



A murine model of wheat versus potato allergy: Patatin and 53kDa protein are the potential allergen from potato

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ABSTRACT

Wheat allergy is the most common around the world as gluten is the potential allergen. People diagnosed with wheat allergy were mainly substitute with other novel food such as potato though it is also being reported for allergenic manifestations. Thus there is an increasing demand for developing a BALB/c mice model to empathize the allergic properties of potato protein and its fractions. Purified potato protein showed lower IgE-binding capacity (474.39 ± 0.6 ng/mL) even in higher concentration (30 mg/mL) compared to wheat gluten (1418.28 ± 0.17 ng/mL, 5mg/mL). Immediate active cutaneous hypersensitivity reaction, vascular leakage, intestinal permeability and lung's inflammatory cell infiltration was also ascertained comparatively lower in potato protein than wheat gluten. Furthermore, patatin (43 kDa) and protease inhibitors (~21 kDa) were purified and separated, and patatin exhibited higher hypersensitivity score than that of protease inhibitors. Immuno-detection assays indicated that patatin and 53 kDa protein in potato protein showed specific Ig-E binding capacity, and 53 kDa was adenosyl homocysteine identified by LC-MS/MS.

1. Introduction

A hypersensitivity reaction induced by normally harmless food components can cause severe symptoms of food allergy through immunologic mechanisms. Prevalence of food allergy ranged from 3%–35% for self-reported allergies, and from 2%–5% for reporting based on symptoms and sensitization (Rona et al., 2007). The hypersensitivity reaction is generally influenced by regional factors, genetics, customs and traditions, and is also related to dietetic exposure to new allergenic food products over the world, e.g., peanut allergy is rare in Israel, Italy and Singapore as compare to France, UK, and Switzerland (Dalal et al., 2002). Numerous studies suggested that the prevalence of food allergy was increasing (Sicherer, 2011). Foods adverse reactions based on antibody response are immunoglobulin E (IgE)-mediated relying on mast cells activation in specific tissues, e.g., respiratory tract, skin, gastrointestinal or cardiovascular system, and can be limited from mild irritation to life-threatening anaphylaxis in different individuals to the same allergen (Bischoff and Crowe, 2005). Young children are mostly known to be affected by IgE-mediated food allergies to causal foods including cow's milk, eggs, tree nuts, peanuts,

fish, shellfish, soy and wheat (Sampson, 2004).

Wheat allergy is the most common phenomenon in children, however, adults can also develop wheat allergy symptoms including asthma, hives (urticaria), rhinitis, atopic dermatitis, swelling or other anaphylaxis within minutes or up to 2 h after consumption of wheat food, and may also lead to abdominal pain, diarrhea, vomiting and eczema (Palosuo, 2003; Niggemann et al., 2011; Battais et al., 2005). Gluten composed of high and low molecular weight subunits is one of the potential wheat allergen. People are diagnosed with a wheat allergy need to substitute with other novel food and avoid products that contain wheat ingredients specially wheat gluten. In some western countries, gluten-free-labeled breads and other products are available which are processed by wheat starch or some other materials which have no gluten protein (Sotkovský et al., 2011).

Potato is the fourth most crucial food crop in the world with a production of 381 million tonnes in 2014 after wheat, rice and corn. China is the world's largest potatoes consumer, it is estimated that 50% of the increased food production which will be needed to meet the demands for the next 20 years will come from potato (Faulkner, 2012). Potato is beneficial as a source of energy, it also contains high amounts

Abbreviations: P1, protease inhibitors; P2, patatin; WG, wheat gluten; PBS, phosphate buffer saline; PP, potato protein; TBS, Tris buffer saline; TBST, Tris buffer saline with tween 20

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of protein, essential amino acids, vitamins and minerals, the quality of protein have been shown to be equally nutritious as egg and soy proteins, and it can be used as a source of lysine (Waglay and Karboune, 2016; Vreugdenhil et al., 2007), thus potato can be regarded as a good substitute for wheat products for preventing from wheat allergies.

In contrast to other foods, potato is widely consumed as one of the first complementary foods for young children and the sensitivity of potato is reasonably rare. Furthermore, potato proteins are also capable to exhibit some good clinical aspects in treatment of peri-anal dermatitis (Embden et al., 2004). Some literatures have revealed that consuming potatoes for food or peeling of raw potatoes may evoke allergic reactions (Quirce et al., 1989; Wahl et al., 1990; Delgado et al., 1996; Beausoleil et al., 2001; de-Lagrán et al., 2009). However, there is no predictive biochemical correlation to explain that what stimulates a susceptible individual's immune system to produce IgE by an otherwise innocuous food protein and whether a protein will act as an antigen or forecast the severity of an allergic reaction (Knippels et al., 2004; Metcalfe et al., 2011). Hypersensitivity to raw potato is probably owing to patatin which is the main storage protein of potato tubers, however, little information about the allergenic potentials of patatin was reported (Nater and Zwart, 1967; Seppälä et al., 1999).

The BALB/c mice is the most common mouse strain used for developing a murine model of food allergy as they are good developer of T helper cell 2 (Th2) and elicit IgE mediated immunological response for antigen challenges, whereas the most substantial barrier is the strong innate tendency in the development of oral tolerance to ingested antigens (Boyce and Austen, 2005). In previous studies, T cell subtype involvement, cytokine profile, and secreted chemical mediators involved in allergic reactions have been well documented (Dearman et al., 2000). The ability to sensitize the animal to a specific food extract through intragastric administration and then elicit an allergic response provides clear ties to the human condition.

Therefore, in order to clarify whether the potato protein is allergic, and what's the difference of potential allergies between potato protein and wheat protein, the effects of wheat gluten, potato protein, purified potato patatin, and potato protease inhibitors on the serum IgE level, serum cytokines, vascular leakage, immediated active cutaneous hypersensitivity (IACH) reaction, and histo-pathological section were analyzed, and the amino-terminal sequencing of potato allergen by LC-MS/MS was also determined, thus providing some theoretical foundation for the hypoallergenic of potato protein.

2. Materials and methods

2.1. Materials and reagents

Wheat flour was purchased from Beijing market. Wheat gluten, Evan's blue dye, formalin solution, and 10% neutral buffer were obtained from Sigma-Aldrich Co., St (Louis, MO). ELISA kits for IgE and cytokines detection were purchased from Abcam plc (330 Cambridge science park, UK) and eBioscience, Inc. Science Dr., (San Diego, USA), respectively. Pierce™ fast western blotting kit, mouse & goat anti-mouse IgE secondary antibody for immunoblotting was bought from Thermo Fisher Scientific, Pierce Biotechnology (Meridian Road Rockford, IL 61,105 USA).

