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The Development Extract Protein of Bambara Nut (*Vigna subterranea* (L.) Verdc.) As a Reagent for Detecting Food Allergies on Skin Prick Test Method

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Abstract: *This study aimed to prepare the protein extract of bambara nut seeds, characterize and apply as a reagent for Skin Prick Test (SPT). Bambara nut protein was extracted from seeds flour of Bambara nut by isoelectric precipitation then analyzed by using SDS-PAGE. The protein extract was formulated into a SPT reagent and met the requirements of the European Pharmacopoeia Monograph on Allergen Products 7 (2010:1063). The SPT reagent was applied on 11 adult subjects who have been suffered food allergies and 9 adult individuals non food allergies. Sera of subjects were collected to measure the IgE total and specific IgE-binding. Immunobloting were also performed on sera of bambara nut allergies. The number of bands were detected in protein extract of bambara nut were 14 bands with the molecular weight of 17-122 kDa. The SPT results suggest that diameter of wheal were ≥ 5 mm. Sensitivity of extract bambara nut protein was 91% and a negative error of 9%, whereas specificity of those was 100% and an error rate of positive diagnosis occurrence at 0%. Two sera subjects of these bambara nut allergic participants showed the specific binding to allergen of bambara nut.*

Keywords – *bambara nut, skin prick test, spesific IgE-binding, sensitivity, specificity*

I. INTRODUCTION

Bambara nut (*Vigna subterranea* (L.) Verdc.), also was known as bambara groundnut was a member of the family *fabaceae* that an indigenous plant from west Africa. In Indonesia, bambara nut was called as *kacang bogor* which was widely cultivated in the area of Bogor, west Java. Bambara nut protein was one of the important resources of vegetable protein due to high nutritional value. Therefore, in developing countries have paid more attention to develop bambara nut in order to overcoming protein malnutrition [1]. Protein and carbohydrate were the main nutrient content of bambara nut seeds [2], essential amino acid predominantly was lysine and leucine [3].The literature study on food allergy indicates that there have been no reports of bambara nut allergic. However, the potency of allergen was estimated come from protein and carbohydrate contents on bambara nut. We designed this study to answer these allegation, thus the main goal of this research consist of extraction, characterization, application of bambara nut protein extract as a skin prick test reagent and determination of sensitivity and specificity of these reagent.

The skin prick test (SPT) was the most widely test used for detecting allergen that caused IgE-mediated food hypersensitivity. It was safe, invasive, inexpensive, results are immediately available in clinic within 10–15 min [4]. Furthermore, skin prick testing was safe which a very small amount of allergen was pricked on the forearm and carried out by physicians [5]. To date, the best managing and treatment of food allergies were kept off food allergens [6];[7]. Avoiding certain foods should be established according to the result of allergy tests, such as skin prick test and double-blind placebo-controlled food challenges. The positive result of skin prick test have to be confirmed with a challenge test [8]. Allergen extract solution (reagent) on SPT have a high quality for accurate diagnosis which are commonly prepared from fresh or cooked foods [9]. The reagent that are used to diagnostic food allergen have been through a previous allergenic potency assessment by the *in vivo* and *in vitro* methods. They are often first characterized by identification of their component proteins using SDS-PAGE and protein concentration by one of several assays [10]. The *in vivo* test was done by skin test to assay the allergenic potency[11]. Furthermore, the reagent should have a high sensitivity since SPT was usually the first diagnostic procedure applied when food allergy was suspected.

Protein extraction methods is crucial on SPT reagent, their containing adequate concentrations of all major and minor allergenic determinants, because not all patients are allergic to any antigen in the extract [4]. The structures and properties of extract components may be influenced by external factors including allergen source, pre-extraction procedures, extraction conditions, post-extraction processing steps and storage conditions [12]. They are sometimes defatted, tend to be crude allergenic mixture and put into 50% of glycerin to minimize degradation during storage [11].

