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Quality performance of protein allergen isolates for allergy diagnostic test (Case: Indonesian soybeans *(Glycine max)* and peanuts *(Arachis hypogaea)*)

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Abstract

Allergen isolate is used in skin prick test to assess the type of offending food for an allergic person. Peanut and soybean are some of the main food allergens. Some researchers showed that different cultivars and environmental conditions could alter IgE binding proteins profiles when tested against plasma from allergic individuals. Consequently, the use of local soybean and peanut as allergen isolates for allergy diagnostic test (such as skin prick test) needed an experiment to characterize these raw materials for showing and proving that they were feasible as a sensitizer. This study aimed to isolate local soybean and peanut proteins and to characterize their allergic reactivity for using as allergen isolate. The protein isolate reactivity was tested by using SDS-PAGE electrophoresis, immunoblotting, and ELISA, involving food allergic human sera containing IgE. It was found that the allergic proteins in soybean and peanut were specific for each individual. This is one of the benefits of using crude protein as an allergen diagnostic tool since a different person may have an allergy to different allergen proteins. Thus, allergen isolates should be in a crude protein form. The results from ELISA analysis showed that the two protein isolates had a good sensitivity in detecting specific IgE, thus seemed to be promising to be produced as an allergen isolates.

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Introduction

A food allergy is an adverse reaction involving the immune system of food allergic individuals. It is caused by food protein, called an allergen. Food allergen is able to induce a specific immune response in genetically predisposed individuals through IgE-mediated mechanisms. The eight most common food allergens cause more than 90% of all food allergic reaction (International Food Information Council Foundation, 2014). The top eight food allergens are milk, egg, fish, soybean, shellfish (crustacean and mollusks), wheat, peanut, and tree nuts (e.g. walnuts). Peanut and soybean allergies are some of the most life-threatening food allergies, whereas both of them are consumed by large people in Indonesia due to their high nutrition.

Some experiments had been conducted to reduce allergenicity of peanut and soybean, including genetic engineering (Dodo *et al*., 2007); oral immunotherapy with peanut (Jones *et al*., 2009; Nurmatov *et al*., 2012; Ismail and Tang, 2012; Factor *et al*., 2012; Sheikh *et al*., 2012; Vickery *et al*., 2013) and treatment using recombinant human interleukin-12 and Toll-like receptor 9 (Bryan *et al*., 2000; Zhu *et al*., 2007); modifying of peanut allergens (Bannon *et al*., 2001; Rabjohn *et al*., 2002; King *et al*., 2005); as well as allergen non-specific therapies with Chinese herbal medicine (Li *et al*., 2001; Song *et al*., 2010; Srivastava *et al*., 2012). However, they do not spontaneously resolve all forms of food allergy encountered in clinical practice and are still needed to investigate the acceptability and long-term effectiveness. Currently, the only treatment available is to avoid the suspected food (Thyagarajan and Burks, 2009), because no well-established therapy or treatments are available to cure or provide long-term remission from food allergy (Singh and Bhalla, 2008). Therefore, several diagnostic tests were developed, and skin prick test is the most commonly used in hospitals to confirm sensitization to specific foods. In this method, a diluted extract of the suspected food (food allergen) is placed on the volar aspect of the forearm, and the skin is then punctured. When a wheal surrounded by redness with a diameter at least 3 mm or greater than the reading in the negative control forms within 15 minutes, then the skin test is positive and the person may be allergic to the tested food. Commercially

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prepared extracts from many fruits and vegetables are often inadequate for using in the test because of the lability of the responsible allergen (Sicherer and Hugh, 2010). Rosen *et al.* (1994) reported that all patients tested demonstrated negative responses to skin prick test with the commercial food extracts but positive responses to skin prick test with natural food extracts which a possible explanation was related to the stability of the allergen during the manufacture of the extract. Therefore, the protein isolated from local food may be used to evaluate IgE-mediated food allergy by using skin prick test. Furthermore, some researches showed that different cultivars, environmental conditions (e.g. temperature, moisture, insect/pathogen load, stress conditions and/or soil nutrient levels) could alter IgE binding proteins profiles when tested against plasma from allergic individuals (Xu *et al*., 2007; Kottapalli *et al*., 2008; Stevenson *et al.*, 2012; Panda *et al*., 2013; Fonseca *et al.*, 2014). Consequently, the use of local soybean and peanut as allergen isolates for allergy diagnostic test (such as skin prick test) needed an experiment to characterize these raw materials for showing and proving that they were feasible as a sensitizer. A previous research showed that several purified major peanut allergens (Ara h 1, Ara h 2, and Ara h 3) and one minor allergen (Ara h 6) possessed a little intrinsic immune-stimulating capacity to induce sensitization in contrast to whole peanut extract (van Wijk *et al*., 2005). Hence, allergen isolates should be in a crude protein form.

