

Enzymatic Activity in Body and Fecal Extracts of the Storage Mite *Chortoglyphus arcuatus*

Jerónimo Carnés^b Manuel Boquete^a Francisco J. Carballada^a Víctor Iraola^b
Maria Teresa Gallego^b Enrique Fernández-Caldas^b

^aHospital Xeral de Calde, Allergology Service, Lugo, and ^bLaboratorios LETI, S.L., R & D Department, Madrid, Spain

Key Words

Chortoglyphus arcuatus • Enzymatic activity, allergen • Mites

Abstract

Background: *Chortoglyphus arcuatus* has been described in many countries. Many allergens are potent enzymes, which may promote a Th2 immune response. The aim of this study was to evaluate the enzymatic activity of body and fecal extracts of *C. arcuatus*. **Material and Methods:** Feces and bodies of full-grown *C. arcuatus* cultures were separated by sieving, extracted in PBS, dialyzed and lyophilized. The antigenic profile of both extracts was determined by SDS-PAGE. Immunoblot experiments were conducted using a pool of sera from allergic individuals residing in Galicia, a region of Spain, where this species is abundant. The enzymatic activity of the extracts was evaluated by the zymogram technique. Serine and cysteine protease activity was measured using in vitro methods. The API Zym[®] system was used to determine the enzymatic properties of the extracts. **Results:** The antigenic profile showed that the body extract contained more and better defined bands than the fecal extract. Allergens were detected in both extracts in a molecular weight range between 14 and 100 kDa. Gelatinolytic gels confirmed that fecal extracts contain more hydrolytic enzy-

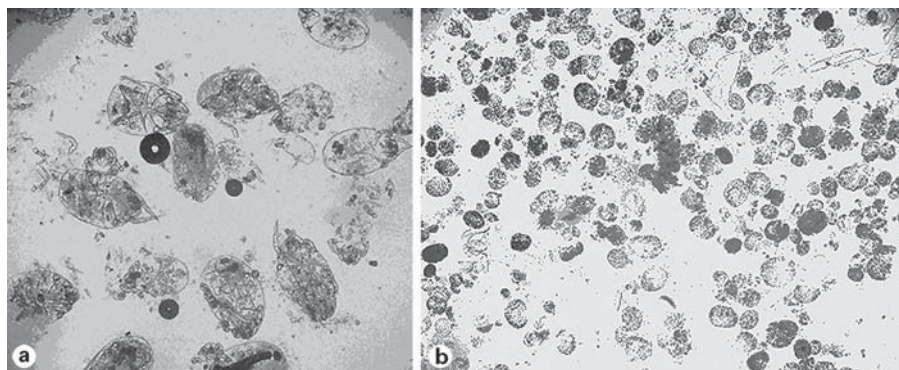
matic activity than body extracts. Serine protease activity in fecal extracts was higher than in body extracts (5.98 vs. 2.701 IU of trypsin/mg of freeze-dried material). No cysteine protease activity was detected. **Conclusion:** *C. arcuatus* extracts contain several allergens and proteins with high enzymatic activity, especially in the feces. Some of these allergens may be enzymes. Fecal extracts have more enzymatic activity than body extracts.

Copyright © 2007 S. Karger AG, Basel

Introduction

The storage mite *Chortoglyphus arcuatus* (Chortoglyphidae) is an abundant mite species in the domestic environment of humid and rural regions of Spain, especially in the northwest (Galicia) [1]. The allergenicity of this mite has been confirmed using in vivo and in vitro methods [2]. We have recently cloned the first allergen of this mite species, which has sequence homology to tropomyosin [3]. This mite species has also been described in several countries, including the USA, and is thought to have a worldwide distribution. Sensitization to their allergens has also been demonstrated in Wisconsin farmers [4] and residents of Ohio [5]. It has a low degree of crossreactivity with the *Dermatophagoides* spp. [6].

Fig. 1. Light-microscopic pictures of *C. arcuatus* bodies (a, $\times 40$) and feces (b, $\times 400$).



