

Quality control of house dust mite extracts by broad-spectrum profiling of allergen-related enzymatic activities

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Abstract

Background: Diagnosis and immunotherapy of allergy against mites is based on complex extracts from large-scale cultures. However, the analysis of their composition using specific antibodies is limited. By taking advantage of the prevailing enzymatic nature of mite allergens, we have developed a broad-spectrum biochemical method for the standardization of native mite products.

Methods: Microplate-based assays have been implemented for thirteen *Dermatophagoides pteronyssinus* enzymatic activities, associated with Der p 1, 3, 4, 6, 8, 9, 15 and 20 allergens. The dynamics of these activities along culture growth, and their profile in purified fractions (bodies and faeces) and international reference standards (WHO/IUIS, two CBER/FDA), have been characterized. The stability of enzymatic activities and major allergens under stress conditions (40°C) has been assessed in the presence/absence of specific protease inhibitors.

Results: The analysis of enzymatic activities revealed distinct profiles along culture growth and between fractions (bodies and faeces). Remarkable differences were found when comparing international reference standards, being consistent with their source material (purified bodies or whole cultures). After 72 h at 40°C, only trypsin and alpha-amylase maintained high activity. Notably, the prominent role of trypsins in the hydrolytic degradation of major allergens is demonstrated by the use of inhibitors.

Conclusions: Our method offers a robust approach to assess the complexity of mite extracts and highlights the critical importance of source materials for the composition and stability of finished products. The implementation of this approach in industry-based quality control procedures would contribute to the standardization of allergenic extracts used for diagnosis and immunotherapy.

House dust mites (HDMs) are a primary source of indoor sensitization being associated with atopic dermatitis, perennial rhinitis and asthma. Among HDMs, *Dermatophagoides pteronyssinus* (*Dp*) is the most widespread species (1). Allergen immunotherapy (AIT) is the only potential disease-modifying treatment for allergic subjects (2). Although novel approaches such as recombinant antigen technology show promise, AIT and diagnosis still rely on complex extracts from natural sources. HDM extracts are produced from large-scale mite cultures being their standardization a main challenge for the industry (3). Source materials of biological nature are inherently variable and mite culture systems (e.g.

diet, climatic regime) may greatly impact allergen production (4–6). In addition, allergen components may to some extent be altered during manufacture processes of active pharmaceutical ingredients (APIs) (7). Finally, eventual chemical modification and formulation of finished products, as well as storage and stability, may affect allergenicity. Official regulatory bodies have introduced detailed step-by-step regulations aiming to counter this complexity (7–9). Although having substantial differences (10), a common ground of the different regulations is that manufacturers are prompted to establish robust in-process control methods to ensure batch-to-batch consistency and quality. However, in spite of

considerable efforts undertaken in recent decades, the international allergy community still pinpoints standardization as an unmet need for AIT (2). Actually, several studies have stressed the great variability observed in allergen composition and allergenicity of commercially available *Dp* extracts (11–13).

Group 1 and group 2 allergens are designated as major allergens based on their recognition by >50% of the population of mite-allergic subjects, and their content generally shows high correlation with total biological potency of the extracts (10). As a result, the main routine HDM standardization methods are (i) quantification of group 1 and 2 allergens by immunoassay with stress on their relative proportion and (ii) *in vitro* determination of total allergenic activity by competitive IgE-binding test using serum pools of allergic patients. Standardization is then accomplished by comparison with established standards, for example manufacturer's in-house reference preparations (IHRP, in EU) or CBER/FDA references (in United States), in base of which biological potency units are assigned. Remarkably, minor allergens are not specifically quantified in the quality control of AIT products, although their clinical importance might be significant (11, 14–16). Improved safety and efficacy of HDM allergy diagnosis and AIT would require additional methodologies that broaden the range of allergen components covered by standardization. In this regard, mass spectrometry shows great promise (17). Nevertheless, the high investment and technical expertise required still limit its adoption by the industry.