In the recent study, both of protein isolates were extracted from raw food materials, because some studies reported that skin testing with raw food gave higher sensitivity (Norgaard *et al*., 1992; Rance *et al*., 1997; Beyer *et al*., 2001). To evaluate the quality of the protein isolates used, a characterization the protein isolates from local soybean and peanut was needed to show the immune stimulating capacity of these protein isolates, and it could be established by SDS-PAGE, immunoblotting, and ELISA. Immunoblotting provided insight in the patient specificity towards the individual peanut or soybean allergens (Koppelman *et al.*, 2004), whereas ELISA showed the sensitivity of peanut or soybean protein isolate.

Materials and Methods

Preparation of defatted soybean and peanut flour

Soybean and peanut were purchased from local market. Soybean seeds were manually peeled and dried-milled to pass through a 60-mesh sieve. Peanut seeds were peeled and ground due to a very high-fat content. The two samples were defatted by n-hexane extraction (sample/hexane = 1:5, v/v) for 1 h at room temperature (Liu et al., 2007). After centrifugation (3200 x g, 15 min, 4°C), the supernatant was discarded and the precipitate was extracted twice more. The defatted flour was collected for protein isolation.

Isolation of soybean and peanut proteins

Each defatted flour (soybean and peanut) was suspended in distilled water (1:10 w/v) and the pH was adjusted to 8.0 (for soybean) and 8.5 (for peanut) with 1.0 M sodium hydroxide (NaOH). They were stirred for 90 min and centrifuged at 3200g for 30 min at 4°C. The pH of the obtained supernatants was adjusted to 4.5 with 1.0 M hydrochloric acid (HCl) to precipitate the protein recovered by centrifugation at 3200g for 20 min at 4°C. The supernatants were discarded and the precipitates (proteins) were dried by freeze drying (Wu *et al*., 2009; Speroni *et al*., 2010). Their protein content was determined by Bradford method (Bradford, 1976). The isolation of soybean and peanut proteins were done in triplicate.

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

SDS-PAGE (using a Bio-Rad mini-gel system, Biorad USA) was performed on a discontinuous buffered system according to the method of Laemmli (1970), using 12% separating gel and 5% stacking gel. Forty microliters of the diluted extract containing 12 µg proteins were mixed with 10 µL of reducing sample buffer (Tris-HCl pH 6.8, SDS, 5% v/v β-mercaptoethanol). The mixture was heated for 5 min. Ten microliters of the sample (equivalent 3 µg of protein) were cooled to room temperature and then were loaded per well. SDS-PAGE was performed at 70 V constant voltages for 3 hours. Gels were stained with Coomassie brilliant blue R-250 (0.1%) dissolved in methanol-acetic acid-distilled water (45:10:45 v/v/v) and destained in the same solution without the dye. Low molecular weight protein markers (Fermentas®) that contained Beta-galactosidase (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) were used as standards to estimate the molecular weight ranges of polypeptides in the sample. The analysis was done in triplicate.

Serum IgE

Without food (soybean or peanut) intervention, blood was collected from thirty adult volunteer informed donors with the positive case history of

food allergy and one non-allergic healthy individual, ranging in age from 20 to 35 years old. A structured questionnaire was used to interview the donors and the obtained information from this interview was used as an allergic patient source of IgE sera. The donors were carefully informed of the scope of the study, and after receiving all the information, they signed the written informed consent form. Serum was separated by centrifugation and stored in aliquots at -20°C until use. The total serum IgE level of thirty sera was measured by ELISA. Each serum was diluted in carbonate-bicarbonate buffer 0.05 M pH 9.6 (1:10), and then applied into the plate, in triplicate and incubated overnight at 4°C, washed five times with phosphate buffer saline-Tween 20 0.05% (PBST) and detected by 100 µL/well of horseradish peroxidase conjugated monoclonal mouse anti-human IgE (purchased from Immunology Consultans Laboratory Inc., 1:6000 in PBST). Serum obtained from a non-allergic healthy individual was used as a negative control. The color was developed with 3,3',5,5'-tetrametilbenzidin substrate (Sigma) for 5 min at room temperature. The reaction was stopped by addition of 2 M sulfuric acid (H2SO4) (100 µL/well). The plate was read with an automatic microplate reader (Labsystem multiscan EX) at 450 nm. This research was purely an observational study, in which there was no food intervention to the subjects who donated their blood.

