ 1 minute before laid the side-up strip onto the back plate of the SDS-PAGE gel above the IPG well. The protein ladder was added to the filter pater (0.5 x 1 cm.) and it was placed near the strip. After this step, a melted overlay agarose (Bio-Rad, USA) was pipetted into the IPG well of the gel.The proteins were separated using the constand current at 10 mA for 60 minutes and 15 mA for 100 minutes. Protein gel was stained with Coomassie brilliant blue R 250. Each distances of bands was measured by ImageJ (IJ 1.45m) for calulated molucular weight.

3.6 Elution of shrimp allergens from SDS polyacrylamide gels

After electrophoresis was done, a sterile scalpel was used to excise the molecular weight marker and the first lane of shrimp extract protein. The cut strip of gel was stained with Coomassie blue (this strip was used as guides to excise band from unstained gel). The rest of the gel was kept on glass plate, covered with plastic wrap at 4 °C. The stained strip of gel with the unstained gel were aligned and were excised the bands of interest. After excision, the remaining gel was stained to determine the accuracy of excision. Excised band pieces were minced into small pieces to increse the surface area and the minced pieces were transfered in to the microcentrifuge tube. The minced pieces were equilibrated in elution buffer (Appendix D) and incubated on a shaker (Eppendorf, ThermoMixer, USA) at 25 °C overnight. After equilibration, protein was centrifuged at 14,000 x g for 5 minutes and carefully moved into a new microcentrifuge tube. An aliquot of the supernatant were tested for the presence of protein by using SDS-PAGE. After elution of proteins from SDS polyacrylamide gels, the supernatant were filled to the dialysis cassette (Thermo, Slide-A-Lyzer™ G2 Cassettes, gamma-irradiated, 10K MWCO). The cassette was floated vertically in the dialysis buffer (Appendix A) for 2 hours at 4°C for 2 times and overnight by stired gently. On the next day, the sample was removed from the cassette by slow aspiration. Then, three milliliters of the dialyzed sample were added to the centrifugal filter devices (Pall, USA, 3K MWCO) and centrifuged at 4,000 × g for 10 minutes at 25 °C. The concentrated sample was transfered to new tube.

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3.7 Protein mass spectrometry (MS)

Identification of protein from SDS-PAGE excised bands by MS was performed by Dr. Sittiruk Roytrakul, National Center for Genetic Engineering and Biotechnology (BIOTEC). The ESI-QUAD-TOF was selected instrument for peptide fragmentation.

3.8 Statistical analysis

The Wilcoxon signed rank test and the unpaired t-test were used to compare between groups. Differences between groups were considered to be significantly with the *P* value of less than 0.05. Analysis was performed using GraphPad Prism version 5.03.

CHAPTER IV

RESULTS

4.1. Specific serum IgE reactivity to raw and cooked shrimp proteins by indirect ELISA

Screening of the 24 shrimp allergic patients by ELISA indicated significant differences in reactivity to the raw and cooked shrimp extracts (*P*= 0.0093; Wilcoxon test) (Figure 4). Serum IgE reacted stronger to raw shrimp extract than cook shrimp extract. The sera from subject No. 2 and 21 showed highest IgE reactivity to both extracts (Figure 5).

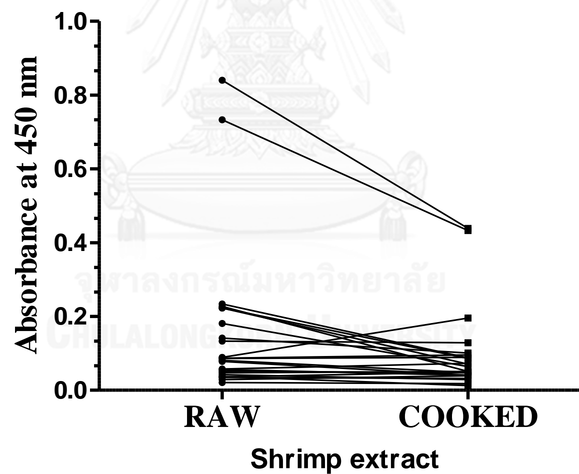
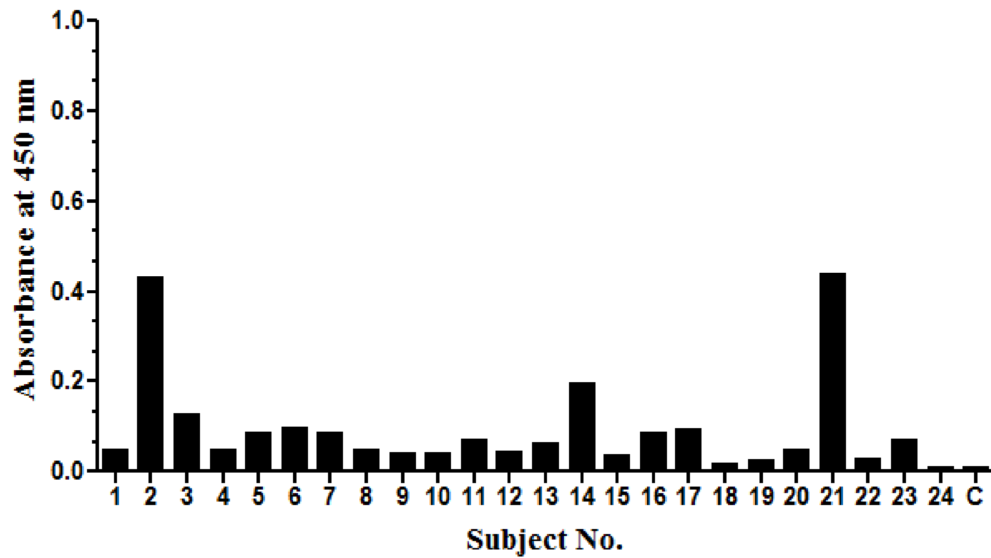


Figure 3. Comparison of serum IgE reactivity to raw and cooked shrimp extracts in shrimp allergic patients by indirect ELISA.

The Wilcoxon test was used to compare serum IgE reactivity between raw and cooked shrimp extracts (n=24, *P*= 0.0093).

A. Cooked shrimp extract



B. Raw shrimp extract

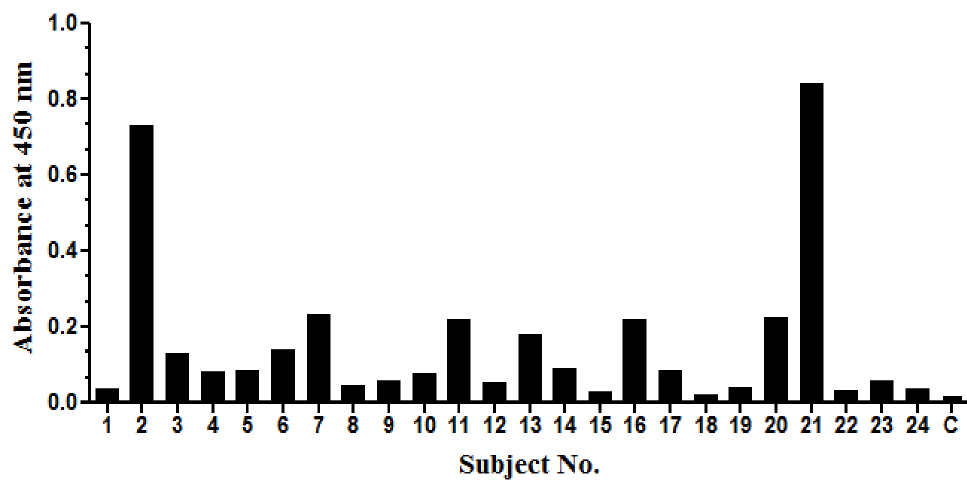


Figure 4. Serum IgE reactivity to cooked (A) and raw (B) shrimp extracts by indirect ELISA.