Numerous method of protein extraction from the plant have been conducted, they were : isoelectric precipitation, alcohol precipitation, isoelectric precipitation with alcohol precipitation and alkali solution with isoelectric precipitation [13]. Isoelectric precipitation and various combination of physicochemical parameters are used to extract protein of bambara nut [14]. However, protein prepared by isoelectric precipitation and isoelectric precipitation combined with alcohol precipitation had better in gel properties [13]. Bambara nut flour was made from seeds [14] without deffated and protein extract was prepared by isoelectric precipitation method [15]. In order to standardize the allergenic extract have to be utilized *in vivo* and *in vitro* test. A real picture of biological activity are *in vivo* techniques is skin prick test. The enzyme-linked immunosorbent assay (ELISA) techniques is the best solution to *in vitro* test in connection with molecular-biological approaches [16]. The study was approved by local Ethics Committee and all subjects gave written informed consent before join with the study as participants.

II. MATERIALS AND METHODS

2.1 Materials : Bambara nut (*Vigna subterranea* (L.) Verdc.) was obtained from local market at Bogor, west Java. BSA (bovine serum albumin), acrylamide, glycine, 2-Mercaptoethanol, 0.05 M carbonate-bicarbonate buffer at pH 9.6, coomasie brilliant blue R-250, coomasie 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'-methylene bisacrylamide, SDS-PAGE weight standard low range, BioRad (containing 9 types of protein, which are myosin (MW:200 kDa), β-galactosidase (MW:116 kDa), phosphorylase b (MW:97 kDa), serum albumin (MW:66.2 kDa), ovalbumin (MW:45 kDa), carbonic andhydrase (MW: 31 kDa), trypsin inhibitor (MW: 21 kDa), lysozyme (MW: 14.4 kDa), and aprotinin (MW: 6.5 kDa).

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, the polyvinylidene fluoride membrane for blotting pore size 0.45 µm, size 15 cm x 15 cm (Sigma N8267), pH meter, sonicator, vortex mixer, stirrer, 0.5 µL to 1000 µL micropipettes, 0.2 µm SFCA syringe filter, Whatman no. 1 filter paper, and other glasswares.

2.3 Preparation of Bambara Nut Extracts : Bambara nut seeds were washed and rinsed in deionized water. They were dried in an oven at 50^{o} C for 72 h and hulled by manually. The dried seeds were ground into flour, passed through 75 µm mesh sieve [14]. Flour was dispersed in distilled water (1:10 w/v) and the pH was adjusted to 8.0 with 1N sodium hydroxide to facilitate protein solubility. It was stirred for 1 h at room temperature. The suspension was centrifuged at 3500g, 4^{o} C for 30 min. The pH of the supernatant was adjusted to 4.0 with 1N hydrochloric acid and stirred at room temperature for 1 h in order to clear acid and to precipitate the protein, then was centrifuged at 3500g, 4^{o} C for 30 min. The precipitate was collected and dried using freeze dryer [13];[15]. The Bradford assay was performed to get a protein content.

2.4 SDS-PAGE Analysis : Electrophoresis was conducted according to Laemmli [17] (apparatus from BioRad laboratories). The sample was applied to 12% (w/v) of separating gel and 5% (w/v) stacking gel. Aproximately 40 µL (1µg/µL) sample were boiled at 95^{o} C for 5 minutes in sample buffer containing bromophenol blue, glycerol, SDS and 2-mercaptoethanol. Then, 15 µL sample were loaded to gel and run at 90 V (constant voltage) for 1.5 hours. The gel was stained with coomasie brilliant blue R-250.

2.5 Subjects : Eleven adult participants who is suffered food allergies and 9 adult individuals non food allergies to be included in this study. These volunteers will be dropped with extract protein of bambara nut on their volar side of the forearm. Before SPT would be done, 10 mL blood were colleted from 20 subjects then centrifuged for 20 minutes at 4^{o} C and 1,250 g. The sera will be used to determine total IgE and spesific IgE-binding.

