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Original Article

Efficacies of immunotherapy with polypeptide vaccine from *ProDer f* 1 in asthmatic mice

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Abstract: Allergic asthma is associated with the major house dust mite group 1 allergens *Der p* 1 and *Der f* 1, which belongs to the papin-like protease family and is the most potent of indoor allergens and allergen-specific immunotherapy (SIT), is seen as effective intervention for the entity. The current study was designed to verify the SIT efficacies of the enzymatic hydrolysates (papain and trypsin) in mice with asthma. We initially developed the asthmatic mouse models with *ProDer f* 1, and respectively applied recombinant *ProDer f* 1 protein and the two kinds of enzymatic hydrolysates for SIT. The results were verified by measuring the contents of IL-4, IL-10, IL-17 and IFN-γ changed in both bronchoalveolar lavage fluid (BALF) and supernatant of splenocyte culture as well as level changes of specific IgE and IgG_{2a} in the serum. After SIT intervention, the symptoms of allergic inflammation was alleviated significantly in mice treated with *ProDer f* 1 protein and the two enzymatic hydrolysates via detection of the lung tissue sections, and infiltration of inflammatory cells was also notably depressed as compared with the models, though the epithelial structure in airways remained similar with the PBS group. In addition, we observed lower serum contents of the specific IgE antibody and lower levels of IL-4, IL-17 in BALF and splenic cells in mice undergone SIT, whereas specific IgG_{2a}, IFN-γ and IL-10 in BALF and supernatant of splenocyte culture were higher as compared to the asthma group. The findings suggest the SIT using the above two kinds of hydrolysates may effectively inhibit the allergic inflammation in the airways of mouse models sensitized with *ProDer f* 1 protein.

Keywords: *Dermatophagoides farinae, ProDer f* 1 protein, protease, asthma, immunotherapy

Introduction

Allergic asthma, an important cause of chronic morbidity and mortality, tends to increase annually, and has been the most concerns because of its significant impact on public health worldwide [1, 2]. *Dermatophagoides farinae* (*Der f*) and *Dermatophagoides pteronyssinus* (*Der p*) are the most potent of indoor allergens responsible for allergic disorders, for their discharges, secretions, and body degradation products and lysates of the dead bodies can be strong allergens [6, 7], especially the allergens of major group 1 (*Der f* 1 and *Der p* 1) coexist in most geographical regions [8-10]. In order to find a perfect solution to treatment of the allergic disorder (such as allergic asthma) on allergen-specific immunotherapy (SIT) basis, bioactive peptide obtained by the enzymatic hydrolysis has been the interest of study in this field. Yet few studies are available on the formation of peptide library and application of such peptide to SIT based on dust mite allergen *ProDer f* 1 synthesized by enzymatic hydrolysis, especially application of the peptide to therapy of the allergic asthma resulting from dust mites. The current study was designed to prepare the peptide vaccine for treatment of the allergic mice with the recombinant *ProDer f* 1 protein that was hydrolyzed respectively with trypsin and papain through evaluation of the therapeutic efficacies by observing the pathological changes of pulmonary tissue sections of a mouse and levels of IL-4, IL-10, IL-17 and IFN-γ in the broncholaveilar lavage fluid (BALF) and supernatant of splenocyte culture (SSCS) as well as serum IgE and IgG_{2a} specific antibodies in order to pave a novel path to treatment of allergic asthma.

Materials and methods

Experimental animals

A total of 50 male BALB/c mice aged 6 to 8 weeks, weighing 18-22 g, were purchased from the Animal Center for Comparative Medicine, Yangzhou University (License No. SCXK 2007-0001). All animal experiments were performed in accordance with the Chinese regulations for animal protection and in adherence with the experimental guidelines and procedures.

Major reagents

Recombinant *ProDer f* 1 allergen was a preservation undergone prokaryotic expression and purification in our laboratory. ELISA kit for determining mouse IgE, IgG_{2a}, IL-4, IL-10, IL-17 and IFN-γ was purchased from R&D (U.S.A). Liu's haematocyte stain was a product of Basco Diagnostics Inc. (Zhuhai, China), and trypan blue were obtained from Sangon Biotech (Shanghai, China). The remaining analytical reagents were domestic products.

