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Enzymatic Activity of Allergenic House Dust and Storage Mite Extracts

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ABSTRACT Proteases are involved in the pathogenicity of allergy, increasing epithelial permeability and acting as adjuvants. Enzymatic activity is therefore important for the allergenicity of an extract and also affects its stability and safety. However, the enzymatic activity of extracts is not usually evaluated. The objective of this study was to evaluate the enzymatic activity of the most allergenic mite extracts and to investigate their allergenic properties. Extracts from nine allergenic mite species (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* Hughes, *Euroglyphus maynei*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae* (Schrank), *Glycyphagus domesticus* (DeGeer), *Acarus siro* L., *Chortoglyphus arcuatus*, and *Blomia tropicalis*) were characterized. Protein and allergen profiles were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blot, respectively. Gelatinolytic activity was evaluated with a zymogram and the activity of other enzymes (cysteine, serine proteases, and esterases) was evaluated individually or with the API-ZYM system. The main differences in protease activity were found between house dust mites and storage mites. House dust mites presented higher cysteine protease activity while storage mites presented higher serine protease activity. These differences are in line with their trophic specialization. A wide range of different activities was found in all the extracts analyzed, reflecting the fact that the extracts preserve the activity of many enzymes, this being necessary for a correct diagnosis. However, enzymes may act as adjuvants and, therefore, could lead to undesirable effects in immunotherapies, making this activity not suitable for treatment products. Modified extracts with lower enzymatic activity could be more appropriate for immunotherapy.

KEY WORDS allergen extract, allergen immunotherapy, enzymatic activity, mite

Protease activity from allergen sources contributes to the pathogenesis of allergies by: increasing the permeability of epithelial cells, facilitating *trans*-epithelial allergen delivery (Herbert et al. 1995), and interaction with antigen-presenting cells; and increasing the tissue distribution of the allergens, digesting the extracellular matrix and modulating the function of a range of immune cells. Moreover, it has also been demonstrated that chronic exposure to enzymatic allergens can gradually compromise the integrity of the airway epithelium in asthma.

Storage and house dust mites are the most important source of indoor allergens, and sensitization to indoor allergens is strongly associated with asthma. *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* Hughes, *Euroglyphus maynei*, *Lepidoglyphus destructor*, *Glycyphagus domesticus* (DeGeer), *Chortoglyphus arcuatus*, *Tyrophagus putrescentiae* (Schrank), and *Acarus* spp. are the more abundant Astigmata species in Spain, Portugal, and Germany (V. I., unpublished data; Iraola and Fernández–Caldas 2009). *Blomia tropicalis* is also very important in some specific regions (as some European islands).

Twenty four different families of allergens have been described in mites until now, including four groups with protease activity (group 1 are cysteine proteases; group 3, trypsins; group 6, chymotrypsins; and group 9, collagenolytic serine proteases) and three groups with glycosidase activity (group 4, alpha-amylases; groups 15 and 18, chitinases).

Group 1 allergens degrade endogenous protease inhibitors (Brown et al. 2003) and surfactant proteins (Deb et al. 2007), increasing the access of allergens to dendritic cells beneath the epithelial barrier. Der p 1 cleaves cell surface molecules involved in the control of IgE synthesis such as FcεRII (Sarfati and Delespesse 1988, Schulz et al. 1995), CD25 (Schulz et al. 1998), CD40 (Ghaemmaghami et al. 2002), DC-SIGN (CD209), and DC-SIGN receptor (Furmonaviciene et al. 2007), stimulates the expression of IL-8 (Adam et al. 2006) and promotes Th2 differentiation. Moreover, Der p 1 enhances total IgE production (Gough et al. 1999) and is able to influence the balance between IL-4 and IFN-γ against an irrelevant antigen, acting as an adjuvant (Comoy et al. 1998).

Mite serine proteases such as allergen groups 3, 6, and 9, are capable of activating the kallikrein-kinin system in normal human plasma (Takahashi et al. 1990) that is involved in inflammation (Polosa 1993).

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Moreover, Der p 3 and Der p 9 activate PAR-2 (Sun et al. 2001), stimulating IL-6, IL-8, and prostaglandin E_2 release from epithelial cells (Asokananthan et al. 2002), and Der f 3 activate the complement system to produce anaphylatoxins (Maruo et al. 1997). Lastly, chitinases may play an important role in the pathogenesis of asthmatic Th2 inflammation and IL-13 effector pathway activation (Elias et al. 2005).

