

Extraction of Squid (*Photololigo Duvaucelii*) Myofibrillar and Sarcoplasmic Proteins to Create A Skin Prick Test Reagent In The Diagnosis of Food Allergy

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Abstract: The major and minor allergens in fish and shellfish that have been identified are broadly spread in the myofibrillar and sarcoplasmic proteins. This study aimed to create the SPT reagents of squid myofibrillar and sarcoplasmic proteins to improve sensitivity and specificity diagnosis. Myofibrillar protein fraction was composed of 11 protein bands with the molecular weights of 18.2-108.4 kDa and sarcoplasmic protein fraction was composed of 13 protein bands with the molecular weights of 9.5-119.3 kDa. The allergenic myofibrillar protein has the molecular weights of 8.9-122.7 kDa while the allergenic sarcoplasmic protein has the molecular weights of 11.8-143.9 kDa. Each allergen extract was formulated into a SPT reagent met the requirements of the European Pharmacopoeia Monograph on Allergen Products 7 (2010:1063) for the parameters of moisture content, protein content, sterility and microbiology. A SPT to the respondents indicated that the sensitivity value of squid myofibrillar reagent was 86% and squid sarcoplasmic reagent was 64% with a negative error rate for each myofibrillar and sarcoplasmic reagents was 14% and 36%, respectively. The result diagnosis of squid myofibrillar and sarcoplasmic would generate sensitivity value at 100%. The separation of squid proteins into myofibrils and sarcoplasm for SPT could improve its sensitivity value and lowered the negative misdiagnosis. The specificity of each myofibrillar and sarcoplasmic proteins was 100% with an error rate of positive diagnosis occurrence at 0%.

Keywords - allergy diagnosis, myofibrillar, sarcoplasmic, squid, skin prick test.

I. INTRODUCTION

Indonesia's squid (*Photololigo duvaucelii*) production volume in 2012 was 140.924 tonnes and has increased by 18.83% since 2007 [1]. Indonesian people's consumption level for fishery products, including squid, also increased by 5.21% in 2007 to 2012. This increased consumption is due to squid's nutrient content and preferred taste. In some people, squid consumption can cause type 1 allergic reaction (hypersensitivity) mediated by IgE antibodies and can be fatal. Allergic reaction is mainly triggered by allergen content in the squid. Squid is one of the top seven major causes of allergic reaction in the world [2]. The incidence of allergies from consuming squid in Indonesia is high, especially in adults.

Skin prick test (SPT) is a reliable method for diagnosing allergies that are mediated by IgE antibodies in patients with rhinoconjunctivitis, asthma, urticaria, anaphylaxis, atopic eczema, drug and food allergies [3]. SPT method was first published by Helmtraud Ebruster in 1959 and has been used as a primary diagnostic method for detection of type I hypersensitivity reactions [4]. The main material used for the SPT is allergen extract or SPT reagent. Allergen extract is a mixture of a variety of different proteins, glycoproteins and polysaccharides. Allergen extract should contain major and minor allergens because not all patients are allergic to any antigen in the extract. Each manufacturer produces different quality and specifications of SPT reagent, although derived from the same allergen. This matter will cause different SPT results although derived from the same allergens but produced by different manufacturers [3]. The variations of SPT reagent quality caused by differences in the raw materials used, extraction methods, and formulation during the manufacture of SPT reagent [5]. Raw material factors that could affect the quality of SPT reagent are the origin of raw materials, varieties, transportation, food processing, and changes during storage [6]. The same species but derived from different regions have different allergen compositions and also different IgE reactivity [7]. Extraction method that is widely used for animal products such as eggs and marine products are generally using PBS solution. An isolation of snow crab meat protein using PBS solution (pH 7.2, 0.01 mol/L) [8].

The major and minor allergens in fish and shellfish that have been identified are broadly spread in the myofibrillar and sarcoplasmic proteins. The examples are allergens in the north sea shrimp (*Crangon Crangon*). Based on Allergen Nomenclature, allergens in the north sea shrimp (*Crangon Crangon*) can be grouped into myofibrillar and sarcoplasmic proteins. Allergens in myofibrillar group are Cra c 1 (MW 38 kDa), Cra c 5 (MW 17.5 kDa), Cra c 6 (MW 21 kDa) Cra c 8 (MW 28 kDa) whereas allergens in sarcoplasmic group are Cra c 2 (45 kDa) and Cra c 4 (25 kDa) [9]. This study aimed to create the SPT reagents with separating myofibrillar and sarcoplasmic proteins to be expected to improve the sensitivity and selectivity of the diagnosis, thereby reducing the misdiagnosis occurrence. The quality requirements of SPT reagent referred to European Pharmacopoeia 7 Monograph on Allergen Products (2010:1063) standard [10].