2.2. Preparation of potato protein (PP)

PP was isolated from potato fruit juice (PFJ). After washing and peeling, fresh potatoes were sliced and placed in a 50 mM anti-browning agent sodium bi sulfite (NaHSO₃) for 5–10 min and ground. The slurry was thereafter clarified at 8000 g for 1 h, the supernatant was collected and ultra-filtered (Yadonghejishuzhi ultra filtration unit, Shanghai, China), dialyzed (membrane cut-off Mw 10 kDa), lyophilized and stored at –20 °C until used. Purity of protein was determined by the Kjeldhal method using Foss Tecaor automatic protein analyzer

(2300 Kjeltex analyzer unit) after digestion by multiplying an indirect nitrogen-to-protein conversion factor of 6.25 (AOAC, 2006).

2.3. Recovery of potential potato allergens (patatin and protease inhibitors)

Potato allergens were isolated from crude potato proteins according to the procedure which was reported previously with some modifications (Andre et al., 1998). 4 g of lyophilized potato protein was solubilized in 150 mL deionized distilled water and then centrifuged at 8000 g for 20 min at 4 °C to obtain clear supernatant. The extract was then applied to a DEAE Sepharose™ Fast Flow (1 mL; GE Healthcare Bio-Sciences, AB Uppsala Sweden) anion exchange column (Pharmacia Biotech) on AKTA protein pure M1 system (Pharmacia Biotech, Uppsala, Sweden) with unicorn software. The column was equilibrated with 200 mL 0.05 M phosphate buffer (pH 6.0) at 4 °C before 120 mL extract was loaded. The column was subsequently rinsed with 200 mL 0.05 M phosphate buffer (pH 6.0) and the bound protein was eluted with a 50 mM phosphate buffer (pH 6.0) containing 1 M NaCl. The proteins elution was obtained with a flow rate of 5 mL/min at 280 nm. NaCl-eluted protein was collected in fractions of 5 mL and pooled as appropriate. Two pools were obtained, pool 1 containing 10–21 kDa protein fractions (protease inhibitors) was named as P1, pool 2 containing patatin was named as P2. Samples were dialyzed against deionized distilled water, lyophilized as described previously and stored at –20 °C prior to further analysis.

2.4. Animal experiment

BALB/c mice (6–8 weeks of age) were purchased from Vital River Laboratories (VRL) (Beijing, China) and were kept under conventional conditions on a 12 h light/ 12 h dark cycle in a temperature controlled room (24 ± 2 °C) with a relative humidity between 40% ± 10% for 1 week prior to experimental use. Feed pellets and disinfected tap water were provided *ad libitum*. All experiments were carried out according to the regulations of the Experimental Animal Welfare and Ethics Committee, Chinese Association for Laboratory Animal Sciences (CALAS).

2.5. Intragastric sensitization and challenge

Three groups of potato, wheat and PBS (sham sensitized) sensitized (n = 18) BALB/c mice were included in this study. The protocol was as follows, which was performed according to the method of Xie et al. (2017) with some modifications. Briefly, for the stage of basic sensitization, 80 mg/mL freshly homogenized wheat flour and 100 mg/mL freshly homogenized potato flour (equivalent to 11 ± 0.5 mg protein content) in PBS were prepared, and the mice were sensitized daily by intragastric gavage with a final volume of 0.03 mL/g body weight for one week. For the stage of excite sensitization, the mice were fasted overnight and challenged with intragastric gavage with 30 mg/mL (high dose) of freshly homogenized potato protein (PP) or 5 mg/mL (low dose) wheat gluten (WG) in PBS from the 2nd week up to 42 days at weekly intervals, the final volume was also 0.03 mL/g body weight. Furthermore, 50 µL PP, WG, protease inhibitors, and potato patatin (2.5 mg/mL) was also used to study the local acute sensitization test after the 6th week. An equal volume of PBS was received by control mice for daily sham sensitization and challenge (Fig. 1).

2.6. Blood collection and serum isolation

Mice were bled immediately from the submandibular vein after each challenge. Blood was allowed to clot, centrifuged at 8000 g for 10 min and pooled on a treatment group basis. Obtained serum was stored at –20 °C until analysis.

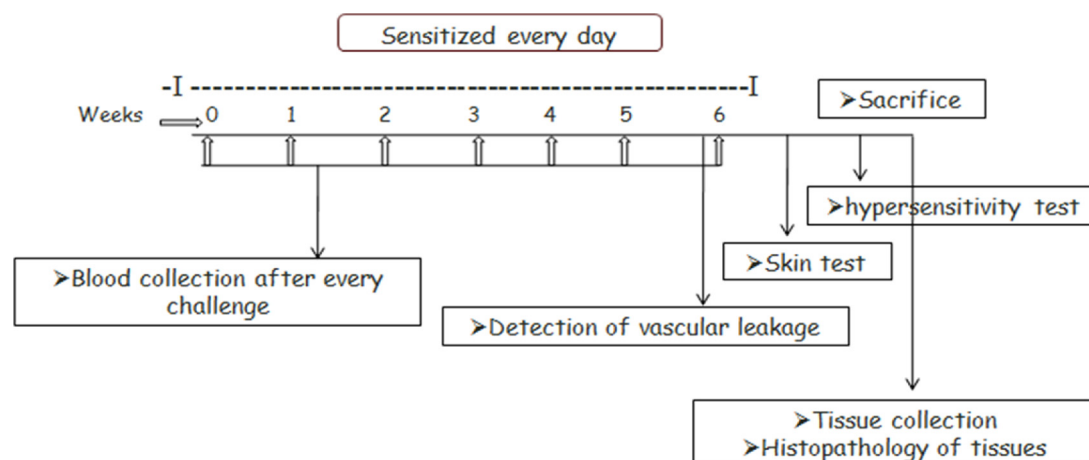


Fig. 1. Schematically represented diagram for sensitization and challenge.