The presence of several proteins with catalytic properties in 8 different mite species has been demonstrated [7]. Allergens with enzymatic activity have been included in mite allergen groups 1, 3, 4, 6, 8, 9 and 15 [8]. Other mite species, e.g. *Glycyphagus domesticus* [9], *Acarus farris* [10], *Tyrophagus putrescentiae* [11], *Blomia tropicalis* [12–14], *Psoroptes cuniculi* [15] and *Aleuroglyphus ovatus* [16], also contain potent enzymes. Comparative studies have been conducted to analyze the presence of enzymes in mite bodies and spent media. It seems that serine proteases (trypsin and chymotrypsin) are more abundant in fecal than in whole body extracts [17, 18].

This enzymatic activity seems to be important in the allergenicity of certain mites, since it can facilitate the access of the allergen to the immune system, playing an adjuvant proallergic role in immunogenicity [19]. The enzymatic activity is present in body and mainly fecal extracts, indicating their role in digestion. It has been suggested that the presence or absence of certain digestive enzymes reflects trophic specialization, showing different ‘enzyme patterns’ among house dust and storage mites.

The aim of this study was to evaluate the enzymatic activity in body and fecal extracts of *C. arcuatus*.

Material and Methods

Extract Preparation

C. arcuatus was cultured on an autoclaved animal source food medium and ground in particles $< 80 \mu\text{m}$. Mites were reared in plastic flasks and incubated at 25°C and a humidity level $> 75\%$. The whole culture medium was frozen to kill the mites, and sieved using mesh sizes of 75 and $125 \mu\text{m}$. The fraction $> 125 \mu\text{m}$ comprised the mite bodies (fig. 1a) with a richness (number of mite particles/total particle number $\cdot 100$) $> 90\%$ (‘body fraction’). Most of the fraction $< 75 \mu\text{m}$ was composed of mite feces (fig. 1b), with richness $> 90\%$ (‘fecal fraction’). Extracts were obtained after incubation and storage of the raw materials (1:10 wt/vol) in

0.01 M PBS (pH 7.4) for 10 h. Thereafter, the extracts were centrifuged at $16,000 g$ at 4°C for 30 min and the supernatants were collected. The remaining raw material was again reconstituted in 1:10 wt/vol PBS and extracted overnight at 4°C under continuous magnetic stirring. Afterwards, the extract was centrifuged, the supernatant separated and mixed with the first extraction, sterile-filtered and dialyzed against bidistilled water in dialysis membranes of 3.5 kDa (CelluSep Membrane; Seguin, Tex., USA), filtered, frozen and freeze-dried.

SDS-PAGE and Immunoblot

SDS-PAGE analyses were used to determine the antigenic profile of both extracts. The samples were run in SDS-PAGE gels with 2.67%C, 15%T (where %T = total concentration of acrylamide and bisacrylamide and %C = percentage of cross-linker relative to the total concentration). One hundred micrograms of protein were loaded on the gels. Gels were silver stained. The gel was electrophoretically transferred to P-Immobilon membrane (PVDF; Millipore, Bedford, Mass., USA) and dried at room temperature for 4 h. The membrane was incubated overnight with the sera of 10 individuals who were skin test positive to *C. arcuatus*, diluted 1:5 vol/vol in PBS 0.01 M Tween 2%.

Enzymatic Activity

Zymogram

Samples were run on SDS-PAGE gels with 2.67%C, 15%T polyacrylamide containing 0.1% gelatine at 4°C . One hundred micrograms of lyophilized material were dissolved in a buffer solution containing β -mercaptoethanol. Subsequently, the gels were incubated for 90 min in a 0.06% solution of L-cysteine (Sigma, St. Louis, Mo., USA) at 37°C . Gels were stained with Coomassie brilliant blue R-250 [20].

API Zym® Studies

The API Zym assay (bioMérieux, France) was used to determine the enzymatic activity of 19 different enzymes in body and fecal extracts following the manufacturer’s instruction. API Zym is a semiquantitative micromethod designed for the research of enzymatic activities. Values ranged from 0 to 5, with 0 being negative and 5 being assigned the maximum intensity. Results have been interpreted according to the instructions of the manufacturer and previous publications [7, 12, 14].