Allergenic extracts used for diagnosis and treatment are usually characterized according to their immunochemical properties and little is known about the enzymatic activity. This parameter is not routinely evaluated although it could affect the quality of the product, modifying the composition or the total allergenicity, safety, and efficacy. In fact, according to the EMEA Guideline on Allergen Products (EMEA/CHMP/BWP/304831/2007), differences between the enzymatic activity of allergens, even in the same homologous group, should be taken into account because they may affect the stability of the allergen extract. The objectives of this study were therefore to characterize the enzymatic activity of allergenic extracts from the nine most important mite species from the allergologic point of view and to investigate their allergenic properties.

Materials and Methods

Allergen Extracts. Extracts from three house dust mites species including *D. pteronyssinus*, *D. farinae*, and *E. maynei*, and six storage mites, *L. destructor*, *T. putrescentiae*, *G. domesticus*, *A. siro* L., *C. arcuatus*, and *B. tropicalis*, were manufactured using standardized internal protocols (Laboratorios LETI, S.L.U., Madrid, Spain).

Briefly, extracts were prepared with semipurified mite bodies (>90% mite bodies). This material was extracted in 0.01 M phosphate-buffered saline (PBS; 1:10 wt:vol) for 4 h at 4°C under continuous magnetic stirring. After homogenization, the extracts were centrifuged at 16,000 × *g* for 30 min at 4°C and the supernatants collected. The remaining raw material (pellet) was again reconstituted in 0.01 M PBS (1:10 wt:vol) 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, dialyzed against double-distilled water with dialysis membranes of 3.5 KDa (CelluSep Membrane; Seguin, TX), filtered, frozen, and freeze-dried. The protein content was measured by the Lowry–Biuret method (Sigma, St. Louis, MO) following the manufacturer's instructions.

Protein and Allergenic Profile. The protein profile was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with gels prepared at different polyacrylamide concentrations (8% and 15% T). Forty micrograms of protein from each sample were denatured with β-mercaptoethanol at 100°C for 10 min and loaded onto the gels. Lastly, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA).

The allergenic profile was determined by western-blot. After SDS-PAGE (15% T; 40 μg of protein), proteins were electrotransferred to a nitrocellulose membrane. They were then incubated with a specific pool of sera (Plasmalab, WA). Western-blots were developed by a colorimetric reaction consisting on anti-IgE labeled with peroxidase (Ingenasa, Madrid, Spain) and 3,3′,5,5′-tetramethyl-benzidine (Sigma) as substrate.

Enzymatic Activity. *Gelatinolytic Activity.* Gelatinolytic activity was evaluated using zymogram gels. Gels (15% T) were prepared with 0.1% gelatin and samples (50 μg of protein) were loaded in reducing and nonreducing conditions. After running the gels, they were incubated with 2.5% Triton X-100 (Bio-Rad Laboratories) for 30 min, then incubated with a 0.06% L-Cys (Sigma) solution for 90 min at 37°C. Lastly, the gels were stained with Coomassie Brilliant Blue R-250.

API-ZYM Determinations. The API-ZYM system (BioMerieux, France) was used to evaluate the activity of 19 different enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Samples were prepared at 1 mg of protein/ml in 0.01 M PBS. Results were interpreted optically according to the instructions manual ranging from 0 to 5 (0 means no color, five very strong color). Results of two or below were considered negative.

Cysteine Protease Activity. Pyr-Phe-Leu-*p*-nitroanilide peptide (Bachem, Offenburg, Germany) was used to determine the cysteine protease activity using papain from papaya latex (Sigma) as standard. Serial dilutions were prepared in 0.2 M sodium acetate/acetic acid pH 4.0. Then, 40 mM L-Cys/40 mM EDTA and the substrate were added. The reactions were incubated for 30 min at 37°C, stopped with 50% W/W acetic acid and read at 405 nm. The final result was expressed in papain equivalents per milligram of protein.

Kallikrein-Like Protease. H-D-Val-Leu-Arg-*p*-nitroanilide di-acetyl hydroxide (Bachem) was used as substrate and trypsin as standard. Reactions took place in 0.1 M Tris-HCl, pH 8.4, at 37°C over 30 min, were stopped with 50% W/W acetic acid and read at 405 nm. The final result was expressed as trypsin equivalents per milligram of protein.