Immunoblotting

Immunoblotting was performed as previously described with some modifications (Song *et al*., 2008), and the analysis was done in triplicate. Proteins were separated by gel electrophoresis under denaturing conditions (SDS-PAGE). Unstained gels were soaked for 15 min in transfer buffer pH 8.3, which consisted of 20% (v/v) methanol, 23.9 mM tris base, 193 mM glycine. A western blot sandwich was assembled by placing a sponge, a filter, the gel, the membrane, another filter and sponge, avoiding the formation of bubbles. The separated proteins were electrophoretically transferred at 90 V constant for 1 h. After the transfer was completed, the membrane was then carefully saturated by incubation in PBST containing skim milk 5% for 1 h, followed by washing it three times in PBST. Serum obtained from the donor was diluted 1:2 in PBST and applied on the membrane and then incubated for 3 h at room temperature. The membrane was washed three times in PBST, followed by 1 h incubation at room temperature in a 1:2000 dilution of horseradish peroxidase conjugated monoclonal mouse anti-human IgE (Immunology Consultans Laboratory Inc.) prepared in PBST. The membrane was washed three times for 5 min with PBST and detected using 3,3'-diaminobenzidine (DAB) substrate (Sigma) solution following manufacturer's instruction.

IgE Immunoreactivity by ELISA

ELISA was performed in order to study the soybean and peanut IgE immunoreactivity using sera obtained from donors suffering from food allergies. For ELISA, 100 µL/well containing 10 µg of the soybean or peanut protein extract (antigen) in coating buffer (carbonate-bicarbonate buffer 0.05 M pH 9.6) were applied first onto the 96-well microplate, in triplicate and kept overnight at 4°C. The unoccupied space on the plates was blocked for 1 h at room temperature with 250 µL/well of PBST containing 5% of skim milk to diminish the nonspecific binding. Between each step in the procedure, the plates were washed five times with PBST. A 100 µL/well of serum obtained from donors (1:10 dissolved in PBST) was applied onto the plate, incubated for 1 h at 37°C and washed again. Serum obtained from a non-allergic healthy individual was used as a negative control. Bound IgE was detected using a 100 µL/well of horseradish peroxidase conjugated monoclonal mouse anti-human IgE (Immunology Consultans Laboratory Inc., 1:6000 in PBST), and allowed to incubate in the plate for 1 h at 37°C, and washed to eliminate any remaining unbound antibody. A subsequent reaction for peroxidase activity was detected by addition of 100 µL/well of 3,3',5,5'-tetrametilbenzidin substrate (Sigma) for 5 min at room temperature. The reaction was stopped by addition of 2 M H_2SO_4 (100 µL/well). The plate was read with an automatic microplate reader (Labsystem multiscan EX) at 450 nm. Uncoated plates incubated with food allergic patient sera were performed as controls.

Results and Discussion

Characteristic of soybean and peanut protein

Figure 1 shows the electrophoretic patterns of the soybean and peanut proteins. Each batch of protein isolates gave the same protein composition in SDS-PAGE analysis (data not shown). At least 8 soybean protein bands with the molecular weight ranging from 20 to 83.7 kDa and 12 peanut protein bands with the molecular weight ranging from 14.8 to 66 kDa were identified in SDS-PAGE analysis. The electrophoretic profile of soybean protein presented three high-intensity bands of protein with molecular weight 68 kDa, 40.4 kDa, and 20 kDa, indicating that these proteins were the most abundant in soybean.