2.6 Reagents Prepation for SPT : Reagent of SPT was made by 0.2 grams protein extract of bambara nut was dissolved with 2 mL phosphate-buffered saline (PBS), pH 8 and sonicated 5 times (1 minute each) and centrifuged at 11,780 *g* for 15 minutes. The supernatant was filter-sterilized 0.22 µm. Protein concentrations were determined by Bradford method (Bradford, 1976). Extract were reconstituted in a phenolated saline solution (0.9% sodium chloride, 0.4% phenol) containing 50% glycerol (v/v) [18];[19]. This reagent have to be free contaminant and skin test were carried out with 1mg/mL bambara nut concentrations.

2.7 Skin Prick Test Procedure : Skin prick test was done by physician on 11 food allergies subjects and 9 subjects non food allergies. Subjects must have avoided antihistamines and other interfering drugs at least 2 days before [20]. The subjects underwent extract protein of bambara nut, negative (saline) and positive (histamine dihydrochloride, 1 mg/mL) controls on their forearm. The first: mark the skin with the initial letter of each reagents. Each site should be a minimum of 2 cm apart. Small amounts of each SPT reagent drop on area that have marked using the brown marrow needle then pricked slowly untill substance was transfered into the epidermal layer without causing bleeding. Each of reagent did not allow to run onto the next prick site. The results read after 15 minutes by measuring the developed wheal. The wheal on skin 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 (3-5 mm) and "+3" if the wheal size is equal to the histamine (5-7 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 [18].

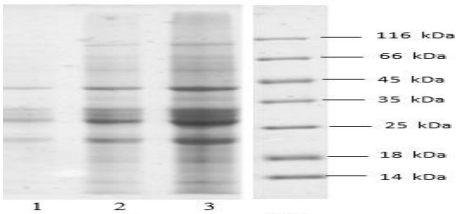
2.8 Total IgE Detection : Detection of total IgE on subjects serum was done by ELISA. Serum was diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6 (1:10). 100 µL serum/well were coated on microtiter plate and incubated for over night at 4 °C. Normal human sera were used as control. After washing five times with 300 µL/well phosphate-buffered saline tween (PBST), the plate were blocked with 5% skim milk in PBST for 1 hour at 37 ° C (200 µL/well). After washing five times with PBST (300 µL/well) the plate were added with 100 µL/well of HRP conjugated monoclonal mouse anti-human IgE antibody (1:6000 in PBST) and incubated at 37 °C for one hour. The plates were washed five times with PBST (300 µL/well). The reaction was developed blue color by adding TMB substrate (100 µL/well). The reaction was stopped by the addition 2M sulfuric acid (25 µL/well), the bright yellow color that developed was read using ELISA reader at 450 nm [21]. Mean ± 2SD of normal controls was taken as cut-off for ELISA positive results [22]

2.9 Specific IgE Detection : Specific IgE-binding to bambara nut extract was determined by ELISA. Microtiter plates were coated at 4 °C for over night with 100 mL/well of bambara nut extract which was diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (10 µg/mL) and 5% skim milk in coating buffer as control. After washing five times with 300 µL/well with PBST, the plate were blocked 1 hour at 37° C with 5% skim milk in PBST (200 µL/well). Furthermore, it was washed five times with PBST (300 µL/well). 100 µL/well serum of subjects (1:10 in PBST) was added and incubated for 1 hour at 37° C. Normal human sera were used as control. The microtiter plates were washed five times with PBST (300 µL/well), then incubated for 1 hour at 37° C with 100 µL/well of HRP conjugated monoclonal mouse anti-human IgE antibody (1:6000 in PBST). After washing five time with PBST (300 µL/well), the reaction was developed blue color by adding TMB substrate (100 µL/wel). After the reaction were stopped by adding 2 M sulfuric acid (25 µL/well), the solution would turn bright yellow. Optical density (OD) was measured using ELISA reader at 450 nm [23]. Mean ± 2SD of normal controls was taken as cut-off for ELISA positive results [22]