II. MATERIALS AND METHODS

2.1 Materials

The main material used in this study was fresh squids (*Photololigo duvaucelii*) obtained from the Muara Angke fish market, North Jakarta. The main chemicals used were BSA (bovine serum albumin), acrylamide, glycine, 2-Mercaptoethanol, 0.05 M carbonate-bicarbonate buffer at pH 9.6. coomassie brilliant blue R-250, coomassie brilliant blue G-250, IgE antibody, anti-IgE human antibody labeled with HRP (Horseradish Peroxidase) enzyme, DAB (3,3'-Diaminobenzidine) substrate, TMB (3,3',5,5'-Tetramethylbenzidine) substrate, N,N'-methylenebisacrylamide, low molecular weight proteins (LMW) Fermentas® (containing 7 types of protein, which are β -galactosidase standards (MW: 116 kDa), bovine serum albumin (MW: 66.2 kDa), ovalbumin (MW: 45 kDa), lactase dehydrogenase (MW: 35 kDa), REase BSP 981 (MW: 25 kDa), β -lactoglobulin (MW: 18.4 kDa), and lysozyme (MW: 14.4 kDa). Other chemicals were purchased from Sigma.

2.2 Equipments

The equipments used were high speed microcentrifuge, SDS-PAGE Bio-Rad Mini-Protean II tool, immunoblotting Mini Trans-Blot® Electrophoretic Transfer Cell Bio-Rad tool, Costar® 96-well ELISA microplates, LabSystem Multiskan EX ELISA reader, UV-VIS spectrophotometer, freeze drier, nitrocellulose membranes for blotting pore size 0.45 μ m, size 15 cm x 15 cm (Sigma N8267), pH meter, sonicator, vortex mixer, stirrer, 0.5 μ L to 1000 μ L micropipettes, 0.2 μ m SFCA syringe filter, Whatman #1 filter paper, and other glasswares.

2.3 Extraction of Squid Myofibrillar dan Sarcoplasmic Proteins

20 grams of cleaned squid was put into a blender, added with 200 mL of phosphate buffer (pH 7.5) with ionic strength (I) = 0.05 M (15.6 mM Na_2HPO_4 , 3.5 mM KH_2PO_4) then added with 0.5 mL aprotinin (protease inhibitor) and homogenized 3 x @ 1 minute. The formed mixture was centrifuged at 4 °C with 5000 \times g speed for 25 min. The centrifuge result consisted of liquid at the top layer (supernatant) and pellet (solid) at the bottom layer. The supernatant was collected in a 50 mL centrifuge tube and the pellets remained in a centrifuge tube were re-added with 200 mL of phosphate buffer (pH 7.5, I = 0.05 M) to be re-extracted. The supernatant was then combined in a 50 mL centrifuge tube (hereinafter called the sarcoplasmic protein fraction), while the pellets were further processed for myofibrillar protein extraction. The pellets were added with 200 mL of phosphate buffer (pH 7.5, I = 0.5 M; 0.45 M KCl, 15.6 mM Na_2HPO_4 , 3.5 mM KH_2PO_4) and homogenized 3 x @ 1 minute. The formed mixture was centrifuged at 4 °C with 5000 \times g speed for 25 min. The obtained supernatant was the myofibrillar protein extracts and the obtained pellets were re-extracted [11].

2.4 Serum Preparation

Serum collection was conducted in two steps. The first step was conducted toward 25 respondents who have food allergy (discovered from interviews). This serum was later used to conduct the allergenicity test of squid myofibrillar and sarcoplasmic proteins using ELISA and immunoblotting methods prior to the SPT. The second step of serum collection was conducted toward 12 respondents with squid allergy and 12 respondents without squid allergy which later used to conduct allergenicity test of SPT reagent. Second-step respondents attended SPT in allergy clinic. Serum collection and SPT were conducted by medical personnel and allergologist physician who have obtained permission from the Health Research Ethics Committee, Faculty of Medicine Indonesia University, Cipto Mangunkusumo Hospital with number of ethical approval is 199/H2.F1/ETIK/2014. 10 mL of each respondent's blood was withdrawn then placed in a bottle containing no EDTA. The withdrawn blood was allowed to stand for 1 hour, then centrifuged for 20 minutes at 2500 rpm (1250 g). The obtained supernatant was a serum containing IgE antibodies and stored at -20 °C [12].

