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RESEARCH

Protease inhibitor treatment decreases allergenicity of *Dermatophagoides pteronyssinus* allergen evidenced by proteome and allergenome analysis

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Abstract

Some of *Dermatophagoides pteronyssinus* (Dp) allergens are proteolytic enzymes which might naturally digest other allergens in the extract. We hypothesized that adding protease inhibitors in mite extract might preserve the allergenic proteins and its allergenic potency. Therefore, this study aimed to investigate the effect of protease inhibitors on Dp allergen by using high-resolution two-dimensional gel electrophoresis -based-proteomics and 2DE-IgE immunoblotting. Each extract was measured for protein concentration then subjected to a nonlinear pH 3-10 first and 2 dimensional electrophoresis. 2DE-IgE immunoblotting was carried out using individual serum of Dp allergic Thai patients. While protein content was not affected by protease inhibitors treatment, SDS-PAGE and Western blot analysis indicated that crude Dp extract without protease inhibitors contained more protein bands than those detected in the other extract. The results in 2DE-IgE immunoblotting showed that the reactive spots in the extract without and with using protease inhibitors were 29 and 21, respectively. IgE reactivities of allergenic proteins form both extracts showed similar outcomes in 1DE and 2DE-IgE immunoblotting assays indicating that protease inhibitors treatment resulted in decreasing IgE reactivity. Our findings demonstrate that protease inhibitors treatments during allergen extraction might interfere with IgE binding epitopes of the allergen. Hence, we discourage the use of protease inhibitors treatment in allergen characterization.

Keywords: House dust mites (HDM), *Dermatophagoides pteronyssinus* (Dp), protease inhibitor, 2DE-IgE immunoblotting.

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Introduction

Allergic diseases have become a major health concern throughout the world as approximately 25-40% of the world population is affected. Besides, their prevalence is dramatically increasing over the last 20-30 years [1,2]. Among the indoor

allergens, house dust mite allergens have been considered to be the most important allergens since 60-80% of the atopic patients are sensitive to them [3,4]. *Dermatophagoides* mites namely *D. pteronyssinus* (Dp) and *D. farinae* (Df) are known as the predominant mite species found worldwide in house dust [5].

Recently, the development of new technology in the field of proteomics has increased rapidly. Since allergen is classified as protein, the characterization of allergens based on proteomic approach has been introduced. Currently, 27 groups of mite allergens have been characterized and established in the IUIS nomenclature database [6]. Among the established allergens, some Dp allergens such as cysteine protease (group 1), trypsin serine protease (group 3), amylase (group 4), chymotrypsin serine protease (group 6), Glutathione-S -transferase (group 8), arginine kinase (group 20) and etc. are enzymes. Hence, crude mite extract containing these enzymatic allergens might cleave peptide bonds or directly destroy other mite allergens and degraded its allergenic potency [8].

To study allergen characterization, all allergens in the extract should be preserved and should not be destroyed by the proteolytic enzymes naturally contained in the extract. Lately, protease inhibitors have been widely used in protein preparation for not only research works but also commercial production in order to protect the integrity of proteins during extraction and purification process [9]. The protease inhibitors might inhibit specific proteolytic enzymes and prevent the split of the target protein into peptides; consequently, the amount of target protein production would not be decreased [10]. Moreover, it has been reported that the addition of protease inhibitors and 1-phenyl-3-(2-thiazolyl)-2-thiourea in cockroach allergen extracts could prevent protein degradation and melanization [11]. Moreover, the addition of protease inhibitors could prolong the shelf life of cockroach extracts [12]. Given that these issues have not been formally addressed, we decided to systematically investigate the effect of protease inhibitors adding to the extract during extraction process on Dp allergen characterization by using high-resolution both one and two-dimensional gel electrophoresis (2-DE)-based-proteomics and 2DE-IgE immuno-blotting. From the data presented, we conclude that protease inhibitor treatment in fact deteriorates the IgE reactivity of *Dermatophagoides pteronyssinus* mite allergen

Material and Methods:

Serum Collection

The research participants attending the Allergy Clinic at Siriraj Hospital were divided into 2 groups; allergic patients in subject group and healthy volunteers in normal group. The subject group in this study must be diagnosed with allergic rhinitis (AR) and reacted with *D. pteronyssinus* and/or *D. farinae* by skin prick test while the healthy volunteers without any chronic disease, the skin prick test with HDM and other common allergens showed negative results. In addition, all sera were measured specific IgE levels to *D. pteronyssinus* allergen by using ImmonoCAP system (ImmunoCAP® 100E Automate, Sweden) for confirm the participant sera. This study was approved by the Ethics Committees of the Faculty of Medicine Siriraj Hospital, Mahidol University (No. Si224/2010).

Preparation of Crude *D. pteronyssinus*

Fresh purified (99%) Dp mites (100 mg) were washed with 0.1 M phosphate buffered saline (PBS), pH 7.4 to remove any contaminants before process. After washing, mites were ground by tissue grinder on ice.

Protease inhibitor namely complete ULTRA tablets (Roche Applied Science, Switzerland) was used in this study. The main components contained serine, cysteine, and acidic proteases and other inhibitors. The preparation for one protease inhibitors tablet was added in 350 µl of distilled water to suspend (1 tablet = 20x) and using into the extraction buffer 50 µl per 1 ml of sample preparation buffer.

Crude Dp extracts were prepared as previously described [13]. In each protocol, 1 g of ground

Dp mite was added with extraction buffer without protease inhibitor or buffer containing protease inhibitor at ratio 1:4 w/v. The preparation was homogenized by sonicator (LABSONIC®P, France) at 30% amplitude, 0.5 cycles for 10 minutes. Then, the suspensions were centrifuged at 12,000g, 4°C for 15 minutes. After centrifugation, the supernatant of each extract was harvested and protein amount was measured by Bradford's method. Crude extract preparations were kept in small aliquots at -80°C until used.

One-dimensional gel electrophoresis (1-DE)

1-DE of mite extracts were carried out as described previously [13]. Briefly, crude Dp extracts of both preparations were separated individually in a 4% stacking, 12.5% separating poly-acrylamide gels using a Mini-PROTEAN® 3 Cell (Bio-Rad, CA, USA). The amount of protein loaded was 10 μg per lane equally. The separated protein bands in each gel were visualized by staining with Coomassie Brilliant Blue G-250 (CBB).

Two-dimensional gel electrophoresis (2-DE)

2-DE of Dp extracts of both protocols were performed as described previously [13]. Briefly, each extract was cleaned with 2D-clean-up kit (GE Healthcare Bioscience, UK) before applying into a strip holder (Ettan IPG PhorElectrofocusing System; GE Healthcare). Then, each IPG strip (GE Healthcare) was put in to the extract with 600 μl of PlusOne™ Dry Strip Cover Fluid (GE Healthcare) was covered on top. The amount of protein loaded was 60 μg per gel. After rehydration, the first dimensional electrophoresis (1DE) was performed at 300 Volts for 30 minutes, 1,000 Volts for 30 minutes and 5,000 Volts for 72 minutes. Two equilibration steps were performed using 10 ml SDS-equilibration buffer containing 100 mg DTT at 25°C for 15 minutes followed by 10 ml of the equilibration buffer containing 250 mg IAA at 25°C for 15 minutes. Next, the strips were placed onto a 12.5% gel cast in the HoeferTM SE 260 system (Amersham Bioscience) after washing with electrode buffer. SDS-PAGE was operated at 10 mA/gel during the first 15 minutes and at 20 mA/gel until the tracking dye reached the lower gel edge. After that, one gel was stained by CBB dye whereas the other gels were transferred to two NC membranes for 2DE-immunoblotting.

Western blot analysis

The proteins separated by 1-DE and 2-DE were electro-transblotted onto a nitrocellulose (NC) membranes in transfer buffer by applying 100 Volts of electric power for 90 minutes. The NC blot was probed primarily with patient's sera. Anti mouse Ig-alkaline phosphatase (AP) conjugate (DakoCytomation, Denmark) and BCIP/NBT substrate (KPL, MD, USA) were used as the band revelation reagents.