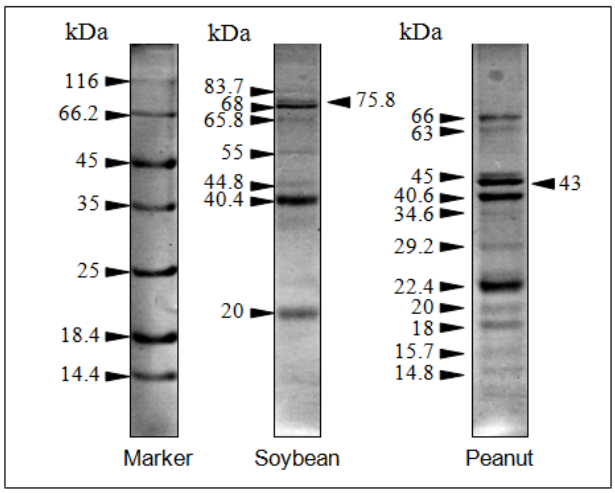

Figure 1. Electrophoretic pattern of the soybean and peanut protein. SDS-PAGE gel of marker (left) showed seven protein bands as standards to estimate the molecular weight of protein bands in soybean (middle) and peanut protein isolate (right)

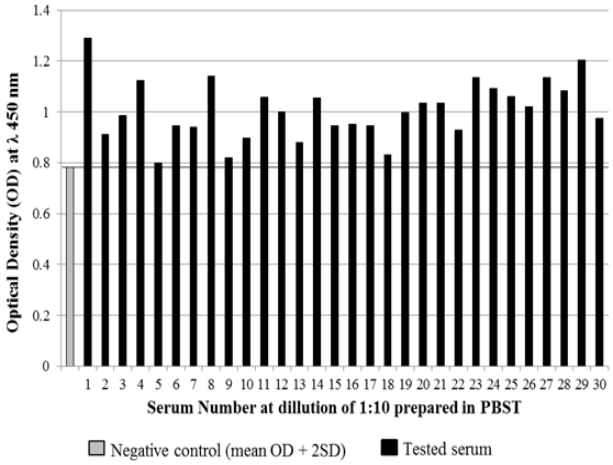

Figure 2. Measurement of serum total IgE. Serum was diluted 1:10 in PBST (based on the result in optimization of serum ditlution) and then analyzed by ELISA. Total IgE of each serum was compared to serum total IgE that was obtained from a non-allergic healthy individual (negative control)

A protein band with molecular weight 68 kDa was predicted as a major allergen, namely Gly m Bd 68K (Tsuji *et al.*, 1997). Whereas the presence of allergens about 30 kDa predicted as Gly m Bd 30K and 26 kDa predicted as Gly m Bd 28K could not be seen clearly.

Seventeen peanut proteins had been identified and characterized as major or minor allergens, and officially termed Ara h 1-17 (IUIS Allergen Nomenclature Sub-Committee, 2016). A less intense protein band with molecular weight 63 kDa in electrophoretic pattern of peanut protein isolate was possibly Ara h 1. The 9-10 peanut protein bands with molecular weight 18 kDa and 20 kDa might be related to Ara h 2 which was characterized as a doublet on SDS-PAGE (Burks *et al.*, 1991; de Jong *et al*., 1998; Chung and Champagne, 2011), and the band at 45 kDa was related to the Ara h 3 (Piersma *et al*., 2005). The four of peanut protein bands that appeared with high intensity, i.e. protein bands with molecular weight 66 kDa, 43 kDa, 40.6 kDa and 22.4 kDa, were not the major allergen.

Respondents food allergic history

Sera used to characterize soybean and peanut protein isolates were obtained from thirty food allergic donors, and one serum was collected from a non-allergic healthy individual as the negative control. The allergy information of those donors was obtained by self-reported food allergy questionnaires and interview about the food responsible for the reaction, the quantity of the suspected food ingested, the details of the prior history of allergic reactions and symptoms, as well as the length of time between food ingestion and development of symptoms. This information was required to make sure that all sera were obtained from food allergic donors.

The thirty sera were coded 1 to 30, with serum 1-6 were obtained from nut allergic donors, whereas serum 7-30 were obtained from seafood allergic donors such as fish, shrimp, shell, and crab. Table 1 shows the history of allergic reactions of the donors participated voluntarily in this research. The use of these difference food allergic sera aimed to show nut protein isolates sensitivity.