2.10 Immunobloting of Extract Protein with Sera Subjects : Immunobloting was performed with patient sera from 10 subjects with bambara nut allergy for detection of IgE binding to allergen of extract. The unstained gel from electrophoresis result was transferred to polyvinylidene fluoride membrane using transblotting equipment with 90 V voltage for 150 minutes. After electroblotting, the polyvinylidene fluoride membrane were cut into strips of 0.5 cm. The strips were blocked with 5% skim milk in PBST at room temperature for 1 hour, washed three times with PBST (5 minutes for each). Each strip was incubated with 2 mL serum at 4^{o} C for overnight (1:10 in PBST). Furthermore strips were washed three times with PBST, then incubated for 1 hour with HRP conjugated monoclonal mouse anti-human IgE antibody (1:3000 dilution in PBST). After that, the membrane was re-washed three times with PBST, and added with DAB substrate. Positive detection results were marked with the formation of brown-colored band on membrane [24] [18]

III. RESULTS AND DISCUSSION

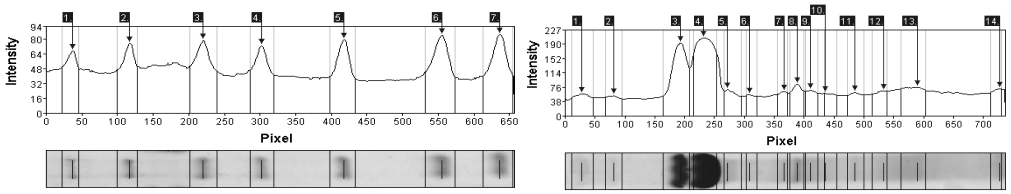
3.1 Protein Profiles of Bambara Nut Extract : The composition of protein, fat, moisture content, ash and carbohydrate were determined in bambara nut seeds. Proximate levels were presented in percentage (%), i.e.: protein (20.8±0.12), fat (6.2±0,78), moisture (7.6±0.36), ash (5.4±0.03), and carbohydrate (59.6 ± 1.29). The profile of the bambara nut extracts on SDS-PAGE at several concentrations were shown in Figure 1.



1

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of bambara nut extract (A), there were loaded by 3 µg lane 1, 10 µg lane 2 and 30 µg lane 3 respectively and marker protein (M)

The identification results using GelAnalyzer 2010a software showed that bambara nut extracts protein consisted of 14 bands with different intensities (Figure 2).



M A

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of bambara nut extract (A) and marker (M)

Result of SDS-PAGE showed that numerous molecular weights were detected from bambara nut protein. They were 122 kDa, 92 kDa, 54 kDa, 46 kDa, 39 kDa, 35 kDa, 29 kDa, 27 kDa, 26 kDa, 24 kDa, 22 kDa, 21 kDa, 19 kDa, and 17 kDa. The extract had two major bands with molecular weight of 54 kDa and 46 kDa. SDS gel electrophoresis of bambara nut with an extraction buffer containing Tris-HCl (pH 7.4) consisted of 14 bands [25]. Proteins of bambara nut were isolated using alkaline extraction had three major bands at 35.0, 43.0, and 112.0 kDa [26].

3.2 Formulation and Quality Requirements of Allergen Products for SPT : The SPT reagent in this study was a product of glycerinated extract containing 50% glycerol and 0.4% phenol. The requirements from European Pharmacopoeia 7 Monograph on Allergen Product (2010:1063) and the results of allergen products analysis of bambara nut extract were shown in Table 1. The results showed that allergen products of bambara nut used for the diagnosis of nut allergy with SPT method have met the requirements of 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 from Bambara Nut