The concentration of shrimp extracts were 250 ug/ml. Allergic sera were diluted at 1:50. C was control non-allergic serum.

4.2. Detection of IgE reactivity to shrimp extracts by Western blot

4.2.1. Analysis of raw and cooked shrimp proteins by SDS-PAGE

The shrimp extracts were separated by SDS-PAGE and the overall protein patterns of raw and cooked extracted were compared (Figure 6). The protein components of shrimp extracts showed various bands ranging between 15 to 230 kDa. The presence of prominent bands at approximately 83, 77, 41, 38 and 19 kDa were seen in both extracts. The band at 41, 38 and 18 kDa possibly corresponding to arginine kinase, tropomyosin, and myosin light chain, respectively, were detected. Several protein bands that were found in the raw extract were not visible in the cooked extract. Moreover, the amount of the raw shrimp extract as judged in all extracts by band intensity, were stronger than those in the cooked shrimp extract. This result suggested that most proteins were degraded by heat treatment.

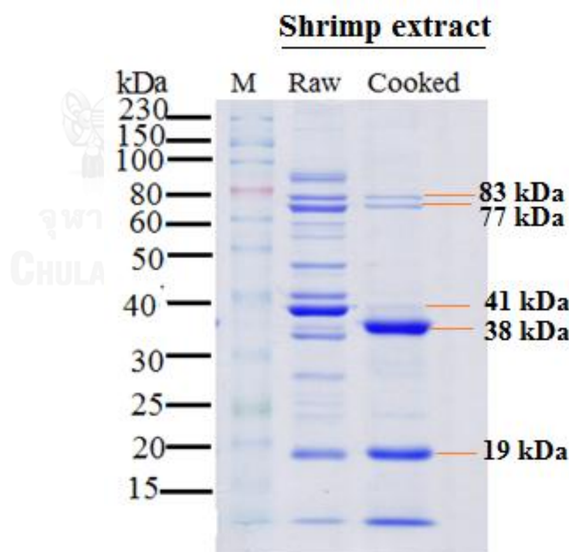


Figure 5. SDS-PAGE of shrimp extracts.

The black tiger shrimp extracts (25µg/lane) were separated on SDS-PAGE and stained with Coomassie brilliant blue R-250.

4.2.2. IgE reactivity to raw and cooked shrimp extracts detected by Western blot

The shrimp allergic patient IgE reactivity pattern of raw and cooked shrimp were determined by Western blot. The reactive bands appeared at various band molecular weights (Figure 7A and 7C). In raw shrimp extract, IgE reactivity were obvious. The numbers of band were higher in the raw shrimp extract than those of the cooked shrimp extract. A significant difference between raw and cooked shrimp extract showed that IgE binding reactive to raw shrimp extract was higher than cooked shrimp extracts (Figure 7B and 7D). From the 24 shrimp allergic patients, two major groups of allergen band at 32-39, 15-26 kDa in both raw and cooked shrimp extract were observed. Eighteen of 24 patients had specific IgE to the 32-39 kDa in cooked allergens were observed (75%) while all patients had specific IgE to the 32-39 kDa in raw allergens (100%) (Figure 7B and 7D). Two allergen band group at 15-26 and 91-230 kDa in cooked shrimp extract were IgE reactive to more than 40% of the patient's sera. Four patient (No. 2, 14 17 and 21) had several IgE reactive bands to both raw and cooked shrimp extracts. Interestingly, 3 patient (No. 4, 8 and 19) showed no IgE binding to cooked shrimp extract. Therefore, the raw shrimp extract will be usd to screen for novel shrimp allergens in the next experiment.

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A. Patterns of IgE reactivity to cooked shrimp extract

B. Frequency of specific IgE reactivity to cooked shrimp extract

Protein MWs (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	C	Frequency (%)
91-230																										42
80-90																										8
71-79																										13
60-70																										21
40-56																										21
32-39																										75
15-26																										42

C. **Patterns of IgE reactivity to** raw **shrimp extract**

D. Frequency of specific IgE reactivity to raw shrimp extract

Protein MWs (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	C	Frequency (%)
91-230																										75
80-90																										46
71-79																										54
60-70																										38
40-56																										96
32-39																										100
15-26																										88

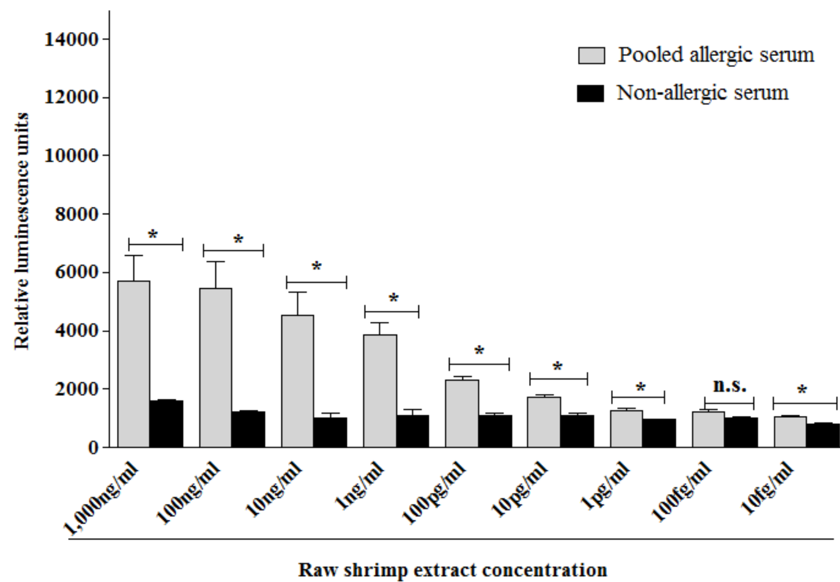
Figure 6. Sera IgE reactivity patterns to cooked (A) and raw (D) shrimp extracts by Western blot.

All sera were diluted at 1:100. Lane M, molecular weight marker. Lane R, raw shrimp extract by SDS-PAGE and Coomassie staining. Lane C, cooked shrimp extract by SDS-PAGE and Coomassie staining. C was control non-allergic serum and 1-24 were the number of patient sera. The left part of the panels shows molecular weight marker of protein and the right part shows frequencies of specific IgE binding of sera to cooked (B) and raw (D) shrimp extract by Western blot. Gray filled boxes indicated allergens recognized by patient sera IgE.

4.3. RS-ATL8 reporter cell line for detection of shrimp allergen proteins

To detect IgE cross-linking allergens, the RS-ATL8 cells were sensitized with 1:100 of pooled sera from 5 shrimp allergic patients (patient No. 5, 7, 17, 21 and 24) that showed high IgE bind to the specific band of allergen on Western blot and non allergic serum overnight. They were stimulated with serial dilution of mixture of raw and cooked shrimp extracts for 3 hours as described in materials and methods. The reaction were measured by detecting luminescent signal. A dose response curve of RS-ATL8 were shown in Figure 8. This experiment indicated a wide range of allergen concentrations ranging from 1,000 ng/ml to 10 fg/ml in raw and cooked shrimp extracts that to induce reporter activation. The minimum concentration to induce reporter activation was 10 fg/ml in raw shrimp extract and 100 fg/ml in cooked shrimp extract, respectively. The raw and cook extract at 100 fg/ml and 10 fg/ml, respectively, did not give significant IgE cross-linking. Therefore, the RS- ATL8 cell lines had ability to detect shrimp allergen protein from 1,000 ng/ml to 10 fg/ml in raw shrimp extracts and 1,000 ng/ml to 100 fg/ml in cooked shrimp extracts. Moreover, cooked shrimp extract with lower concentration induced reporter activity than the raw shrimp extract.