Serine Protease Activity. Serine protease activity was evaluated using D-Ile-Pro-Arg-*p*-nitroanilide dihydrochloride (Sigma) as substrate and trypsin from bovine pancreas (Thermo Scientific, Kanagawa, Japan) as standard. Reactions took place in 0.1 M Tris-HCl, pH 8.4, over 30 min at 37°C, were stopped with 50% W/W acetic acid and read at 405 nm. The final result was expressed in trypsin equivalents per milligram of protein.

Collagenase Activity. Nonspecific proteinase activity was determined using insoluble diazotized colla-

gen (azocoll; Sigma) according to previously reported methods (Chavira et al. 1984) and manufacturer information. Trypsin (Sigma) was used as standard. Briefly, azocoll was dissolved at 0.5% W/V in 0.1 M potassium phosphate buffer, pH 7.8, at 37°C and incubated with samples for 15 min at 37°C. Afterwards, the reactions were filtered through 0.45 nm syringe filters and optical density at 520 nm was measured. The final result was expressed in trypsin equivalents per milligram of protein.

Esterase Activity. Esterase activity was evaluated using N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE; Sigma) as substrate adapting manufacturer instructions. BAEE reacts enzymatically with water leading to N-α-benzoyl-L-arginine and ethanol and, consequently, the pH decreases. The volume of 0.1 M NaOH needed to keep the pH constant at pH 7.0 in a 48 mM BAEE solution is calculated. Results were expressed as units per milligram of protein, calculated as follows:

$$U/mg\ protein = \frac{Molarity\ of\ NaOH\ (mM) \times Volume\ of\ NaOH(\mu l) \times 1{,}000}{Time(min) \times mg\ protein}$$

Acid Phosphatase Activity. Acid phosphatase activity was evaluated using 4-nitrophenyl phosphate disodium salt hexahydrate as substrate and potato acid phosphatase as standard (Sigma). Reactions took place in 0.1 M citric acid/0.2 M sodium phosphate, pH 5.0, over 30 min at 37°C, were stopped with 0.1 N NaOH and read at 405 nm. The final results were expressed in acid phosphatase equivalents per milligram of protein.

Alkaline Phosphatase Activity. Alkaline phosphatase activity was evaluated using 4-nitrophenyl phosphate disodium salt hexahydrate as substrate and alkaline phosphatase from *Escherichia coli* as standard (Sigma). Reactions took place in 0.1 M Tris-HCl, pH 8.4, over 30 min at 37°C, were stopped with 0.1 N NaOH and read at 405 nm. The final result was expressed as alkaline phosphatase equivalents per milligram of protein.

Results and Discussion

Allergen extracts consist on a mixture of allergenic and nonallergenic proteins and several other biological substances. These extracts are commonly used for allergy diagnosis and immunotherapy. Because these allergen extracts are administered in allergic individuals, they must be fully characterized and their immunochemical properties correctly defined to avoid undesirable effects in patients.

One of these undesirable properties, mainly in mites, is the enzymatic activity that all these extracts exhibit in native conditions. Most mite allergens are enzymes with different activities (Hubert 2004). In fact, >95% of mite allergens are associated with faecal particles, suggesting a role in the digestion of food (Tovey et al. 1981, Sanchez–Ramos et al. 2004, Carnés et al. 2008). Previous studies have demonstrated that the different digestive enzymes expressed are related to their diet. These differences were minimized in our study because all the species included were grown in the same media (internal procedures of Laboratorios LETI, SL).

Table 1. Families, species, and protein content

Group	Species	μg prot/mg
Pyroglyphidae	*Dermatophagoides pteronyssinus*	383.0
	Dermatophagoides farinae	424.0
	Euroglyphus maynei	283.0
Glycyphagoidea	*Lepidoglyphus destructor*	365.0
	Glycyohagus domesticus	324.0
	Chortoglyphus arcuatus	408.0
	Blomia tropicalis	472.0
Acaridae	*Tyrophagus putrescentiae*	432.0
	Acarus siro	432.0

In this study, enzymatic activity of allergenic three house dust mites (*D. pteronyssinus*, *D. farinae*, and *E. maynei*) and six storage mite species (*L. destructor*, *T. putrescentiae*, *G. domesticus*, *A. siro*, *C. arcuatus*, and *B. tropicalis*) was evaluated.