Cysteine Protease Activity

Cysteine protease activity was determined using the L-1460 peptide (Pyr-Phe-Leu-p-nitroanilide, 10 mM; Bachem, Bubendorf, Switzerland) as substrate and papain (Sigma) as standard (results expressed in IU of papain/mg of freeze-dried material). Briefly, 100 µl of serial dilutions of the samples, or papain, diluted in 0.2 M sodium acetate/acetic acid buffer, pH 4, were placed in plastic microtiter plates (Nunc, Roskilde, Denmark). Afterwards, 50 µl of 40 mM L-cysteine/EDTA and 50 µl of the substrate were added. The reaction was incubated for 30 min at 37°C and stopped with 50 µl of 50% acetic acid and read at 405 nm. The results were extrapolated from the regression line obtained with papain.

Serine Protease Activity

Serine protease activity was determined using the S-2288 peptide (H-D-Ile-Pro-Arg-p-nitroanilide dihydrochloride, 1.5 mM; KabiVitrum, Franklin, Ohio, USA) as the substrate and trypsin (Sigma) as the standard. Briefly, 150 µl of serial dilutions of the samples and the standard were diluted in 0.1 M Tris/HCl (pH 8.4). Subsequently, 50 µl of the substrate were added and incubated for 30 min at 37°C. The reaction was stopped with 50 µl of 50% acetic acid and read at 405 nm. Results were expressed in international units of trypsin per milligram of freeze-dried material.

Results

SDS-PAGE and Immunoblot Experiments

The antigen profile of the extracts showed that the body extract contained more and better defined bands than the fecal extract (fig. 2). Several proteins in the molecular weight range of 9–100 kDa were detected. The most prominent bands corresponded to molecules with approximately 14, 16 and 30 kDa. These proteins could be related with the group 2 and 1, respectively, of mite allergens. Fecal extract showed several poorly visualized bands in the range of 9–60 kDa.

Several IgE binding bands were visualized in body and fecal extracts in the molecular weight range of 20–100 kDa (fig. 3). Specific IgE binding to the fecal extract was more consistent among the patients than the body extract. Specific IgE binding to allergens present in the body extract was more variable among patients.

Enzymatic Activity

Gelatinolytic gels (zymogram) confirmed that fecal extracts contain more enzymatic activity than body extracts (fig. 4). The enzymatic activity was mainly detected in proteins with molecular weights >25 kDa, and especially in a band of approximately 27 kDa and to two bands of 40 and 47 kDa. Serine protease activity in fecal extracts was higher than in body extracts (5.98 vs. 2.701 IU of trypsin/mg of freeze-dried material). No cysteine

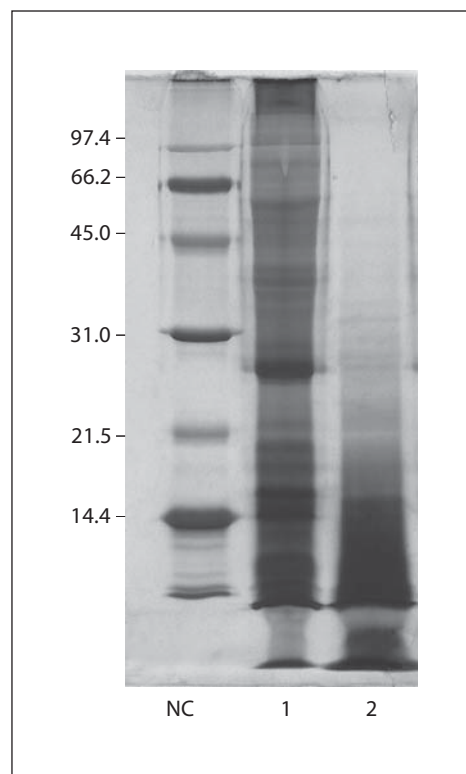


Fig. 2. SDS-PAGE of 100 µg *C. arcuatus*. NC = Normal control; lane 1 = body extract; lane 2 = fecal extract.

protease activity was detected. API Zym studies demonstrated reduced enzymatic activity in fecal extracts in most of the 19 enzyme assays conducted (fig. 5; table 1).

