



Isolation of winged bean (*Psophocarpus tetragonolobus* L.) allergen protein to create skin prick test reagent

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ABSTRACT

Any type of food that contains protein can cause allergies. The winged bean (*Psophocarpus tetragonolobus* L.) is a type of legume plant that contains high protein, and it is quite abundant in West Java, Indonesia. There are no specific studies discussing allergies to winged bean plants. This study aimed to isolate the protein of the winged bean (*P. tetragonolobus* L.) and characterize the allergenicity of the protein isolate using ELISA and immunoblotting and test the reactivity by the skin prick testing method. The fresh winged bean (FWB) protein profile had 13 protein bands (22–89 kDa). The boiled winged bean (BWB) contained 10 bands (22–73 kDa). The results of immunoblotting of 10 allergy-positive respondents whose serum was taken showed that the FWB protein can bind to specific IgE antibodies in respondent 1 in the protein band with a molecular weight (MW) of 24 kDa, respondent 5 with a MW of 219 kDa, respondent 7 with MWs of 29 and 23 kDa, and respondent 9 with a MW of 17 kDa. Meanwhile, the BWB protein can bind to specific IgE antibodies in respondents 7 and 8. In the BWB sample, the allergen protein was bound to specific IgE only in respondent 7 with MWs of 31 and 24 kDa and respondent 8 with MWs of 31 and 23 kDa.

INTRODUCTION

Allergies are immune dysfunctions that affect quality of life and lead to death if not treated properly (Guillamón *et al.*, 2008). The World Allergy Organization estimated that the prevalence of allergies in the entire world population ranges from 10% to 40% (Pawankar *et al.*, 2013). Based on ISAAC's study, the prevalence of allergic rhinitis among 13–14 years old is from 1.4% to 39.7% (Nurhutami *et al.*, 2020). Food allergy is a condition caused by an IgE reaction to food ingredients (chemical compounds) and is a concerning type of allergy with the highest prevalence observed in infancy and early childhood (Chapman *et al.*, 2006). One of the symptoms that arise from food allergies is allergic rhinitis that can cause nasal obstruction, causing a decrease in quality of life

(Zachreini *et al.*, 2015). IgE antibodies are usually synthesized in small amounts to optimize defense against microbes and other helminths by stimulating the release of inflammatory mediators by mastocytes (Zakaria *et al.*, 1992a). An allergic reaction mediated by IgE is a health problem in developing and developed countries (Lestari Eka *et al.*, 2016). In allergic reactions, IgE synthesis is unregulated and can be detected in large amounts in the blood. Not all dietary proteins trigger IgE synthesis. However, specific proteins will trigger their specific IgE antibodies, which can be used to determine the specific allergen-inducing protein (Zakaria *et al.*, 1992b). IgE recognition of its protein allergens is the basis of most allergy analyses and determinations that can identify a single protein allergen in various foods (Wijaya *et al.*, 2014), and it is the principle of the skin prick test (SPT) for allergy diagnosis (Astuti *et al.*, 2018).

The winged bean is a local Indonesian plant that can be used as a raw material that is rich in protein sources (Wahyuni, 2015). The winged bean has a high protein content ranging from 33.83% to 38.31%, so it can be used as an alternative source

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of vegetable protein (Amoe *et al.*, 2006). Protein contents significantly affect growth (Rachmawati and Samidjan, 2016).

The winged bean belongs to the family Fabaceae. The winged bean is a nonconventional legume crop that is similar to soybean in lipid and protein contents and one of the protein sources that are palatable but highly degradable in the rumen (Adiwimarta *et al.*, 2018). According to Faeste *et al.* (2010), the Fabaceae family has potential as an allergen. Jimenez Lopez *et al.* (2015) and Guillamon *et al.* (2010) reported that a Fabaceae family plant, i.e., *Lupinus angustifolius* L., can cause allergies.

This study aimed to isolate the winged bean's allergen protein and to characterize the allergen protein isolate from the SDS-PAGE, ELISA, and immunoblotting assays. The risk of allergy symptoms has not been assessed in Indonesia (Priyantini *et al.*, 2020). To avoid allergies, people tend to avoid foods that are suspected of causing them. Therefore, an allergy diagnosis test is needed. Allergy tests that are carried out to find out the type of food that causes a person to be allergic are the skin prick test or skin prick test.