The protein content of the manufactured extracts (Table 1) ranged from 283 μg/mg of freeze-dried material (*E. maynei*) to 432 μg/mg (*T. putrescentiae* and *A. siro*). All the extracts showed a wide variety of proteins, mainly smaller than 100 KDa (Fig. 1).

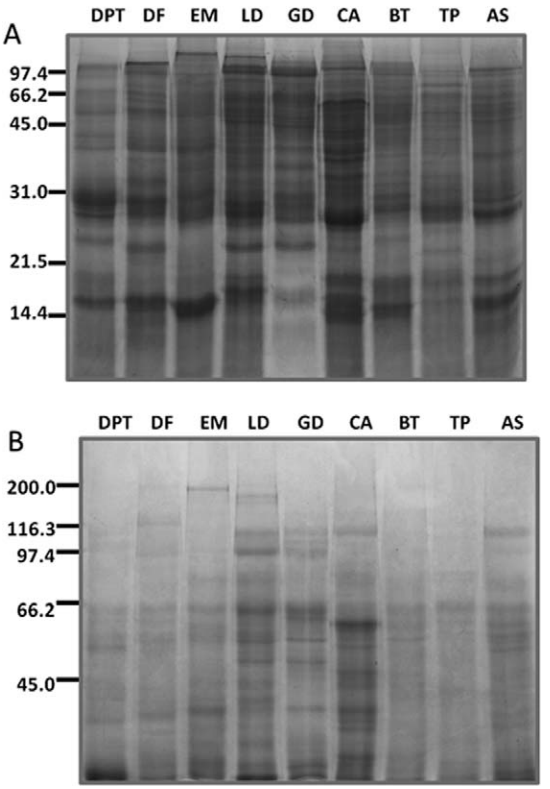

Fig. 1. SDS-PAGE 15% T (A) and 8% T (B) of *D. pteronyssinus* (DPT), *D. farinae* (DF), *E. maynei* (EM), *L. destructor* (LD), *G. domesticus* (GD), *C. arcuatus* (CA), *B. tropicalis* (BT), *T. putrescentiae* (TP), and *A. siro* (AS) extracts.

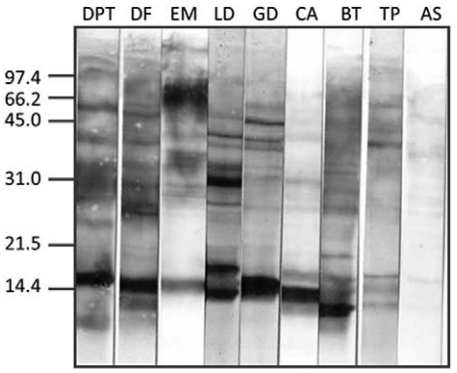

Fig. 2. Immunoblot (SDS-PAGE 15%T) of *D. pteronyssinus* (DPT), *D. farinae* (DF), *E. maynei* (EM), *L. destructor* (LD), *G. domesticus* (GD), *C. arcuatus* (CA), *B. tropicalis* (BT), *T. putrescentiae* (TP), and *A. siro* (AS) extracts.