Discussion

Patients with mite sensitization and allergic asthma often react to a variety of allergens present in mite bodies, feces and secretions [21, 22]. The presence of proteolytic activity in fecal extracts is of great clinical importance, since fecal pellets are more easily becoming airborne and inhaled, thus reaching the respiratory mucosa. It has been proposed that the proteolytic activity of mite allergens plays a proallergic role, influencing their immunogenicity by enabling allergens to breach the integrity of the airway epithelial barrier. As a consequence, allergic inflammatory responses are promoted. Furthermore, by disrupting tight epithelial junctions, the enzymatic activity facilitates the transport of allergens across the epithelium [23].

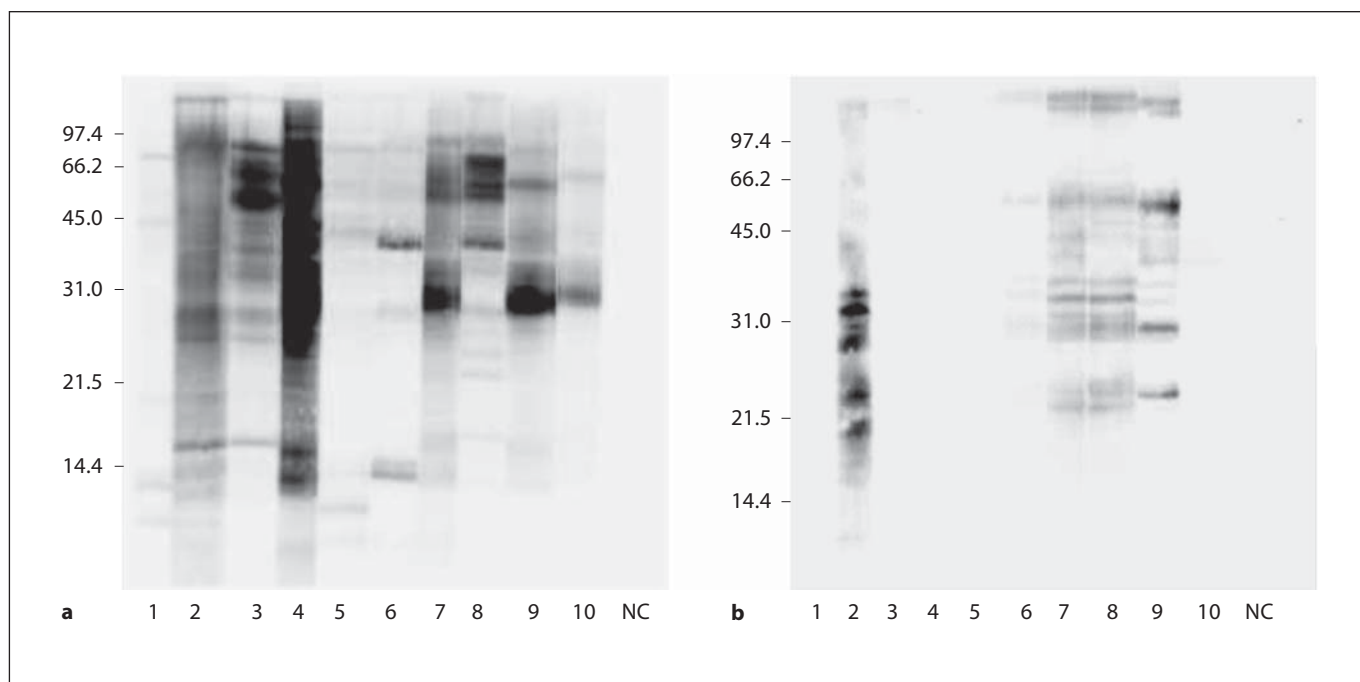


Fig. 3. Immunoblot experiments using 10 individual sera. **a** Body extract. **b** Fecal extract. NC = Normal control.