The SPT was the method for diagnosing IgE-mediated allergic diseases. The SPT is an easy, inexpensive, and relatively safe *in vivo* examination procedure (Sari *et al.*, 2014). The main ingredient used for the SPT reagent in this study was an allergen protein extract from the winged bean (*Psophocarpus tetragonolobus* L.).

MATERIALS AND METHODS

Isolation of winged bean seed protein

The winged bean (*P. tetragonolobus* L.) was treated with two treatments of fresh and boiled samples (Setiawan *et al.*, 2019; Wijaya *et al.*, 2014; Wu *et al.*, 2009). The winged beans were obtained from plantations in the Ciawi area, Bogor Regency, Indonesia. The winged bean seeds used are seeds that were harvested when the plants were 8 weeks old and soaked in water for 18 hours. The skin of the fresh winged bean (FWB) that had been soaked was peeled using an abrasive peeler, dried in a drying oven at 50°C, and pulverized. The flouring was carried out using a pin disc mill with a sieve size of 60 meshes. In the boiling process, the winged bean seeds were boiled for 30 minutes, and the skin of the winged bean was also peeled using an abrasive peeler, dried in a drying oven at 50°C, and mashed. The flours of FWBs and boiled wing beans, each as much as 100 g, were extracted using n-hexane (1:5) for 2 hours at room temperature and centrifuged at 4,000 rpm at 40°C for 15 minutes. The precipitate was dried in a drying oven at 50°C for 2 hours. The sample was mixed with distilled water (ratio 1:10 w/v), 1 N NaOH was added to raise the pH to 8–8.5, and the mixture was stirred at 40°C for 90 minutes and centrifuged at 4,500 rpm for 30 minutes at 4°C. The pH of the obtained supernatant was lowered to 3.45 using 1 N HCl; then, it was centrifuged at 4,500 rpm and 4°C for 20 minutes. The sample was dried with a freeze drier and analyzed for protein by the Bradford method.

Protein isolate profile analysis with SDS-PAGE

Winged bean protein isolates were characterized by SDS-PAGE using acrylamide gel (Wijaya *et al.*, 2014). A whole 40 µl sample was transferred into a 0.5 ml microtube, and 10 µl

of sample buffer was added. The tube was then heated in boiling water at 100°C for 5 minutes. The resulting gel was transferred to a closed container that already contained a dye of Coomassie Brilliant Blue G-250.

Serum preparation of allergy patients

A total of 10 food allergy patients had their serum taken by medical personnel from the Indrajana Allergy Clinic in Jakarta (Research Code of Ethics No. 168/IT3.KEPMSM-IPB/SK/2019) (Astuti *et al.*, 2016; Wijaya *et al.*, 2014). The blood was placed in a tube containing no EDTA and was centrifuged at 2,500 rpm (1,250 g) for 20 minutes. The serum was stored at –20°C.

Total serum IgE analysis by ELISA method

100 µl of serum which had undergone a 1:10 dilution (in a 0.05 M carbonate-bicarbonate buffer with pH 9.6) was tied to a microtiter plate and was incubated overnight at 4°C (Wijaya *et al.*, 2014). As a negative control, normal serum was used. The remaining samples were discarded, and the microtiter plate was washed five times using PBST (250 µl/well). Then, 200 µl of 5% skim milk in PBST was added to the microtiter plate and was incubated at 37°C for 1 hour. After washing five times using PBST (250 µl/well), HRP-conjugated monoclonal mouse anti-human IgE 1:6,000 antibody was added to PBST at 100 µl/well and was incubated at 37°C for 1 hour. Then, the microtiter plate was washed using PBST (250 µl/well) 10 times, and then 100 µl/well of a TMB substrate was added. Positive results were indicated by the appearance of a blue color. After 5 minutes, the reaction was stopped using 2 M H₂SO₄ of 100 µl/well, and the solution turned bright yellow. Optical density (OD) was measured using an ELISA reader at a wavelength of 450 nm.