Preparation of ProDer f 1 and its concentration measurement

Our previous orthogonal experiment confirmed that the optimal conditions for enzymolysis of trypsin and papain were: pH 6.5; temperature 60°C; hydrolysis time 4 h; enzyme dosage, 4000 U/g for papain, and pH 8; temperature 45°C; hydrolysis time 4 h; enzyme dosage, 5000 U/g for typsin. Thus, *ProDer f* 1 protein (0.825 mg/ml, 5 mg) was undergone purification and enzymolysis with trypsin and papain for obtaining the tryptic and papain hydrolysates.

Fabrication of the standard curve for tetrapeptide Gly-Gly-Tyr-Arg

5% trichloroacetic acid (TCA) was successively filled in a 10 ml volumetric flask by volume of 0.0, 0.4, 0.6, 0.2, 0.8, 1, 1.2, 1.4, 1.6 and 1.8 mg/ml, respectively, to prepare the standard solution for Gly-Gly-Tyr-Arg. Then 6.0 ml standard solution was taken respectively, and 4.0 ml biuret reagent was added. The solution was mixed evenly in an eddy mixing apparatus, and centrifuged at 2000 r/min for 5 min after standing for 10 min. The supernatant was taken to measure OD value at 540 nm (The first tube was used as blank control). The standard curve of Gly-Gly-Tyr-Arg was fabricated based on the concentration of tetrapeptide as X-axis (mg/ml), the OD value as the Y-axis.

Measurement of the polypeptide in protein hydrolyzate of ProDer f 1

2.5 ml sample solution was taken and added into the flask containing 2.5 ml 10% (W/V) TCA, and thoroughly mixed in the vortex mixer. After standing for 15 min, the solution was centrifuged at 4000 r/min for 15 min. The supernatant was transferred into a 50 ml volumetric flask that was metered to the volume by adding 5% (W/V) TCA and evenly shaken. Then 6.0 ml of the solution was moved into a small flask and 4.0 ml biuret reagent was added (sample solution: biuret reagent = 3:2, V/V, and its preparation was described above). The polypeptide concentration in the samples was measured by reference to the previous standard curve of tetrapeptide Gly-Gly-Tyr-Arg.

Allergen-specific immunotherapy

Fifty BALB/c mice were randomized into PBS group (group A), asthma group (group B), *ProDer f* 1 treatment group (group C), immunotherapy group with either papain hydrolysis (group D) or trypsin hydrolysis (group E) (n = 10 for each group). Mice in PBS group were sensitized with 200 μl PBS solution (pH 7.2)/per caput, and the remaining 4 groups, with 200 μl solution [10 μg *ProDer f* 1 protein and 4 mg Al $(OH)_3$ were dissolved in PBS; pH (7.2)] by intraperitoneal injection at day 0, 7 and 14, respectively. By day 21, the PBS group was challenged with aerosol inhalation of PBS suspension containing 4% Al $(OH)_3$ for 30 min/per time, once a day for consecutive 7 days, and the records were maintained on the asthma attack. Between day 25 and 27, mice in group C were additionally administered with intraperitoneal injection of *ProDer f* 1 solution [10 μg Der f 1 and 4% (w/v) Al $(OH)_3$ were dissolved in PBS; pH (7.2)] in dose of 100 μl/ml, 30 min prior to aerosol inhalation for specific immunotherapy. Mice in group D and E were given exclusive papain hydrolysis and trypsin hydrolysis in dose of 0.2 ml via intraperitoneal injection in accordance with the previous dosage and procedures. 24 h after the final intervention, mice in the four groups were sacrificed and undergone the following test.

Measurement of the specific cytokine in BALF

Five mice were randomly selected from each group, and anesthetized with intraperitoneal