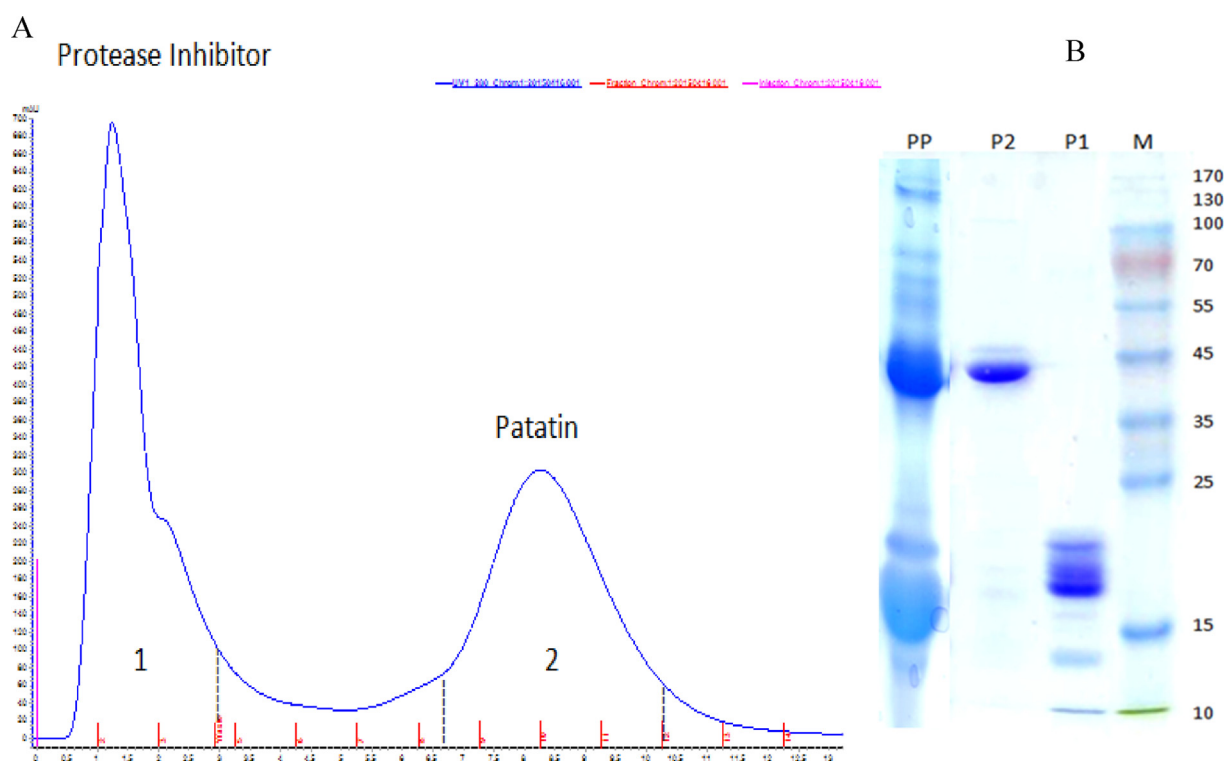


Fig. 2. (A) Fractions obtained after crude potato protein elution through AKTA PURE, (B) SDS-PAGE pattern of purified potato protein fraction.

2.7. Determination of serum IgE levels

Levels of serum IgE in different groups were measured with an ELISA kit (Abcam plc, 330 Cambridge Science Park, UK) according to the manufacturer's instructions. Pooled sera from each sensitized or control mice group was collected and diluted 1:50 with 1X diluents solution. Mouse IgE standard calibrator was prepared at the concentration of 0–400 ng/mL. 100 μ L sera sample or standards was added to polystyrene micro titer well (96 well plate pre-coated with anti-IgE antibodies) in duplicate and incubated at room temperature for 30 ± 2 min. Following incubation, well content was aspirated and washed three times with wash buffer. Serum IgE was detected by addition of 100 μ L of anti-mouse IgE antibodies conjugated with horseradish peroxidase (HRP) for an additional 30 ± 2 min at room temperature in dark. After washing three times with wash buffer as before, the reactions were developed with 100 μ L of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution in the dark for 10 min at

room temperature, and the reaction was stopped with the addition of 1 N H_2SO_4 and then the optical density was read at 450 nm with a microplate reader (HIDEX, plate chameleon V with Micro Win 2000, software version 4.46). Coefficients of variance (CV) % greater than 10 were repeated to assure a high accuracy. Data was analyzed with GraphPad prism 5 (GraphPad Software, Inc. San Diego, CA) referring to the standards for data transformation and nonlinear regression (curve fit) and data were expressed as mean $\pm$ standard deviation (SD).

2.8. Determination of serum cytokines

Levels of interleukin-4 (IL-4), interleukin-5 (IL-5) and interferon- γ (IFN- γ) were assessed by ELISA kits (eBioscience, Inc. Science Dr., San Diego, USA) following the manufacturer's instructions. The limit of detection for IL-4 and IL-5 were < 4 pg/mL, respectively, and IFN- γ was 15 pg/mL.

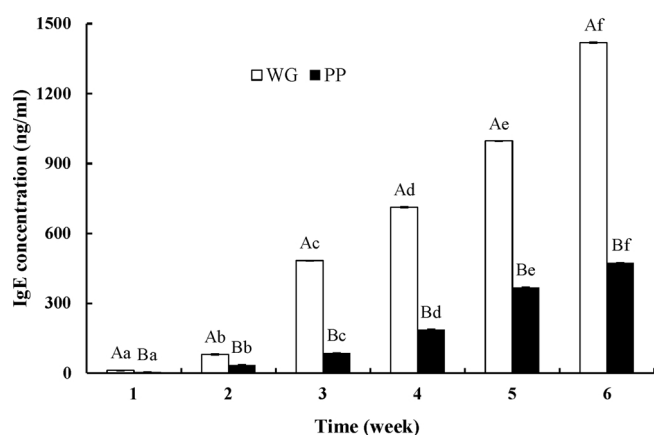


Fig. 3. Serum level of allergenic protein-specific IgE of mice which were sensitized with WG and PP (n = 18 mice per group).

2.9. Detection of vascular leakage

Subsequently prior to the 6th intragastric challenge, the mice were received 100 μ L 0.5% (w/v) Evan's blue dye through submandibular vein injection to evaluate vascular permeability. Visible blue color on footpads of mice was recorded for sign of vascular leakage within 30–40 min after dye/antigen administration. Test was conducted in duplicate.

2.10. Intradermal skin tests

IACH reaction was tested by intradermal skin test after the 6th week of sensitization, as previously described with a slight modification (Proust et al., 2008). Briefly, abdominal skin of 2–3 mice from each group was shaved carefully before one day and marked four different sites (three repeated experimental sites and one negative control site). A 100 μ L 0.5% (w/v) Evan's blue dye intravenously injected to each mice through submandibular vein, followed by 50 μ L of PP, P1, P2, or WG (2.5 mg/mL) in PBS at the marked experimental sites in each mice, PBS was applied as negative control. The visible blue color was assessed after 15–20 min of the dye-antigen administration and the reaction was scored as positive if it resultant with a blue wheal > 0.3 cm in diameter in any direction.