2DE IgE immunoblotting

2DE IgE immunoblotting was performed as described previously [12]. Briefly, the Dp allergens blotted-NC membrane was placed in a blocking solutions (3% BSA in Tris buffer, pH 7.4) for 1 hour to block the empty sites of the strip. After washing with PBS-T (0.05% Tween-20 in PBS, pH 7.4) to remove the blocking reagent, the proteins on the membrane were probed with individual serum of the mite-sensitive patient or non-allergic control (diluted 1:10 in PBS-T) at 4°C overnight. The NC membranes were incubated with the secondary antibody (Mouse anti-human IgE antibody AP conjugated; Southern Biotech, USA) at 25°C for 1 hour. Then, NC membranes were incubated with BCIP/NBT chromogenic substrate (KPL, USA) in dark for 10 minutes. The IgE reactive spots were visualized by using ImageQuant LAS 4010 (GE Healthcare).

Results

Protein amount in both extracts were repeatedly analyzed for five times to compare the effect of protease inhibitors. Average protein level in the extract lack of protease inhibitors was 35.76 mg/g of Dp mite which was not different from that of extract containing protease inhibitors (36.80 mg/g).

From SDS-PAGE analysis, the protein molecular masses ranged from 10-177 kDa

as shown in Fig 1. The number of detected bands in Dp extract without protease inhibitors treatment (Fig 1 A) was 54, while those of Dp extract with protease inhibitors (Fig 1 B) was 50. The prominent bands of the extract without protease inhibitors treatment were seen in the part of molecular weight ranging from 26-72 kDa. On the other hand, the noticeable bands of the extract with protease inhibitors treatment were observed in the area of lower MW (between 10-20 kDa). The other parts of the extract with protease inhibitors treatment showed paler staining than the stained bands of the extract without using protease inhibitors.

Study subjects recruited in this study comprised of 5 allergic patients and 5 normal controls. Mean of *D. pteronyssinus* specific IgE levels in the patient's group was 97.08 KAU/L whereas that of control group was <0.35 KAU/L. The characteristics of all study subjects were shown in Table 1.

Comparing with protein patterns of both extraction protocols revealed by SDS-PAGE, similar outcomes were also shown in 1DE-IgE immunoblotting (Fig 2). The pattern of reactive bands of the extract without using protease

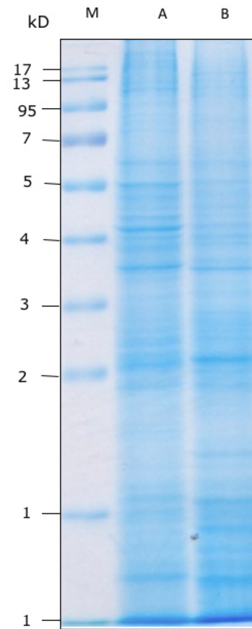

Fig 1 Protein patterns of *Dp* extract without protease inhibitors (lane A) and *Dp* extract with protease inhibitors (lane B) revealed by SDS-PAGE stained with CBB dye.
Lane M: protein molecular weight standard. Numbers at the left are protein masses in kilo Daltons.

Table 1 Characteristics of 5 house dust mite allergic patients (subjects) and the 5 non allergic patients (normal controls)

Patient/ Control No.	Sex	Age (years)	Diagnosis	Skin prick test positive to crude extract of	Specific IgE to *D. pteronyssinus* (KAU/L)
P1	M	32	AR	Dp,Cat,Dog,Ant,CR,MQ,Pollen	99.00
P2	F	27	AR	Dp,Cat	86.40
P3	F	20	AR	Dp,Dog,Cat,Dog	>100
P4	M	36	AR	Dp	>100
P5	F	25	AR,Atopic dermatitis	Dp,Cat,CR	>100
C1	F	27	-	-	<0.35
C2	M	26	-	-	<0.35
C3	F	53	-	-	<0.35
C4	M	26	-	-	<0.35
C5	F	30	-	-	<0.35

Abbreviation: M, Male; F, Female; AR, Allergic rhinitis; P, Patient; C, Control; Dp, *Dermatophagoides pteronyssinus*; MQ, Mosquito; CR, Cockroach;.