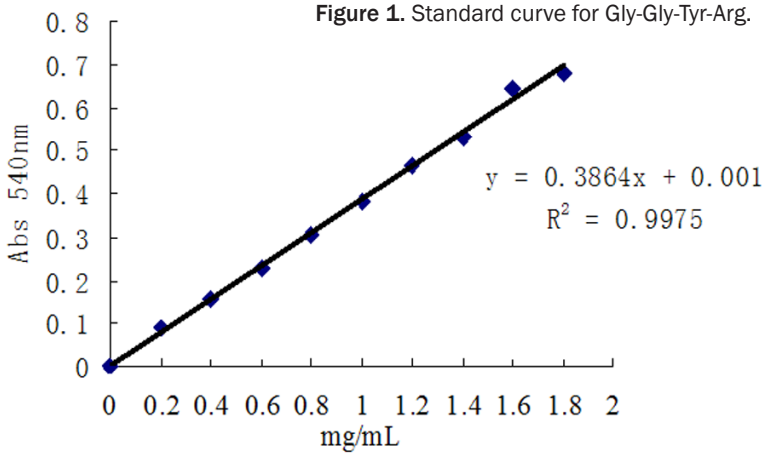

Figure 1. Standard curve for Gly-Gly-Tyr-Arg.

injection of 10% chloral hydrate (0.2-0.3 ml). After that, the mouse was fixed on its back, and the trachea was cut open with an ophthalmic scissors to expose the airway. Then a self-made needle-like tracheal cannula was inserted into the trachea and fixed approximately down at 1 cm. 1 ml of phosphate buffer (PBS, sterile and pre-cooled) was applied to harvest the BALF by three repeated aspirations in order to ensure recovery rate over 80%. Then the BALF was centrifuged 500 r/min 5 min at 37°C, and the supernatant was transferred into another clean EP tube and frozen in -80°C. ELISA was performed to measure the contents of IL-4, IL-10, IL-17 and IFN-γ in BALF.

Measurement of the serum specific IgE, IgG_{2a}

The blood samples were taken in individual mouse via removal of its eyeballs, remained static for 30 min and centrifuged at 4°C by 1000 r/min for 5 min. The supernatant was obtained and stored at -80°C for spare use. Antigen specific IgE and IgG_{2a} content was determined with ELISA technique in accordance with the user's instructions.

Culturing of the splenocytes

After the blood was obtained, all mice were sacrificed by cervical displacement, and sterilized by immersing in 75% ethanol for 5 to 10 min. The spleen was aseptically taken and cut into pieces, and made to pass through the 300-mesh stainless steel cell sieve that was placed over a sterile culture dish containing D-Hank's solution for preparation of the single cell suspension. Then the preparation was transferred into a 15 ml centrifuge tube, where the erythrocyte was lysed with EDTA-NH_4Cl by standing for 5 min and centrifuged at 1000 r/min for 5 min. RPMI-1640 (10% fetal bovine serum, 100 U/ml penicillin and 100 μg/mL streptomycin) was used to precipitate the cell pellets that were subjected to resuspension to harvest the spleen cells. The cell concentration was adjusted to 1×10^6 cell/ml. Trypan blue staining was performed and live cell count > 90% was eligible for subsequent experiment. The adjusted spleen cell suspension was applied to the 24-well plate by 1 ml for each well, in which the purified and sterilized *ProDer f* 1 protein solution was added and adjusted to a final concentration by 25 μg/ml, and incubated in CO_2 incubator (37°C, 5% CO_2) for 72 h. Then the supernatant was taken and the content of IL-4, IL-10 and IFN-γ were determined by ELISA assay.

Hematoxylin & eosin (HE) staining of the pulmonary tissues

Lung tissues were taken from each group of mice, fixed in 4% formaldehyde solution at 4°C overnight. The tissues were then embedded in paraffin, sectioned in conventional technique, stained with H&E, and observed under light microscope for examination of the EOS infiltration, incidence of edema and bronchial epithelial damage. This work was entrusted to the Pathology Department of Affiliated Hospital of Wannan Medical College.

Statistical analysis

Data were expressed as the mean ± SD ($\bar{x} \pm s$), and analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used for statistical analysis to determine differences between groups. $P < 0.05$ was considered as statistical significance.