Serum specific IgE analysis by ELISA method

The protein sample (10 µg/ml) was dissolved in a carbonate-bicarbonate buffer (0.05 M, pH 9.8), and then it was incubated overnight at 4°C. The plates were then washed five times using PBST of 250 µl/well, blocked with 5% skim milk in PBST of 200 µl/well, and incubated at 37°C for 1 hour. Then, the microtiter plate was washed using PBST (250 µl/well) five times. The serum of allergy patients that had been diluted 1:10 in PBST was added, as much as 100 µl/well, and then incubated at 37°C for 1 hour. After incubation, the plate was washed using PBST (250 µl/well) five times; then, 100 µl/well of HRP-conjugated monoclonal mouse anti-human IgE 1:6,000 antibody in PBST was added and then incubated at 37°C for 1 hour. The plate was washed using PBST (250 µl/well) 10 times, and 100 µl/well of a TMB substrate was added. Positive results were indicated by the appearance of a blue color. After 5 minutes, the reaction was stopped using 2 M H₂SO₄ of 100 µl/well, and the solution turned bright yellow. OD was measured using an ELISA reader at a wavelength of 450 nm.

Immunoblotting

The unstained electrophoresis gel was transferred to a nitrocellulose membrane (0.45 m) (Wijaya *et al.*, 2014). Blotting was performed at 90 V for 90 minutes and the membrane was soaked or fixed with 50% methanol for 2 minutes and then blocked with 5% skimmed milk in PBST at room temperature for 1 hour.

The membranes were washed using PBST three times, added with serum from allergy patients (1:10) in PBST, and incubated at room temperature for 2 hours. Washing was performed again using PBST three times, and then HRP-conjugated monoclonal mouse anti-human IgE antibody (1:3,000 dilution in PBST) was added and incubated for 1 hour while shaking. The membrane was washed again using PBST three times, and a DAB substrate was added. The positive detection result of the allergen protein complex with serum was indicated by the formation of a brown band on the nitrocellulose membrane.

Skin prick test

The SPT was performed by an allergologist at the Allergy and Asthma Clinic, DR. Indrajana, Jakarta (Jeong *et al.*, 2013; Maleki *et al.*, 2010; Wijaya *et al.*, 2014; Wijaya *et al.*, 2015). The research subjects for this SPT were 10 people with allergies. The skin prick test was performed on the volar part of the forearm, which was previously disinfected with alcohol and marked with a ballpoint pen. As a positive control, histamine 1 $\mu\text{g}/\mu\text{l}$ was used, and as a negative control, a 50% glycerol-saline solution was used. The result was declared 0 if the size of the bumps was the same as the negative control (no bumps formed). The result was declared +1 if the size of the bumps was 25%–50% larger than the negative control (<3 mm). The result was declared +2 if the size of the bumps was 50%–75% larger than the negative control (3–5 mm), +3 if the size of the bumps was the same as the positive control (5–7 mm), +4 if the size of the bumps was 25%–50% larger than the positive control, and >+4 if the size of the bumps was more than 50% of the positive control.

RESULTS AND DISCUSSION

Isolation of winged bean seed protein

The concentration of the boiled winged bean (BWB) protein extract was 2,187 mg/l, while the total concentration of the FWB protein extract was 2,106 mg/l (Fig. 1). The protein concentration of BWBs was higher than that of FWBs. This could be due to the boiling process.

The heating process causes changes in protein solubility so that it affects the amount and type of protein that can be extracted in the protein isolation process (Nirbaya *et al.*, 2020; Nirbaya *et al.*, 2021). The treatment also caused the winged bean's

allergenicity to change. According to Vissers *et al.* (2011), the thermal process of a protein can cause changes in protein structure that can cause changes in its allergenicity. Protein allergenicity can be decreased or increased by treatment because it can change the structure of the allergen so that the allergen is not recognized by antibodies (Suseno *et al.*, 2016; Zakaria *et al.*, 1992a).

The SDS-PAGE profile of winged bean extracts is presented in Figure 2. In SDS-PAGE, protein bands were formed consisting of many proteins with different band thicknesses indicating the high and low concentrations of the proteins in a sample (Ma *et al.* 2009). The area of the protein band thickness was calculated using the GelAnalyzer 2010a software.

The results of the GelAnalyzer 2010a software showed that the FWB had 13 protein bands with molecular weights of 89, 68, 48, 35, 27, 24, and 23 kDa and 6 bands with a molecular weight of 22 kDa. Meanwhile, the BWB had 10 protein bands with molecular weights of 73, 51, 36, 24, and 23 kDa and 5 bands of 22 kDa (Fig. 3).