2.5 SDS-PAGE Electrophoresis and Immunoblotting

Electrophoresis was conducted according to Laemmli [13] with 12% concentration of separating gel and stained with coomassie brilliant blue R-250. The unstained gel from electrophoresis result was transferred to 0.45 µm nitrocellulose membrane using transblotting equipment with 90 V voltage for 90 minutes. After being transferred, the membrane was removed from equipment and soaked with 50% methanol for 2 minutes, then blocked with 5% skim milk in PBST (Phosphate Buffered Saline with 0.05 % Tween® 20) for 1 hour at room temperature. The membrane was washed with PBST 3 times, each for 5 minutes. After being washed, the membrane was added with the allergic patient serum with 1:10 dilution in PBST, then incubated for 2 hours at room temperature. Washing was conducted again (3 times) with PBST, each for 5 minutes, then added with HRP conjugated monoclonal mouse anti-human IgE antibody (1:3000 dilution in PBST) and incubated for 1 hour while shaken. After that, the membrane was re-washed with PBST (3 times) each for 5 minutes, and added with DAB substrate. Positive detection results were marked with the formation of brown-colored band on nitrocellulose membrane [14].

2.6 Determination of Total IgE with ELISA Method

100 µL serum/well with a 1:10 dilution (in 0.05 M carbonate-bicarbonate buffer at pH 9.6) was attached to the microtiter plates and incubated for 1 night at 4 °C. Normal human sera (NHS) were used as control. The remaining sample was removed and the microtiter plates were washed 5 times with PBST (250 µL/well). After that, 200 µL/well of 5% skim milk in PBST was added and incubated for 1 hour at 37 °C. Furthermore, it was washed 5 times with PBST (250 µL/well) and added with 100 µL/well of HRP conjugated monoclonal mouse anti-human IgE antibody (with 1:6000 dilution in PBST), then incubated at 37 °C for 1 hour. The microtiter plates were washed with PBST (250 µL/well) for 10 times, then added with 100 µL/well of TMB substrate. Positive results were marked when blue color developed. After 5 minutes, the reaction was stopped by adding 100 µL/well of 2 M H₂SO₄, and the solution would turn bright yellow. Optical density (OD) was measured using ELISA reader at 450 nm. Mean ± 2SD of normal controls was taken as cut-off for ELISA positive results [12]; [15].

2.7 Determination of Specific IgE with ELISA Method

100 µL/well of each squid myofibrillar and sarcoplasmic proteins 10 µg/mL (in 0.05 M carbonate-bicarbonate buffer, pH 9.6) was attached to the microtiter plates, then incubated at 4 °C for 1 night. The remaining sample was removed and the microtiter plates were washed for 5 times with PBST (250 µL/well). After that, 200 µL/well of 5% skim milk in PBST was added and incubated for 1 hour at 37 °C. Furthermore, it was washed 5 times with PBST (250 µL/well). 100 µL/well of allergic patient serum with 1:10 dilution in PBST was added and incubated for 1 hour at 37 °C. Normal human sera (NHS) were used as control. After incubation, the microtiter plates were washed with PBST (250 µL/well) for 5 times, then added 100 µL/well of HRP conjugated monoclonal mouse anti-human IgE antibody (diluted 1:6000 in PBST), then incubated for 1 hour at 37 °C. Microtiter plates were washed with PBST (250 µL/well) for 10 times, and 100 µL/well of TMB substrate was added. Positive results were marked when blue color developed. After 5 minutes, the reaction was stopped by adding 100 µL/well of 2 M H₂SO₄, and the solution would turn bright yellow. Optical density (OD) was measured using ELISA reader at 450 nm. Mean ± 2SD of normal controls was taken as cut-off for ELISA positive results [12]; [15].

2.8 Creating Skin Prick Test Reagent

Each 0.2 g of myofibrillar and sarcoplasmic proteins isolates was dissolved in 2 mL of phosphate buffer saline (PBS) at pH 7.4, sonicated 5 x @ 1 minute in cold condition and then centrifuged at 11780 g for 15 minutes. The obtained supernatant was filtered using 0,2 µm syringe filter for sterilization in order to obtain a sterile protein stock solution. The procedure was conducted in a sterile and aseptic room. The protein content of stock solution was determined using the Bradford method (Bradford, 1976). Stock solution was then dissolved in 50% glycerol-saline solution containing 0.4% sterile phenol, thus the concentration became 1 mg/mL. The obtained solution was then called “squid myofibrillar and sarcoplasmic SPT reagent” which then tested chemically, sterilitically and microbiologically. If it was qualified from chemical, sterility and microbiological tests, the reagent would be used for SPT in humans [16].