Results

Fabrication of standard curve for Gly-Gly-Tyr-Arg and measurement of polypeptide concentration

The standard curve for tetrapeptide Gly-Gly-Tyr-Arg was created by its concentration as X-axis

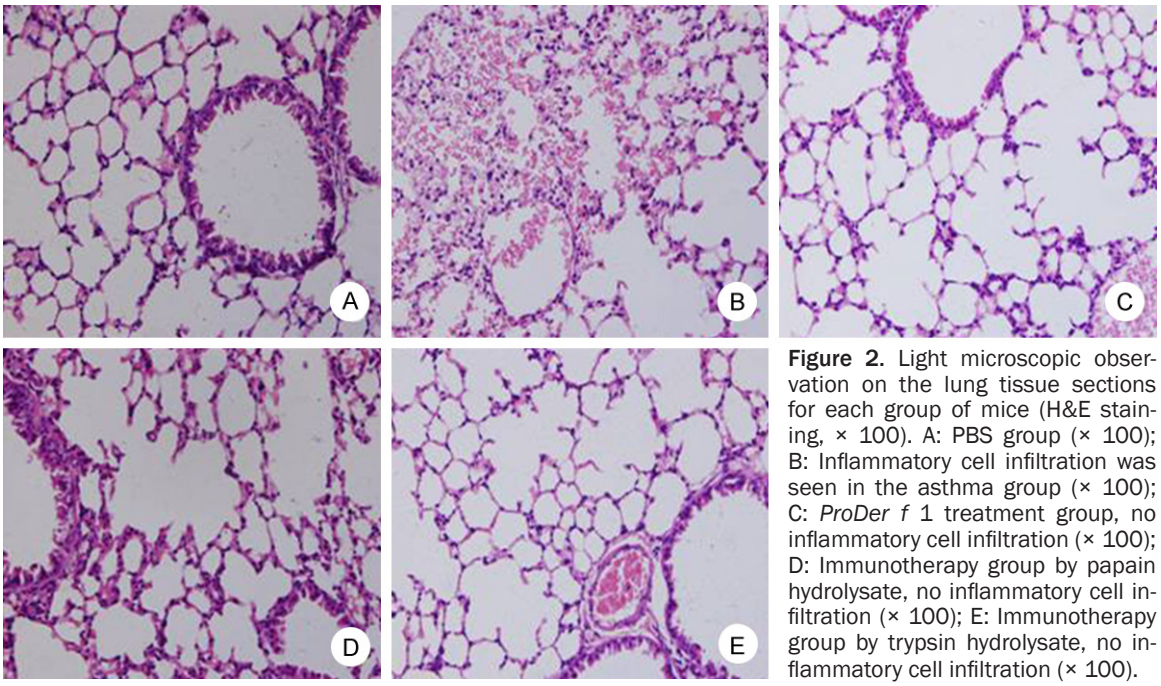

Figure 2. Light microscopic observation on the lung tissue sections for each group of mice (H&E staining, × 100). A: PBS group (× 100); B: Inflammatory cell infiltration was seen in the asthma group (× 100); C: *ProDer f* 1 treatment group, no inflammatory cell infiltration (× 100); D: Immunotherapy group by papain hydrolysate, no inflammatory cell infiltration (× 100); E: Immunotherapy group by trypsin hydrolysate, no inflammatory cell infiltration (× 100).

(mg/ml) and the OD value as Y-axis, which led to a regression equation of $y = .3864x + 0.001$, $R^2 = 0.9975$ (**Figure 1**). The measured concentrations were 0.69 mg/ml and 0.95 mg/ml, respectively for papain hydrolysate and trypsin hydrolysis.

Change of the symptoms and signs in each group of mice

The mice in asthma group and each treatment group developed characteristic symptoms of asthma attack at day 21 (the first of challenge) upon 10 to 15 minutes of inhalation, including intense restlessness, deep and fast breath, nodding like breathing, convulsions, scratching against its face and nose as well as incontinence. The symptoms started earlier with the increased dosage of inhalation excitation, yet were relived after SIT for the mice in the treatment group. The major asthmatic symptoms faded away once the inhalation was withdrawn, and were recovered to normal condition in a few minutes, though the gasping may persist for a while. Minor symptoms such as restlessness and scratching against the head appeared in the negative controls only at the irritation stage, and no evident wheezing and nodding like breath were present. Briefly, the asthma symptoms died away in all mice undergone specific immunotherapy.