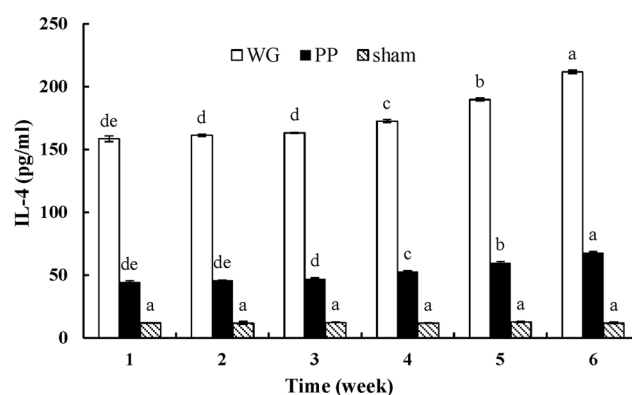
2.11. Assessment of hypersensitivity reactions

Mice were observed for hypersensitivity symptoms within 1–1.5 h after intragastric challenge up to five weeks. At day 42 from initial sensitization, mice (n = 5) in WG and sham group were chosen randomly and challenged intraperitoneally (n = 5) with 50 μ L (5 mg/mL) of WG, and PBS, furthermore, the mice in PP group were also randomly divided into 3 groups (5 mice in each group), and challenged intraperitoneally with 50 μ L (5 mg/mL) PP, P1, and P2, respectively. The systemic anaphylaxis symptoms were assessed by the scoring system as described previously, and scored as follows (Laemmli, 1970):

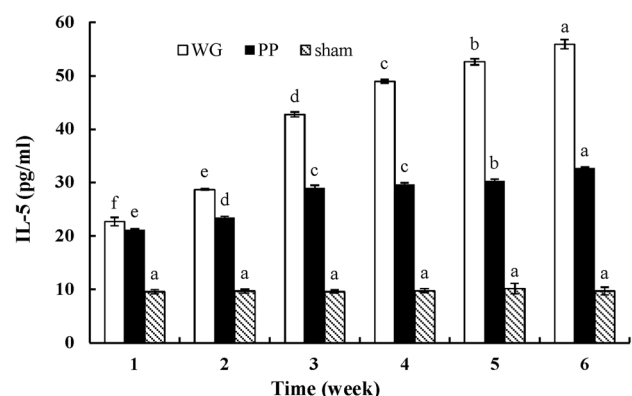
0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, and/or decreased activity with increased respiratory rate; 3 = wheezing, labored respiration, and cyanosis around the mouth and the tail; 4 = no activity after prodding or tremor and convulsion; and 5 = death.

2.12. Histo-pathological analysis

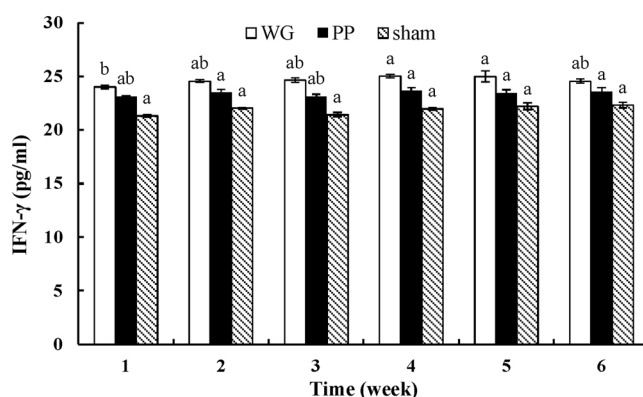
Lungs and intestinal tissues of mice were immediately collected after death to assess the pathologic alterations, the tissues were fixed in 10% formalin neutral buffer (Sigma-Aldrich Co., St. Louis, MO) for 24 h to dehydrate, and 3–5 micron paraffin embedded sections were stained



(A)



(B)



(C)

Fig. 4. Serum level of IL-4 (A), IL-5 (B), and IFN- γ (C) of mice which were sensitized with WG and PP (n = 18 mice per group).

with hematoxylin and eosin (H&E) and photographed using a Nikon Eclipse 80i image-processing system (Nikon Inc., Melville, NY, USA) under 100 magnification, and at least 10 areas of each tissue slice were examined.

2.13. Immuno-detection assays

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

To separate fractions of protein, SDS-PAGE was implemented under reducing conditions on 10% polyacrylamide separating and 4.5%

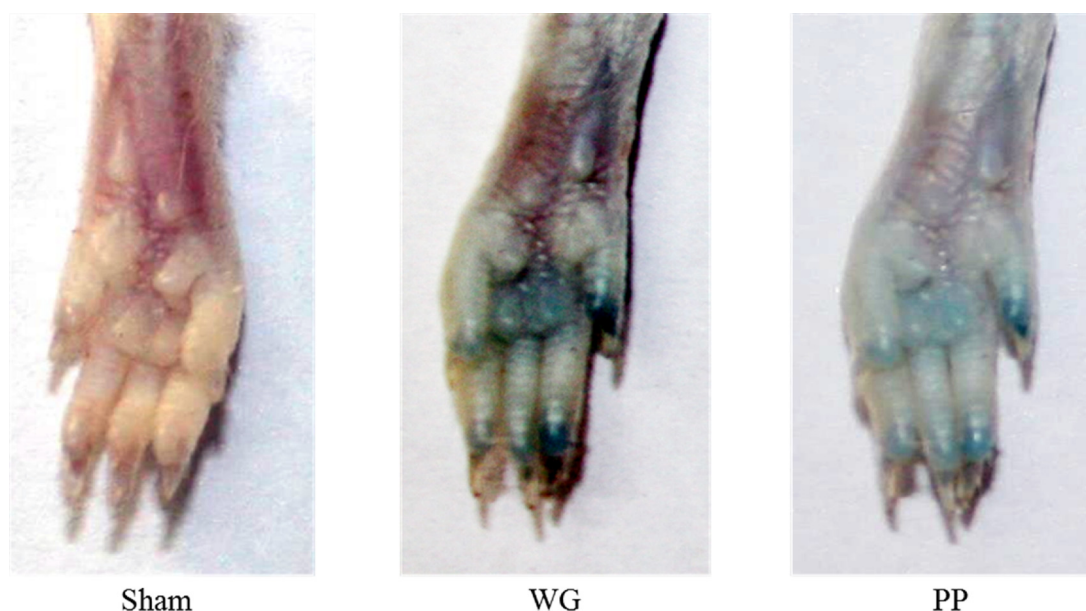


Fig. 5. Vascular leakages of all groups of mice induced by WG and PP (n = 18 mice per group).

Table 1

Results of immediate active cutaneous hypersensitivity (IACH) reaction in different groups (n = 6 mice per group).

Groups sensitized	Diameter (mm)	Positive reaction sites (n/total)
WG	8.98 ± 1.3*	6/6
PP	6.54 ± 2.6*	6/6
P1	1.92 ± 1.6	0/6
P2	7.68 ± 1.4*	6/6
Sham	0.70 ± 0.34	0/6

a) WG: wheat gluten, b) PP: potato protein, c) P1: protease inhibitors, d) P2: potato patatin.