Before serum was used in nut protein isolates characterization, all sera including serum from negative control were analyzed by ELISA for ensuring that the donors had the food allergy and their IgE level was higher than the negative control. The IgE measurement by ELISA was total serum IgE analysis that was not specific for peanut and soybean allergen. The cut-off for the positive result was taken as 2 S.D. above the mean OD_{450nm} (Optical Density) scores of negative control (de Leon *et al.*, 2007; Kumar *et al*., 2010). Therefore, the negative control could give higher ELISA OD mean value than the blank. It was necessary to compare the ELISA OD mean value of allergic individual's serum to the negative control. The ELISA analysis was considered appropriate if no blank had ELISA OD mean value greater than 0.060. The results showed that thirty donors participated in this research were allergic (Figure 2).

Immunoblotting of soybean and peanut protein isolates

The amount of specific IgE in serum contributes significantly to immunoblotting result because the positive result in immunoblotting is identified

Table 1. The history of allergic reactions of the donors participated voluntarily in this research

Donor No.	Age	Sex	The food responsible for the reaction	Symptoms after food ingestion	The length of time between food ingestion and development of symptoms.	Quantity of the suspected food ingested
1	27	F	Peanut, soybean	Rosacea	Hours	Large amounts
2	27	F	Peanut, soybean, almond	Rosacea (severe)	Hours	Small amounts
3	29	F	Peanut	Rosacea	Hours	Large amounts
4	29	F	Peanut, soybean	Burning and hives (severe)	A few minutes	Small amounts
5	28	F	Soybean	Rosacea	Hours	Large amounts
6	29	M	Peanut, soybean	Burning and hives	A few minutes	Small amounts
7	21	F	Seafood	Hives	Hours	Large amounts
8	21	F	Shrimp	Hives, rosacea	Hours	Large amounts
9	21	F	Seafood	hives	Hours	Large amounts
10	35	F	Seafood	hives	Days	Large amounts
11	30	F	Shrimp	hives	Hours	Small amounts
12	21	M	Shrimp, crab	breathlessness	Days	Large amounts
13	22	M	Shell	red bumps	Hours	Large amounts
14	21	F	Seafood	red bumps	Hours	Large amounts
15	22	M	Shrimp	red bumps	Hours	Large amounts
16	21	F	Seafood, egg	breathlessness	Hours	Small amounts
17	21	F	Shell	red bumps	Days	Large amounts
18	22	M	Shrimp	red bumps	Hours	Large amounts
19	21	F	Shrimp, crab	red bumps, runny nose	Hours	Large amounts
20	20	M	Shrimp	cough	Hours	Large amounts
21	20	F	Fish	Burning and hives	Hours	Large amounts
22	21	M	Seafood	red bumps	Hours	Large amounts
23	21	M	Shrimp, fish	red bumps	Hours	Large amounts
24	21	F	Seafood	headache	Hours	Large amounts
25	22	M	Seafood	hives	Hours	Large amounts
26	20	M	Seafood	red bumps	Hours	Large amounts
27	20	F	Seafood	headache	Hours	Small amounts
28	20	M	Shrimp	runny nose	Hours	Large amounts
29	20	F	Shrimp	red bumps, hives	Hours	Large amounts
30	21	F	Shrimp	Breathlessness	Hours	Large amounts

by the visual assessment. At higher specific IgE concentration, color resulted by reaction between secondary antibodies and DAB substrate will appear and can be detected visually. Otherwise, at low specific IgE concentration, the produced color can not be detected, leading to the error in drawing conclusions. Therefore, immunoblotting was performed using sera 1-6 (serum was taken from nut allergic donors). Although not all of sera had high-level of total serum IgE, but these sera were expected to contain large amounts of nut-specific IgE antibodies, based on a history of allergic donors presented in Table 1. However, based on research of Batanero *et al.* (1996) which showed that serum IgE antibodies from people allergic to a particular food type could also bind to epitope in other food allergens, immunoblotting had been done using serum taken from donor with allergy to seafood for showing the presence of nut-specific IgE in the serum. Serum 8 was suspected to contain nut-specific IgE antibodies because the donor reported the same symptom of nut allergy manifested by rosacea (inflammatory skin condition of adults that looks somewhat like acne). Therefore, based on this history of allergy, serum 8 was selected for testing immunoblotting to determine the possibility of nut-specific IgE in that serum.