Pathological changes of the pulmonary tissues

By pulmonary sections, mice in asthma group demonstrated evident bronchitis and vasculitis associated with EOS and other inflammatory cell infiltration as well as fractured or shed bronchial epithelial cells in certain locations (**Figure 2B**), where partial mucus plugs and evident edema on the vascular walls were seen. In contrast, *ProDer f* 1 protein (**Figure 2C**) and the hydrolysis product (**Figure 2D**, **2E**) had effectively inhibited infiltration of the inflammatory cells into the airways. As with the lung sections of group D and E, the inflammatory reaction was also alleviated notably, and infiltration of inflammatory cells was lessened significantly compared to the asthma models. Although epithelial structure in the airways remained similar to that of PBS group (**Figure 2A**), yet recovery of the epithelial structure in the airways of group C was not so well as group D and E.

Specific cytokines in BALF

ELISA assay of IL-4, IL-10, IL-17 and IFN-γ (**Figure 3**) demonstrated significant higher levels of IL-4 (295.81 ± 16.71 pg/ml *vs*. 162.09 ± 11.64 pg/ml) and IL-17 (273.16 ± 13.38 pg/ml *vs*. 117.94 ± 6.74 pg/ml) in asthma group than PBS group ($P < 0.01$). This suggested that BALF in asthma group was dominated by IL-4 in Th2

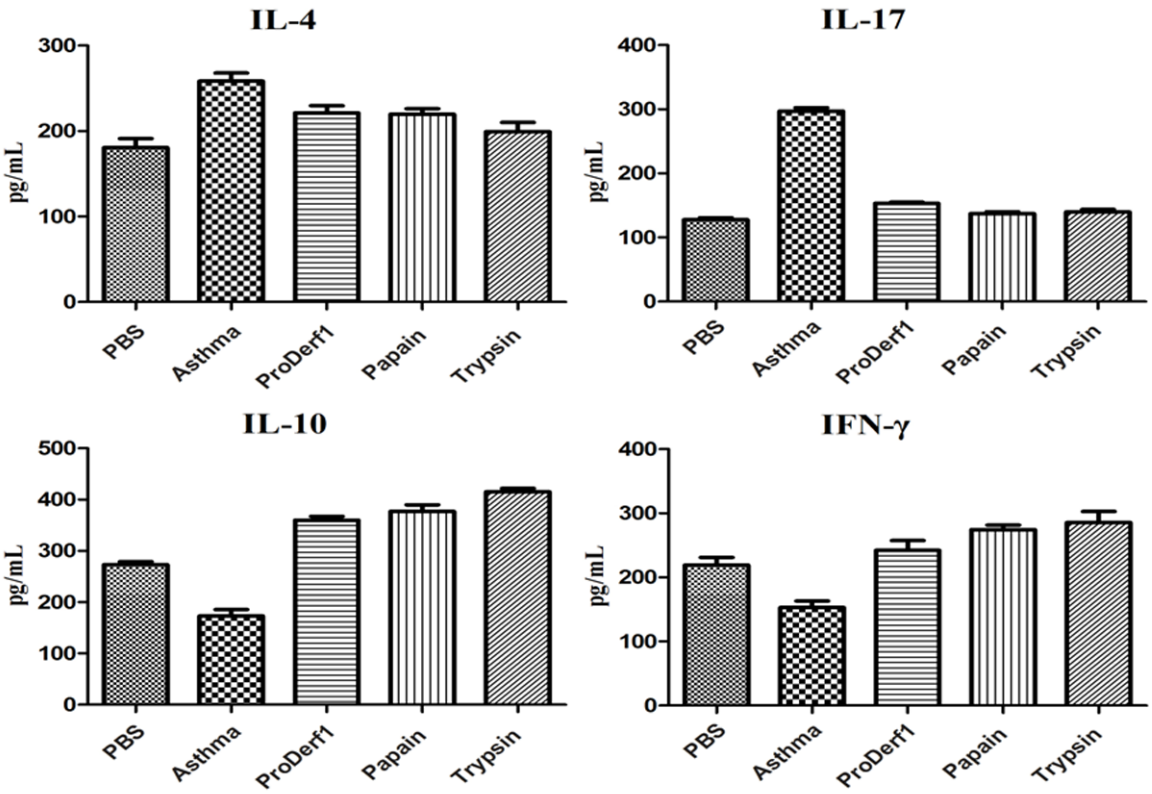

Figure 3. IL-4, IL-10, IL-17 and IFN-γ level measured in BALF.