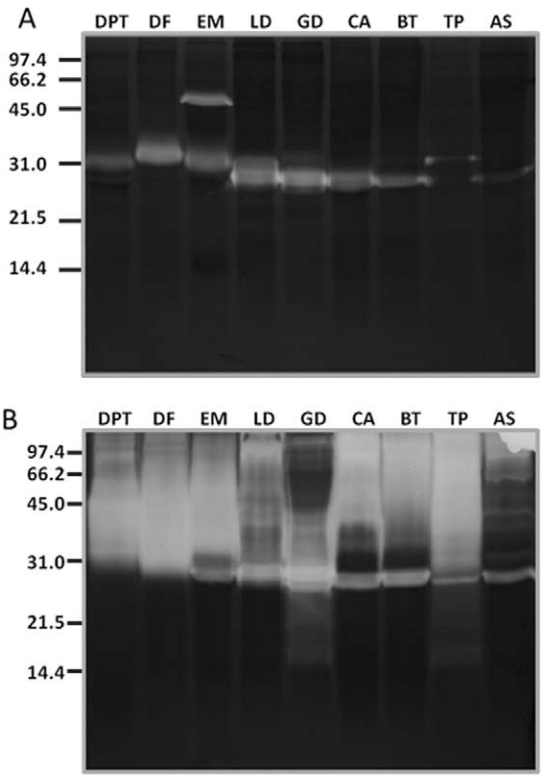

Fig. 3. Zymogram (SDS-PAGE 15%T) in reducing (A) and nonreducing conditions (B) of *D. pteronyssinus* (DPT), *D. farinae* (DF), *E. maynei* (EM), *L. destructor* (LD), *G. domesticus* (GD), *C. arcuatus* (CA), *B. tropicalis* (BT), *T. putrescentiae* (TP), and *A. siro* (AS) extracts. Standard used in reducing conditions in both zymograms.

Related species had similar protein profiles, determined by 1D SDS-PAGE. In the Pyroglyphidae family, the two species of *Dermatophagoides* had a very similar protein pattern, while *E. maynei* presented some differences (the *Dermatophagoides* species had one protein of ≈25 KDa, and lacked of a high molecular weight band compared with *E. maynei*). The different species of the Glycyphagoidea group were very similar to each other, specially between *L. destructor* and *G. domesticus*, although *G. domesticus* had fewer low molecular weight proteins. Lastly, the protein profile among the Acaridae family presented slight differences in high molecular weight proteins. All the extracts showed intense bands at around 15 and 30 KDa.

The allergenic profile showed a wide variety of allergens (Fig. 2). All the extracts presented one to three bands of around 15 KDa, possibly corresponding to group 2 allergens. However, the recognition pattern of bigger proteins presented higher variability.

Enzymatic activity was evaluated by gelatinolytic activity (Fig. 3), Api-ZYM system, which allows a semiquantitative determination of the main enzymatic activities (Fig. 4), and, finally, some activities were evaluated individually (Table 2). Although all the species included in the study are associated with allergic sensitization, enzymatic properties differed significantly, mainly in terms of protease activity.

No differences were observed in collagen or gelatin degradation between groups. Collagen is degraded by cysteine proteases and serine proteases, with different optimum pH (acid or basic, respectively). Collagen degradation was detected in all mite species (Table 2). This activity ranged from 4 to 9 μg trypsin/mg protein in most extracts, except in the case of *G. domesticus*, which presented the lowest activity (1.9 μg/ml). Gelatinases are metalloendopeptidases (EC 3.4.24) that hydrolyze gelatin (derived from collagen) into its subcompounds. Gelatinolytic activity was visualized in all the extracts (Fig. 3). Most of the allergen extracts lost their enzymatic activity after reduction. Only one band of 32 KDa was observed in *D. farinae*, two bands of 31 and 48 KDa in *E. maynei*, the 31 KDa one also present in *D. pteronyssinus* and *T. putrescentiae* extracts, while a band of 29 KDa was identified in the Glycyphagoidea group. In nonreducing conditions gels, gelatinolytic activity was markedly high with *D. farinae* and *T. putrescentiae*, while Glycyphagoidea (*L. destructor* and *G. domesticus*) and *A. siro* presented the lowest activity. It has been suggested that gelatinases could be used to digest collagenous substrates in mammal skin cells (King et al. 1996), so it would seem more reasonable that this activity was higher in Pyroglyphidae. However, collagenase activity was measured in a basic environment. Moreover, the ability to digest collagenous material has also been suggested for storage mites (Hubert 2004).

Phosphatases (EC 3.1.3) remove phosphate groups from phosphorylated substrates, allowing their absorption. All the extracts were positive to all phosphatases evaluated. Acid phosphatase activity was the most pronounced in all mite species studied (Table 2; Fig. 4A), as has also been observed in other mites such us *Dermanyssus gallinae*, *Psoroptes ovis*, *Tetranychus urticae*, and *Sarcoptes scabiei* (Nisbet 2000, Morgan and Arlian 2006). Acid phosphatase activity ranged from 451.2 (*T. putrescentiae*) to 1,235.8 μg acid phosphatase/mg protein (*A. siro*), even higher than the