The treated winged bean sample (BWB) had fewer protein bands than the sample without a treatment (FWB). The greatest intensity in FWB was the protein with a BM of 68 kDa at 13.7%, while the greatest intensity in BWB was the protein with a BM of 22 kDa at 19.5%. Based on the WHO/IUIS Allergen Nomenclature database, winged beans have no allergen data.

Analysis of Total IgE and Specific IgE

The results of the total IgE analysis of 10 positive food allergy respondents are described in Figure 4. All the results of the total IgE analysis of the respondents showed that all respondents were allergy patients with different levels of allergies. The greater total IgE of a person indicates the higher level of allergy (Zakaria *et al.*, 1998). Figure 4 shows that the respondent who had the highest level of allergy was respondent 5 and the respondent who had the lowest level of allergy was respondent 6. IgE antibodies in serum that can bind to protein isolates are specific IgE antibodies to isolate proteins as allergens. A high specific IgE value indicates a strong binding of IgE in allergic subjects with specific allergen proteins (Chalid *et al.*, 2019).

The analysis of specific IgE extracted from the winged bean in the 10 respondents was performed using the ELISA method because it is a fairly good method to show specific IgE reactivity with serum IgE at low concentrations (Astuti *et al.*, 2015).

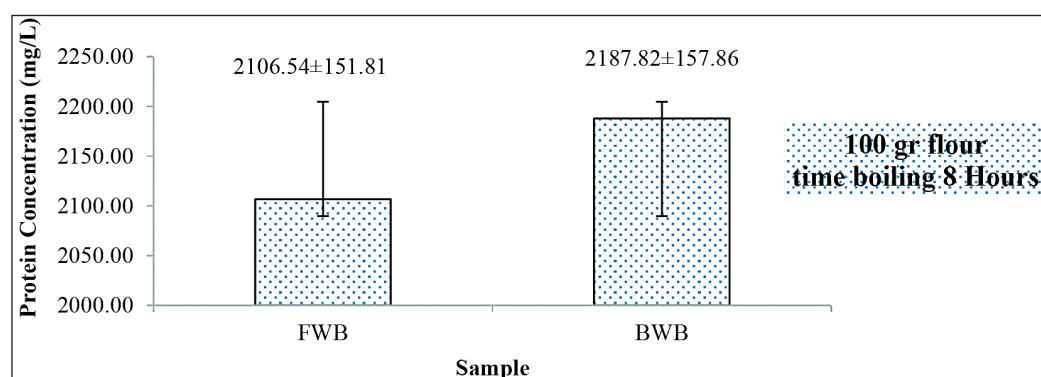


Figure 1. FWB and BWB proteins.

The results of the specific IgE analysis from the winged bean sample showed that, from 10 respondents, only 9 respondents were positive for allergies to fresh and BWBs, i.e., respondents 2, 3, 4, 5, 6, 7, 8, 9, and 10 (Fig. 5).

Total IgE results were not positively correlated with specific IgE results (Astuti *et al.*, 2018). Total IgE only shows if a person is suffering from allergies, but it cannot show the type of allergen. This was indicated in respondent 1, who had positive total IgE, but the specific IgE was negative. It is possible that respondent 1 had an allergy to a type of food other than winged beans.

The immunoblotting results of 10 allergy-positive respondents, whose serum was taken, were also analyzed using the GelAnalyzer 2010a software. The results showed that the FWB protein could bind to specific IgE antibodies in respondents 1, 5, 7, and 9. Meanwhile, the BWB protein could bind to specific IgE antibodies in respondents 7 and 8. The allergen protein of the FWB, which could bind to specific IgE in respondent 1, was a protein band with a BM of 24 kDa. In respondent 5, it was a protein band with a BM of 219 kDa. In respondent 7, the positive IgE was bound to protein bands in FWB samples at 29 and 23 kDa.