type cytokines and inflammatory factor IL-17. Further comparison with the asthma group, IL-4 and IL-17 levels were observably down-regulated ($P < 0.01$), yet the expression trend of IFN-γ and IL-10 was in reverse manner. All indicators presented with statistical significance except for IL-10 levels identified between *ProDer f* 1 treated group (359.53 ± 16.68 pg/ml) and papain hydrolysate intervention group (376.44 ± 29.33 pg/ml) ($P > 0.05$). However, the difference was significant when comparing the indicators of *ProDer f* 1 treatment group with trypsin intervention. These findings suggested that whether *ProDer f* 1, hydrolysates from papain or trypsin were able to improve the contents of IL-10 and IFN-γ in BALF, and to inhibit the cytokine expression of IL-4 and IL-17 in asthmatic mice. Nevertheless, the hydrolysates of papain and trypsin had similar therapeutic effect, and better than *ProDer f* 1 did.

Antigen specific IL-4, IL-10, IL-17 and IFN- γ contents measured in the spleen cells

According to ELISA results, The cytokine levels of IL-10 and IFN-γ were significantly increased in all treatment groups compared with asthma group (172.95 ± 28.07 pg/ml; 153.21 ± 22.36 pg/ml) ($P < 0.01$), whereas IL-4 and IL-17 levels (153.21 ± 4.73 pg/ml) were notably decreased ($P < 0.01$), and IL-4 decrease (221.02 ± 19.40 pg/ml) was also seen in *ProDer f* 1 treatment group ($P < 0.05$). Apart from the difference was not significant by comparison of IFN-γ level in *ProDer f* 1 treatment group with PBS group (242.15 ± 34.26 pg/ml *vs*. 219.03 ± 27.51 pg/ml), the difference was significant for all immunotherapy groups ($P < 0.01$). Moreover, the content of I IFN-γ was significantly decreased in asthma group compared to PBS group ($P < 0.01$) (**Figure 4**). The results showed that the supernatant of spleen cells in asthma group mainly expressed IL-4, one of Th2 cytokines; in contrast, treatment group primarily expressed Th1 cytokines, such as IFN-γ.

Content of ProDer f 1-specific IgE and IgG_{2a} measured in the sera

ELISA detection of the antigen specific IgE and IgG_{2a} in the sera of mice demonstrated significantly higher level of IgE in asthma group

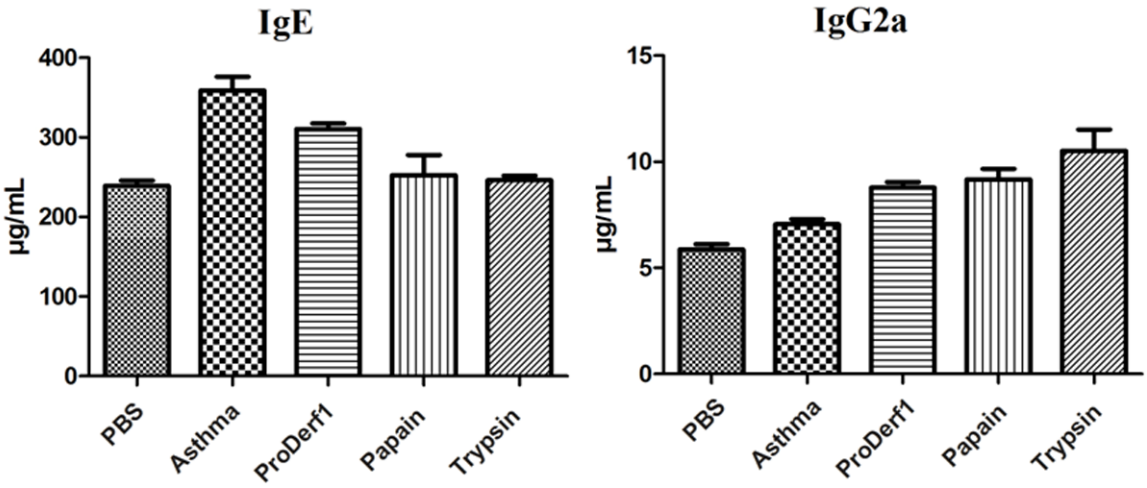

Figure 4. IL-4, IL-10, IL-17 and IFN-γ levels measured in the supernatant of splenocyte culture.