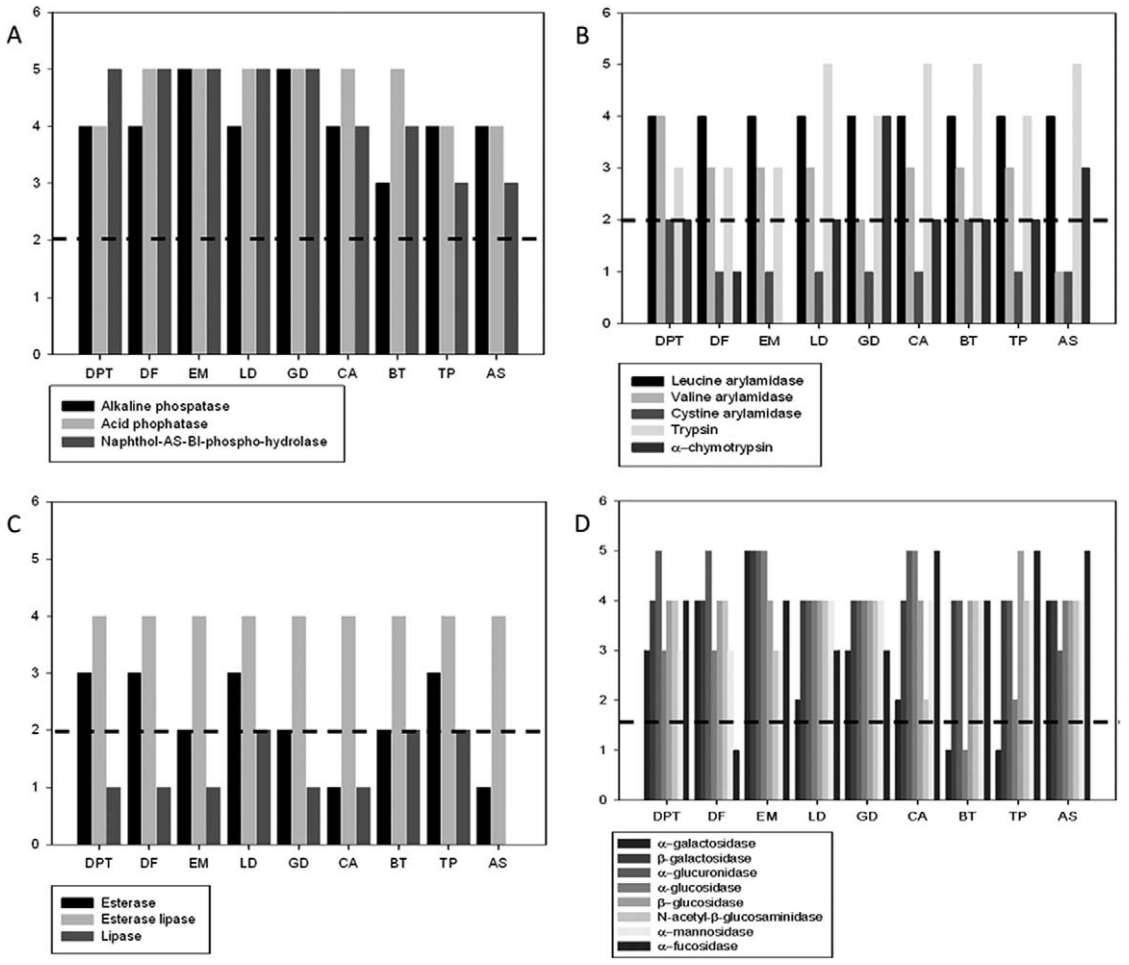

Fig. 4. Enzymatic activity of *D. pteronyssinus* (DPT), *D. farinae* (DF), *E. maynei* (EM), *L. destructor* (LD), *G. domesticus* (GD), *C. arcuatus* (CA), *B. tropicalis* (BT), *T. putrescentiae* (TP), and *A. siro* (AS) extracts determined with API-ZYM system: (A) phosphatases; (B) proteases; (C) lipases; and (D) glucosidases. Activities lower than or equal to two (dotted line) were considered negative. Units are expressed in a visual scale.

standard used (Table 2). The average was 755.8 μg acid phosphatase/mg protein. Moreover, it reached the maximum API-ZYM score in six of the nine species (*D. farinae*, *E. maynei*, *L. destructor*, *G. domesticus*, *C. arcuatus*, and *B. tropicalis*). Lastly, alkaline phosphatase ranged from 0.8 μg alkaline phosphatase/mg protein (*D. farinae*) to 19.6 μg (*A. siro*). This activity was also especially high in the case of the Glycyphagidae family.

