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Enzymatic Activity of *Dermatophagoides pteronyssinus* Extracts after Acidic Treatment

Enrique Fernández-Caldas Mayte Gallego Jerónimo Carnés Victor Iraola

Research and Development, Laboratories LETI, Madrid, Spain

Key Words

Enzymatic activity · *Dermatophagoides pteronyssinus* · Depigmentation · Der p 1 · Der p 2 · Asthma

Abstract

Background: Mite extracts contain potent enzymes. These enzymes, especially Der p 1, may affect the bronchial homeostasis and the amplification of the allergic response. The objectives of this study were to determine how depigmentation affects the enzymatic activity of allergen extracts of *Dermatophagoides pteronyssinus* and to verify if these depigmented extracts retain their in vitro allergenic properties. ***Methods:*** Four native extracts were manufactured from 4 different batches of raw material of *D. pteronyssinus*. Once extracted, native extracts were reconstituted and modified by adding increasing quantities of 2 M HCl to the solution and dialyzed against double-distilled water. The enzymatic activity of these 8 extracts (4 native and 4 depigmented) was evaluated using in vitro methods. The allergenic potency was evaluated by human specific IgE and IgG ELISA inhibition experiments. The major allergen content (Der p 1 and Der p 2) was measured with monoclonal antibodies. ***Results:*** Protease, phosphatase, lipase and glycosidase activity was detected in native extracts. After depigmentation, all the enzymatic activities showed a significant decrease. SDS-PAGE reveals the same protein profile in both types of extracts. The results of ELISA inhibition confirmed that depigmented extracts preserved their antigenic and allergenic capacity. Der p 2 levels increased in depigmented extracts, while the detection capacity of Der p 1 decreased. ***Conclusions:*** The depigmentation process significantly reduced the enzymatic activity of these mite extracts, while preserving their allergenicity and antigenicity. No significant differences were observed in the antigenic profile of native and depigmented extracts.

Introduction

Important airborne allergens may possess hydrolytic enzymatic activities, such as proteases, glycosidases and ribonucleases. Several of these enzymes are important allergens, including cysteine and serine proteases and glycosidases (amylase) in mites and moulds and ribonucleases in grass and tree pollens and moulds. House dust mite allergen extracts contain more than 20 IgE-binding molecules [1–3]. Some of them with enzymatic activity included groups 1, 3, 4, 6, 8, 9 and 15 [4, 5]. Other mite species, such as *Glycyphagus domesticus* [6], *Acarus farris* [7], *Tyrophagus putrescentiae* [8], *Blomia tropicalis* [9, 10], *Psoroptes cuniculi* [11] and *Aleuroglyphus ovatus* [12] also contain allergens with enzymatic activity. Serine proteases (trypsin and chymotrypsin) seem to be more abundant in faecal than in whole body extracts [13, 14]. Enzyme allergens are widely present in other allergen sources such as honey bee venom [15], imported fire ant [16], vespids [17] and *Candida albicans* [18–20].

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Correspondence to: Dr. Jerónimo Carnés
Laboratorios LETI, S.L.
Calle del Sol No. 5
ES–28760 Tres Cantos, Madrid (Spain)
Tel. +34 91 771 1790, Fax +34 91 804 0919, E-Mail jcarnes@leti.com

It has been widely demonstrated that allergenic mite extracts have enzymes capable of degrading a wide range of substances, including other proteins and allergens, and can have negative effects on the efficacy and stability of therapeutic extracts. The protease activity of allergens may induce a range of inflammatory effects modulating the adaptive immune system by cleavage of CD23 and CD25, including induction of vascular permeability, oedema, activation of neural reflex leading to bronchoconstriction and stimulation of gland secretion and cough [21, 22]. Der p 1 has additional effects on the innate defence mechanisms of the lung by inactivating in vitro and ex vivo the elastase inhibitors in humans. Der p 1 may also increase the susceptibility of patients with allergic inflammation to infection [23]. Der p 1 can damage bronchial epithelium, increasing its permeability and detaching cells in vitro. The resulting breach of the epithelial barrier may increase exposure to antigen-presenting cells as well as the likelihood that IgE will be raised against proteins in mite faecal pellets. Allergens such as Der p 3 and Der p 9, with serine protease activity, may also induce a non-allergic inflammatory response in the airways through the release of proinflammatory cytokines from the bronchial epithelium [24].