* $p < 0.01$ versus sham sensitized mice.

stacking gel following the standard method (Astwood et al., 1996). 20 μ l 5 mg/mL of WG or PP in sample buffer and 5 μ l of pre-stained protein marker (Thermo Fisher Scientific) loaded to individual well and allowed to run at a constant range of 30 mA/gel for approximately 1.5 h at 20 °C (Mini slab gel, BioRad Laboratories, Hercules, CA, USA). Gels were either stained with Coomassie brilliant blue (CBB) G-250 or probed with mice sera on poly vinylidene difluoride (PVDF) membrane for immuno detection.

2.13.2. Immuno-blotting

Pierce™ fast western blotting kit (Thermo Fisher Scientific) was used to characterize the mice serum IgE binding capacity to WG or PP. Fractionated proteins were transferred to PVDF membrane, 0.2 μ m in a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc. Germany) and was set at 100 V for 60 min in Pierce Western Blot Transfer Buffer (TBS) (Pierce Biotechnology, Rockford, US) with 20% methanol. To assure the transfer and proper binding quality of protein on PVDF membrane, polyacrylamide gel from each pair were stained with Amido-black and CBB stain. Blot were washed briefly in TBST to remove transfer buffer and placed in blocking solution (5%, w/v, Difco™ Skim milk)(Difco Laboratories, Becton, Dickinson and Company Sparks, MD USA) in TBST for 1 h at room temperature with continuous shaking. The blot was then washed 3 times with TBST and incubated with 100 μ l of pooled mice serum in 10 mL of fast western antibody diluent, overnight at 4 °C. Blots were then incubated with 10 μ l goat anti-mouse IgE secondary antibody (1 μ g/mL) in 10 mL antibody diluent for 30 min followed by optimized HRP reagent diluted 1:10 for 10 min at room temperature with continues shaking. Three to four washes with 20 mL

TBST were repeated after each step. WG or PP specific IgE binding components were detected by adding super signal west pico luminol/enhancer solution and super signal west stable peroxide solution at 1:1 and bound IgE was visualized using Gel Doc Image analysis system (Bio-Rad Laboratories Inc.) with a chemiluminescence image detector (Image Quant LAS 4000 min.).

2.14. Determination of potato allergen through LC-MS/MS

The amino acid sequencing and mass fingerprinting of potato allergens, identified through western blot was carried out by LC-MS/MS. The targeted protein were excised from gel (0.5 mm³-1 mm³) with a razor blade, washed with 25 mM ammonium bicarbonate with 50% (v/v) acetonitrile (ACN), reduced with 10 mM dithiothreitol (DTT) for 1 h at 56 °C, alkylated with 100 mM iodoacetamide (IAM) at room temperature for 40 min in dark and were incubated with sequencing-grade modified trypsin at 37 °C overnight. To extract the digested peptides, 0.1% trifluoroacetic acid in ACN was added, the samples were sonicated for 5 min and the separated supernatant was dried under vacuum.

LC-MS/MS analysis was carried out by using mass spectrometer Q-Exactive (Thermo, USA) and chromatographic equipment EASY-nLC 1200 with Reversed-phase C18 column (75 μ m x 25 cm, Thermo, USA) for 90 min with flow rate of 300 nL/min (A: 2% ACN with 0.1% formic acid; B: 80% ACN with 0.1% formic acid). The spectra were analyzed with data acquisition software (Thermo Xcalibur 4.0, Thermo, USA) and searched against solanum tuberosum species information available in the UniProt Database, which website was <http://www.uniprot.org/taxonomy/?query=Solanum+tuberosum&sort=score>. In addition, the protein was identified according to the molecular weight, the number of spectra matched to the peptide segment (PSMs), protein groups, and score sequet HT. The higher PSMs, protein groups, and score sequet HT, the more accurate the protein identification.

3. Results

3.1. Recovery of potential potato allergens (patatin and protease inhibitors) and screening through SDS PAGE

PP was recovered from PFJ as described in the method section and the protein purity was 87.82 ± 0.99% on a dry basis. Protease inhibitors (P1) and patatin (P2) was purified using anion exchange

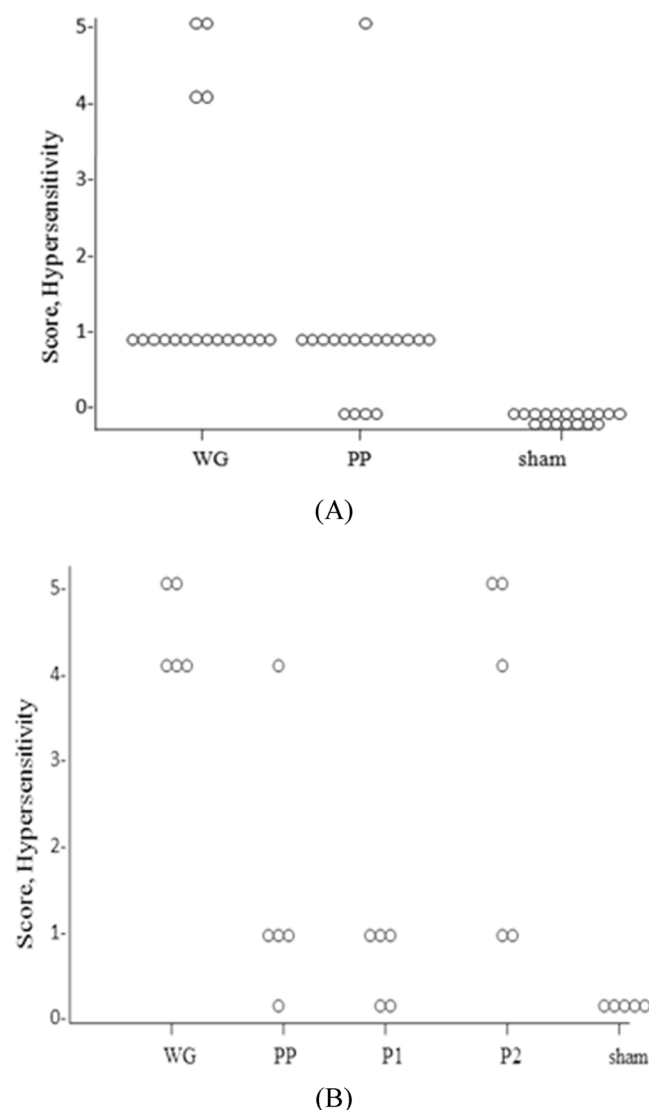


Fig. 6. Symptom scores for assessment of hypersensitivity reaction. (A) Hypersensitivity symptoms scored on a scale from 0 to 5 up to 5 weeks from initial challenge with WG and PP ($n = 18$ mice per group), (B) Hypersensitivity symptoms recorded after challenged with WG, PP, P1, and P2 at the 42nd day ($n = 6$ mice per group).

chromatography through AKTA Pure and resolved on 10% polyacrylamide gel in reducing condition. Electrophorogram obtained by SDS-PAGE in Fig. 2(B) showed that the P1 included 21, 20 and 16 kDa protease inhibitors fractions, P2 was potato allergen patatin and included 43 kDa fractions, while PP showed the presence of 43, 21, 20 and 16 kDa fractions, and some other fractions. In addition, Rizwan (2017) in our lab also confirmed the presence of protease inhibitors and patatin in potato protein by sequencing using LC-MS/MS, besides SDS-PAGE.