Table 2. Enzymatic activity of the nine different mite species

Species	Cysteine protease (μg papain/mg prot.)	Kallikrein protease (μg trypsin/mg prot.)	Serine protease (μg trypsin/mg prot.)	Colagenase (azocoll) (μg trypsin/mg prot.)	Esterase (BAEE) (U/mg prot.)	Acid phosphatase (μg acid phosphatase/mg prot.)	Alcaline phosphatase (μg alcaline phosphatase/mg prot.)
D. pteronyssinus	24.4	6.1	0.1	5.7	442.8	779.3	1.9
D. farinae	20.3	4.9	0.1	8.8	350.8	542.5	0.8
E. maynei	9.5	3.8	1.4	4.5	147.7	891.1	3.4
L. destructor	8.7	28.5	5.4	6.2	544.0	916.6	6.7
G. domesticus	11.7	12.7	3.3	1.9	441.0	838.4	4.6
C. arcuatus	8.7	56.4	8.0	8.2	800.3	656.5	3.6
B. tropicalis	3.0	46.2	9.9	8.3	584.1	490.8	1.0
T. putrescentiae	7.8	70.3	14.2	8.4	713.5	451.2	1.7
A. siro	9.3	18.3	4.3	5.0	919.2	1,235.8	19.6

All the extracts presented aminopeptidase activity, although no cysteine arylamidase activity was found in any of the extracts (Fig. 4B). Leucine arylamidase activity was similar in all the extracts, with a level of 4. Valine arylamidase was positive for seven species (all except *G. domesticus* and *A. siro*). House dust mites showed higher cysteine protease activity than storage mites, while storage mites presented a considerably higher serine protease activity.

Cysteine proteases or thiol proteases (EC 3.4.22) present a nucleophilic cysteine thiol in the catalytic site. This activity was 8–10 μg papain/mg protein in most species, although it was especially high in *Dermatophagoides* spp. (20–24 μg papain/mg protein) and low in *B. tropicalis* (3.0 μg papain/mg protein). It has been suggested that house dust mites express cysteine proteases to digest the skin cells through the hydrolysis of collagen and keratin (Stewart 1995). Group 1 allergens have not been isolated from *Acarus*, *Tyrophagus*, *Glycyphagus*, and *Lepidoglyphus* spp. One reason could be that storage mites feed on seeds and the molds that live on them, rich in protease inhibitors. It may be that storage mites express proteinases that are not inhibited by them. In fact, there is a preponderance of serine proteases in storage mites, although they have also been reported in house dust mites.

Serine proteases or serine endopeptidases (EC 3.4.21) are proteases that present a serine residue in the catalytic site. All the extracts presented serine protease activity (Table 2), although it was higher in SM, reaching *T. putrescentiae* as much as one hundred times the activity of *Dermatophagoides*. Moreover, kallikrein-like protease activity was also higher in storage mites than in house dust mites, ranging from 3.8 μg trypsin/mg protein (*E. maynei*) to 70.3 μg trypsin/mg protein (*T. putrescentiae*).

Some differences were found in determinations of serine protease activity depending on the substrate used. They include protein-digesting enzymes such as trypsin-like proteins, chymotrypsin and collagenolytic serine proteases. Trypsin-like proteins preferentially cleave protein chains on the carboxyl side of positively charged amino acids (Lys, Arg, or His). Chymotrypsins are serine endopeptidases that preferentially cleave protein chains on the carboxyl side of aromatic amino acids (Phe, Tyr, or Trp). Collagenolytic serine proteases preferentially cleave substrates containing C-terminal phenylalanine or leucine substrates.

Trypsin activity was detected in all the studied species (Fig. 4B), although, in line with previous studies, it was higher in storage mites than in house dust mites (Morgan and Arlian 2006). Chymotrypsin activity was only detected in *G. domesticus* and *A. siro* extracts (Fig. 4B), although Blo t 6, Der f 6, and Der p 6 are described as chymotrypsins. Other authors have similarly found that this activity was not detected by the API-ZYM method in the rest of the species (Nisbet 2000, Morgan and Arlian 2006).