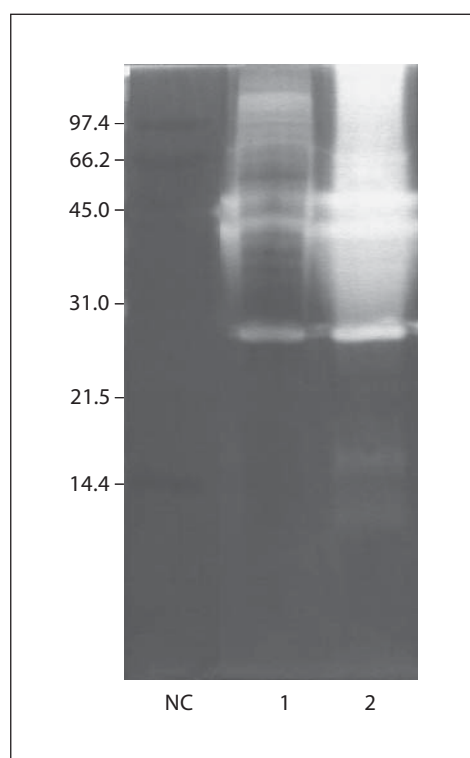


Fig. 4. Zymogram of 100 µg *C. arcuatus*. NC = Normal control; lane 1 = body extract; lane 2 = fecal extract.

An influence on the innate mechanism of defense has also been described. The proteolytic activity of mite feces interacts with pulmonary elastase, inactivating a human elastase inhibitor in vivo and ex vivo. Because these elastase inhibitors have antimicrobial as well as antielastase activity, the inactivation of these innate components of the lung defense system by proteolytic enzymes present in mite feces may increase the susceptibility of patients with allergic inflammation to infection and, therefore, produce a possible exacerbation of allergic respiratory diseases [24]. Following exposure of the bronchial mucosa to fecal pellets of house dust mites, the mite proteases can access the lung interstitium and promote Th2 responses.

Chronic exposure to mites is an important risk factor for the development of sensitization, allergic respiratory diseases and chronic lung inflammation [25]. While this relationship was primarily established for *Dermatophagoides* spp., it has become evident in recent years that other mite species are also the source of potent enzymes.

Analyses of immunoblots revealed a different specific IgE recognition pattern between body and fecal extracts. Individual immunoblots revealed that most patients are mainly sensitized to allergens present in the body extracts. However, 5 of these individuals also



Fig. 5. Results of the API Zym® assay comparing bodies (B) and feces (F) of *C. arcuatus*.

showed IgE binding to allergens in the fecal extract. The allergens recognized in the fecal extracts corresponded to allergens with higher molecular weights. Although specific IgE binding to allergens in the body extract varied greatly between patients, a similarity in the pattern of recognition could be observed for bands with 22, 30, 32, 34 and 62 kDa, and bands >95 kDa. Patients tended to react to various bands, suggesting a high degree of sensitization.

The results obtained from the immunoblots suggest that extracts derived from bodies are better indicators of *C. arcuatus* sensitization. However, there seems to be a subgroup of patients who will also react to fecal extracts. The results also suggest that body and fecal extracts may differ with respect to the allergens detected. Currently, experience in the diagnosis of *C. arcuatus* sensitization is lacking, and large population studies are needed to elucidate which type of extract is better for diagnosing and even treating *C. arcuatus*-sensitized patients. Eventually, both extracts may be required.