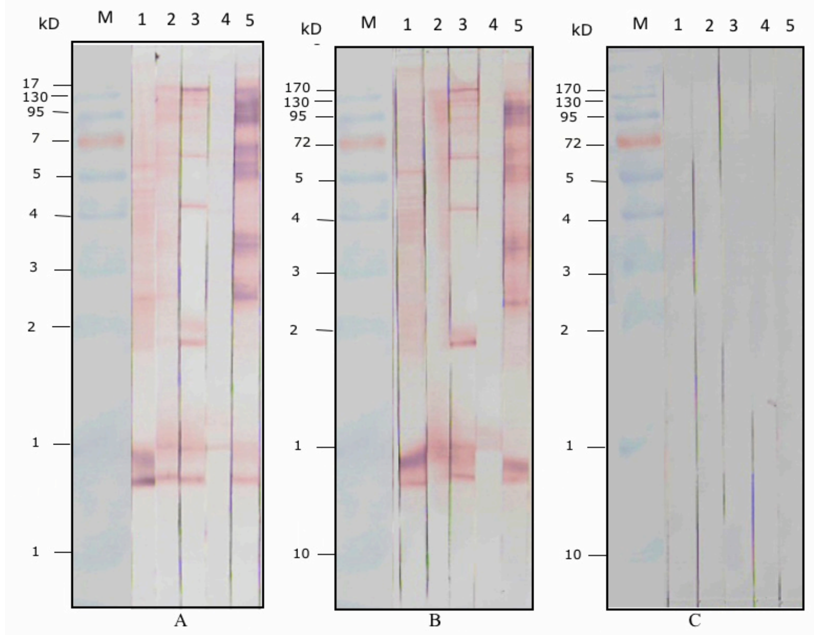

Fig 2 1DE-IgE immunoblotting of allergic patients and normal controls

2A: 1DE-IgE immunoblotting of 5 allergic patients sera on *Dp* proteins in the extract without protease inhibitors

2B: 1DE-IgE immunoblotting of 5 allergic patients sera on *Dp* proteins in the extract with protease inhibitors

2C: 1DE-IgE immunoblotting of 5 negative patients sera on *Dp* proteins in the extract without protease inhibitors

Table 2 Comparison of IgE-reactivity of each reactive *Dermatophagoides pteronyssinus* protein-band with its relative molecular weight matched to the allergen database in the extract without protease inhibitors and those in the extract with protease inhibitors revealed by Western blot analysis

Band No.	Relative molecular weight (MW; kDa)	IgE-reactivity of *Dp* allergic patient sera	
		Dp extract without protease inhibitors	*Dp* extract with protease inhibitors
1	177	40%	20%
5	103	40%	40%
17,18	60	60%	20%
41	36	40%	20%
46,47,48	31	80%	20%
50	29	60%	20%
51	27	60%	0%
53	25	60%	40%
54	24	80%	60%
63	15	100%	100%
64,65,66,67,68	14	80%	60%

inhibitors (Fig 2A) to IgE in Dp allergic patient sera are quite similar to those of extract with using protease inhibitors (Fig 2B).

The comparison of frequency and percentage of IgE-reactivities of each Dp protein-band in both protocols revealed by 1DE-IgE immunoblotting analysis were demonstrated in Table 2. Only band No. 5 (~MW 103 kDa) and No. 63 (~MW 15 kDa) had equal IgE reactivity in both extracts. The rest reactive bands in the extract without protease inhibitors treatment showed higher percentage of IgE reactivities than those of the other extract.