3.2. Determination of serum IgE levels

To assess the dynamics of IgE production, serum total IgE was monitored weekly by ELISA kits after sensitization. From Fig. 3, we can see that the content of antigen-specific IgE in the mice which were sensitized by a low dose of WG (5 mg/mL) increased significantly after the third week (482.75 ± 0.24 ng/mL) and reached the highest after 6 weeks (1418.28 ± 0.17 ng/mL) from initial sensitization. While in the group of mice which were sensitized with a much higher dose of PP (30 mg/mL), the content of antigen-specific IgE was much lower

(474.39 ± 0.60 ng/mL) after 6 weeks. In addition, the mice which were fed with PBS (sham group) exhibited no antigen-specific IgE content (0 ng/mL), which could not be shown in the Fig. 3.

3.3. Determination of production of Th1 and Th2 serum cytokines

Level of serum cytokines, such as IL-4, IL-5, and IFN- γ was determined by ELISA kits, which results were shown in Fig. 4A–C, respectively. A significant ($p < 0.01$) rise in serum IL-4 and IL-5 level was detected at the day 21st and reached the highest at 6th week, the content of IL-4 and IL-5 was ranging from 158.47 to 211.8 pg/mL, and 22.7–55.9 pg/mL for wheat sensitized group, respectively, while it was 44.6–67.9 pg/mL and 21.2–32.7 pg/mL for potato allergic group, respectively. However, the level of IFN- γ in wheat and potato sensitized group was similar, and was little higher than that of the sham group.

3.4. Detection of vascular leakage

Vascular leakage induced by WG and PP antigen was determined, and the results were shown in Fig. 5. After Evan's blue dye administration, footpads of mice in each group were photographed. Bluish coloring of the footpads in wheat and potato sensitized group showed that vascular leakage was markedly higher induced by WG than that of PP, even though the concentration of WG (5 mg/mL) was much lower than that of PP (30 mg/mL). In addition, no vascular leakage was detected in footpads of sham sensitized control group.

3.5. Induction of IACH reaction

IACH reaction for visible blue color was assessed after 15–20 min of the dye-antigen administration, the reaction was scored as positive if the resultant wheal diameter was > 0.3 cm in any direction. In Table 1, the results indicated that WG, PP, and P2 showed significant larger diameter appearance of wheal formation which was 8.98 ± 1.3 mm, 6.54 ± 2.6 mm, and 7.68 ± 1.4 mm, respectively. However, P1 was not able to be sensitized by dermal mast cells and did not give rise significant wheal diameter in response.

3.6. Assessment of hypersensitivity reactions

Mice were observed for hypersensitivity symptoms scored from 0 to 5 as described in the method section within 1–1.5 h after each challenge up to five weeks and the 42nd day. In Fig. 6A, the results showed that all mice sensitized with wheat gluten exhibited hypersensitivity reaction, the hypersensitivity score of 14 mice was 1, two mice was 4, and another 2 mice was 5. Whereas in the PP group, four mice were asymptomatic (score was 0), and the hypersensitivity score of 13 mice was 1, only 1 mouse had the highest score. What's more, all mice in control group exhibited no hypersensitivity symptoms.

At the end of the experiment (at the 42nd day), the surviving mice ($n = 5$) were finally challenged intraperitoneally injection with WG, PP, P1 and P2, the results were shown in Fig. 6B. Mice sensitized with WG exhibited the most severe hypersensitivity symptoms due to that all mice had the score ≥ 4 . Among these three proteins in potato, P2 elicited significant hypersensitivity with symptoms of systemic anaphylaxis, while mice tested with P1 showed the lower hypersensitivity reaction (all score ≤ 1) and two out of five were negatively symptomatic, mice tested with PP showed that four mice out of five exhibited hypersensitivity symptoms.

3.7. Histo-pathological analysis

Lungs and intestinal tissues were collected from groups of mice after challenge with WG or PP. Hematoxylin and eosin (H&E) stained tissues were photographed for the occurrence of histo-pathological changes, which were shown in Fig. 7. It was observed that in intestinal tissues,