In general, 3 methods are used to inactivate enzymes in various substrates, namely enzyme inhibitors, heat and acidic pH. Herein, we describe a process by which we reduce the enzymatic activity of mite extracts. This method, also known as depigmentation, consists of a controlled acid treatment and dialysis. It inactivates the enzymatic activity of mite extracts and removes irrelevant material. Immunotherapy with this type of extract (depigmented), followed by a polymerization process, was shown to be clinically effective and safe [25]. In a previous study, we have also confirmed that these enzymatically inactive extracts retain their in vivo allergenicity [26].

The objectives of this study were to determine how this acidic treatment affects the enzymatic activity of *Dermatophagoides pteronyssinus* allergen extracts and to verify if these depigmented extracts retain their in vitro allergenic properties.

Material and Methods

Allergen Extracts

Native Extracts. Four different native extracts of *D. pteronyssinus* were manufactured; 3 extracts were prepared with semi-purified mite bodies (more than 80% mite bodies, extracts 1, 3 and 4) and another extract with purified (sieved) mite culture spent medium, containing a high proportion of faecal particles (more than 80%, extract 2). One hundred-gram aliquots of these 4 raw materials were extracted individually (1:10 weight/vol) in 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 4 h at 4°C under continuous magnetic stirring. After homogenization, the extracts were centrifuged at 16,000 *g* at 4°C for 30 min and the supernatants collected. The remaining raw material was again reconstituted in 1:10 weight/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 double-distilled water in dialysis membranes of 3.5 kDa (CelluSep Membrane; Seguin, Tex., USA), filtered, frozen and freeze-dried.

Depigmented Extracts. Lyophilized native extracts were reconstituted in double-distilled water and the pH was adjusted to 2 with 2 M HCl. The extract was kept under continuous magnetic stirring for 15 min at room temperature. Afterwards, the extracts were dialyzed against double-distilled water in dialysis membranes (3.5 kDa) for 17 h. The pH of the solution was neutralized after adjusting the pH value to 7.3–7.4 by addition of 1 M NaOH. The extracts were sterile filtered, frozen and freeze-dried.

The protein content of all the extracts was evaluated by the Lowry-Biuret method (Sigma, Bedford, Mass., USA). Bovine serum albumin was used as standard.

Der p 1 and Der p 2 Measurements

Der p 1 and Der p 2 content was measured in native and depigmented extracts using the kit of Indoor Biotechnologies (Charlottesville, Va., USA) and following the manufacturer's instructions. Plates were coated with monoclonal antibodies 10B9 for Der p 1 and 1D8 for Der p 2. As a secondary antibody, the biotinylated monoclonal antibodies 5H8 for Der p 1 and 7A1 for Der p 2 were used. The standards used to quantify Der p 1 and Der p 2 extracts consisted of calibrated *D. pteronyssinus* extracts preserved in 1% bovine serum albumin/50% glycerol/PBS (pH 7.4).

Specific IgE and IgG ELISA Inhibition Experiments

Allergenic potency of the extracts was calculated by ELISA inhibition experiments using a serum pool of *D. pteronyssinus* allergic individuals following methods previously described [25, 26]. Each native extract was used as standard to compare the immunochemical characteristics of its corresponding depigmented extract.

Protein Profile

The protein profile of the extracts was determined by SDS-PAGE. The samples were denatured at 100°C for 10 min and centrifuged for 1 min at 16,000 *g*. Forty micrograms of freeze-dried material (native and depigmented extracts) was loaded per lane. Gels were stained with silver and Coomassie Brilliant Blue R-250 (BioRad Laboratories, Hercules, Calif., USA).

Enzymatic Analyses of the 8 Extracts

Gelatin Zymography

Samples were run in SDS-PAGE gels with 2.67% C, 15% T polyacrylamide containing 0.1% gelatin at 4°C. One hundred micrograms of lyophilized material was dissolved in a solution containing 0.05 M Tris-HCl, glycerol, SDS, Br-phenol blue and β-mercaptoethanol. After running, 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 [27].