Each of sera 1-6 and 8 was tested to soybean and peanut protein isolates, and the result showed that each batch of protein isolates had the same IgE-binding protein pattern in the immunoblotting analysis to the tested sera (data not shown). The results of immunoreactivity by immunoblotting against human sera showed a great deal of variabilities, and not all of serum IgE could bind to each nut protein (Figure 3). Serum 3 exhibited the positive response to soybean protein isolate. Sera 1, 4, 5 and 8 demonstrated positive responses to peanut protein isolate, serum 2 showed the positive response to both of protein isolates, whereas no immunoreactivity was detected when serum 6 was used in immunoblotting.

Immunoblotting soybean protein isolate against serum 2 showed intense immunoreactive bands (Figure 3A), and the most potent allergenicity of allergens presented in protein bands with molecular weight 30 kDa and 26 kDa. When it was compared to the electrophoretic pattern of soybean proteins (Figure 1), these two allergen bands were completely undetectable. It suggests that allergenicity is not substantiated in the concentration of allergens in protein isolates. The 30 kDa and 26 kDa bands found in soybean were known as Gly m Bd 30K and Gly m Bd 28K respectively, and both of these proteins were a major allergen (Tsuji *et al*., 1997). There was significantly less IgE binding to assumed major allergen Gly m Bd 68K (protein band with molecular weight 68 kDa) in soybean protein isolate (Figure 3A) compared to the high concentration of the protein band (Figure 1). Additionally, IgE binding was also detected to other proteins in soybean of differing molecular weight which could be minor allergens. Unlike serum from donor 2, incubation of soybean protein isolate with serum 3 only showed an immuno-recognition with assumed major allergen Gly m 68K (protein band with molecular weight of 68 kDa).

Figure 3B showed the immunoreactivity against

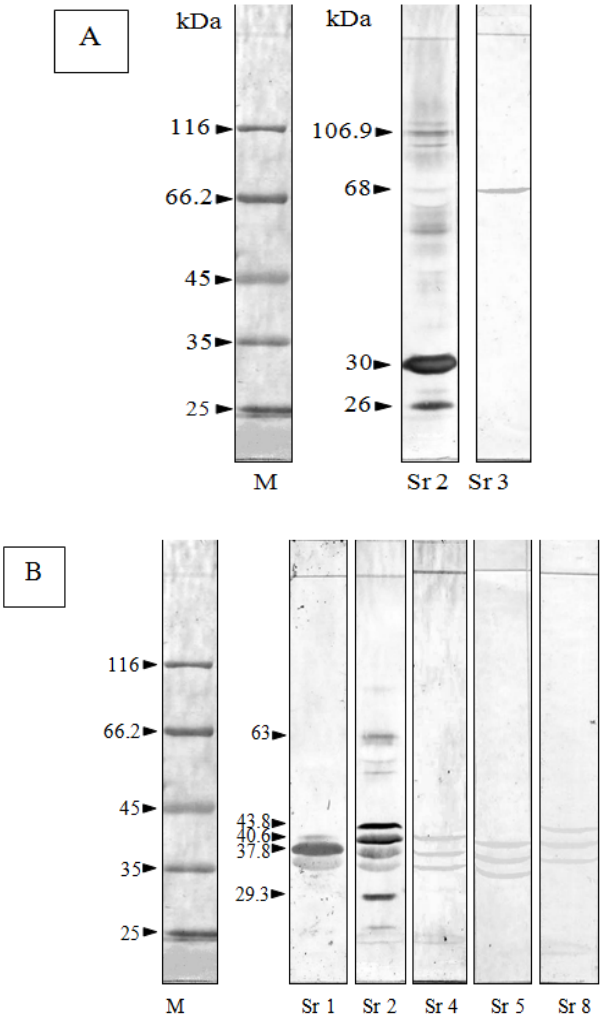

Figure 3. Detection of allergenic: (A) soybean proteins, and (B) peanut proteins by immunoblotting analysis. In soybean protein, the positive results only appeared when it was incubated by serum 2 (Sr 2) and serum 3 (Sr 3) respectivelly. In peanut protein, the positive results only appeared when it was incubated by serum 1 (Sr 1), serum 2 (Sr 2), serum 4 (Sr 4), serum 5 (Sr 5) and serum 8 (Sr 8). Membrane of marker (M) was used to estimate the molecular weight of allergic proteins in soybean and peanut protein isolates