2.9 Skin Prick Test

The SPT was conducted by allergologist physician who has received permission from the Health Research Ethics Committee with number of ethical approval is 199/H2.F1/ETIK/2014. SPT was conducted on the volar forearm. The area that dropped with reagent was marked with a ballpoint and disinfected with alcohol. 1 mg/ml histamine was used as a positive control and 50% glycerol-saline solution was used as a negative

control. Positive and negative controls were dropped on the opposite area then other reagents were dropped. Skin prick that has been dropped with histamine, control buffer, and allergen extracts using the brown marrow needle. The pricking process was conducted slowly with 45° slope the penetrated the epidermal layer without causing bleeding. The diameter of the wheal (urtica) on the skin was measured after 15-20 minutes by measuring the developed wheal (urtica). The test results were transferred to the millimeter block paper by making line surrounding the wheal border with marker pen (size 0.2), taped with masking tape and masking tape was affixed to the millimeter paper block. Each circle diameter on the tape was then measured. The result expressed as “0” when the wheal size is equal to the negative control (no wheal formed), “+1” if the wheal size is 25% -50% greater than the negative control (<3 mm), “+2” if the wheal size is 50%-75% greater than the negative control (3 mm-5 mm) and “+3” if the wheal size is equal to the histamine (5 mm-7 mm), and “+4” if the wheal size is 25%-50% greater than the histamine and “>+4” when the wheal size is more than 50% greater than the histamine. From the SPT results, sensitivity and selectivity of each reagent were measured [16].

III. RESULTS AND DISCUSSION

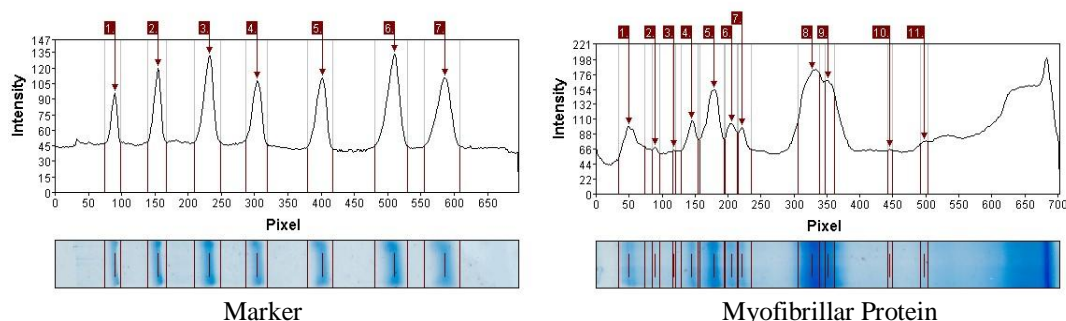
3.1 Analysis of Total IgE and Specific IgE Antibodies

Analysis of total IgE antibodies in the respondents serum aimed to determine the status of the respondent, whether they have positive or negative allergy. The selected respondents must have food allergy history based on the interview results. The analysis result showed that 25 respondents (respondent 1-25) have positive allergy and suitable for allergenicity test. Analysis of specific IgE antibodies aimed to determine the ability of squid myofibrillar and sarcoplasmic proteins to bind with specific IgE antibodies in the serum and also to determine whether the respondents have positive or negative squid allergy. 9 of 25 respondents (36%) have positive squid allergy and 11 respondents (44%) were allergic to squid myofibrils.