The allergenicity of individual Dp allergenic spot in the extracts without and with protease inhibitors treatment performed by 2DE-IgE immunoblotting assay were exhibited in Table 3. There were 14 reacted spots having equal IgE reactivites from both preparations whereas

Table 3 Allergenicity (serum IgE reactivity) of each *Dermatophagoides pteronyssinus* allergenic protein with its relative molecular weight matched to the allergen database in the extracts without and with protease inhibitors treatment demonstrated by 2DE-IgE immunoblotting assay

Spot No.	Relative molecular weight (MW; kDa)	IgE-reactivity of *Dp* allergic patient sera	
		Dp extract without protease inhibitors	*Dp* extract with protease inhibitors
1	180	25%	0%
4	115	25%	25%
5,6	105	25%	25%
7	95	50%	50%
8,9,10	90	50%	25%
11,12	82	75%	25%
13	80	50%	0%
14	68	75%	25%
15	65	50%	25%
17	60	50%	50%
22	56	25%	0%
24	53	25%	25%
25,26	52	25%	0%
29	50	25%	0%
31,32	44	25%	25%
34	41	25%	0%
38,39,40	40	25%	0%
42,43	36	25%	25%
46	32	25%	25%
47	31	50%	50%
48,49	30	25%	25%
50	28	50%	50%
52	26	50%	75%
53,54	25	50%	75%
55,56,57	23	25%	25%
58,59,60	21	25%	0%
63	16	50%	50%
66,67,68	14	25%	25%
69	13	50%	50%

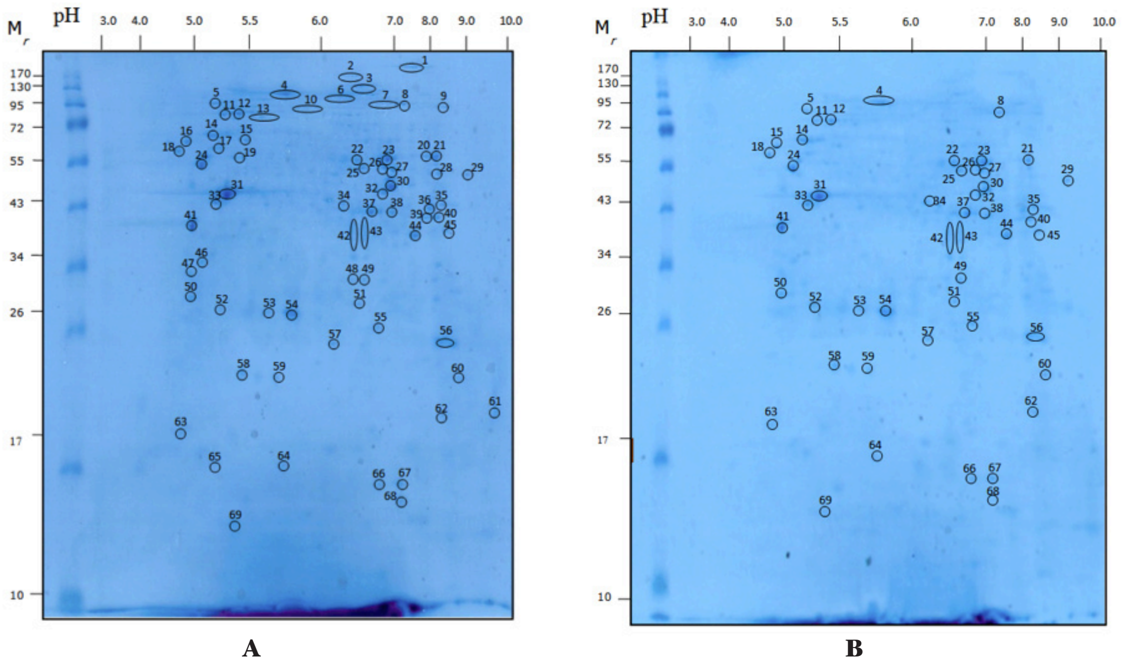

Fig 3 Coomassie Brilliant Blue G-250-stained proteins of *Dp* crude extract after 2D gel electrophoresis

The *Dp* extract without protease inhibitor showed 69 protein spots (A) and the *Dp* extract contained protease inhibitor showed 49 protein spots (B).