Nearly half of the nineteen allergenic proteins identified so far in *Dp* are enzymes (www.allergen.org), being their enzymatic activity experimentally validated in most cases. They may participate in proteolytic digestion, such as group 1 cysteine protease (18) and group 3 trypsin (19), group 6 chymotrypsin (20) and group 9 elastase-like/collagenolytic serine protease (21, 22); carbohydrate metabolism, such as group 4 alpha-amylase (23) and group 15 chitinase; and aminoacid metabolism (group 20 arginine kinase) and detoxification (group 8 glutathione S-transferase, GST), being the function of the later three allergens only deduced from sequence homology (24). Some of these enzymes are accumulated in faecal pellets where they remain functionally active when inhaled by the allergic subject (1). In this regard, an emerging concept exists on the important role that proteolytic allergens might play on the high allergenicity of HDM, although the clinical relevance of this contribution is still largely unknown (1). Multiple IgE-independent mechanisms have been suggested for these allergens, from direct airway damage to late-phase innate immune responses (25). Moreover, evidence suggests that other proteases beyond already known allergens might also contribute to such responses (13). Thus, consideration of other nonallergenic enzymes in HDM extracts could be clinically relevant.

In this study, we intend to take advantage of the prevailing enzymatic nature of *Dp* allergens to develop a biochemical method to specifically monitor enzymatic activities being related to allergens (i.e. same enzymatic or functional group) that could be of use in the evaluation of source materials,

intermediate manufacturing stages, APIs or any preparation containing native allergens to be used in diagnosis or AIT. The dynamics of these activities during mite culture growth and their profile in purified culture fractions (bodies/faeces) and in *Dp* international reference standards have been characterized. The compatibility of the method with common excipients used in diagnosis and AIT products (phenol, glycerol) has been demonstrated. Finally, the stability of the enzymatic activities under stress conditions and the impact of proteolytic enzymes on Der p 1 and Der p 2 degradation have been studied.

Methods

Mite extracts

The origin of the laboratory strain of *D. pteronyssinus*, culture conditions, and the method to count mites in cultures have been previously described (6). The reference standards E10-Dp and E11-Dp from the United States Food and Drug Administration (FDA) were provided by the Center for Biologics Evaluation and Research (CBER, Silver Spring, Maryland, US). The *D. pteronyssinus* international standard 82/518 from the World Health Organization/International Union of Immunological Societies (WHO/IUIS) was provided by the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, UK). Detailed methods for the preparation of mite extracts, including reference standards, are described in Data S1.1.

Total protein, Der p 1 and Der p 2 contents

Protein concentration was estimated by the Lowry method using a BSA standard curve and the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA), except for *Dp* reference standards due to the presence in CBER/FDA E11-Dp of phenol that interferes with this method. The Bio-Rad Protein Assay kit (Bio-Rad) was used in this case, with bovine gamma globulin as standard. The compatibility of this kit, based on the Bradford method, with phenol (0.5% w/v) and glycerol (50% v/v) added to mite extracts was confirmed (data not shown).

Quantification of Der p 1 and Der p 2 allergens was performed using the Der p 1 ELISA kit (EL-DP1A) and Mite Group 2 ELISA kit (EL-D2) (Indoor Biotechnologies, Charlottesville, VA, USA). Addition of phenol up to 0.05% (w/v) or glycerol up to 5% (v/v) to aqueous *Dp* extracts directly applied to ELISA microplates (far above the concentrations actually required for this study) showed no substantial impact on the estimation of Der p 1 content (data not shown).

Enzymatic activity measurements of *Dermatophagoides pteronyssinus* extracts

Detailed analytical methods are described in Data S1.2 and summarized in Table S1. The activity profile of the proteolytic enzymes was further investigated in order to determine the reaction conditions to maximize assay specificity. The

analysis comprised the determination of optimal pH for substrate hydrolysis (Fig. S1) and study of inhibition profiles (Table S2). The basal enzymatic activity of the mite-free fresh rearing diet (1:1 mixture of fish food flakes and brewer's yeast flakes) was analysed confirming that any of the enzymatic assays showed measureable activity, with the exception of alpha-amylase that showed negligible activity (12-fold lower activity than the lowest activity extract of our study). The interference of phenol and glycerol and the effect of dilution were assessed as described in Data S1.3.