A. Raw shrimp extract



B. Cooked shrimp extract

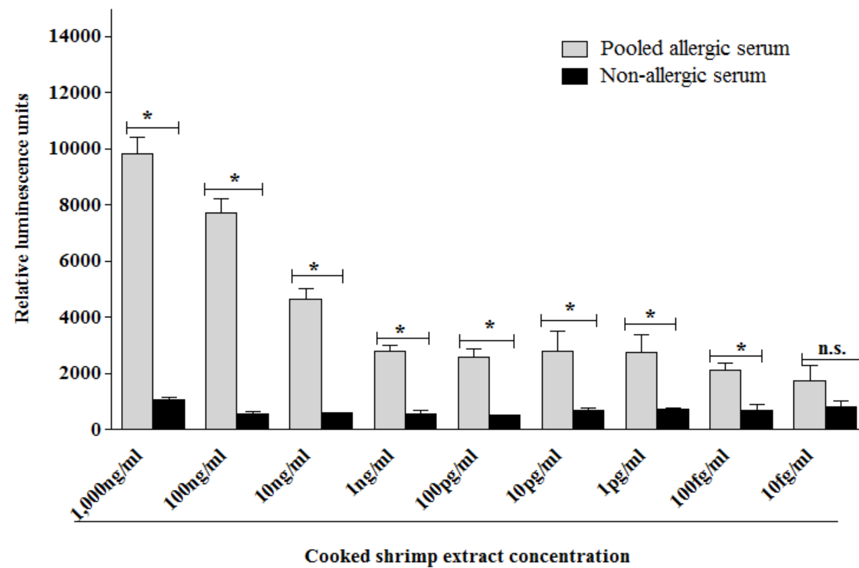


Figure 7. The sensitivity of RS-ATL8 reporter cell line to detect shrimp allergen proteins.

RS-ATL8 cells are sensitized with diluted pooled sera (1:100) from shrimp allergic patients and non-allergic healthy control serum overnight. Cells were stimulated with 1µg/ml to 10 fg/ml of raw and cooked shrimp extracts. Positive controls included in

this experiment (IgE + anti-IgE) are not shown. Pooled allergic serum is shown by the gray columns and non-allergic healthy control serum is shown by black columns. Data are mean ± SD of the readings of triplicates. T-test results indicate *P*<0.05 significance for pooled allergic serum compared non-allergic healthy control serum. * : *P*<0.05, n.s., not significant difference.

4.4. Two-dimensional gel electrophoresis (2-DE)

Shrimp extracts were separated by 2-DE. The results revealed many protein spots of raw shrimp extracts with pI ranging from 3 to 10 and molecular weight from ranging 11 to 135 kDa (Figure 9A). The ten protein spots that showed clear separation were collected and excised for reporter cell line analysis indicated by circles in Figure 9B and Supplementary Figure 16C in the appendix F. The separated gel was stained by Coomassie blue and used as reference for excision of spots (Figure 9A). After this treatment, all ten spots were eluted in elution buffer and use to treat RS-ATL8 cell lines. The results of the luciferase activity revealed that all ten spots did not induce detectable reporter activity and the crosslinking of IgE (data not shown).

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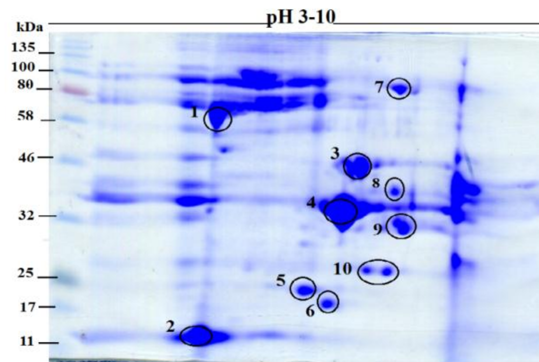
A. The reference gel from Western blot



B. The excised gel

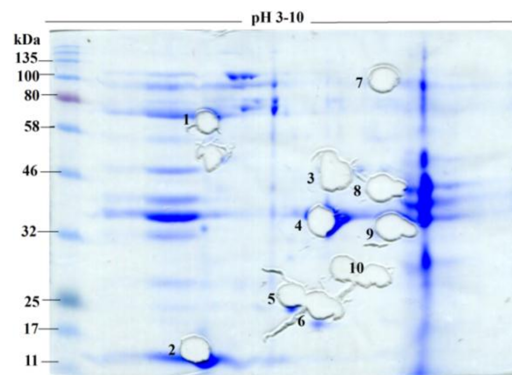


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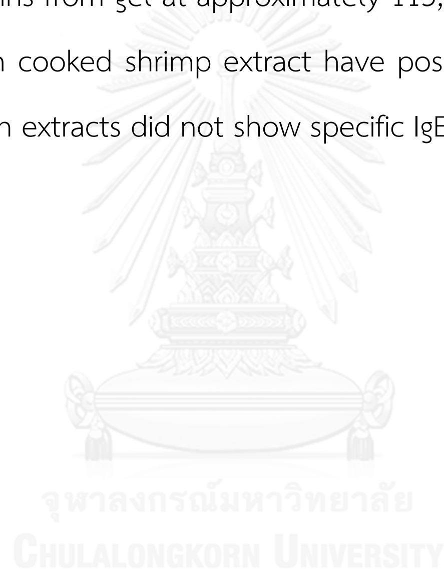
Figure 8. Two-dimensional gel electrophoresis (2-DE) and Western blot analysis. (A), the raw shrimp extract were separated by 2-DE using 7 cm, pH 3–10, nonlinear followed by 10% SDS-PAGE and stained with Coomassie brilliant blue R-250. The spots selected from the reference gel and Western blot (numbers beside the circles) were used for assay by RS-ATL8 cell lines (B).

4.5. Preparation of proteins from 1-DE

As shown above, the spots from two-dimensional electrophoresis did not induce the cross-linking of IgE. This may be due to insufficient amout of antigens. Therefore, we next attempted to identify shrimp allergen that induce the cross-linking of IgE by using the eluted protein from 1-DE. Four protein bands (115, 41, 38 and 19 kDa) and two protein bands (38 and 19 kDa) within the major IgE-binding

regions of the allergenic shrimp from raw and cooked shrimp extracts, respectively, were excised and eluted. The eluted protein bands were confirmed by SDS-PAGE (Figure 10A and 10C). The results showed that the bands matched the molecular weight of interested protein area (Figure 10A and 10C). In order to confirm that the eluted proteins retained an IgE-binding ability, the eluted protein from polyacrylamide gel was used to detect IgE binding by Western blot using pooled allergic serum from 5 shrimp allergic patients which shown high IgE binging to the specific band of allergen on Western blot (Figure 10B and 10D). The results showed that the eluted proteins from gel at approximately 115, 41, and 38 kDa in raw shrimp extract and 38 kDa in cooked shrimp extract have positive reaction. In contrast, the 19 kDa protein in both extracts did not show specific IgE binding.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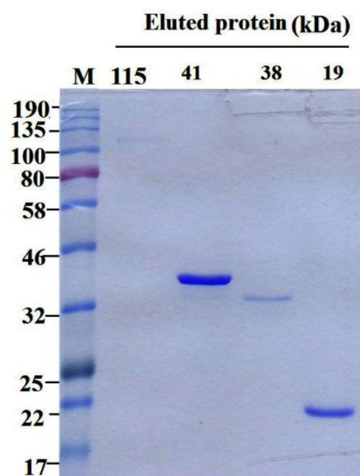
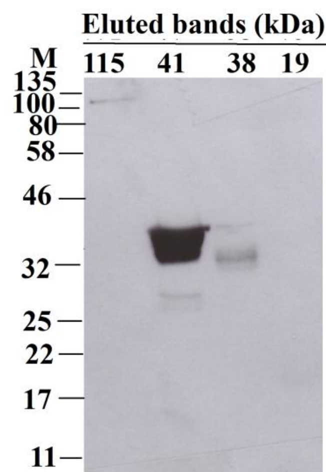
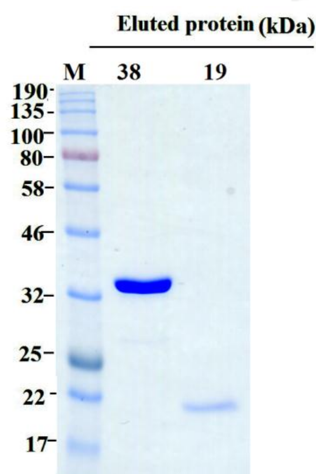
A. SDS-PAGE of raw shrimp extract