Table 1. Main characteristics of the analysed extracts

	Extract 1		Extract 2		Extract 3		Extract 4	
	native	depigmented	native	depigmented	native	depigmented	native	depigmented
Yield[a]	4.2	68.5[b]	1.65	80.5[b]	4.7	72.6[b]	5.76	77.1[b]
Protein content[c]	686	659	221	260	843	943	838	772
Der p 1[c]	30	0.24	188.4	6.4	136	1.68	80.4	1.88
Der p 2[c]	10.7	17	62.2	88.2	45.13	11.5	58.9	123.8
50% inhibition IgE[d]	0.035	0.04	0.042	0.092	0.086	0.179	0.052	0.080
50% inhibition IgG[d]	0.082	0.05	0.048	0.026	0.05	0.028	0.013	0.039

[a] Percent recovery of freeze-dried extract from raw material. [b] Percent recovery from the native extract. [c] Micrograms per milligram of freeze-dried material. [d] Micrograms of freeze-dried material needed to reach the 50% inhibition.

Api Zym® Studies

The Api Zym (BioMérieux, France) assay was used to determine the enzymatic activity of 19 different enzymes in native and acid-treated extracts following the manufacturer's instruction. Samples were previously dissolved in 0.01 M PBS at a concentration of 1 mg/ml. Api Zym is a semi-quantitative micromethod designed for the research of enzymatic activities.

Determination of Protease Activity

Protease activity was determined using 2 non-specific substrates: the insoluble diazotized collagen substrate Azocoll (Sigma) and benzoyl-L-arginine-ethyl ester (BAEE; Sigma). The assays were conducted according to previously published reports [28] and the recommendations of the manufacturer (Radiometer Copenhagen, Bagsvaerd, Denmark). The results were expressed in international units of trypsin/milligram of freeze-dried extracts for the Azocoll assay and in units per milligram of freeze-dried extract for BAEE.

Cysteine Protease Activity. Cysteine protease activity was determined using the L-1460 peptide (10 mM Pyr-Phe-Leu-*p*-nitroanilide; Bachem AG, Bubendorf, Switzerland) as the substrate and papain (Sigma) as the standard. The results were expressed in international units of papain/milligram 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 A/S, 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 (1.5 mM H-D-Ile-Pro-Arg-*p*-nitroanilide dihydrochloride; 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). Afterwards, 50 μl of the substrate was 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/milligram of freeze-dried material.

Kallikrein Activity. Kallikrein protease activity was determined using the L-1885 peptide (1.5 mM H-D-Val-Leu-Arg-pNa acetate; Bachem AG) as the substrate and trypsin as the standard. Briefly, 100 μl of serial dilutions of the samples and the standard were diluted in 0.1 M Tris-HCl (pH 8.4). Afterwards, 50 μl of the substrate was 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/milligram of freeze-dried material.

Acid Phosphatase Activity. Acid phosphatase activity was determined using the 30 mM 4-nitrophenyl phosphate disodium salt hexahydrate as the substrate and acid phosphatase (Sigma) as the standard. Briefly, 50 μl of serial dilutions of the samples and the standard were diluted in 0.1 M citric acid/0.2 M sodium phosphate dibasic 2-hydrate (pH 5). Afterwards, 100 μl of the substrate was added and incubated for 30 min at 37°C. The reaction was stopped with 50 μl of 1 N NaOH and read at 410 nm. Results were expressed in international units of acid phosphatase/milligram of freeze-dried material.

Alkaline Phosphatase Activity. Alkaline phosphatase activity was determined using the 30.4 mM 4-nitrophenyl phosphate disodium salt hexahydrate as the substrate and alkaline phosphatase (Sigma) as the standard. Briefly, 50 μl of serial dilutions of the samples and the standard were diluted in 0.1 M Tris HCl (pH 8.4). Afterwards, 100 μl of the substrate was added and incubated for 30 min at 37°C. The reaction was stopped with 50 μl of 1 N NaOH and read at 410 nm. The results were expressed in international units of alkaline phosphatase/milligram of freeze-dried material.

Results

Allergen Extracts

The immunochemical characteristics of the native and depigmented allergen extracts are shown in table 1. The modification (depigmentation) process of the native extracts produced a mean reduction in the yield of the extracts of 25.3%.