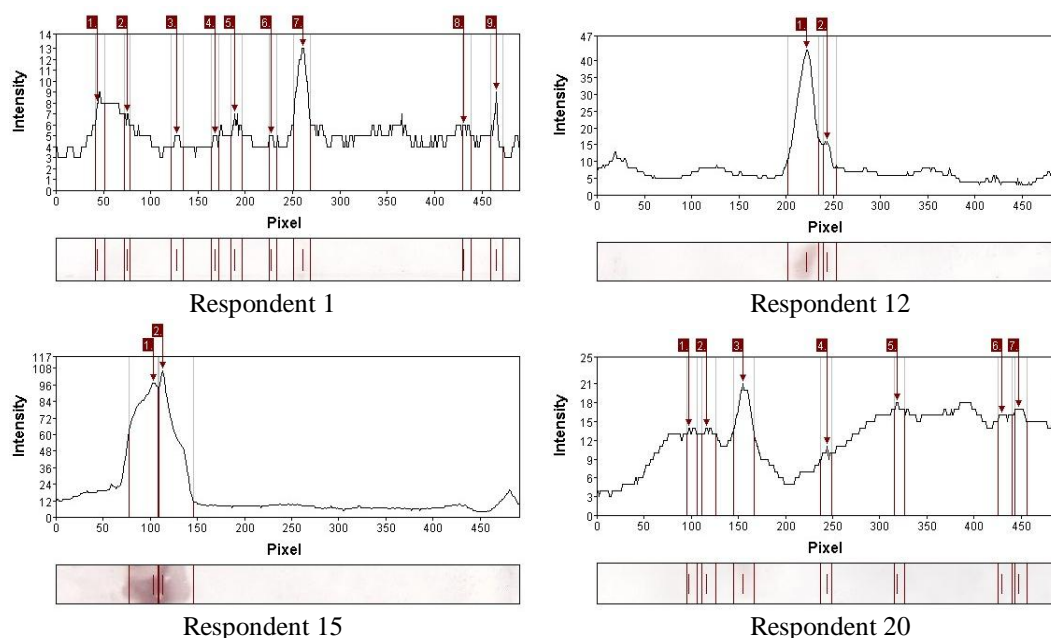
A person who consumes a squid not only eats myofibrillar protein or sarcoplasmic protein, but consumes both. If the diagnosis results indicate that the person is positively allergic to squid myofibrils and not allergic (negative) to squid sarcoplasm, vice versa, then the allergic status to squid is positive. The analysis results showed that respondents who were allergic to squid was 14 respondents or 56% of the total respondents. Serum that has a high allergenicity was used to determine the protein allergens profile with immunoblotting.

3.2 Profiles of Protein and Squid Myofibrillar Allergens

Fig. 1 shows the profile of squid myofibrillar protein from SDS-Page Electrophoresis results (A) and the profile of protein allergens from immunoblotting with allergic respondents serum (B). The identification results using GelAnalyzer 2010a software showed that squid myofibrillar protein consisted of 11 bands with different intensities. The detected molecular weights of myofibrillar protein were 108,4 kDa, 92,2 kDa, 82,9 kDa, 74,1 kDa, 64,8 kDa, 58,4 kDa, 54,8 kDa, 35,8 kDa, 32,5 kDa, 22,5 kDa and 18,2 kDa. with the greatest intensities were protein with molecular weight of 35.8 kDa (22.9%), 64.8 kDa protein (18.3%) and 108.4 kDa protein (13.6%). According to Ashie and Simpson (1997), the molecular weight of myofibrillar protein ranges from 17 kDa to 1000 kDa. 17-18 kDa proteins are suspected as troponin-C, 20-24 kDa proteins are suspected as troponin-I, while 65-70 kDa proteins are suspected as tropomyosin.



A. Electrophoresis results of squid myofibrillar protein

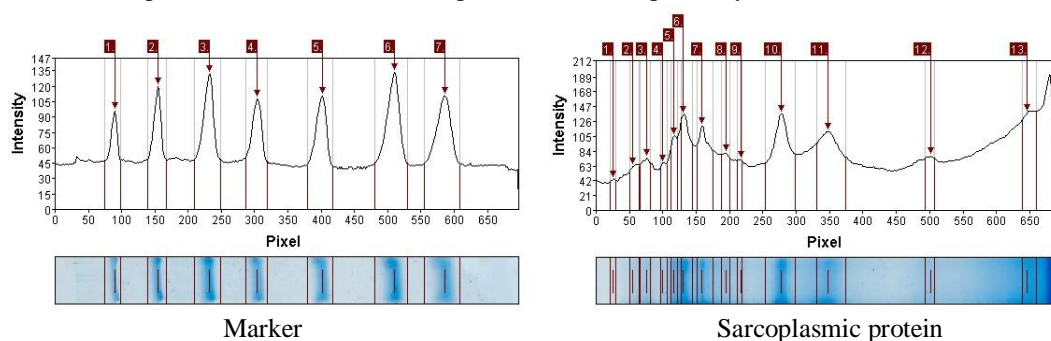


A. Immunoblotting results of squid myofibrillar protein
 Figure 1. SDS-Page Electrophoresis results (A) (marker and squid myofibrils) and immunoblotting (B) (Respondent 1, 12, 15 and 20)