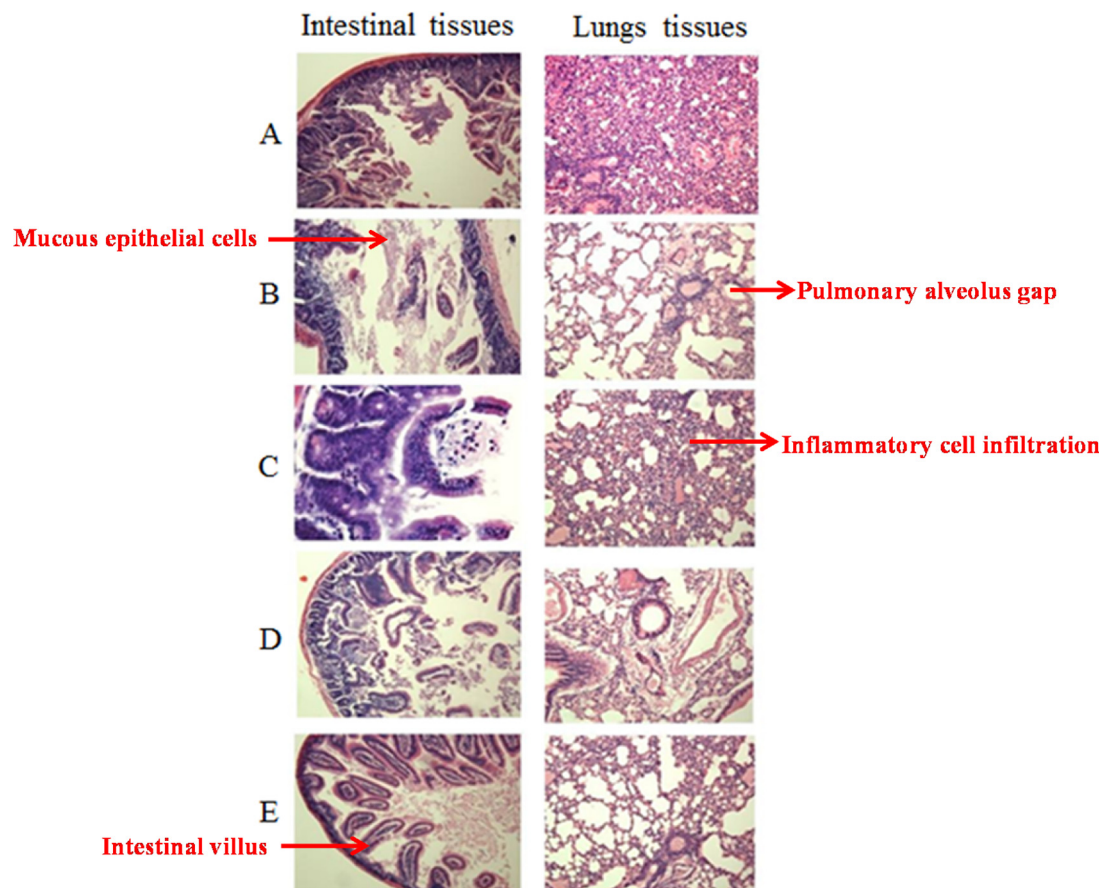


Fig. 7. Histo-pathological analysis of intestinal and lung tissue of mice which were sensitized with PBS (A), PP (B), P2 (C), P1 (D), and WG (E) ($n = 6$ mice per group).

the villus was broken in all sensitized groups, the mucous epithelium cell was abscised and the intestinal mucous was denatured. While lung tissues of all sensitized groups showed broadening of pulmonary alveolus gap, a large amount of exudate was in the airway lumen, and inflammatory cell infiltration. No any histopathological changes were observed in sham sensitized group tissues.

3.8. Immuno-detection assays

Western blot was carried out for the detection of wheat or potato induced IgE antibody in mice serum. 5 mg/mL of WG or PP were resolved by 10% SDS-PAGE gel electrophoresis, transferred to PVDF membrane and probed with mice serum followed by goat anti-mouse IgE polyclonal secondary antibody as discussed in the method section. Immuno-blot of wheat protein showed the binding of gluten specific IgE to wheat protein with higher intensity than PP. Coomassie stained SDS-PAGE pattern of wheat protein showed 16 protein fractions (Fig. 8A) with 7 immunogenic gluten fractions (Fig. 8B) ranging from 25 to 70 kDa. To overcome false positive binding, test was repeated three times with the same results.

Together with P1 and P2, a 53 kDa molecular mass protein was detected from the CCB-stained gel, showed IgE-binding ability by immune-blotting with relatively higher intensity. Another 71 kDa potato allergen was also identified through western blot. At this point, identified IgE-binding potato allergens were subjected to LC-MS/MS for amino-terminal sequencing and mass fingerprinting. The amino-terminal sequencing of the 53 kDa band yielded primary amino acid sequencing which could be identified as Adenosyl-homocysteinase of potato tuber while 71 kDa allergen was an uncharacterized protein. Results of LC-MS/MS are shown in Table 2.

4. Discussion

In our knowledge, this is the first report of a murine model of potato allergy in which IgE mediated response induced by oral sensitization without adjuvant. It elicits multiple symptoms involving several target organs, such as skin, pulmonary allergic inflammation of lungs and gastrointestinal tract with increased intestinal permeability and vascular leakage. Elevated serum cytokines IL-4 and IL-5 provide the evidence for the role of Th2 cell in the development of IgE mediated responses. In this study, oral gavage was preferred to establish a model which reflects more accurately applicable route of human exposure. Although oral exposure to PP was associated with low grade of IgE production as compared to WG, potato is hypoallergenic than wheat.

Our model also demonstrated that in vivo immunogenic capacity of wheat allergen through oral administration is higher than potato. However, potato allergens were also capable to provoke IgE mediated hypersensitivity response. It is well known that allergenic proteins are comparatively stable in simulated gastrointestinal fluids (SGF) (Hanson, 1981). PP causes in vivo immunogenic susceptibility through ingestion most probably because of patatin, although the proportional content of patatin in fresh potato is very small. The complex form of PP induces tolerance and is hypoallergenic comparatively to purified form of potato allergen such as patatin. Some previous studies indicated that peanut allergy has a relationship with doses, thus dose depended studies are also required (Laemmli, 1970).

It is also reported that oral tolerance in mice is influenced by many factors, for example, the age of animal, the nature and dose of sensitizing protein (Strobel and Ferguson, 1984; Strobel, 1996; Li et al., 2000). In contrast to WG and purified P2, protein fractions ranging from 10–21 kDa which exhibit allergenic capacity as individual protein

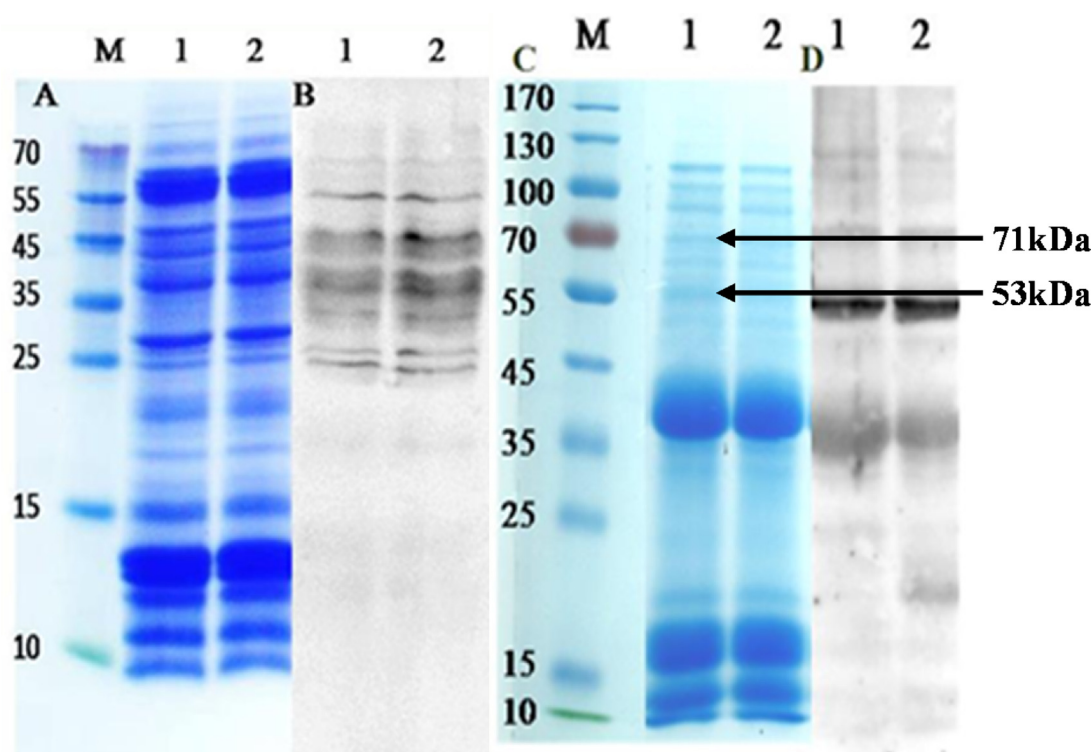


Fig. 8. Comparison of mouse IgE antibody binding to wheat protein and PP.