B. IgE reactivity of raw shrimp extract



C. SDS-PAGE of cooked shrimp extract



D. IgE reactivity of cooked shrimp extract

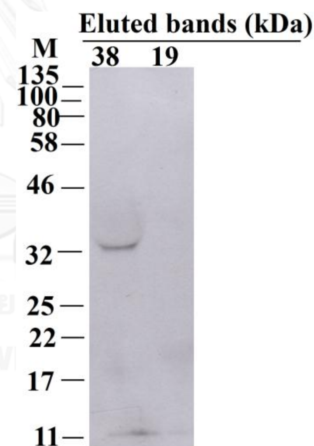
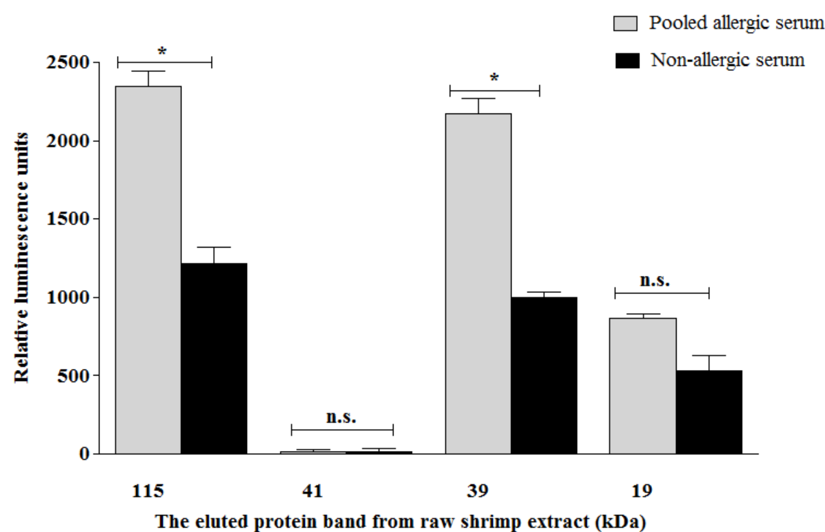


Figure 9. Detection of the excised protein band of raw and cooked shrimp extract from SDS-PAGE and Western blot by pooled sera. (A), the excised protein band of raw shrimp extract were analysed by SDS-PSGE. lanes 2-5, the eluted protein bands. (B), the eluted protein was recognized from pooled sera IgE by Western blot. (C), the excised protein band of cooked shrimp extract were analysed by SDS-PSGE . lane 2 and 3, the eluted protein bands. (D), the eluted protein was recognized from pooled sera IgE by Western blot.

4.6. IgE cross-linking activity of eluted protein bands from 1-DE

Pooled sera from shrimp allergic pateints, who had high IgE reactivity to raw and cooked shrimp extract by ELISA and Western blot were used to sensitized RS-ATL8 cells. Cells were sensitized with 1:100 of shrimp allergic patient sera and non-allergic serum overnight. The eluted protein bands from raw shrimp extract at apporoximately 115, 41, 38 and 19 kDa and cooked shrimp extract at 38 and 19 kDa, respectively were used to stimulate cells (Figure 11). The results indicated that the eluted protein bands at approximately 115, 38 and 19 kDa from raw shrimp extract had a ability to induce IgE cross-linking. In contrast, the eluted protein bands at 38 and 19 kDa from cooked shrimp extract could not induce IgE cross-linking.

A. The eluted proteins from raw shrimp extract



B. The eluted proteins from cooked shrimp extract

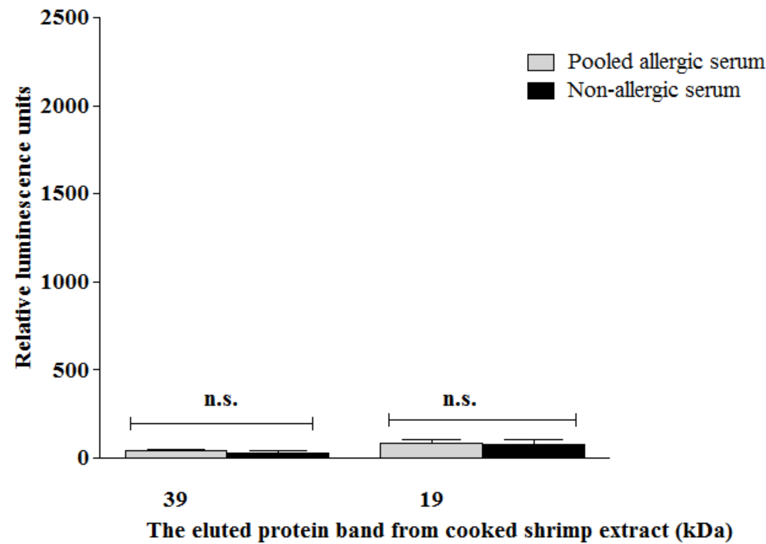


Figure 10. The IgE crosslinking ability of the elute protein bands from shrimp extracts.

RS-ATL 8 cells were sensitized with diluted pooled sera (1:100) from shrimp allergic patient and non-allergic healthy control serum overnight. Cells were stimulated with eluted protein bands of raw (A) and cooked shrimp extracts excised from 1-DE (B). Positive controls included in this experiment (IgE + anti-IgE) are not shown. The results of pooled allergic serum were shown in the gray columns and non-allergic healthy control serum were shown in black columns. Data are mean ± SD in triplicates. T-test results indicate $P<0.05$ significance for pooled allergic serum compared non-allergic healthy control serum. * : $P<0.05$, not significant difference.

4.7. Protein identification by MS

The allergen protein band from raw shrimp at 115 and 38 kDa that had ability to induce IgE cross-linking (Figure 10A) were excised from 1D-PAGE (Figure 9A). The protein in the bands were analysed by MS. Table 1 and 2 summarized the results of all identified proteins from black tiger shrimp in each band at 115 and 38 kDa. Five

major proteins myosin heavy chain type 1, myosin heavy chain type 2, myosin heavy chain type 3, myosin heavy chain type 6A and ubiquitin-activating enzyme (E1) (protein score 561, 118, 511, 511, 68 respectively) were identified in the 115 kDa band (Table 1). Similarly, three major proteins were identified in the 38 kDa band as glyceraldehyde-3-phosphate dehydrogenase, arginine kinase and crustacyanin C2 (protein score 1426, 108, 18, respectively) (Table 2). Among these proteins, ubiquitin-activating enzyme (E1) at 115 kDa and crustacyanin C2 at 38 kDa are a novel shrimp allergen candidate.

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Table I. Proteins with molecular weight of 115 kDa identified by MS in black tiger shrimp.

No	Protein candidate	Protein score	Nominal mass	No. of peptide matches
1	myosin heavy chain type 1	561	220933	33
2	myosin heavy chain type 2	118	220231	21
3	myosin heavy chain type 3	511	34519	10
4	myosin heavy chain type 6a	511	33075	10
5	ubiquitin-activating enzyme E1	68	116441	8

Table II. Proteins with molecular weight of 38 kDa identified by MS in black tiger shrimp.