Fig. 1 shows the protein profile of squid myofibrillar allergens that can bind to specific IgE antibodies from 4 serums of squid allergic respondents. The serum of allergic patients was selected with a high allergenicity level of squid myofibrils that has been determined by ELISA method. Protein allergens that could bind to the specific IgE antibodies in 1, 12, 15, and 20 respondent serums were 9 bands, 2 bands, 2 bands and 7 bands, respectively. Squid myofibrillar protein allergens that could bind to serum 1 specific IgE were 122,7 kDa, 100,6 kDa, 72,7 kDa, 56,4 kDa, 49,4 kDa, 38,6,0 kDa, 31,5 kDa, 10,9 kDa, and 8,9 kDa. Specific IgE antibodies in serum 12 that could bind to squid myofibrillar protein were 40.0 kDa and 35.9 kDa, whereas in respondent serum 15 were 83.9 kDa and 78.7 kDa and in respondent 20 were 87,6 kDa, 77,8 kDa, 61,0 kDa, 35,1 kDa, 22,1 kDa, 11,0 kDa, and 9,9 kDa. The 38.6 kDa protein which was detected using serum 1 was suspected as a major allergen contained in the squid, namely tropomyosin proteins (Tod p 1) according to Allergen Nomenclature ([9]; [17]; [18]). Other major allergen found in squid is tropomyosin protein with the molecular weight of 36 kDa and the one that could be detected using serum 12 has 35.9 kDa molecular weight [19].

3.3 The Profiles of Squid Sarcoplasmic Protein and Allergen

The number of detected sarcoplasmic proteins were more than myofibrillar proteins. The detected sarcoplasmic proteins were 119,3 kDa, 105,4 kDa, 97,2 kDa, 88,1 kDa, 82,2 kDa, 77,3 kDa, 69,1 kDa, 59,6 kDa, 54,4 kDa, 42,6 kDa, 32,2 kDa, 17,1 kDa, and 9,5 kDa with the greatest intensities were 42.6 kDa protein (19.2%), 32.2 kDa protein (18.5%) and 9.5 kDa protein (13%), respectively.



(A) SDS-PAGE Electrophoresis results of squid sarcoplasmic protein

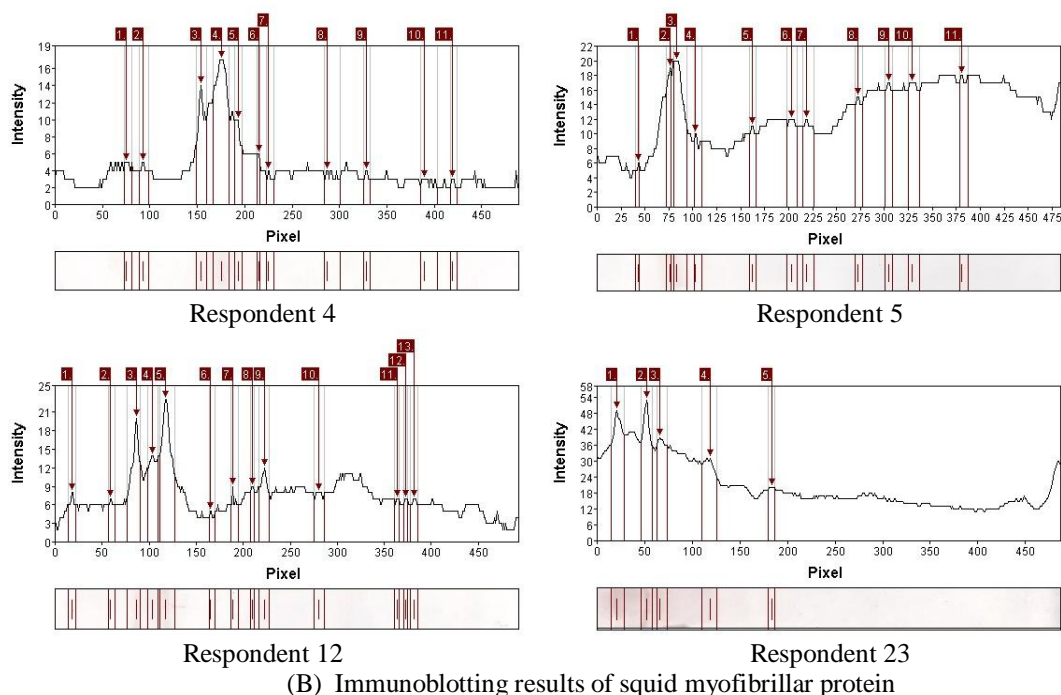


Figure 2. SDS-PAGE Electrophoresis results (A) (marker and squid sarcoplasm) and immunoblotting (B) (Respondent 4, 5, 12 and 23)