8 non-reacted spots in the extract with protease inhibitors treatment. Only 2 reactive spots in the preparation lacking protease inhibitors had lower IgE reactivities than those of spots in the formulation contained protease inhibitors. The rest spots in the extract without protease inhibitors showed higher percentage of allergenicity than those from the other protocol and this outcomes were in consistent to the results from 1 DE-immunoblotting assay.

By using the 2DE proteomic approach, 69 reactive spots in the extract without using PI (Fig 3 A) were detected on the CBB-stained gel, but only 49 spots could be isolated from allergenic extract with using protease inhibitors (Fig 3 B).

Allergenomes of both extract protocols were identified by 2DE-IgE Immunoblotting as shown in Fig 4. No reactive spot was discovered after probing the blots with the normal sera. Nevertheless, there were in total 29 spots reactive to IgE in the sera of allergic patients in extract without protease inhibitors treatment whereas only 21 reacted spots were revealed in extract with protease inhibitors treatment. Generally, the results in 1 DE and 2DE-IgE immunoblotting indicated that Dp extract without using protease inhibitors had more protein reacted bands/spots than those detected in the extract with protease inhibitors.

Discussion

Proteolytic enzymes are crucial for a variety of biological processes in organisms ranging from lower (virus, bacteria, and parasite) to the higher organisms (mammals). Proteases can cleave proteins into smaller fragments by catalyzing peptide bonds hydrolysis [9]. Some of insect allergens such as mite and cockroach as well as fungal allergen are proteolytic enzymes [14,15]. No systematic study on the global effect of protease treatment during allergen preparation has to our knowledge been published. We therefore undertook to analyze the effect of protease inhibitors on allergenicity of Dp allergen.

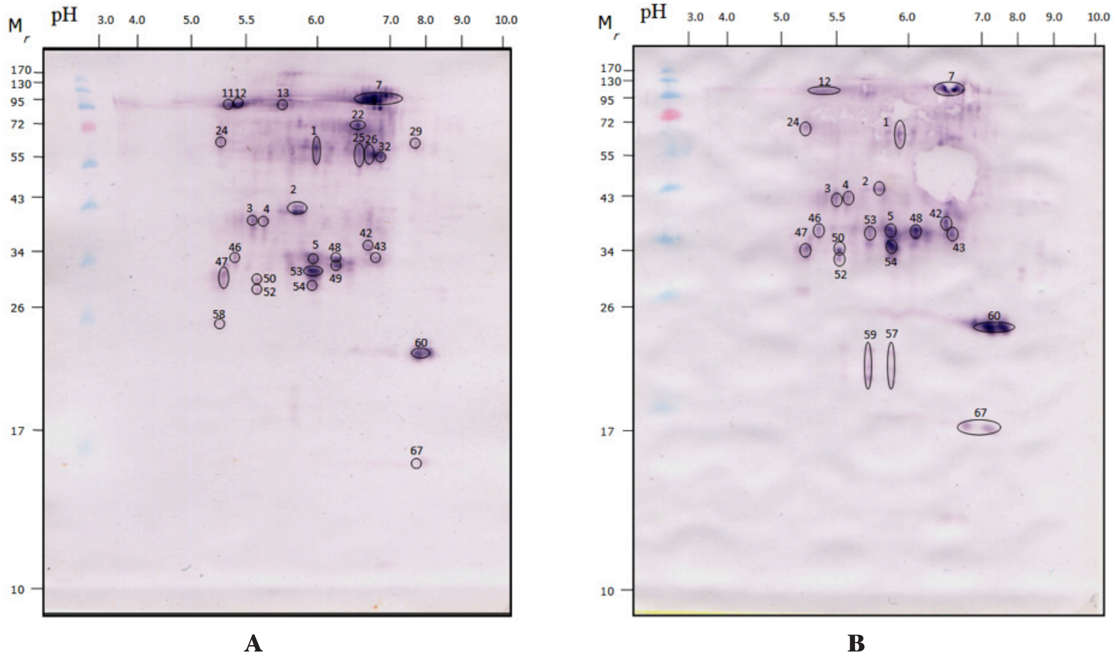

Fig 4 Representative results of 2DE-IgE immunoblotting (Patient No. 5)