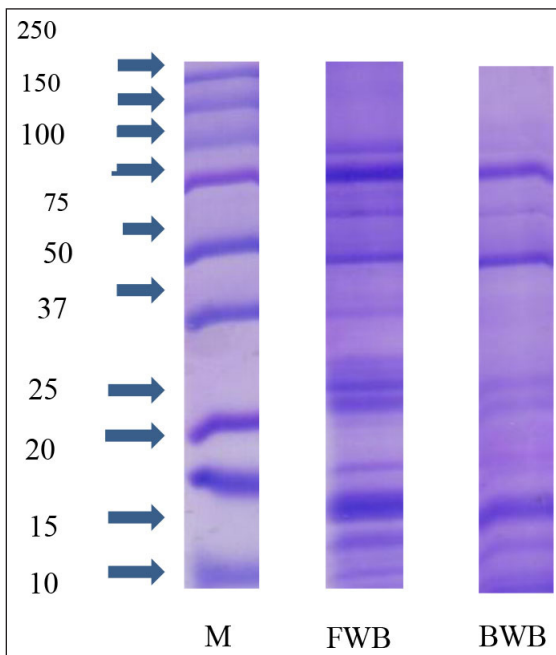


Figure 2. Profile of SDS-PAGE of FWB and BWB extracts.

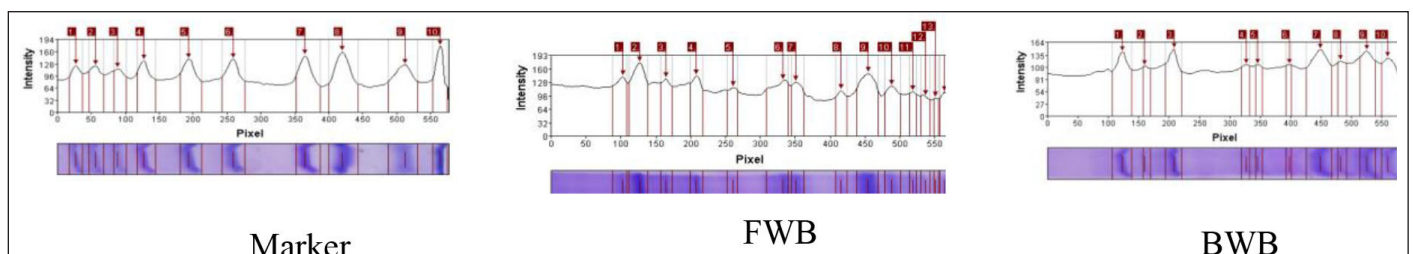


Figure 3. SDS-PAGE protein marker (M), FWB protein, and BWB after analysis using GelAnalyzer 2010a software.

In respondent 9, positive IgE was bound to the protein band in the FWB samples at 17 kDa (Fig. 6).

In the BWB samples, the allergen protein was bound to specific IgE only in respondents 7 and 8. The allergen protein of the BWB that was bound to specific IgE in respondent 7 was in the protein band with BM of 3 and 24 kDa. Meanwhile, in respondent 8, the allergen protein of the BWB was bound to specific IgE in the protein band with BM of 31 and 23 kDa.

The results of immunoblotting analyzed with the GelAnalyzer 2010a software were different from those in SDS-PAGE. The protein band in SDS-PAGE was more abundant than the protein band in immunoblotting. This is because not all proteins in the sample can bind to specific IgE in the serum. This means that not all protein fractions in the extract can produce IgE if ingested and not all winged bean protein fractions can cause allergies. Therefore, for use as a SPT reagent, it is better to use whole extracts and it is not necessary to separate the winged bean protein to produce SPT reagents (Wijaya *et al.*, 2014). For the manufacture of SPT reagents, the use of whole protein extracts without protein fractionation is much safer because with whole extracts the presence of all protein fractions is guaranteed to be in the whole extract.

Skin prick test

In Table 1, the total IgE results show that 10 respondents were allergy sufferers. Specific IgE results from FWBs showed 9 people had positive results and only 1 person had negative specific IgE results. The results of the SPT showed that respondent 1 was negative for winged bean-specific IgE but showed positive results for the SPT in the FWB (+1) and BWB (+1) samples. Respondents 4, 5, and 8 had negative results on the SPT for all samples, both FWB and BWB, even though the specific IgE results were positive. Respondents 6, 7, 8, 9, and 10 showed varying results between specific IgE and SPT results. Respondents 6, 9, and 10 had a positive specific IgE, but the SPT results only showed a positive result (+1) for FWB, while they showed a negative result for BWB. In respondent 7, the SPT with a positive result was only the BWB (+1).

The SPT results showed the appearance of red bumps on the skin (Fig. 7). This occurred because of the cross-link between the rice eel allergens used and the IgE bound to the mastocyte cells of allergy sufferers. This further caused the release of histamine and other mediators from the mastocyte cells, which resulted in vasodilation and increased blood vessel permeability, resulting in redness of the skin and bumps (Wijaya *et al.*, 2015). The results of total IgE and specific IgE of all subjects showed a positive reaction.