Fig. 2 shows the protein could bind to the specific IgE each in 4, 5, 12 and 23 respondents were 11 bands, 11 bands, 13 bands and 5 bands, respectively. Sarcoplasmic proteins that could bind to specific IgE antibodies in serum 4 were 100,6 kDa, 89,8 kDa, 61,4 kDa, 53,8 kDa, 48,2 kDa, 42,0 kDa, 39,5 kDa, 26,9 kDa, 20,8 kDa, 14,2 kDa, and 11,8 kDa protein while in serum 5 were 122,7 kDa, 99,7 kDa, 95,5 kDa, 85,0 kDa, 58,5 kDa, 45,2 kDa, 42,3 kDa, 29,4 kDa, 24,1 kDa, 20,6 kDa, and 15,0 kDa proteins. Sarcoplasmic proteins that could bind to specific IgE antibodies in serum 12 were 143,9 kDa, 111,6 kDa, 94,3 kDa, 85,0 kDa, 77,8 kDa, 57,8 kDa, 49,7 kDa, 42,8 kDa, 40,2 kDa, 28,2 kDa, 16,7 kDa, 15,8 kDa, and 15,0 kDa protein, while in serum 23 were 140,4 kDa, 115,8 kDa, 106,3 kDa, 76,3 kDa, and 51,0 kDa protein.

Several seafood sarcoplasmic proteins which can cause allergens is the sarcoplasmic calcium binding protein with 20 kDa molecular weight [20], hemocyanin with 72 kDa molecular weight [21], arginine kinase with 42 kDa molecular weight [22] and amylase [23]. Protein with a molecular weight of 42 kDa is suspected as arginine kinase that can bind to specific IgE antibodies in 4, 5 and 12 serums. Protein with 20 kDa molecular weight is suspected as sarcoplasmic calcium binding protein that can bind to specific IgE antibodies in serum 5.

3.4 Formulation and Quality Requirements of Allergen Products for SPT

Allergen products or reagent made in this study was a product of glycerinated extract containing 50% glycerol. Table 1 shows the requirements from European Pharmacopoeia 7 Monograph on Allergen Product (2010:1063) and the analysis results of allergen products of squid myofibrils and sarcoplasm. The analysis results showed that allergen products of squid myofibrils and sarcoplasm used for the diagnosis of squid allergy with SPT method have met the requirements set by the European Pharmacopoeia 7 Monograph on Allergen Products (2010:1063).

Table 1. Requirements of European Pharmacopoeia 7 Monograph on Allergen Product (2010: 1063) and the analysis results of allergen products of squid myofibrils and sarcoplasm

No.	European Pharmacopoeia 7		Analysis Results	
	Parameters	Requirements	Myofibrils	Sarcoplasm
1	Moisture content (%)	Maximum 5% for freeze-dried products and can be more than 5% for liquid products	60,20 ± 0,35 (liquid products)	63,50 ± 0,06 (liquid products)
2	Protein content (µg/µl)	80-120% from the stated concentration (1 µg/µl)	0,86	0,90
3	Sterility	Sterile, if it is not sterile then	Sterile	Sterile

refers to chapter 5.1.4. on European Pharmacopoeia 7 01/2011:50104				
4	Total plate count*	Max 10 ² CFU/g or CFU/mL	0,00 ± 0,00	0,00 ± 0,00
5	<i>Staphylococcus aureus</i> *	Not detected in 1g or 1 mL	0,00 ± 0,00	0,00 ± 0,00
6	<i>Pseudomonas aeruginosa</i> *	Not detected in 1g or 1 mL	0,00 ± 0,00	0,00 ± 0,00
7	Fungi*	Max 10 ¹ CFU/g or CFU/mL	0,00 ± 0,00	0,00 ± 0,00
8	Yeast*		0,00 ± 0,00	0,00 ± 0,00

*According to European Pharmacopoeia 7 01/2011:50104)

3.5 Skin Prick Test Results

Table 2 shows that 14 test respondents were allergic patients based on total IgE test results by ELISA method. Analysis of specific IgE of squid myofibrils showed that 13 respondents gave positive allergic results to squid myofibrils and 6 respondents gave positive allergic results to squid sarcoplasm. Overall, all respondents were positively allergic to squid. The results of SPT in Table 2 shows that there is a variation in the results between the myofibrillar protein and sarcoplasmic protein. For example, respondent code 4 showed negative result toward myofibrillar protein (0) but showed very strong positive allergic result (+4) toward sarcoplasmic protein. Contrarily, respondent code 11 gave positive result toward myofibrillar protein (+2) but gave negative result to sarcoplasmic protein (0). There were 7 respondents (50%) who showed positive allergic results to the myofibrillar protein and sarcoplasmic protein. Based on the SPT, all respondents in Table 2 are positively allergic to squid although some of them showed negative results on myofibrillar and sarcoplasmic proteins.