No	Protein candidate	Protein score	Nominal mass	No. of peptide matches
1	glyceraldehyde-3-phosphate dehydrogenase	1426	13922	72
2	arginine kinase	108	40427	7
3	crustacyanin C2	18	19816	4

CHAPTER V

DISCUSSION

An accurate diagnosis of food allergy is essential for proper treatment of allergic symptoms. One of the important problems in management of shellfish has been the lack of a definite diagnostic assays because of the poor characterization of biological and immunological properties of the allergens. The primary objective of this study is to compare the IgE reactivity of allergic subjects to raw and cooked protein of black tiger shrimp. Our ELISA results showed that the pattern of serum IgE reactivity in shrimp patients to raw extract was higher than that against the cooked extracts (Figure 4). The similar findings were demonstrated by other groups on other food allergens [20, 52, 53]. A study by Paschke et al. [54] showed that heating of cow's milk for 10 minutes could decrease the allergenecity of bovine allergens. This may be due to epitope structures modification from cooking process; likewise, Nakamura et al. [55] found that the Maillard reaction could cause reduction in the allergenecity of squid tropomyosin (TM) in some epitopes. Our results are consistent with this hypothesis that reduction in shrimp allergenecity is caused by thermal treatment [56].

In SDS-PAGE analysis, the most protein bands were revealed in raw shrimp extract. Other studies have also reported similar observation [16, 31, 52] (Figure 6). These bands corresponding to 38, 41 and 19 kDa were revealed in both extracts. From the estimate molecular weight, they are likely to be tropomyosin, arginine kinase and myosin light chain, respectively. Several protein bands that were found in the raw extract were not detectable in the cooked extract due to the cooking

process. Protein may be degraded and/or a loss of secondary and tertiary protein structures while retaining their primary structure [16, 53]. From Western blot reactivity, 100% of patients showed IgE binding to raw extract at approximately region 32-39 kDa and also 75% in cooked extract. In various studies, they identified this protein in this area as tropomyosin that is a heat-stable and water-soluble [20, 30, 36, 53, 56-59] (Figure 7). Moreover, a 40-56 kDa, arginine kinase, was identified as a minor allergen in raw (96%) and cooked (21%) shrimp extract [36, 60]. Interestingly, the raw shrimp extract showed high IgE reactivity in both ELISA and Western blot. The IgE binding of non allergic control at 33 kDa in raw shrimp extract could be explained by non-specific binding of secondary antibody (Supplementary Figure 15 in the appendix F). However, for the three patients (No. 4, 8 and 19) there were no IgE reactivity detected by Western blot (Figure 7C). These patients may not have specific IgE against cooked shrimp proteins. This may be because it depends on indivadual IgE reactivity to the protein or cooking has decrease the allergenicity of the shrimp extract [31, 53].

ELISA and Western blot are immunological method that depend on the binding of specific IgE in sera of allergic patients to allergens. The result using this method often does not correlate with data obtained from clinical history and *in vivo* test (skin prick test) [11, 39]. Furthermore, they do not provide any information on the capacity of protein to promote IgE-immediated hypersensitivity reaction [39, 61]. Therefore, the results could lead to a false positive test [49]. In contrast, the assay using the activation of mast cell requires two or more epitope on the allergen to cross-link specific IgE molecules that bound its receptor (FcεRI) on the surface of mast cells [61]. Skin prick test or oral food challenges can increase risk of severe symptoms. In recent years, basophil activation tests (BAT) have been used for *in vitro* diagnosis of IgE-immediated hypersensitivity reaction based on cellular mechanisms

and the histamine release [41, 62, 63]. This test is not used as standard diagnosis due to restriction that it needs to be performed within one day after whole blood sampling [41]. Recently, it was reported that rat basophillic leukemia (RBL) derived mast cell line was developed for assessment the the cross linking capacity of allergens in phase of mesurement allergen-specific IgE only [11, 39, 64]. The reporter cell line (RS- ATL8) was used to this study. The crude of raw and cooked shrimp extracts that induce IgE cross-linking using this cell line have not been reported. The results showed in Figure 8 demonstrated that RS-ATL8 reporter cell line has the high sensitivity to detect crude of raw and cooked shrimp allergens at 10 fg/ml and 100 fg/ml, respectively. This assay is high sensitivity and directly determine the biological activity of the binding between IgE and allergens [11]. Therefore, it can be used for screening the level of allergen that induce IgE cross-linking.

In recent years, the identification of the food allergens used proteomic analysis [47]. In various studies, they have identified allergen in black tiger prawn by a combination of Western blot, two-dimensional electrophoresis (2-DE) and mass spectrometry [20, 52, 65], but they did not test on ability of allergens to induce IgE cross-linking . In this study, we applied the 2-DE and reporter cell line for identifying shrimp allergens. Several protein spots from shrimp extract of molecular weight ranging from 11 to 135 kDa extracts with pI from 3 to 10 were collected. We found that all ten spots did not induce IgE cross-linking (data not shown) (Figure 9). This is probably due to insufficient amount of protein in the eluted protein that it is not enough to induce IgE crosslinking or may be due to other molecules that interfere with the system.

Using 1-DE and Western blot, we selected four single bands from raw shrimp extract and two single bands from cook shrimp extract that clearly interacted with IgE antibodies from patient sera for treatment of reporter cell line. The 115 and 38

kDa bands from raw shrimp extract have the ability to induce an IgE cross-linking (Figure 11A). In addition, Western blot results in there regions correlated with the reporter cell lines results. On the other hand, the 115 and 38 kDa band of raw shrimp extract revealed a weak reactivity but they are sufficient to induce an IgE cross-linking (Figure 11) and could be recognized by pooled serum from shrimp allergic serum in the result of Western blot (Figure 7B). Interestingly, the 41 kDa band from raw shrimp extract had clear intensity that stronger than those in shrimp extract bands in Western blot, but the eluted band did not induce an IgE cross-linking (Figure 11A). The cooked shrimp extract bands at 38 and 19 kDa did not induce an IgE cros-slinking (Figure 11B). The IgE binding to raw extract at approximately at 33 kDa was non-specific (Supplementary Figure 15 in the appendix F). The reporter cell line technique is sensitive, specific and reproducible which can detect allergens with less than 1ng of crude shrimp extract (Figure 8) [11]. Western blot and reporter cell line confirmed the allergenicity of two protein bands from raw shrimp extract in this study. However, further analyses the eluted protein bands should be identified by mass spectrometry and optimized the amounts of protein. The eluted protein band in this study may be used *in vitro* technique to confirm traces allergens in food or to develop the diagnosis.

From the reporter cell line results, we identified proteins in the two protein bands from raw black tiger shrimp extract by MS (Table I and II). The results indicated that 115 kDa had 5 major proteins sush as myosin heavy chain type 1, myosin heavy chain type 2, myosin heavy chain type 3, myosin heavy chain type 6A and ubiquitin-activating enzyme. The 38 kDa contained 3 major proteins sush as glyceraldehyde-3-phosphate dehydrogenase, arginine kinase and crustacyanin C2. Ubiquitin-activating enzyme E1 and crustacyanin C2 have not been reported to be shrimp allergens. Myosin heavy chain (MHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

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were reported as an allergen in banana shrimp (*Fenneropenaeus merguiensis*) [58]. The study by Cristina el al. reported the protein allergens in *Solenocera melantho* shrimp by using MS as α-actinin, β-actin, arginine kinase, biphosphate aldolase, fructose, sarcoplasmic calcium-binding protein, and ubiquitin [59]. Ubiquitin-activating enzyme E1 is enzyme for activating ubiquitin [66] and Crustacyanin is a carotenoprotein pigment that can be modification of lobster shell colour [67]. These proteins have not been reported as shrimp allergens. Therefore, using RS-ATL8 cell line may lead to identify novel shrimp allergen that can cross-link IgE upon binding.