Stability of *Dermatophagoides pteronyssinus* extracts

A sample of a five-week-old whole culture was homogenized in ice-cold 0.15 M NaCl, diluted to 20 mg/ml of soluble protein and stocked at -80°C after dividing into aliquots, as described in Data S1.1. In a first experiment, three experimental replicates were obtained by the dilution of mite extracts to 4 mg/ml of protein extract in PBS into sealed screw-cap microtubes, and incubated for either 72, 48, 24, 4 or 1 h at 40°C with agitation. For each time point, independent samples were prepared from stock and shifted to make the collection time converge. Samples were then centrifuged at 10 000 g for 5 min at 4°C to recover supernatants, aliquoted and stored at -80°C . Fresh aliquots were thawed for each independent assay. Enzymatic activities were estimated as described in Table S1 and Data S1.2 and expressed in terms of specific activity on the basis of the initial extract protein content (4 mg/ml). The effect of protease inhibitors (all from Sigma Aldrich, St Louis, MO, USA) on stability was also explored. Three treatments (two experimental replicates) and control were performed: (i) 2 μM STI (soybean trypsin inhibitor), (ii) 10 μM E-64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane) and 1 mM PMSF (phenylmethylsulfonyl fluoride) and (iii) 10 μM E-64 and 4 mM TLCK (N-alpha-p-tosyl-L-lysine chloromethyl ketone). Incubation and sample handling were performed as described above. Time points at 72, 24, 4 and 0 h were analysed.

Data analysis

Statistical analysis to compare the enzymatic activity in purified culture fractions was performed using unpaired *t*-test. The growth curve experiments were analysed by repeated-measures one-way ANOVA coupled with Tukey's post-test. Correlation between Der p 1 content and cysteine proteases specific activity was assessed by the Pearson's correlation coefficient (*r*). Data from the first stability experiment were adjusted to a one-phase exponential decay equation model to calculate activity/allergen half-life time (HL); the goodness of fit was defined by the R^2 parameter. Enzymatic data from the second stability experiment were adjusted to a one-phase exponential decay equation model and compared by extra sum-of-squares F test, and ELISA data from this experiment were analysed by one-way ANOVA coupled with Tukey's post-test. Statistical analyses were computed using GraphPad PRISM v6.00 (GraphPad Software, La Jolla, USA).

Results

Development of an allergen-related enzymatic method

Biochemical assays were optimized for the microplate-based quantification of thirteen *Dp*-derived enzymatic activities. The specificity of each proteolytic assay was maximized by the identification of the optimal pH for each substrate (Fig. S1) as well as its inhibition profile in the presence of protease inhibitors (Table S2). Such an approach revealed that three of the substrates commonly used to target cysteine endoproteases and one substrate targeting cathepsin-D were indeed being additionally hydrolysed by other enzyme groups in *Dp* extracts. Thus, default addition of specific inhibitors (STI and E-64, respectively) was required to selectively target the enzymes of interest. Extended details can be found in Supporting Information (Data S2). All enzymatic activities could be measured in diluted (0.25 mg/ml) extracts, differing their specific activity estimates in $<25\%$ with respect to those of an undiluted control extract (4 mg protein/ml), except for legumain and endochitinase. The presence of 50% glycerol or 0.5% phenol in mite extracts did not interfere ($<25\%$ variation) with the estimation of enzymatic activities, with the exceptions of trypsin (1.4-fold increase with glycerol) and exochitinase (1.3-fold increase with phenol) (Table S3).

Enzymatic profile in purified mite bodies (PMB) and faecal pellets (PFP)

PFP showed significantly higher ($P < 0.05$) activity than PMB for the following enzymes, in descending order of relative activity (Table 1): chymotrypsin > trypsin > Der p 1-like protease > cathepsin-L > cathepsin-B. Conversely, PMB showed significantly higher ($P < 0.05$) activity than PFP for the following enzymes: arginine kinase > GST > cathepsin-D > endochitinase > exochitinase > elastase > alpha-amylase. Legumain activity did not significantly vary between fractions.

The Der p 1 content estimated by ELISA was higher in PFP than in PMB (3.12 ± 1.16 $\mu\text{g}/\text{mg}$ of soluble protein and 0.87 ± 0.12 $\mu\text{g}/\text{mg}$, respectively; mean $\pm$ SE, $n = 3$), whereas the Der p 2 content showed the opposite pattern (3.90 ± 0.94 $\mu\text{g}/\text{mg}$ and 0.91 ± 0.07 $\mu\text{g}/\text{mg}$, in PMB and PFP, respectively). A highly significant positive correlation was found between Der p 1 content (by ELISA) and Der p 1-like protease specific activity of purified mite fractions ($r = 0.9569$, $P = 0.0027$, $n = 6$).