Table 2. Total IgE (tIgE), specific IgE (sIgE) and skin prick test result of squid myofibrils and sarcoplasm on the respondents with squid allergy

No	Resp. Code	tIgE Result	sIgE result			SPT Result		
			Myofibrils	Sarcoplasm	Diagnosis result	Myofibrils	Sarcoplasm	Diagnosis result
1	1	+	+	+	+	0	+1	+
2	2	+	+	-	+	+1	0	+
3	3	+	+	+	+	+3	+3	+
4	4	+	-	+	+	0	+4	+
5	5	+	+	-	+	+1	0	+
6	11	+	+	-	+	+2	0	+
7	12	+	+	+	+	+1	+1	+
8	14	+	+	-	+	+1	+2	+
9	17	+	+	-	+	+1	0	+
10	19	+	+	+	+	+1	+4	+
11	21	+	+	-	+	+1	0	+
12	22	+	+	+	+	+3	+3	+
13	30	+	+	-	+	+3	+3	+
14	31	+	+	-	+	+4	+3	+

+: Positive, - and 0: Negative, Resp. code: respondent code

Table 3. Total IgE (tIgE), specific IgE (sIgE) and skin prick test result of squid myofibrils and sarcoplasm on the respondents without squid allergy

No	Resp. Code	tIgE Result	sIgE Result			SPT Result		
			Myofibrils	Sarcoplasm	Diagnosis result	Myofibrils	Sarcoplasm	Diagnosis result
1	8	+	-	-	-	0	0	-

2	15	+	-	-	-	0	0	-
3	16	+	-	-	-	0	0	-
4	18	+	-	-	-	0	0	-
5	20	-	-	-	-	0	0	-
6	24	+	-	-	-	0	0	-
7	25	-	-	-	-	0	0	-
8	26	-	-	-	-	0	0	-
9	27	-	-	-	-	0	0	-
10	29	-	-	-	-	0	0	-
11	34	+	-	-	-	0	0	-
12	35	+	-	-	-	0	0	-

+: Positive, - and 0: Negative, Resp. code: respondent code

Table 3 shows that the SPT results of all respondents who were not allergic to the proteins of myofibrillar and sarcoplasmic proteins is negative. It is shown that although based on total IgE the respondents have allergy, but it is not necessarily allergic to squid. Specific IgE results of squid myofibrils and sarcoplasm by ELISA method and SPT indicates that the respondents were not allergic to squid.

Table 4. Sensitivity and specificity of squid reagents for skin prick test

No	SPT Reagent	Allergy, Positive SPT, Sum (%) Sensitivity)	Allergy, Negative SPT, Sum (%) Negative Error)	Non-allergy, Positive SPT, Sum (%) Positive Error)	Non-allergy, Negative SPT, Sum (%) Specificity)
1	Myofibrils	12/14 (86)	2/14 (14)	0/12 (0)	12/12 (100)
2	Sarcoplasm	9/14 (64)	5/14 (36)	0/12 (0)	12/12 (100)
	Diagnosis result	14/14 (100)	0/14 (0)	0/12 (0)	12/12 (100)

Table 4 shows the sensitivity and specificity of squid reagent for SPT test which is measured based on the SPT results in Table 2 and 3. The sensitivity results showed that squid myofibrillar reagent (86%) was higher than squid sarcoplasmic reagent (64%) and the error rates for myofibrillar and sarcoplasmic reagents were 14% and 36%, respectively. If the the sensitivity results of squid myofibrils and sarcoplasm are combined, then the sensitivity value of squid protein isolate is 100%. It can be concluded that the separation of squid proteins into myofibrils and sarcoplasm for SPT test could increase the sensitivity value and decrease the negative diagnosis error. The specificity value of myofibrillar and sarcoplasmic proteins was similar, each one was 100% with the error rate of positive diagnosis occurrence at 0%.

IV. CONCLUSION

Squid myofibrillar and sarcoplasmic proteins extracts may be used to create SPT reagent in accordance to the requirements of European Pharmacopoeia 7 Monograph on Allergen Products (2010:1063). The separation of squid proteins into myofibrils and sarcoplasm for SPT reagents could improve its sensitivity and specificity value and lowered the negative and positive misdiagnosis. For the purpose of allergy testing practicality in the hospital or allergy clinic, research to calculate the sensitivity and specificity of combined myofibrillar and sarcoplasmic proteins need to be conducted.

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