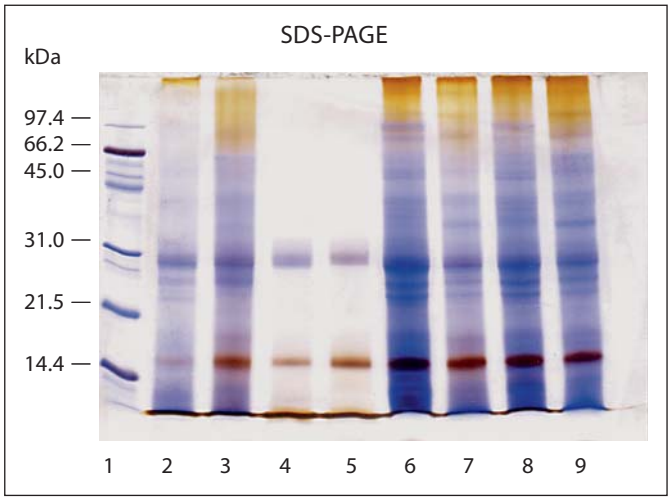

Fig. 1. SDS-PAGE of 4 *D. pteronyssinus* extracts. Lanes 2, 4, 6 and 8 = Native extracts; lanes 3, 5, 7 and 9 = depigmented extracts.

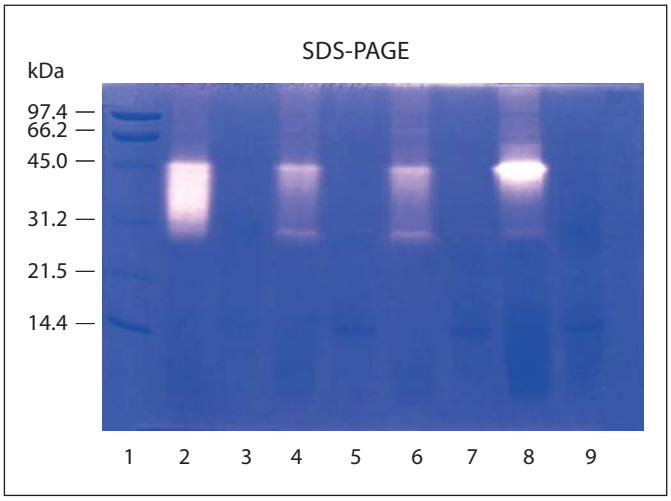

Fig. 2. Gelatin zymography of 4 *D. pteronyssinus* extracts. Lanes 2, 4, 6 and 8 = Native extracts; lanes 3, 5, 7 and 9 = depigmented extracts.

The protein content was similar in the native and acid-treated extracts. Mean values were 647 ± 293.2 μg/mg of freeze-dried material for the native extract versus 658.5 ± 299.2 μg/mg of freeze-dried material for acid-treated extracts. Non-statistically significant differences were found ($p = 0.774$).

SDS-PAGE Analysis of Native and Depigmented Extracts

Figure 1 shows the SDS-PAGE analysis of 4 batches of native and depigmented *D. pteronyssinus* extracts. Lanes 2, 4, 6 and 8 are native and lanes 3, 5, 7 and 9 depigmented extracts. The faecal extract is shown in lanes 4 and 5. Similar band profiles were observed in all cases, when native and depigmented extracts were compared, suggesting no major changes in protein composition.

Specific IgE and IgG ELISA Inhibition Experiments

In table 1, the 50% inhibition point values obtained with the 4 groups of extracts, determined for IgE and IgG, are represented. Native and depigmented extracts showed a very comparable IgE- and IgG-binding capacity. All depigmented extracts experienced a reduction in the IgE-binding capacity and needed more protein to reach the 50% inhibition point. Three depigmented extracts needed less protein concentration to reach the 50% inhibition point with IgG. Although some differences were observed, the overall allergenicity and the antigenicity of the extracts remained within acceptable limits, taking into consideration the 50–200% acceptable range for ELISA assays.

All curves had a correlation coefficient (r value) >0.95. The test for parallelism between native extracts versus respective depigmented extracts (4 groups) did not show statistically significant differences (t test; $p > 0.01$), indicating that they passed the test for parallelism using the reference (native extracts) as standards.

The percentage of inhibition between native and depigmented extracts was very similar for the 4 extracts. The highest value of the maximum inhibition was 96.1% and the lowest 86.6% for IgE inhibition studies, and 95.3 and 77.6% for IgG inhibition studies, respectively.