Using zymogram experiments, we have demonstrated that *C. arcuatus* extracts contain several enzymes with proteolytic activity, especially in the feces. Allergens with the capacity to hydrolyze the gelatine present in gels after incubation in an adequate substrate show a molecular weight range from 25 to 90 kDa. These enzymes, which are present in different quantities in both extracts, seem to be responsible for the enzymatic activity in body and fecal extracts. However, according to these results, fecal extracts have more proteolytic serine protease activity than body extracts. These results are in concordance with those obtained using other methods, which included trypsin as a standard. The absence of cysteine protease activity is in agreement with other studies carried out in storage mites. We have observed that the proteolytic ac-

Table 1. Enzymatic activity results obtained with API Zym (values ranging from 0 to 5, with 0 being no and 5 maximal activity)

	Bodies	Feces
Control	0	0
Alkaline phosphatase	5	1
Esterase (C4)	2	2
Esterase-lipase (C8)	4	1
Lipase (C14)	0	0
Leucine arylamidase	5	3
Valine arylamidase	3	0
Cystine arylamidase	0	0
Trypsin	0	4
α -Chymotrypsin	1	0
Acid phosphatase	4	3
Naphthol-AS-BI-phosphohydrolase	5	1
α -Galactosidase	1	0
β -Galactosidase	5	2
β -Glucuronidase	4	1
α -Glucosidase	3	0
β -Glucosidase	2	0
N-Acetyl- β -glucosaminidase	5	4
α -Mannosidase	2	0
α -Fucosidase	5	2

tivity detected in *C. arcuatus* is similar to the one described in other species, and, therefore, it can be speculated that the immune response after inhalation of *C. arcuatus* enzymes is similar to that experienced by patients when they inhale allergens/enzymes of other mites such as *Dermatophagoides* spp. or *B. tropicalis*. Using API Zym analyses, a qualitative method to determine enzymatic activity, the highest proteolytic activity was determined in fecal extracts in comparison to body extracts. How-

ever, phosphatase, lipase or glycosidase activities are more important in body extracts.

In conclusion, based on the present investigation, we believe that *C. arcuatus* is a mite species of allergological importance, not only due to its widespread distribution and allergenicity, but also due to the enzymatic activity

present in feces, which corresponds to that found in other mite species. *C. arcuatus* extracts contain several important allergens, especially in the body extracts. Therefore, body extracts are better indicators in the diagnosis of in vivo and in vitro sensitization to *C. arcuatus*.