No	*European Pharmacopoeia 7*		Analysis Results of reagent
	Parameters	Requirements	
1	Moisture content (%)	Maximum 5% for freeze-dried products and can be more than 5% for liquid products	60.77
2	Protein content (µg/µl	80-120% from the stated concentration (1 µg/µl)	(1 µg/µl)
3	Sterility	Sterile, if it is not sterile then refers to chapter *5.1.4.* on *European Pharmacopoeia 7 01/2011:50104*	Sterile
4	Total plate count*	Max 10^2 CFU/g or CFU/mL	0.00 ± 0.00
5	*Staphylococcus aureus**	Not detected in 1g or 1 mL	0.00 ± 0.00
6	*Pseudomonas aeruginosa**	Not detected in 1g or 1 mL	0.00 ± 0.00
7	Fungi*	Max 10^1 CFU/g or CFU/mL	0.00 ± 0.00
8	Yeast*		0.00 ± 0.00

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

3.3 Skin Prick Test Results : Skin prick tests (SPT) are most frequently used as the first test to screen for specific IgE on foods. SPT technique was widely used to demonstrate an IgE response to food. Allergens eliciting a wheal at least 3 mm larger than that produced by the negative control are considered as positive, indicating the possibility that the patient has symptomatic reactivity to the specific food [27]. The picture of wheal one of subject bambara nut allergies was presented in Figure 3. Wheal diameter of subject are 7 mm for study extract and 5 mm for positive control and 2.5 mm for negative control, respectively.

A B C

Figure 3. Characterization of wheal subject bambara nut allergy (A), positive control (B) and negative control (C)

The results shown that the value of SPT did not depend on the large size of wheal diameter (Tabel 2). For example, subjects number 24 and 26 have the same wheals diameter (5 mm) but SPT results are different. The SPT results are relative to negative control. A positive result of a specific allergen is indicated by a mean wheal diameter measuring 3 mm or more greater than the negative control. The presence of the wheal indicates that a person has been sensitised to specific allergen while flare or erythema is not used as a gauge of allergic sensitisation [28].

Table 2. Skin Prick Test (SPT) Results on Ten Bambara Nut-Sensitive Subjects

No	Subjects No./sex/age	Allergen history (interview)	Wheal diameter (mm)	SPT results
1	02/F/ 20 y	tuna, dust	7	>+4
2	04/F/19 y	sea food	4	+1
3	17/F/22 y	peanut, soybean	6	+1
4	23/M/20 y	peanut	4	+1
5	24/F/21 y	cold	5	+1
6	26/F/24 y	peanut	5	+2
7	29/F/18 y	shrimp, dust	8	>+4
8	38/F/20 y	nuts	5	+2
9	59/M/20 y	sea food	4	+1
10	66/F/24 y	nuts, dust	5	+1

Abbreviations: N, number; y, years; mm, millimeter; SPT, skin prick test

The interview document stated none of allergic subjects to bambara nut but the SPT results shown that they were positive SPT. In Indonesia, bambara nut was not as popular as peanut or soybean and its utilization in food was also limited, as ingredients on "sayur asam" that it Javanese ethnic cuisine.

3.4 Immunobloting with Sera of Subjects Bambara Nut Allergy : Western blots were also performed on 10 serum of bambara nut subjects allergy. The results was shown at figure 4.

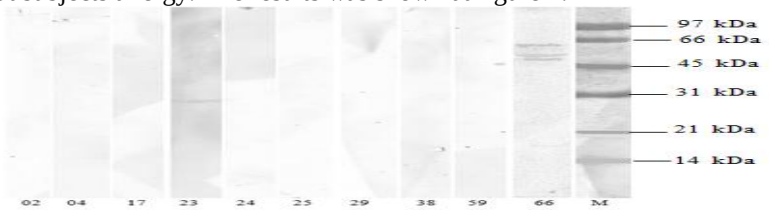


Figure 4. Western blot analysis of skin prick test reagent using serum IgE of subject allergy

Subjects 23 and 66 whose bambara nut allergic exhibited the allergen that binding specific on IgE. Bambara nut extract contain IgE-binding protein in 26 kDa, 48 kDa, 41 kDa and 38 kDa. We could not predict the kind of allergen since there is no data recorded on allergen database. Bambara nut is not novel plant, it is just local food that are well known and originated from tropical Africa. In Indonesia, it was just cultivated on some areas and mainly in the area of Bogor. There are few studies have reported the character of bambara nut protein, the study explained that bambara nut seeds have just five proteins were identical and these are seeds storage protein B, vicilin, beta and alpha isoforms of 8S globulin, and 10kDa protein precursors [29]. Another research reported that bambara nut (*Vigna subterranea*) had the highest similarity with peanut (*Arachis hypogaea*), it means clear indication that they possess phylogenetic relationships [25].