Enzymatic Activity

The enzymatic activity was detected when gelatin was hydrolyzed after the incubation of gels in L-cysteine. Figure 2 shows that native extracts were capable to hydrolyze gelatin, demonstrating the enzymatic activity of several allergens; however, acid-treated extracts did not show enzymatic activity.

Api Zym results are shown in table 2, expressed as negative (value 0), positive (value 1) and highly positive (value 2). The results showed a significant reduction in enzymatic activity, especially in protease and glycosidase activities. In general, protease activity suffered a considerable diminution in all depigmented extracts. No chymotrypsin activity was detected in any case. Depigmented extracts also showed a significant decrease in glycosi-

Table 2. Enzymatic activity determined by the Apy Zim® system of the different extracts

Enzyme	Extract 1		Extract 2		Extract 3		Extract 4	
	native	depigmented	native	depigmented	native	depigmented	native	depigmented
Lipases								
Esterase (C4)	2	2	2	2	2	2	2	2
Esterase lipase	2	2	2	2	2	2	2	2
Lipase (C14)	0	0	0	0	0	0	0	0
Proteases								
Leucine arylamidase	2	0	2	0	2	0	2	0
Valine arylamidase	2	0	1	0	2	0	2	0
Cystine arylamidase	0	0	0	0	1	0	1	0
Trypsin	2	0	2	0	0	0	0	0
α-Chymotrypsin	0	0	0	0	0	0	0	0
Phosphatases								
Alkaline phosphatase	2	0	2	0	2	0	2	0
Acid phosphatase	2	1	2	2	2	2	2	2
Naphthol-AS-BI-phosphohydrolase	2	1	2	2	2	2	2	2
Glycosidases								
α-Galactosidase	2	0	1	0	1	0	1	0
β-Galactosidase	2	1	2	1	2	2	2	2
β-Glucuronidase	2	0	1	0	2	0	2	0
α-Glucosidase	1	0	1	0	1	0	1	0
β-Glucosidase	2	0	2	1	2	2	2	1
N-acetyl-β-glucosaminidase	2	2	2	2	2	2	2	2
α-Mannosidase	2	0	1	0	2	1	2	1
α-Fucosidase	2	0	0	0	2	2	2	2

0 = Negative; 1 = moderate activity; 2 = high activity.

Table 3. Results of the different enzymatic assays

Enzymatic assays	Extract 1			Extract 2			Extract 3			Extract 4		
	native	depig.	% red.	native	depig.	% red.	native	depig.	% red.	native	depig.	% red.
Azocoll, IU trypsin/mg	57.03	0.28	99.50	2,808.32	522.20	81.41	242.79	21.84	91.01	890.35	0	100
Serine protease, IU trypsin/mg	6.30	0.16	97.40	38.65	2.11	94.54	0.43	0.09	78.60	90.42	0.12	71.90
Kallikrein, IU trypsin	16.27	7.31	55.08	109.50	0.38	99.65	1.15	0.53	53.84	1.33	0.70	47.67
Alkaline phosphatase, IU	2.77	0.90	67.86	1.80	0.92	48.94	3.63	1.12	69.07	2.83	1.51	46.56
Acid phosphatase, IU	0.30	0.06	81.67	0.27	0.08	69.34	0.24	0.02	90	0.11	0.04	59.43
BAEE/mg	0.03	0	96.43	0.12	0.01	89.74	0.01	0.01	50	0.01	0.01	45.45

depig. = Depigmented; % red. = % reduction.

dase activity, except in N-acetyl-β-glucosaminidase which remained unaffected. Phosphatase activity was similar in native and depigmented extracts except for alkaline phosphatase activity, which was not detected in depigmented extracts. Lipase activity was unchanged in all cases.

There was a consistent reduction in enzymatic activity of the depigmented extracts when compared with their respective native extracts (table 3). All measured enzymatic activities demonstrated a constant reduction (between 45 and 100%) after the modification process of the extract. Total protease activity measured by the Azocoll method and BAEE showed a significant decrease. When

protease activity was evaluated individually, we found a significant reduction in serine, cysteine and kallikrein activity.

Discussion

The search for methods to modify allergen extracts has been a constant argument in allergy research throughout the years [29]. The main goal is to reduce the allergenicity (IgE binding) while retaining the capacity to bind specific IgG [30]. While most alteration methods have concentrated on the modification of proteins, fewer studies have focused on the reduction in enzymatic activity and on the selective modification of allergens which are active and potent enzymes.