Enzymatic profile along culture growth

Comparison of the specific activity of whole mite culture (WMC) extracts (Fig. 1, panel O) from four time points during the evolution of a culture (days 28, 35, 42, 62) revealed four distinct activity patterns: (a) enzymatic activity significantly increased with time, for Der p 1-like protease (Fig. 1A), trypsin (Fig. 1E) and chymotrypsin (Fig. 1F); (b) maximum enzymatic activity after 35–42 days of culture and no significant change or slight variation at culture saturation (day 62, Fig. 1N), for cathepsin-B (Fig. 1B), cathepsin-L

Table 1 Enzymatic profile of purified mite bodies (PMB) and faecal pellets (PFP) fractions

Enzymatic activity (substrate)		Related Allergen*	PMB (SA [†])	PFP (SA [†])	Ratio [‡]	P-value [§]
Cysteine endoproteases EC3.4.22	Der p 1-like protease (Boc-QAR-AMC)	Der p 1	0.364 ± 0.026	0.770 ± 0.116	-2.1	0.0273
	Cathepsin-B (Z-RR-AMC)	Der p 1	0.305 ± 0.016	0.471 ± 0.027	-1.5	0.0064
	Cathepsin-L (Z-FR-AMC)	Der p 1	4.21 ± 0.06	6.62 ± 0.23	-1.6	0.0005
	Legumain (Z-VAN-AMC)	Der p 1	0.732 ± 0.017	0.763 ± 0.016	1.0	0.2686
Serine endoproteases EC3.4.21	Trypsin (Boc-QGR-AMC)	Der p 3	5.69 ± 0.45	59.86 ± 10.19	-10.5	0.0060
	Chymotrypsin (Suc-AAPF-AMC)	Der p 6	0.039 ± 0.003	0.642 ± 0.032	-16.7	<0.0001
	Elastase (Suc-AAA-AMC)	Der p 9	0.036 ± 0.001	0.011 ± 0.001	3.4	<0.0001
Aspartic endoproteases EC3.4.23	Cathepsin-D (MocAc-GKPILFFRLK (Dnp)-D-R-NH2)	-	3.551 ± 0.068	0.169 ± 0.006	21.0	<0.0001
α-Amylases EC3.2.1.1	Alpha-amylase (starch)	Der p 4	866.5 ± 3.7	404.9 ± 18.8	2.1	<0.0001
Chitinases EC3.2.1.14	Exochitinase (4MU-NAG)	Der p 15/18	1.719 ± 0.028	0.388 ± 0.034	4.4	<0.0001
	Endochitinase (4MU-(NAG) ₃)	Der p 15/18	1.655 ± 0.012	0.213 ± 0.006	7.8	<0.0001
Glutathione S-transferases EC2.5.1.18	Glutathione S-transferase (CDNB & GSH)	Der p 8	88.3 ± 1.8	1.3 ± 0.3	68.6	<0.0001
Arginin kinases EC2.7.3.3	Arginine kinase (ATP & L-Arginine)	Der p 20	552.9 ± 7.2	3.9 ± 0.5	142.1	<0.0001

*Related *D. pteronyssinus* allergen (www.allergen.org).

†Specific activities (SA) are expressed as nmol of hydrolysed substrate / min × mg protein, except for alpha-amylase (μg of hydrolysed starch / min × mg protein), glutathione S-transferase (nmol GSH-CDNB conjugate / min × mg protein) and arginine kinase (nmol L-arginine and ATP converted to N⁰-phospho-L-arginine and ADP / min × mg protein). Figures are mean ± standard error of three biological replicates.

‡Ratio SA of bodies and faeces, negative values indicate higher activity in PFP.

§Unpaired t-test of PMB' and PFP' SA.

(Fig. 1C), legumain (Fig. 1D) and alpha-amylase (Fig. 1I); (c) maximum enzymatic activity after 35–42 days and a substantial decrease at culture saturation, for elastase (Fig. 1G), exochitinase (Fig. 1J), endochitinase (Fig. 1K), GST (Fig. 1L) and arginine kinase (Fig. 1M); and (d) maximum activity at the first time point (day 28) and significant decay from day 35, for cathepsin-D (Fig. 1H). The Der p 1 content estimated by ELISA significantly increased with time (Fig. 1P). Highly significant positive correlation was found between Der p 1 content (by ELISA) and Der p 1-like protease specific activity ($r = 0.9437$, $P < 0.0001$, $n = 12$). By contrast, correlation with the activity of other cysteine endoproteases, cathepsin-B and -L, was not significant ($r = 0.2386$, $P = 0.4551$; $r = 0.5237$, $P = 0.0805$, respectively).