some sera of the peanut protein isolate. IgE binding to peanut protein isolate showed that IgE antibodies that recognize peanut protein were present in five sera (sera 1,2,4,5,8), and IgE binding intensity differed among sera. The serum IgE antibodies in five sera were bound to proteins with the molecular weight ranging from 35 kDa to 45 kDa. IgE antibodies in serum 1 were bound most strongly to a protein with molecular weight of about 37.8 kDa; while IgE antibodies in serum 2 were bound most strongly to proteins with molecular weight of about 63 kDa, 43.8 kDa, 40.6 kDa, 37.8 kDa, and 29.3 kDa. IgE antibodies in serum 4 were bound to proteins with molecular weight of about 40.6 kDa, 37.2 kDa, and 35.2 kDa; IgE antibodies in serum 5 were bound to proteins with molecular weight of about 40 kDa, 37.2 kDa, and 33.3 kDa; whereas IgE antibodies in serum 8 were bound to proteins with molecular weight of about 42.9 kDa, 40 kDa, and 35.8 kDa. Of five sera tested, only IgE antibodies of serum 2 showed reactivity to one of the major peanut allergen, namely the 63 kDa protein, which was known as the major allergen Ara h 1. Serum 2 had the highest immunoreactivity to the protein bands. The weakest IgE binding was seen in serum 8. These results agreed with Ballmer-Weber *et al.* (2007) and Song *et al*. (2008) who reported that the pattern of IgE reactivity against proteins was highly individual among the patients and did not correlate with the severity of symptoms.

Both of immunoblotting results demonstrated the specificity of IgE reactivity and a more diverse recognition pattern of allergens in the donors. The donors showed a different specificity for the individual protein bands of both soybean and peanut. Therefore, protein isolate for diagnosis of allergy could be a crude protein. Differences of allergenic properties of protein were due to the presence of many epitopes within the allergenic protein (Sen *et al*., 2002). A large number of these epitopes resulted in their specificity vary widely on each individual. In other words, what might be high binding affinity of allergen-specific IgE antibody to an epitope for some individuals could be a low binding affinity for others, and its strength was determined by major histocompatibility complex haplotype variability between individuals (Blaser, 1996; Blaser *et al.*, 1998). Comparing with purified allergens, crude protein isolate has a higher sensitivity in inducing immune responses. Nicolaou *et al.* (2010) and Dang *et al*. (2012) have reported that Ara h 2 is discriminative in identifying patients with peanut allergy.

Sensitivity of soybean and peanut protein isolates analyzed by ELISA

The results of the immunoblotting analysis were in accordance with the results obtained by ELISA. In this study, ELISA was conducted to examine the feasibility of soybean and peanut protein isolates for using as an allergen in allergy diagnosis. This was due to allergen isolates must have a high sensitivity. It meant that allergens in both of isolates were able to bind with specific IgE antibodies from people who were allergic to nut alone or multiple foods.

ELISA using the serum from donors with soybean or peanut allergy will surely give a positive test result, because of a large number of nut-specific IgE antibodies in the serum. Hence, predominant IgE-binding proteins are easily identifiable. However, it

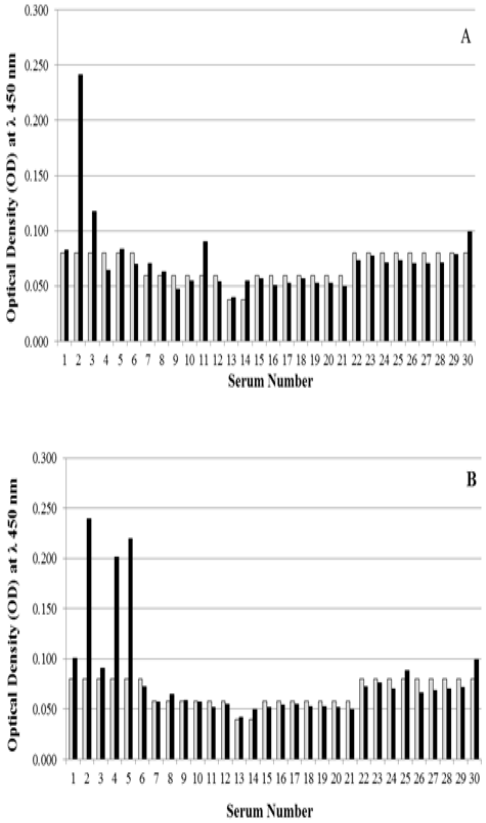

Figure 4. Immunoreactivity of each serum IgE antibodies by ELISA towards (A) soybean protein isolate, and (B) peanut protein isolate. Each protein applied into the 96-well microplate was incubated by thirty sera separately, and ELISA OD level was compared to ELISA OD of protein that was incubated by serum obtained from a non-allergic healthy individual (negative control).