The procedure we have used was initially designed to remove pigments (flavonoids) from pollen grains, and therefore, was termed 'depigmentation'. However, by applying the method to mites, we confirmed an important reduction in the enzymatic activity of these extracts. This beneficial effect has warranted further evaluation of the process. In the present study, we evaluated the enzymatic activity of native and depigmented extracts of *D. pteronyssinus*. We have confirmed previous reports demonstrating the presence of proteases in native extracts of *D. pteronyssinus* [2, 14, 31, 32] and verified that a reduction in the pH of the extracts to pH 2 using 2 M HCl and dialysis is effective in reducing the enzymatic activity with slight changes in their allergenic properties. This procedure further purifies the extracts by releasing approximately 30% of dry weight of irrelevant material [33].

We have evaluated the protease activity of 4 extracts (native and depigmented in each group) by different assays in order to determine the reduction in the enzymatic activity after the depigmentation process. The Azocoll and hydrolysis of BAEE methods used non-specific substrates and can determine the serine and cysteine protease activities. Both methods, which provided similar information, show a high enzymatic protease activity in native extracts. However, this activity is drastically reduced in acid-treated extracts, demonstrating a consistent reduction in the enzymatic activity of the depigmented extracts. Using BAEE, we obtained reduction rates of 45–96% and with Azocoll, 81–100%. We persistently detected less enzymatic activities for other enzymes, including kallikrein, cysteine and serine proteases and acid and alkaline phosphatases. In all cases, activity reduction in depigmented extracts versus native extracts was significant. Reduction in the kallicrein activity rate was 48–100%, in serine protease activity 72–97.4%, with alkaline phosphatase 46–69% and with acid phosphatase 59–90%.

In general, and considering the differences in methodology (quantitative and qualitative), the results obtained with Api Zym assays can be considered as comparative with those obtained in quantitative studies. In the glycosidase group, we observed a reduction in α-galactosidase, β-glucuronidase, α-glucosidase and α-manosidase. In the group of lipases, the results were very similar in native and acid-treated extracts, and no lipase C14 activity was detected in any extract. A significant reduction was obtained in alkaline phosphatase. Finally, in the protease group, a significant activity reduction was observed in leucine arylamidase, valine arylamidase and trypsin; no α-chymotrypsin activity was found in the extracts. All these results have been confirmed by the zymogram experiments, where activity reduction in acid-treated extracts can be visualized. In native extracts, the allergens corresponding to a molecular weight range of 25–90 kDa showed an important enzymatic activity, whereas no hydrolysis in the gels is observed in acid-treated extracts. This method has been previously used to analyse the enzymatic activity in allergenic extracts of different *Blomia* spp. [34].

In spite of these changes in enzymatic activity, the gels (SDS-PAGE) and IgE and IgG inhibition experiments of the native and depigmented extracts retained a similar band pattern and inhibition point. Specific IgE inhibition assays suggest a reduction in specific IgE binding in the depigmented extracts, whereas specific IgG binding is retained or enhanced. In the present study, using monoclonal antibodies, we have demonstrated the presence of Der p 1 in native and depigmented extracts. However, these depigmented extracts experienced a steady reduction in the detection capacity of Der p 1. We believe that the decrease in the detection capacity of Der p 1 after modification is due to the possibility that the commercially available monoclonal antibodies used are no longer capable of recognizing this molecule due to changes in its tertiary structure. Another possibility is that Der p 1 experiences a continuous degradation process while in solution. We established a mean increase of 89% in the levels of Der p 2 in the depigmented extracts. Several reasons may account for this finding: (1) the depigmentation procedure enhances the detection capacity of the monoclonal antibodies by freeing more recognizable epitopes; (2) the acidic pH enhances the solubility of Der p 2, and therefore, it is present in a greater quantity in the extract, and (3) by the fact that we removed 30% of irrelevant mate-

rial, 1 mg of depigmented freeze-dried material contains more Der p 2 per milligram than previously.

In conclusion, we have developed a modification process which alters the enzymatic activity of native extracts of *D. pteronyssinus*. However, native and depigmented extracts retained similar characteristics and allergenic composition. The modification process of mite allergen extracts described herein consistently reduces the enzymatic activity of these extracts.

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