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CHAPTER VI

CONCLUSIONS

ELISA results showed that the pattern of serum IgE reactivity in shrimp allergic patients to raw shrimp extract was higher than the cooked shrimp extracts. In SDS-PAGE, the most protein bands appeared in raw shrimp extract. From Western blot, all patients showed IgE binding to raw shrimp extract in the range of 32-39 kDa of raw shrimp extract and 75% in cooked extract. The minimum concentration of crude shrimp extract to induce IgE cross-linking in RS-ATL8 cell line were 10 fg/ml in raw shrimp extract and 100 fg/ml in cooked shrimp extract. The ten spots from 2-DE did not induce IgE cross-linking in RS-ATL8 cell line. We attempted to identify shrimp allergen that induce the cross-linking of IgE by using the eluted protein from 1-DE. The 115 and 38 kDa bands from raw shrimp extract have the ability to induce an IgE cross-linking in RS-ATL8 cell line and the proteins in these bands were analysed by mass spectrometry (MS). Ubiquitin-activating enzyme E1 at 115 kDa and crustacyanin C2 at 38 kDa from black tiger shrimp were identified which as a novel shrimp allergen.

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APPENDICES

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APPENDIX A

Reagents for tissue culture

1. Complete MEM with serum (100 ml)

MEM with glutaMax I	90	ml
Heat inactivated FBS	10	%
Penicillin	100	U/ml

2. Complete MEM with antibiotic (10ml)

Completed MEM with serum	10	ml
Geneticin	0.5	mg/ml
Hygromycin B	0.2	mg/ml

3. Freezing medium (10 ml)

Complete MEM with serum	9	ml
DMSO	1	ml

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4. FBS inactivation

Commercial FBS which were stored at -20°C was thawed at 4°C for overnight and inactivated at 56°C for 30 min.

5. 1x PBS pH 7.4 (1000 ml)

NaCl	8	g
KCl	0.2	g

Na_2HPO	41.44	g
KH_2PO_4	0.24	g
MilliQ water	1000	ml

Adjust the pH to 7.4 and sterilized by autoclaving at 121°C and pressure 15 psi for 15 min. Store the solution at room temperature

APPENDIX B

Reagents for ELISA

1. 1X PBST solution (500ml)

1X PBS	500	ml
Tween20	0.05	%

Add Tween20 250 µl in 1X PBS 500 ml. The solution was mixed and store at room temperature

2. Blocking solution

5% skim milk in 1XPBST solution

3. TMB buffer (Potassium citrate tri basic monohydrate= K_3citric x $1H_2O$) **(500ml)**

Citric acid	19.69	g
Potassium citrate	33.25	g
MilliQ water	500	ml

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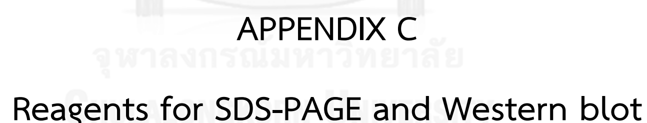
Dissolve citric acid 19.69 g with milliQ water 400 ml. Then add potassium citrate 33.25 g and adjust the pH to 4 with citric acid solution. The solution was added milliQ water up to total 500 ml and sterilized by autoclaving at 121°C and pressure 15 psi for 15 min. Store the solution at 4°C

4. H_2O_2 + TMB (3,3’,5,5’-Tetramethyl benzidine) substrate (10ml)

TMB	2.5	mg
DMSO	250	µl
TMB buffer	9.9	ml
H_2O_2	3.4	µl

Dissolve TMB 2.5 mg in 250 µl of DMSO. Then add TMB buffer 9.9 ml and H_2O_2 3.4 µl and then mix the solution.

APPENDIX C



Reagents for SDS-PAGE and Western blot

<u>Buffers for SDS-PAGE</u>

1. 10% SDS-polyacrylamide gel (8 ml)

Sterile water	3.836	ml
40% Acrylamide and Bis-acrylamide solution	2	ml
1.5 M Tris-HCL, pH 8.8	2	ml
10% SDS	0.08	ml

10% APS	0.08	ml
TEMED	0.004	ml

2. 5% stacking gel (2 ml)

Sterile water	1.204	ml
40% Acrylamide and Bis-acrylamide solution	0.25	ml
1 M Tris-HCL, pH 6.8	0.504	ml
10% SDS	0.02	ml
10% APS	0.02	ml
TEMED	0.002	ml

3. 2x Laemmli buffer (10 ml)

0.5 M Tris-HCL pH 6.8	1.25	ml
10% (w/v) SDS	1	g
glycerol	5	ml
0.01% (w/v) bromophenol blue	0.001	g
Sterile water	3.75	ml
5% (v/v) β-mercaptoethanol	0.5	ml

Aliquoted to 950 µl and stored -20 °C. Immediately before used, add 50 µl of β-mercaptoethanol and the components mixed thoroughly by vortexing.

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4. 6x Laemmli buffer (10 ml)

0.5 M Tris-HCL pH 6.8	7	ml
10% (w/v) SDS	1	g
glycerol	3	ml
0.01% (w/v) bromophenol blue	0.001	g
5% (v/v) β-mercaptoethanol	0.5	ml

Aliquoted to 950 µl and stored -20 °C. Immediately before used, add 50 µl of β-mercaptoethanol and the components mixed thoroughly by vortexing.

5. 5x running buffer (1000 ml)

Trisma-base	15.1	g
Glycine	94	g
SDS	5	g
Distilled water	1000	ml

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6. 1.5 M Tris-HCl, pH 8.8 (1000 ml) (stock buffer for separating gels)

Trisma-base 181.71 g was dissolved in 800 mL distilled water, pH was adjusted into 8.8. with concentrated HCl . Finally volume was adjusted into 1000 ml.

7. 1.5 M Tris, pH 6.8 (1000 ml) (stock buffer for stacking gels)

Trisma-base 181.71 g was dissolved in 800 mL milliQ water, pH was adjusted into 6.8. with concentrated HCl . Finally volume was adjusted into 1000 ml.

8. Coomassie Blue stock solution(200ml)

PhastGel^{TM} Blue R	1	tablet
Absolute methanol	120	ml
Distilled water	80	ml

PhastGel^{TM} Blue R 1 tablet was dissolved in 80 mL of distilled water and stired for 5 to 10 minutes.Then add methanol 120 ml and stir until all of the dye was dissolved. The solution was filtrated through whatman filter paper No.1.

9. Coomassie Blue working solution(10ml)

Coomassie Blue stock solution	1	ml
Destain solution	9	ml

10. Destain solution(100ml)

Absolute methanol	30	ml
Acetic acid	10	ml
Distilled water	80	ml

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11. Gel Drying Solution

Methanol	30	%
Glycerol	5	%

<u>**Buffers for Western blot**</u>

1. Transfer buffer (1000 ml)

Trisma base	5.08	g

Glycine	2.9	g
SDS	0.37	g
Sterile water	800	ml
Absolute methanol	200	ml

Store at 4°C

2. Blocking solution(100ml)

1XPBST	100	ml
Non-fat dry milk	5	g

3. ECL substrate of HRP

90 mM of Coumaric acid was dissolved in DMSO in total volume 10 ml,aliquoted and stored -20°C

250 mM of Luminol was also dissolved in DMSO in total volume 10 ml, aliquoted and stored -20°C.

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Solution A

100 mM Tris-HCL pH8.5(stored at 4°C)	2.5	ml
90 mM coumaric acid	11	µl
250 mM luminol	23	µl

Solution B

100 mM Tris-HCL pH 8.5(stored at 4°C)	2.5	ml

30% H_2O_2	1.5	µl

16. Film developer and fixer

Flim developer and fixer were diluted in tap water at dilution 1:4 in total volume 500 ml.