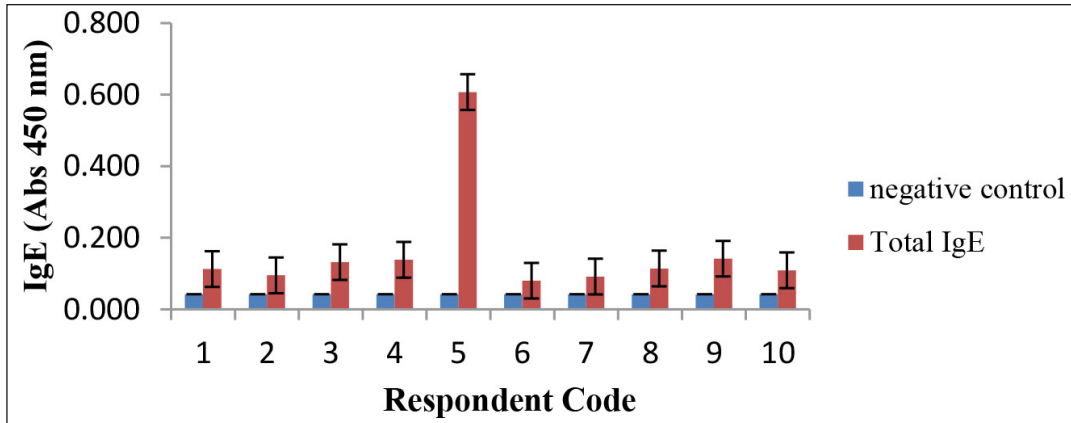


Figure 4. Analysis of total IgE in the serum of 10 respondents with allergies.

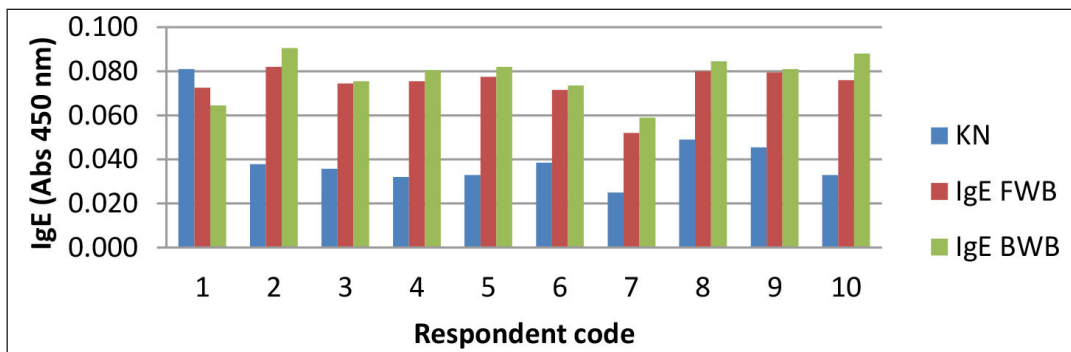


Figure 5. Specific IgE antibodies on samples of FWB and BWB in the serum of 10 allergy patients.