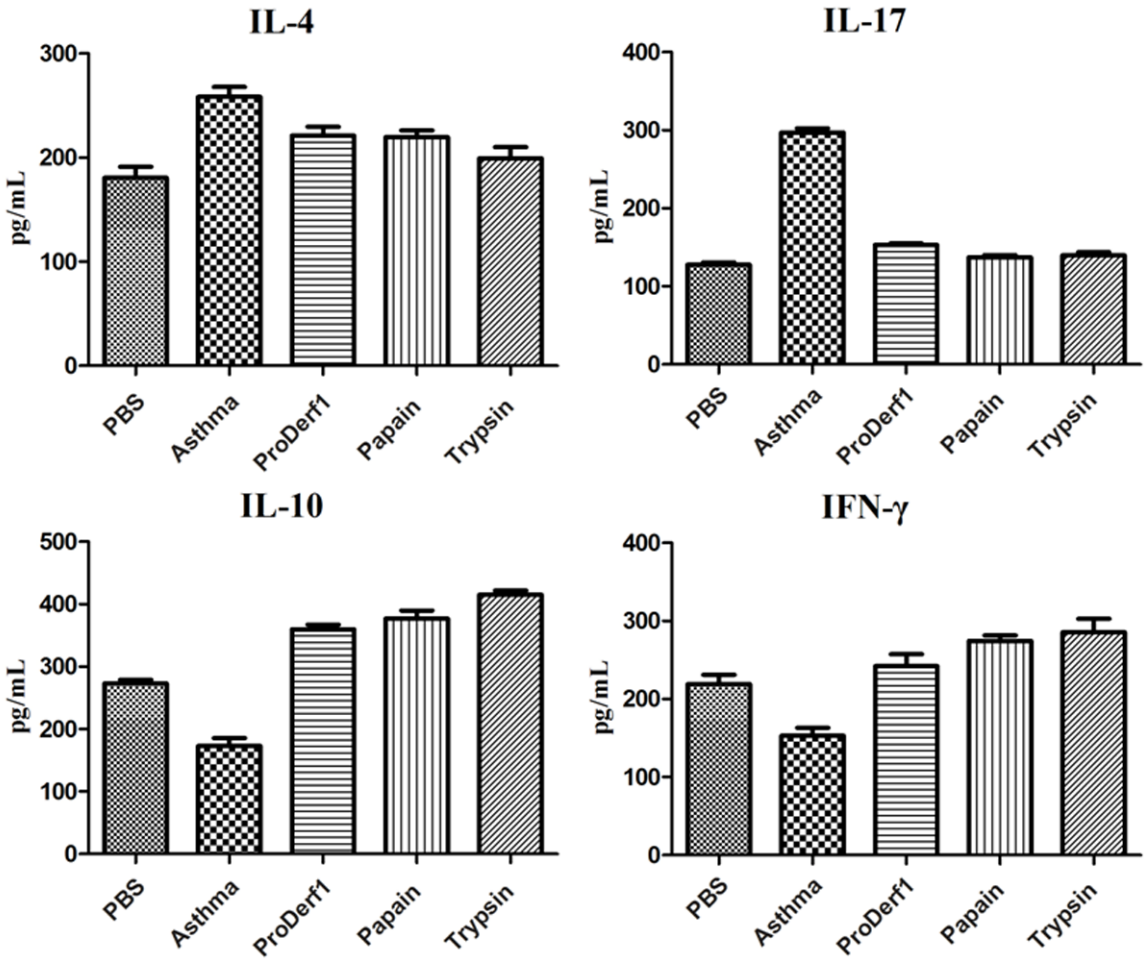

Figure 5. Levels of specific IgE and IgG_{2a} in sera of mice.

(358.82 ± 38.80 μg/ml) than PBS group (239.31 ± 14.24 μg/ml) ($P < 0.01$). This implied that purified *ProDer f* 1 was capable of inducing inflammatory reaction in lung tissues of mice,

resulting in increase of IgE level. Compared with asthma group, IgE contents in immunotherapy groups by papain hydrolysates and trypsin hydrolysates were significantly decreased (252.55 ± 56.51 μg/ml; 246.07 ± 13.21 μg/ml), and IgG_{2a} was increased (9.16 ± 1.13 μg/ml *vs*.10.52 ± 2.22 μg/ml), these differences were significant ($P < 0.01$ or $P < 0.05$). The IgE level (310.54 ± 15.71 μg/ml) in *ProDer f* 1 treatment was decreased ($P < 0.05$), whereas IgG_{2a} level was strikingly increased ($P < 0.01$) (**Figure 5**). These results showed that immunotherapy can inhibit the expression of immune specific IgE antibody, yet boost IgG_{2a}.