APPENDIX D

Reagents for protein elution

1. Elution buffer (30ml)

Trisma-base 0.9 g was dissolved in 30 mL distilled water, pH was adjusted into 6.8. with concentrated HCl and autoclaved at 121°C , pressure 15 psi for 15 min . After sterilization, The solution was added 0.1% of SDS.

APPENDIX E

Reagents for Two-dimensional gel electrophoresis (2-DE)

1. 10% separating gel (5 ml)

Sterile water	2.42	ml
40% Acrylamide and Bis-acrylamide solution	1.25	ml
1.5 M Tris-HCL, pH 8.8	1.25	ml
10% SDS	0.05	ml
10% APS	0.025	ml

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TEMED	0.00165	ml

2. Rehydration buffer (50 ml)

Urea	21	g
Thiourea	8	g
Sterile water	50	ml

The solution was filtrated through whatman filter paper No.1, aliquoted and stored at -20 °C. Immediately before use, dissolve DTT 1.55 and CHAPs 10 mg into rehydration buffer solution 0.25 ml. Then the solution was added Bio-lyte ampholytes 1.25 µl and trace bromophenol blue solution. The components mixed thoroughly by vortexing.

3. Equilibration buffer (100ml)

1.5 M Tris-HCl, pH 8.8	3.3	ml
Urea	36	g
50% (v/v) glycerol	40	ml
20% (w/v) SDS	10	ml

Adjust volume into 100 ml, aliquoted and stored at -20°C

4. Equilibration buffer I

Equilibration buffer	2.5	ml
DTT	0.05	g

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5. Equilibration buffer II

Equilibration buffer	2.5	ml
iodoacetamide	0.0625	g

APPENDIX F

Supplement

1. Optimal concentration of pooled serum from shrimp allergic patients

The RS-ATL8 cells were sensitized with serial dilution of treated and untreated pooled serum (1:10, 1:50 and 1:100) and stimulated with 1 μg/ml of the affinity purified goat anti-human IgE (Bethyl, USA) (Figure 11). Positive control (IgE + anti-IgE) and negative control (pooled serum only) included in this experiment are not shown. There was non statistically significant for dilution between treated and untreated pooled serum at 1:100. Conclude that appropriate concentration of pooled serum was 1:100 dilution and non cytotoxicity.

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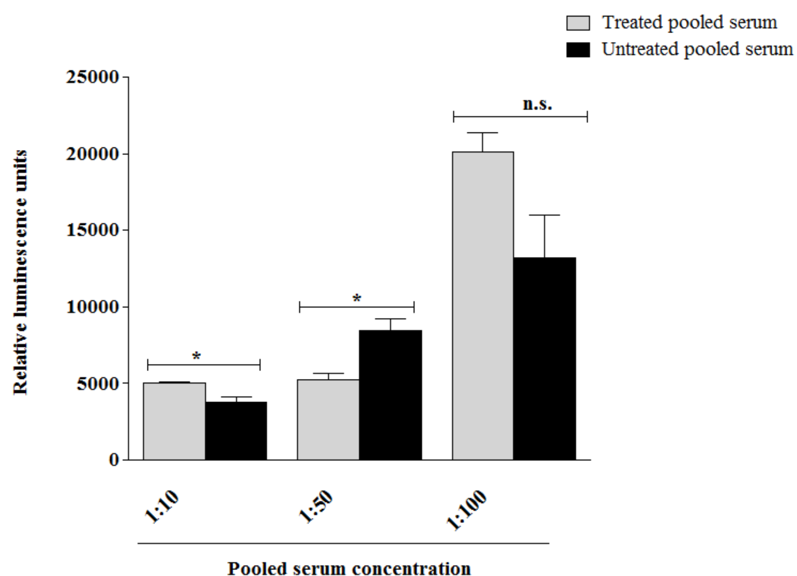

Figure 11. Optimal concentration of pooled serum from shrimp allergic patients. The pooled serum from shrimp allergic patients were treated at 56 ^{o}C for 30 min and untreated. The RS-ATL8 cells were sensitized overnight with various concentrations of pooled serum and stimulated with 1 μg/ml of goat anti- IgE purified. Luminescence was measured 3 hours after stimulation. Data are mean ± SD of the readings of triplicates. T-test results indicate P<0.05 significance for pooled allergic sera compared untreated serum control. * : *P*<0.05, n.s., not significant difference. Treated pooled serum is shown by the gray columns and untreated pooled serum is shown by black columns.

2. Optimal concentration of goat anti- IgE purified for as a positive control

The relative luminescence of the affinity purified goat anti-human IgE (Bethyl, USA) was similar to all concentration (Figure 12). Therefore, we selected 1ug/ml of goat anti human IgE for the treatment

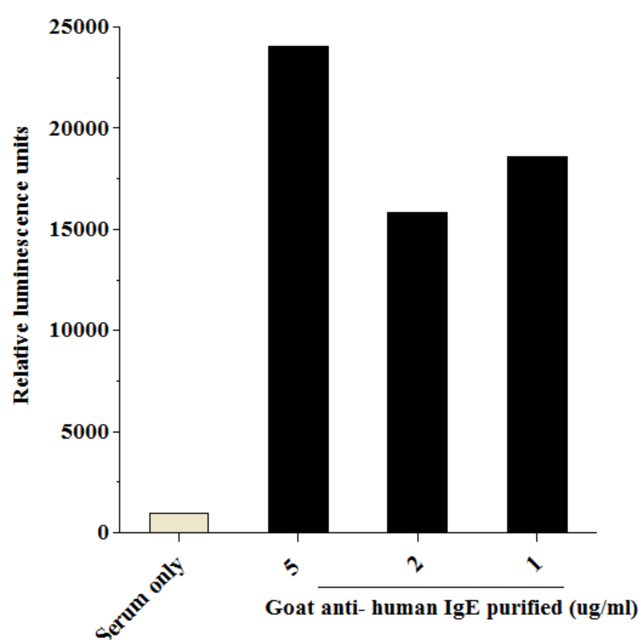

Figure 12. Optimal concentration of goat anti- human IgE purified for as a positive control.

RS-ATL8 cells were sensitized overnight with 1:100 concentration of pooled serum. Cells were then stimulated with different concentration (ranging from 5 to 1ug/ml) of goat anti-human IgE. Luminescence was measured 3 hours after stimulation. Pooled serum is shown by the gray columns (negative control) and pooled serum stimulated with different concentration of goat anti- human IgE purified are shown by black columns.

3. Screening of raw shrimp extract allergenicity with individual shrimp allergic serum

Cells were sensitized with 1:100 of individual shrimp allergic serum and non-allergic serum overnight. They were stimulated with raw shrimp extracts at 1 μg/ml for 3 hours. Ten of 23 patients(No. 1-6, 14,19, 21 and 23) had specific IgE to induce reporter activation after stimulated with the raw shrimp extract that higher than the control serum. A response curve of RS-ATL8 were shown in Figure 13. Positive control (IgE + anti-IgE) and negative control (RS-ATL8 cell lines only) included in this experiment are not shown.