(A) SDS-PAGE gel of wheat protein stained with coomassie brilliant blue, (B) Western blot of wheat protein with allergic mice serum, (C) SDS-PAGE gel of PP stained with coomassie brilliant blue, (D) Western blot of PP with allergic mice serum.

Table 2

Amino-terminal sequencing and mass fingerprinting of IgE-binding potato allergens identified through LC-MS/MS.

Accession ID	Description	AAs	MW (kDa)	Score	number of peptides identified	peptide coverage
M0ZI19	Uncharacterized protein OS = Solanum tuberosum GN = PGSC0003DMG400000444 PE = 3 SV = 1	652	71.29	193.64	19	34.50
Q2XTD0	Adenosylhomocysteinase OS = Solanum tuberosum PE = 2 SV = 1	484	53.01	289.13	22	52.27
Q2MY48	Patatin	387	42.53	94.37	8	18.09
P58515	Serine protease inhibitor	186	20.10	41.72	6	50.54

(P1) fails to effectively give rise the wheal formation in intra dermal skin test. The possible reason could be the age of mice selected in our study were 6–8 week old (adult) may had more tolerance for low molecular weight potato allergens as reported in another study on peanut and cow milk, where cow milk hypersensitivity (CMH) could be induced only by sensitization of 3 week old mice but not in adult mice (Rebecca and Dearman, 2007).

To provide a sensitive model for the detection of potato allergen as it was seen that potato induces low grade of IgE level, the intra peritoneal route was used for allergenic challenge to elicit anaphylaxis (Jalonen, 1991). Mice tested for induced hypersensitivity with intra peritoneal challenge, the initial reactions consisted primarily of cutaneous reactions with puffiness around the eyes and mouth, followed by respiratory reactions, such as wheezing and labored respiration. The most severe reactions provoked loss of consciousness and death. Mice sensitized with the low boosting dose (5 mg/mL) of WG exhibited more severe reactions than those sensitized with the high boosting dose (30 mg/mL) of PP. Fatal or near fatal anaphylaxis occurred with WG or purified potato allergen patatin. No any symptoms of anaphylaxes were observed in sham-sensitized mice. Only potato sensitized mice, challenged with patatin exhibited an anaphylactic reaction at score 4 and 5, similar to that observed with WG challenge, indicated that patatin among other novel potato allergen is more prone to elicit IgE mediated

hypersensitivity through intragastric gavage and more resistant through gastric digestion.

Numerous studies have shown that intestinal permeability increased the food allergic disease (Hatz et al., 1990). Previous findings about animal studies support that IgE-mediated hypersensitivity give rise alterations in gastrointestinal permeability in mice (Perdue et al., 1984; Berin et al., 1997; Bosnjak et al., 2011). Increased vascular permeability, caused by vasoactive mediators in response of IgE-mediated hypersensitivity, is a hallmark of systemic anaphylaxis. In our model, potato and wheat-induced pathologic alterations were found in different tissues, although, vascular leakage and intestinal permeability with WG were comparatively higher than potato allergens, there were no arise in sham sensitized mice.

Histologic examination of the small intestines revealed that the intestinal villus was broken. The results also showed that the mucous epithelium cells were fall off with marked vacuole denaturation, and mucilage denaturation, inflammatory germ cell infiltration were also seen. These data demonstrate that intestinal anaphylaxis was occurred after sensitization with WG or PP. and also support the view that systemic anaphylaxis might be a consequence of intestinal anaphylactic events, which allowed antigen to pass rapidly into the systemic circulation and induce mediator release from mast cells and other cells in extra intestinal sites.

It was reported that IgE-mediated hypersensitivity in food allergy reactions can create asthmatic symptoms in humans (Mestecky et al., 1978). The role of Th2-type cytokines IL-4, and IL-5 in allergic pulmonary inflammation in food allergic diseases had also been well documented. However, the level of IFN- γ was similar to the sham sensitization group, while the level of IL-4 was significantly increased in wheat induced allergy group as compare to potato induced allergy group. Histological examinations of the lungs from wheat and potato allergic group demonstrate the broadening of the pulmonary alveolus gap, and revealed marked endobronchial exudation, and inflammatory cell infiltration was also observed in the development of pulmonary allergic responses. In this model, pulmonary inflammatory cells are most likely stimulated directly by ingestion of these antigens and the migration of antigen-specific intestinal lymphocytes induced endobronchial exudation, which was in accordance with previous reports, which indicated that activated lymphocytes may possibly drive from one mucosal site to another (Mattes et al., 2001, 2002).

It was confirmed that antigen presented in potato and wheat proteins can create a positive skin allergic response which could be reflected through intradermic test. Positive skin test showed that the IACH reaction for WG group was the most serious, and P2 and PP group also exhibited the similar IACH reaction with WG group, while P1 group almost had no positive reaction sites during the skin test. The possible reason can be justified by the immune- blotting results, where P2 shows a clear band of patatin specific IgE binding. Although among low molecular weight potato allergen P1, only 20 kDa allergen showed IgE binding when reduced in water, while reducing with PBS may alter IgE binding site and decrease its binding capacity. Amino-terminal sequencing and mass fingerprinting of IgE binding proteins observed by western blot were confirmed as 71 kDa (an uncharacterized potato protein), 53 kDa (Adenosyl-homocysteinease), 42 kDa (patatin) and 20 kDa (serine protease inhibitor). Potato proteins was identified as Adenosyl-homocysteinease showed IgE binding sites with highest binding capacity as compare to all other potato allergens. By these finding, 71 kDa and 53 kDa potato allergens need to be purified for further characterization.

5. Conclusion

In this study, it is possible to conclude that the oral administration method of wheat or potato protein without adjuvant can trigger IgE mediated hypersensitivity in BALB/c mice. The main reason that why potato is hypoallergenic than other allergic foods may possibly because of its protein digestibility in GIT track or alteration of IgE binding site in the presence of salts. Potato protein in crude form has a complex structure and less allergic to wheat gluten even gluten is also a complex form of protein, while the purified native protein can create hypersensitivity response in low doses. Future experiments are planned to investigate dose depended response of purified protease inhibitors and patatin. The crucial feature of this model is the establishment of time-dependent immunologic responses in wheat and potato allergy without adjuvant and may be beneficial in future studies of clinically relevant immunotherapies of food related allergies.

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