Analysis of international reference standards

All of the enzymatic activities under study, except for elastase, could be detected in at least one of the three analysed references, and remarkable differences were found comparing WHO/IUIS and CBER/FDA standards (Table 2). Volumetric-based specific activity of Der p 1-like and legumain proteases were similar in the three extracts. This result contrasted with the lower estimation of Der p 1 by ELISA in the WHO/IUIS standard. Other cysteine endoproteases, cathepsin-B and -L, were lower in the WHO/IUIS standard.

Stability of aqueous mite extracts under accelerated storage conditions

Estimation of enzymatic activities in aqueous *Dp* extracts incubated at 40°C for up to 72 h revealed significantly different levels of stability. The stability of the enzymes was quantified according to the activity half-life time (time to reduce the enzymatic activity by half), ranging over 72 h (time frame of the study), for trypsin and alpha-amylase, to 0.8 h, for endochitinase, the less stable activity (Fig. 2, panels A to M), in decreasing order: trypsin > alpha-amylase > chymotrypsin > legumain > cathepsin-L > Der p 1-like protease > arginine kinase > cathepsin-B > cathepsin-D > exochitinase > elastase > GST > endochitinase. Likewise, major allergens half-life times, as detected by ELISA, were as follows: 61.7 h for Der p 2 and 46.2 h for Der p 1 (Fig. 2, panels P and O, respectively).

The inhibition of serine proteases by STI significantly ($P < 0.05$) reduced the loss of Der p 1-like protease activity (Fig. 3A), but not cathepsin-L activity (Fig. 3B), suggesting that serine proteases were participating in the loss of Der p 1-like activity. On the contrary, the inhibition of cysteine proteases and chymotrypsins by E64 and PMSF (Fig. 3C), or the inhibition of cysteine proteases and trypsins by E64 and TLCK (Fig. 3D), did not have a significant impact on the loss of trypsin or chymotrypsin activities, respectively. In the light of these results, the degradation of Der p 1