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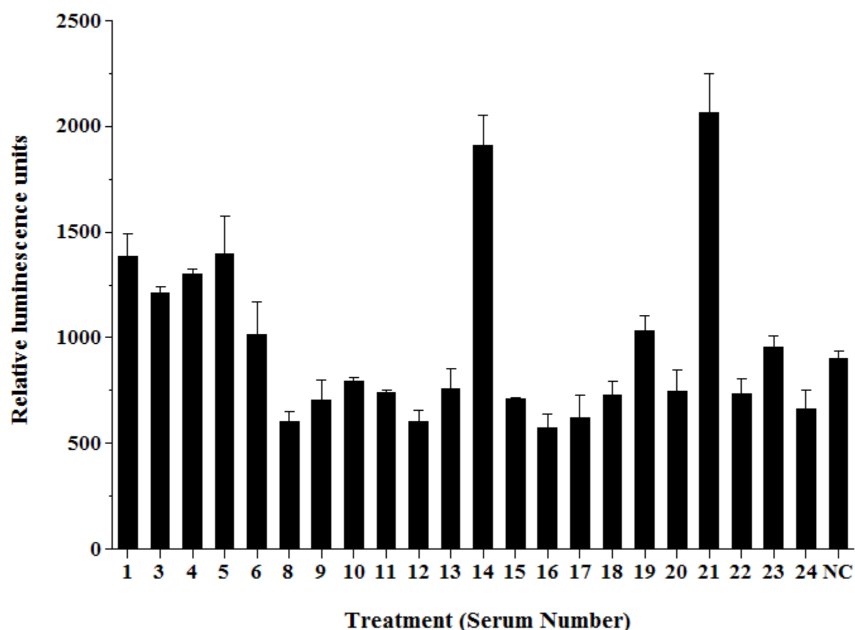

Figure 13. Screening of raw shrimp extract allergenicity with individual shrimp allergic serum.

The RS-ATL8 cells were sensitized overnight individual shrimp allergic serum and stimulated with 1 µg/ml of raw shrimp extract. Luminescence was measured 3 hours after stimulation. Data are mean ± SD of the readings of triplicates. One-24 were the number of patient sera and NC was control non-allergic serum.

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4. Western blot analysis

IgE binding about 33 kDa was non-specific binding (Figure. 14), each strip were incubated with only the secondary antibody, goat anti-human IgE labelled with horse radish peroxidase, at a dilution of 1:5000 (KPL, USA). The result showed that the cross reactive bands was still visible.

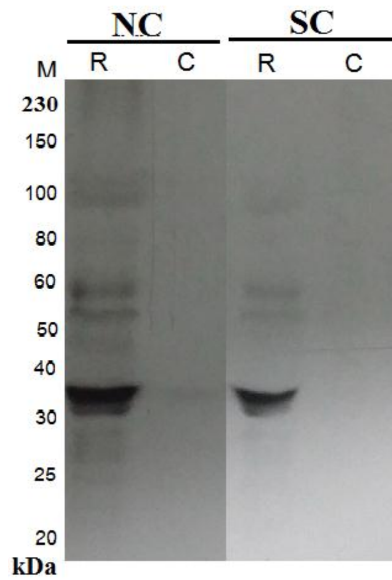
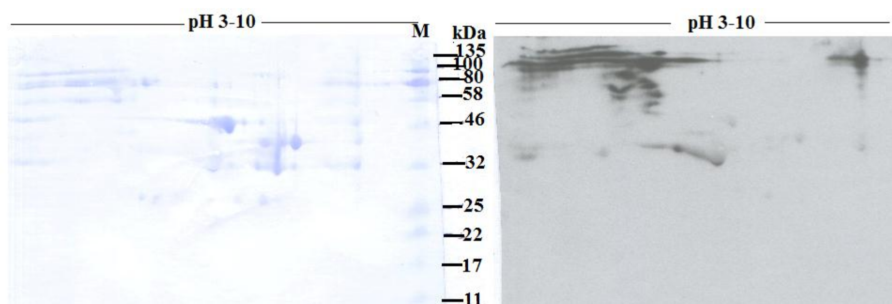


Figure 14. Western blot analysis confirming the binding of non-specific
Reactivity with control non-allergic serum (NC) and secondary antibody (SC). Molecular weight marker was shown left. Lane R, raw shrimp extract. Lane C, cooked shrimp extract.

4. Two-dimensional electrophoresis (2-DE) and Western blot analysis of raw shrimp extract

The raw shrimp proteins were separated by two-dimensional electrophoresis and recognized by Western blot using pooled sera from 5 shrimp allergic patients. The several spots of protein recognized with pooled serum at high molecular weight and pI ≈ 3-5. None of the protein spot recognized with non allergic control serum (data not shown).

A. 2D-PAGE B. 2D- Western blot



C. The reference gel from SDS-PAGE

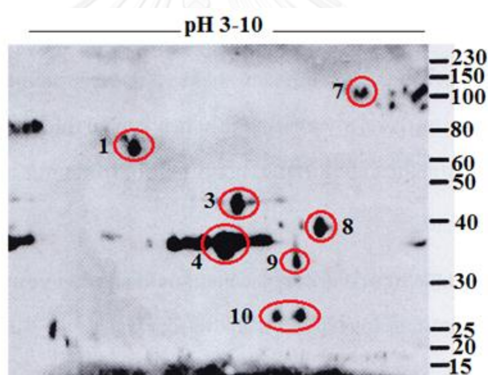


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Figure 15. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) profiles.

The raw shrimp extract was separated by 2-DE using 7 cm, pH 3–10, nonlinear followed by SDS-PAGE 10% and stained with Coomassie brilliant blue R-250 (A). For Western blot, protein spots of raw shrimp extract was interacted with the pooled serum were diluted at 1:100 (B). (C) Western blot with individual serum performanced by Ms. Wanaporn Yimchuen (2013) as a reference spot.

5. Infomation of shrimp allergic patient

Subject	Age	Sex	Symptoms	SPT to shrimp	IgE to shrimp
	(years)				by CAP (kUA/L)
1	31	F	CU	P (raw)	0.29
2	8	M	AE ,U, CU	P	> 100
3	42	F	NP, IT, SN, AR, R, S	P (cook , raw)	0.44
4	34	F	W	P	< 0.35
5	15	M	N/A	N/A	N/A
6	20	F	N/A	N/A	1.14
7	30	F	anaphylaxis	P (raw)	9.78
8	9	M	anaphylaxis	P	13.2
			W, GI, V, CU, AE, LS		
			ES, CT, AN, W		
9	26	F	N/A	N/A	0.07
10	29	F	AE, GI, O	P	0.35
11	11	M	Anaphylaxis	P	9.09
12	11	F	SN, EP	P	0.62
13	17	M	N/A	N/A	7.57
14	N/A	F	N/A	N/A	N/A

15	10	F	Anaphylaxis	P	0.91
16	N/A	F	N/A	N/A	N/A
17	N/A	F	N/A	N/A	N/A
18	17	M	AE, O	P	N/A
19	21	F	SN, NP, S	P	0.11
20	14	F	R, EP, LS, CT	P	1.52
21	13	M	N/A	N/A	N/A
22	54	F	EP, NP, LS, W, N	P	N/A
23	N/A	M	N/A	N/A	N/A
Subject	Age	Sex	Symptoms	SPT to shrimp	IgE to shrimp
	(years)				by CAP (kUA/L)
24	51	M	CU, ES, SN	P	N/A
NC	36	F	-	-	-

Table III. Infomation of 24 shrimp allergic patient and one nonallergic control. **Sex**; M : male , F : female; **Symtoms** : AS, asthma; AN, anaphylaxis; AP, abdominal cramp; AR, allergic rhinitis; AE, angioedema; CU, cutaneous (rash, erythemal); C, conjunctivitis; FA, other food allergy; GI, gastrointestinal symptoms; NC, nasal congestion; NP, nose pruritus; N,nausea; IT, itchy throat ; SN, stuffy nose ; O, oral pruritus ; R, rhinorrhea ; RC, rhinoconjunctivitis; U, urticaria ; S, sneezing ; Sw, difficulty swallowing; T, throat tightness; W, wheezing. **SPT (skin prick test)**; P: positive, NC was control non-allergic serum and 1-24 were the number of patient sera., N/A : not available.

VITA

I graduated from the Department of Biology, Faculty of Science, Narasuan University with a Bachelor's degree in 2006. I started working with Faculty of Veterinary Medicine, Chiang Mai University as scientist for 5 years. After that I moved to Bangkok in 2011 and started working as Scientist at Department of Biology, Faculty of Science, Mahidol University until the present.

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