References

- Boquete M, Iraola V, Fernández-Caldas E, Arenas Villaroel L, Carballada FJ, Gonzalez de la Cuesta C, Lopez-Rico MR, Nuñez Orjales R, Parra GA, Soto-Mera MT, Varela S, Vidal C: House dust mite species and allergen levels in Galicia, Spain: a cross-sectional, multicenter, comparative study. *J Investig Allergol Clin Immunol* 2006;16:169–176.
- Boquete M, Carballás C, Carballada F, Iraola V, Carnés J, Fernández-Caldas E: In vivo and in vitro allergenicity of the domestic mite *Chortoglyphus arcuatus*. *Ann Allergy Asthma Immunol* 2006;97:203–208.
- Fernández-Caldas E, Vailes LD, Troubina O, King EM, Arlian L, Boquete M, Iraola V, Lafosse-Marín S, Chapman MD: Molecular cloning of *Chortoglyphus arcuatus* allergens. *J Allergy Clin Immunol*, in preparation.
- Marx JJ Jr, Twigg JT, Ault BJ, Merchant JA, Fernández-Caldas E: Inhaled aeroallergen and storage mite reactivity in a Wisconsin farmer nested case-control study. *Am Rev Respir Dis* 1993;147:354–358.
- Yadav A, Elder BL, Morgan MS, Vyszynski-Moher DL, Arlian LG: Prevalence of serum IgE to storage mites in a southwestern Ohio population. *Ann Allergy Asthma Immunol* 2006;96:356–362.
- Puerta L, Fernandez-Caldas E, Lockey RF, Caraballo LR: Sensitization to *Chortoglyphus arcuatus* and *Aleuroglyphus ovatus* in *Dermatophagoides* spp. allergic individuals. *Clin Exp Allergy* 1993;23:117–123.
- Morgan MS, Arlian LG: Enzymatic activity in extracts of allergy-causing astigmatid mites. *J Med Entomol* 2006;43:1200–1207.
- Fernández-Caldas E, Iraola Calvo V: Mite allergens. *Curr Allergy Asthma Rep* 2005;5:402–410.
- Bowman CE: Comparative enzymology of economically important astigmatid mites; in Griffiths DA, Bowman CE (eds): *Acarology* VI. Chichester, Ellis-Horwood, 1984, vol 2, p 993.
- Sánchez-Ramos I, Hernandez CA, Castanera P, Ortego F: Proteolytic activities in body and faecal extracts of the storage mite, *Acarus farris*. *Med Vet Entomol* 2004;18:378–386.
- Ortego F, Sanchez-Ramos I, Ruiz M, Castanera P: Characterization of proteases from a stored product mite, *Tyrophagus putrescentiae*. *Arch Insect Biochem Physiol* 2000;43:116–124.
- Montealegre F, Quinones C, Torres N, Goth K: Detection of serine proteases in extracts of the domestic mite *Blomia tropicalis*. *Exp Appl Acarol* 2002;26:87–100.
- Flores I, Mora C, Rivera E, Donnelly R, Montealegre F: Cloning and molecular characterization of a cDNA from *Blomia tropicalis* homologous to dust mite group 3 allergens (trypsin-like proteases). *Int Arch Allergy Immunol* 2003;130:12–16.
- Cardona G, Guisantes J, Eraso E, Serna LA, Martínez J: Enzymatic analysis of *Blomia tropicalis* and *Blomia kulagini* (Acari: Echinomypodidae) allergenic extracts obtained from different phases of culture growth. *Exp Appl Acarol* 2006;39:281–288.
- Nisbet AJ, Billingsley PF: Hydrolytic enzymes of *Psoroptes cuniculi* (Delafond). *Insect Biochem Mol Biol* 1999;29:25–32.
- Edwards TB, Trudeau WL, Fernández-Caldas E, Lee DK, Seleznick MJ, Lockey RF: Proteinases in extracts of the storage mite, *Aleuroglyphus ovatus*. *J Allergy Clin Immunol* 1992;90:129–131.
- Ando T, Homma R, Ino Y, Ito G, Miyahara A, Yanagihara T, Kimura H, Ikeda S, Yamakawa H, Iwaki M, et al: Trypsin-like protease of mites: purification and characterization of trypsin-like protease from mite faecal extract *Dermatophagoides farinae*. Relationship between trypsin-like protease and Der f III. *Clin Exp Allergy* 1993;23:777–784.
- Stewart GA, Lake FR, Thompson PJ: Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: physicochemical characterisation of potential allergens. *Int Arch Allergy Appl Immunol* 1991;95:248–256.
- Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, Ichikawa S, Tokura T, Akiba H, Mitsuishi K, Ikeda S, Okumura K, Ogawa H: Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization toward IgE and IgG responses. *J Immunol* 2006;177:1609–1617.
- Michaud D, Faye L, Yelle S: Electrophoretic analysis of plant cysteine and serine proteinases using gelatin-containing polyacrylamide gels and class-specific proteinase inhibitors. *Electrophoresis* 1993;14:94–98.
- Weghofer M, Thomas WR, Pittner G, Horak F, Valenta R, Vrtala S: Comparison of purified *Dermatophagoides pteronyssinus* allergens and extract by two-dimensional immunoblotting and quantitative immunoglobulin E inhibitions. *Clin Exp Allergy* 2005;35:1384–1391.
- Pittner G, Vrtala S, Thomas WR, Weghofer M, Kundi M, Horak F, Kraft D, Valenta R: Component-resolved diagnosis of house dust mite allergy with purified natural and recombinant mite allergens. *Clin Exp Allergy* 2004;34:597–603.
- Sehgal N, Custovic A, Woodcock A: Potential roles in rhinitis for protease and other enzymatic activities of allergens. *Curr Allergy Asthma Rep* 2005;5:221–226.
- Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C, Lamb J, Sallenave JM: House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol* 2003;29(3 Pt 1):381–389.
- Custovic A, Simpson A, Woodcock A: Importance of indoor allergens in the induction of allergy and elicitation of allergic disease. *Allergy* 1998;53:115–120.