Esterases (E.C. 3.1) hydrolyze ester bonds into an acid and an alcohol. Esterases include a wide variety of proteins, such as phosphatases and lipases. General esterase activity was evaluated using the BAEE substrate (Table 2). Esterase activity was especially low in *E. maynei* (147.7 U/mg protein) and high in *A. siro* and *C. arcuatus* (919.2 and 800.3 U/mg protein respectively), with an average of 549.3 U/mg protein. The fact that this activity was higher in storage mites than in house dust mites may be related to the storage mites' seed-rich diet.

Mites feed on lipids from seeds and the fat of animals. Lipases are responsible for hydrolyzing the hydrogen bonds between the glycerol backbone and the fatty acid side chain of various lipids: C4 esterase removes short-chain fatty acids from water-soluble substrates, whereas C14 lipase removes longer-chain fatty acids. These activities were evaluated with the API-ZYM system using 2-naphthyl butyrate for evaluating the C4 esterase activity, 2-naphthyl caprylate for C8 esterase lipase activity and 2-naphthyl myristate for C14 lipase activity, differing in the length of the hydrocarbon chain (Fig. 4C). All the extracts presented similar esterase lipase activity, with an API-ZYM level of 4. No lipase activity was found and, in line with previous studies, esterase lipase had the highest activity (Morgan and Arlian 2006), being positive in four of the nine extracts (*D. pteronyssinus, D. farinae, L. destructor*, and *T. putrescentiae*).

Glycosidases (E.C. 3.2.1) are enzymes that hydrolyze O- and S-glycosyl compounds, releasing smaller sugars. They differ in the kind of bonds they can break. Although glucosidase activity was evident in all the extracts (Fig. 4), it was observed that not all mites presented the same pattern of glycosidase expression; for example, α-galactosidase and α-glucuronidase were higher in house dust mites, while α-mannosidase and α-fucosidase were higher in storage mites. However, the level of β-glycosidases was very similar between the two groups. Some of these enzymes play important roles; β-glucosidase is involved in cellulose degradation and *N*-acetyl-β-glucosaminidase is a chitinase involved in molting in invertebrates.

Enzymatic activity is not a characteristic unique to mite allergens. Previous studies report that proteases derived from cockroaches (Page et al. 2003), molds (Kheradmand et al. 2002) and pollens also contribute to the pathogenesis of allergies. Pollen proteases, usually involved in pollen germination, can compromise the effectiveness of the epithelial barrier (Runswick 2007, Vinhas et al. 2011). Pollen proteases include aminopeptidases, cysteine proteases, and high-molecular-weight serine proteases (Gunawan et al. 2008, Vinhas et al. 2011).

It has been suggested that proteases act as adjuvants and may therefore be required to overcome the innate resistance of the airway to Th2 activation and allergic inflammation (Kheradmand et al. 2002). The presence of proteases may then also explain the presence of non-IgE-mediated respiratory symptoms, for example during the peak of the pollen season (Vinhas et al. 2011).

In addition to the IgE-mediated reactions of an allergen, the adjuvant effect of the enzymes (allergens or not) should be taken into account in the characterization of extracts used for diagnosis and immuno-

therapy. Inclusion of some enzymatic activity measurements in the production of allergenic extracts may be appropriate as quality control to obtain more homogeneous extracts.

Enzymatic reactions could lead to undesirable effects in immunotherapies. Knowledge of the enzymatic activity of the extracts and the mechanisms to minimize it is therefore essential in terms of obtaining more stable and safer extracts. One possible alternative could be the modification of the extracts to reduce enzymatic activity, by using an acidic treatment for example, minimizing the allergenicity (IgE binding) but not the immunogenicity (IgG binding), thereby increasing the safety of the treatment (Fernandez–Caldas et al. 2008, Gallego et al. 2010). However, other authors suggest that the cysteine protease activity of Der p 1 is essential for eliciting not only IgE but also IgG (Kikuchi et al. 2006).

In summary, we have demonstrated that mite extracts have high enzymatic activity capacity and there is great variability between enzymes expressed by different mite groups, and that the mite species investigated express functional enzymes that could alter the allergenicity of the extracts. Increased knowledge of this enzymatic activity is crucial for ensuring the quality and safety of the extracts.

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