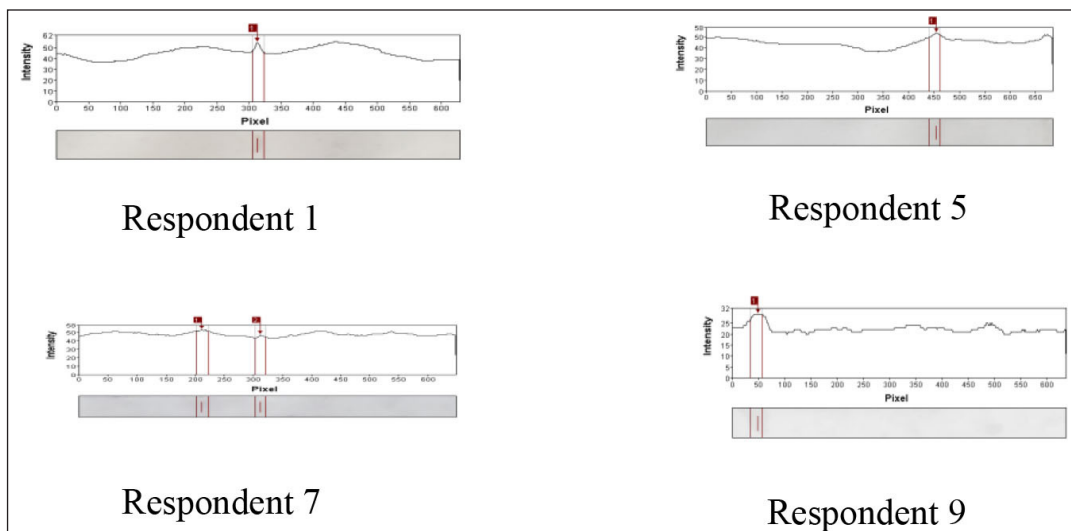


Figure 6. Immunoblotting results of FWB protein on the serum from respondents 1, 5, 7, and 9.

Table 1. Results of total IgE, specific IgE, and skin prick test of winged beans.

Repondent	Total IgE result	Specific IgE result		Skin prick test result	
		FWB	BWB	FWB	BWB
1	+	-	-	+1	+1
2	+	+	+	+4	+2
3	+	+	+	+3	+3
4	+	+	+	0	0
5	+	+	+	0	0
6	+	+	+	+1	0
7	+	+	+	0	+1
8	+	+	+	0	0
9	+	+	+	+1	0
10	+	+	+	+1	0

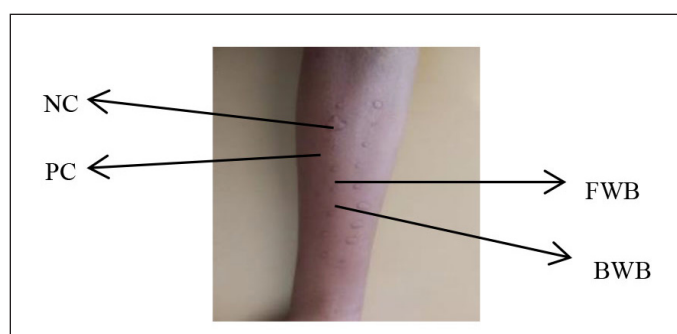


Figure 7. Skin prick test results for fresh and boiled winged bean allergens. Description: NC = negative control, PC = positive control, FWB = fresh winged bean, and BWB = boiled winged bean.

However, the results of the SPT showed varying results. This is presumably due to the protein content of the different samples. However, overall, all subjects showed positive SPT results.

CONCLUSIONS

The SDS-PAGE profile in this study showed that the FWB had 11 protein bands with molecular weights of 89, 67, 47, 34, 24, and 23 kDa and 5 bands of 22 kDa. Meanwhile, the boiled winged bean (BWB) contained 9 protein bands with molecular weights of 73, 35, 24, and 23 kDa with 2 bands and 4 bands of 22 kDa. The protein extract was tested for protein allergenicity using the ELISA method, and it showed the presence of allergenicity. The winged bean protein extract had the ability to bind IgE in the subject's serum, so it can be used for making SPT allergens. The SPT results showed that there was an allergen protein in the fresh and boiled winged bean samples that can be used for the SPT reagent.

The reagent SPT from FWB and BWB complies with the Standardization of Allergen Extracts European Pharmacopoeia Monograph of Allergen Products and has been trialed in humans. For application as a reagent, it is sufficient to use whole protein extracts and there is no need to use protein fractions because as seen in the results of the immunoblotting and SPT not all protein fractions from the protein extracts reacted positively and specifically to the IgE sera of the allergic subjects. The cost of this SPT is relatively low, so people do not mind doing allergy tests so they can easily find out which protein is right for them. It is

hoped that people, especially children, do not have to worry about consuming various proteins. The need for protein is very important considering that children, especially those who are still growing, need adequate amounts of it to prevent protein deficiency due to a condition of lack of nutritional intake for a long time, which results in growth disorders in children, namely, high body height, lower than their age.

In addition, a SPT also needs to be carried out considering the consequences that arise from allergic reactions similar to reactions due to infection with microbes, such as diarrhea, abdominal pain, and vomiting. This is very important because it can prevent the inappropriate use of antibiotics due to frequent misdiagnosis, especially in children.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This research was approved by Indrajana Allergy Clinic in Jakarta (Research Code of Ethics No. 168/IT3.KEPMSM-IPB/SK/2019).

DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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