has been demonstrated by the result of immunoblotting (Figure 3B), that donor allergic to shrimp (serum 8) might react to the nut. From the Figure 4 can be seen that serum 8 had positive result toward both protein isolates, whereas immunoblotting analysis showed positive results only toward peanut protein isolate. It might due to the higher-level of ELISA OD in peanut than that in soybean (data not shown), which made soybean-specific IgE in serum 8 was not enough to give a positive result in immunoblotting analysis. In addition, this phenomenon posed the question as to whether some individuals might have many different specific IgE antibodies to the different type of foods, or protein allergens in peanut were shown to share similar IgE binding epitopes with proteins present in shrimp (suspected food of donor 8) demonstrating that there were some cross-reactive IgE binding epitopes. Therefore, the soybean and peanut protein isolates had to be tested with IgE antibodies from some patients with different food allergy. Consequently, ELISA was performed on 6 sera of donors allergic to nuts, and 24 sera of donors allergic to seafood (fish, shrimp, scallops, crab). These protein isolates should be able to bind with small amounts of specific IgE.

Figure 4A and Figure 4B showed the IgE responses of each serum toward soybean protein isolate and peanut protein isolate, respectively. ELISA of soybean protein isolate to thirty sera showed the positive result in 10 donors, and ELISA of peanut protein isolate showed the positive result in 11 donors. The high-level of ELISA OD mean value indicated the high concentration of serum nut-specific IgE antibodies. Highest positive value, indicated by ELISA OD mean value, were recorded when serum 2 was used (Figure 4), confirming the positive results of immunoblotting for all isolates (Figure 3). It indicated that serum 2 had the high concentration of peanut-specific IgE and soybean-specific IgE antibodies. Based on the result of the interview summarized in Table 1, that donor 1 was allergic to soybean and peanut, confirmed the result of ELISA (Figure 4). Serum 1 contained IgE antibodies that were bound to allergens to both soybean and peanut, but with different levels of ELISA OD mean value. The higher ELISA OD level in peanut than that in soybean showed that peanut-specific IgE in serum 1 was much higher than soybean-specific IgE. Consequently, the donor 1 was more sensitive to peanuts. It was also confirmed by the results of immunoblotting. The positive result of immunoblotting against serum 1 was only obtained when using peanut protein isolate (Figure 3B). This was due to the concentration of soybean-specific IgE in serum 1 was not high enough to be detected, leading to a false-negative result.

The protein isolates of both soybean and peanut also were recognized by serum 3, but showing a low IgE antibody response to peanut compared to the IgE antibody response to soybean protein isolate. It might help explain the result of immunoblotting of serum 3 that was only positive against soybean protein isolate (Figure 3A). Sera 4 and 5 had high ELISA OD level in peanut, according to the positive result of immunoblotting in peanut (Figure 3B). However, serum 5 also had a higher ELISA OD level in soybean isolate than negative control, which characterized the risk of soybean allergy of the donor. ELISA result of serum 6 was not consistent with the information obtained from the donor who claimed allergies to soybean and peanut.

In the case of animal-based foods, multiple IgE-binding proteins were detected. Of the 26 sera with seafood allergy, the sera 8, 13, 14, and 30 recognized protein isolates of both soybean and peanut; the sera 7 and 11 recognized only soybean protein isolate; and the sera 9 and 25 recognized only peanut protein

isolate. These phenomena indicated the presence of soybean or peanut-specific IgE antibodies in their serum, but in very small amount. In addition, the immunological reactivity of protein isolates with soybean or peanut-specific IgE antibodies at low concentration in the serum of donors who are allergic to seafood suggested that these protein isolates were sensitive in detecting the presence of nut-specific IgE, making it feasible to use for diagnosing food allergies.

Conclusion

The crude protein isolates from both local soybean and peanut contained bands similar to the major allergens known. The thirty food allergic donors have high serum IgE level compared to negative control. Immunoreactivity by immunoblotting against human sera showed that allergic protein was specific for each individual. The sensitivity of soybean and peanut crude protein isolates analyzed by ELISA showed that they had high sensitivity, and, therefore, could be used for skin prick test.

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