Discussion

Allergic asthma induced by mites is common clinical prevalence, and its incidence rate is yearly rising. This entity is closely involved in many inflammatory cells (such as eosinophils, mast cell, T lymphocyte) and their secretary cytokines [11], and it pathogenesis appears quite intricate. Previous studies insisted that asthma attack was attributable to imbalance of Th1 and Th2 cells [12], which primarily manifests as over-secretion of Th2 cytokines such as IL-4, IL-5, IL-10, IL-13 and other cytokines, and reduced production of IFN- γ and IL-2 of Th1 cytokines. This imbalance further generates airway hyper-reactivity caused by enhancement of B cell, activation of eosinophils and infiltration of airway [13, 14].

Allergen-specific immunotherapy (SIT), also known as desensitization therapy, is the exclusive current etiology therapy capable of modifying the progress of allergy [15, 16]. This therapy protocol targets disease-modifying by either subcutaneous or sublingual administration of the allergen extracts through gradual increase of the dose of a patient's relevant allergens until immunological tolerance to the allergens is induced, Despite the proven efficacy of SIT for allergic conditions, yet this protocol, primarily depending on the crude extracts from allergens of dust mites, can involve severe anaphylactic adverse reactions because of the complex components of such extracts. In order to find a perfect solution to the clinical option, enzymatic hydrolysis of proteins has become the interest of study, particularly in the field of bioactive peptides. Previous studies confirmed that peptides can be biological activities of variety and optimal natural resources for drug screening [15, 17]. Importantly, immune-inducible active peptide has many physiological roles in not only boosting the body immunity through immunoloregulation, but also stimulating proliferation of lymphocyte and enhancing the phagocytosis of macrophage so that the body resistance to foreign pathogens is improved [18]. Therefore, research on bioactive peptides can be safe alternative and novel option for the treatment of asthma and other allergic diseases.

The current study was undertaken to verify such purpose based on the hydrolysates prepared from papain and trypsin in our previous work through enzymolysis of the purified *ProDer f* 1 protein under optimally established technical conditions. Initially, we established the murine models of asthma by using *ProDer f* 1, and tentatively applied the two hydrolysates to the mice on SIT basis. The results showed that the hydrolysates can reduce the serum antibody-specific IgE levels in the asthmatic mice, and automatically degrade IL-4 level from Th2 cells, IL-17 level from Th17 cells in the supernatant of splenocyte culture and BALF obtained from the experimental animals, whereas upregulated IgG_{2a} content and increased IFN-γ and IL-2 levels from Th1 cells to a certain degree. Further examination on the pulmonary sections by comparison with the positive controls (asthma group) suggested the lung tissues from mice undergone SIT had no significant pathological change, and the bronchial and alveolar structure remained relatively intact, with no obvious inflammatory cell infiltration in around the small bronchi and vessels. Still, the bronchial wall mucosa appeared free of any fracture and epithelial shedding, and the observation is similar to negative controls (PBS group). These findings indicate that the hydrolysates (papain and typsin) obtained from *ProDer f* 1 protein can effective SIT for allergic disorders. However, the efficacy remains to be improved with regard to the options of by using the hysdrosates or *ProDer f* 1.

In conclusion, we successfully established the asthmatic models with BALB/c mouse, and verified the effect of SIT on the murine models with *Der f* 1 protein hydrolysates (papain hydrolysates and trypsin hydrolysates). The results suggest that this protocol can efficaciously alleviate lung inflammation and asthmatic symptoms to a certain extent and our work may facil-

itate re-thinking of a novel vaccine for treatment of allergic asthma induced by acaroid mites. Nevertheless, further research will be necessary in terms of the optimal constituent ratio of different protein hydrolysate and dosage form of polypeptide as well as spatial configuration of polypeptide molecular that affects the outcomes.

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Disclosure of conflict of interest

None.

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