The *Dp* extract without protease inhibitor was separated by 2DE and probed with serum of allergic patient no. P5. IgE in serum of this patient reacted to 28 protein spots in the mite extract without addition of protease inhibitors (A) whereas only 21 reacted protein spots were detected in the mite extract contained protease inhibitors (B)

The average levels of the protein in both preparations were quite similar and not statistical significantly different. This indicates that protease inhibitors might not affect protein concentration which is one of the key indicator for allergen quality. Besides, the effect of protease inhibitors might not be observed as the extracts were analyzed within a month. However, the average amount of protein (35.76 mg/g of mite) in the extract lacking of protease inhibitors was slightly lower than that of the extract containing protease inhibitors (36.80 mg/g). This might be related with the measurement method used in this study namely Bradford technique which is the common procedure in both routine and research laboratories. In Bradford assay, the protein–binding dye bound to primarily arginine on the protein structure [16]. It is possible that some of the cleavage polypeptide products, especially low molecular weight and low content of arginine residue, might not be detected by this assay. Therefore, the protein level in the extract without protease inhibitors treatment was slightly lower than the treated sample performed in this study.

SDS-PAGE analysis was performed to investigate the protein profiles from both types of extracts (Fig 1). Even though the protein patterns of both formulations were quite similar in numbers but the different intensity of the protein bands from each extracts were noticeable (Figs 1 A and 1B). The study of the protease enzymatic activities in allergenic extracts from 3 species of mites causing allergy namely *D. farinae and D. pteronyssinus* and *Tyrophagus putrescentiae* and 3 species of cockroaches (*Blatella germanica, Periplaneta americana*, and *P. fuloginosa*) were studied using ApiZm and EnzCheck assays. Among these 6 preparations, the extract from *T. putrescentiae* mite had the highest protease activity, followed by those of the cockroach extracts, while *D. farinae and D. pteronyssinus* extracts only had weak protease activity [17].

Hence, the treatment with or without protease inhibitors in the extract of *D. farinae and D. pteronyssinus* mites might not affect the protein content. Moreover, the method of SDS-PAGE and Western blot analysis which are the common methods for allergen characterization might not be appropriate to detect the different outcomes especially their enzymatic activities. Therefore, a number of previous studies on allergen characterization did not add protease inhibitors in the extracts [13,18-20].

Generally, in the extract with no protease inhibitors treatment, proteins are cleaved and separated independently. This aspect was clearly demonstrated by Olivievi et al [21]. The breakdown of proteins to form a smaller polypeptides might had a different value of isoelectric point and molecular mass, which are generated the different patterns in 2DE map and IgE – immunoreactive map as shown in Figs 3 and 4, respectively.

The results of reactive bands in each extracts were corresponding to the protein pattern in SDS-PAGE analysis. From Table 2, it clearly demonstrates that the IgE reactivity of most reactive bands of Dp extract without protease inhibitors were higher than those of Dp extract with protease inhibitors. The similar findings were also exhibited in Table 3. Not only more reactive spots but also higher percentages of their allergenicities were identified from the extract without protease inhibitors than those of the extract with protease inhibitors treatment. In fact, the mechanism of protease inhibitors is to block the active site of protein and prevent cleaving proteins into smaller fragments without changing protein structure or its function [9,10]. Blocking the cleaving site by protease inhibitors may inhibit or interfere IgE binding epitopes as this catalytic region is not only for proteolytic activity but also for conformational IgE antibody binding epitopes of Der p 1 and Der f 1 [22]. Therefore, allergens in the extract with protease inhibitors supplement showed lower IgE reactivities than those of allergenic protein in the other type of extract.

We conclude that across all methods employed in this study, adding protease inhibitors in the extraction will interfere with IgE binding epitopes of the allergen. Hence, we discourage the use of protease inhibitors especially in the extract contained protease-rich allergens or having high enzymatic activities. Our findings have wide implications for allergen preparation both for research studies as well as mass specific protein production including for commercialization.

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