3.5 Comparison of SPT and Spesific IgE : SPT and *in vitro* IgE assays are useful methods to demonstrate the presence of food specific IgE antibodies. Normally, the concentration of total IgE in serum patients allergic and parasitic tends to increase [5];[30]. Table 3 shows that 11 subjects were allergic to bambara nut based on value of total IgE. The analysis of specific IgE showed that 10 subjects gave positive allergic. Based on the data of Table 3, the measurement of specific IgE and SPT results were inconsistent on the subject number eighteen. The same case was also found by Maleki *et al.* (2010), the subjects who anaphylaxis to shrimp shown the specific IgE were negative to peanut allergens, but SPT results were positive to commercial and study peanut extract.

Table 3. Total IgE, specific IgE and Skin Prick Test Results of Bambara Nut Extract on the Subjects Allergies

No	Subject No.	Total IgE	Specific IgE-binding	SPT results
1	2	+	+	+
2	4	+	+	+
3	17	+	+	+
4	18	+	+	-
5	23	+	+	+
6	24	+	+	+
7	25	+	+	+
8	59	+	+	+
9	29	+	+	+
10	38	+	+	+
11	66	+	+	+

No, number; - and 0, negative; +, positive; SPT, skin prick test

Table 4. Total IgE, Specific IgE and Skin Prick Test Result of Bambara Nut Extract on the Subjects Bambara Nut Non Allergy

No	Subjects No.	Total IgE	Spesific IgE	SPT results
1	1	+	-	-
2	3	+	-	-
3	5	+	-	-
4	6	+	-	-
5	7	+	-	-
6	8	+	-	-
7	9	+	-	-
8	15	+	-	-
9	16	+	-	-

No, number; - and 0, negative; +, positive; SPT, skin prick test

All of non allergy subjects showed negative skin tests although total IgE test positive (Tabel 4). It means, value of total IgE from all of sera subjects increase but they did not show allergic to bambara nut. The measurement of specific IgE on sera from all of subjects and SPT result were negative. This phenomenon is reason to support the above statement. All of subjects were suspected allergic to other allergens or parasitic infection.

Table 5. Sensitivity and Specificity of Bambara Nut Skin Prick Test Reagent

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)
Bambara nut extract	10/ 11 (91)	1/11 (9)	0/9 (0)	9/9 (100)

Skin prick test reagent should have a high sensitivity since SPT was usually the first diagnostic procedure applied when food allergy was suspected. Most of the existing commercial food extracts are not standardized and the diagnostic efficiency is still unknown [20]. For this reason, we did standardization of bambara nut allergen extract. Sensitivity and specificity of SPT reagent were calculated on the SPT results of subjects allergy and non-allergy. Sensitivity and specifity of bambara nut extract are 96% and 100%, respectively. It mean that SPT reagent which was used in this study had high specificity and good sensitivity.

IV. CONCLUSION

SPT reagent was made from bambara nut seeds and the protein extraction was conducted by isoelectric precipitation. The characterization of bambara nut extract were done by SDS-PAGE to show protein profile, Bradford assay of protein concentration, ELISA and immunobloting of specific IgE-binding. The extract of bambara nut showed 14 bands by SDS-PAGE and immunobloting analysis results 4 protein allergens but we could not decide the kind of protein. Reagent of SPT was prepared appropriate with the protocol of European Pharmacopoeia 7 Monograph on Allergen Products (2010:1063 and applied to allergy and non-allergy subjects. SPT reagent was used in this study had high specificity and good sensitivity.

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