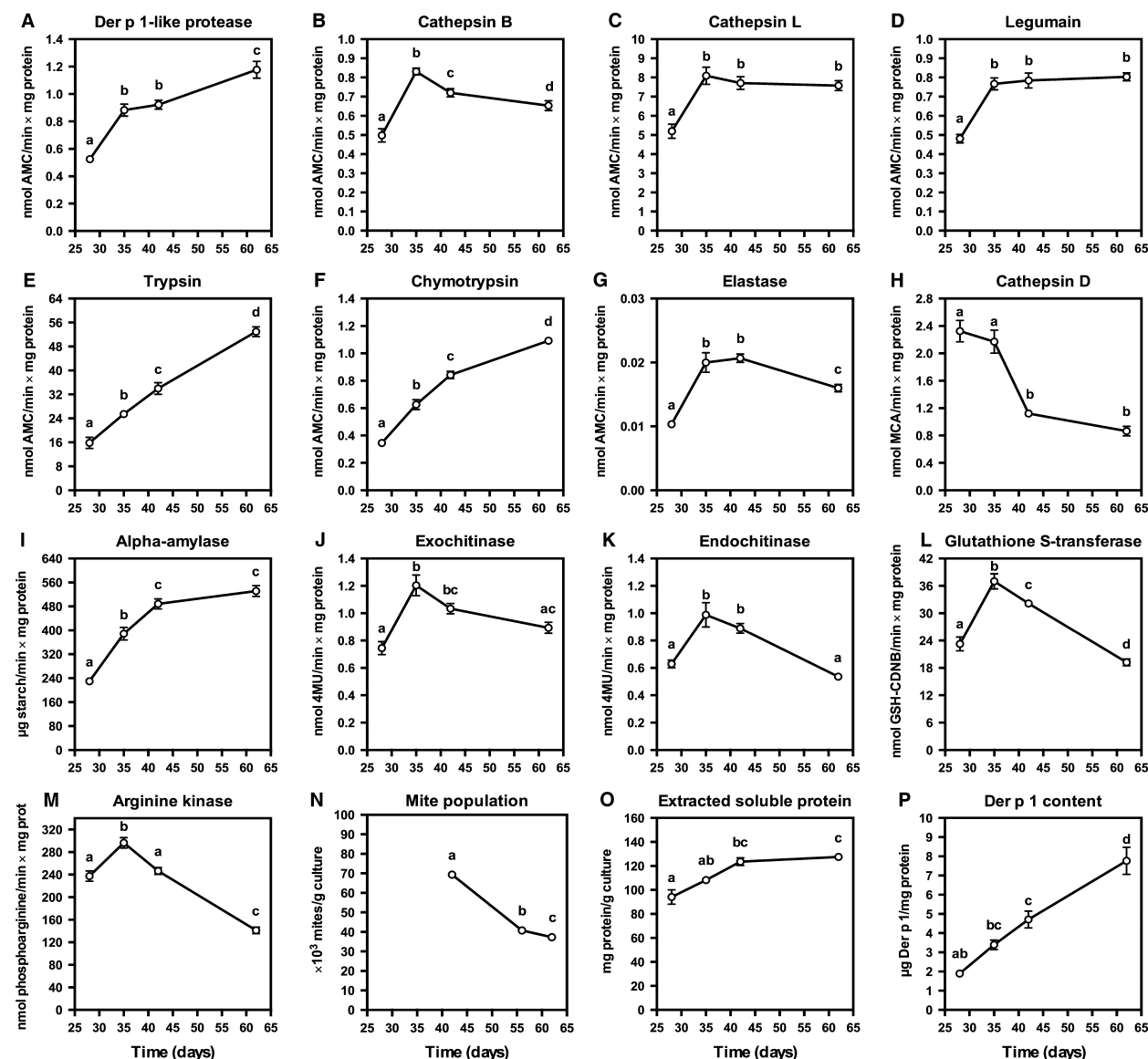


Figure 1 Enzymatic and allergen profile during different phases of culture growth. Whole culture samples were extracted at days 28, 35, 42 and 62 and analysed for specific enzymatic activity (panels A to M), density of living hatched mites (N), yield of protein

extraction (O) and Der p 1 allergen content (P). Data are means and SE of three biological replicates. Different letters in each panel indicate significant differences ($P < 0.05$).

(Fig. 3E) and Der p 2 (Fig. 3F) was also followed in the presence/absence of inhibitors. None of the inhibitor treatments completely prevented the degradation of major allergens, but all showed some degree of protection in relation to control, with significant differences between treatments at 72 h ($P < 0.05$). The highest degradation was observed when trypsin activity remained and cysteine proteases or chymotrypsin activities were inhibited by E-64 and PMSF. These results demonstrated that Der p 1 and Der p 2 are more efficiently hydrolysed by trypsin than by cysteine proteases or chymotrypsins.

Discussion

We report on a method for the quality control of HDM allergenic extracts through the study of allergen-related enzymatic activities. These comprise all known *Dp* allergens with enzymatic properties (www.allergen.org): four proteases (Der p 1, 3, 6 and 9), two glycosylases (Der p 4 and 15) and two transferases (Der p 8 and 20), considering that Der p 18 is a non-functional chitinase (24). In addition, the analysis includes endoprotease activities (cathepsin-B, cathepsin-L and legumain) that, in base to our knowledge on other mite species

Table 2 Characterization of international reference standards

Enzymatic activity (SA*)	CBER/FDA E10-Dp	CBER/FDA E11-Dp	WHO/IUIS 82/518
Der p 1-like protease	1.041 ± 0.008	1.149 ± 0.019	1.287 ± 0.019
Cathepsin-B	0.210 ± 0.007	0.265 ± 0.004	0.108 ± 0.004
Cathepsin-L	3.62 ± 0.07	4.85 ± 0.04	1.23 ± 0.07
Legumain	0.504 ± 0.007	0.639 ± 0.004	0.463 ± 0.007
Trypsin	3.17 ± 0.09	0.73 ± 0.00	152.46 ± 7.17
Chymotrypsin	0.000 ± 0.000	0.000 ± 0.000	1.241 ± 0.010
Elastase	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Cathepsin-D	1.015 ± 0.033	1.337 ± 0.054	0.112 ± 0.009
Alpha-amylase	302.8 ± 3.4	745.8 ± 33.5	133.8 ± 13.1
Exochitinase	0.460 ± 0.004	1.991 ± 0.018	0.020 ± 0.001
Endochitinase	1.512 ± 0.026	1.581 ± 0.009	0.004 ± 0.000
Glutathione S-transferase	17.7 ± 0.5	34.3 ± 0.2	0.0 ± 0.0
Arginine kinase	12.6 ± 0.8	6.8 ± 0.2	3.7 ± 0.4
Protein (µg/ml)†	486 ± 10	1017 ± 24	267 ± 3
Der p 1 (µg/ml)‡	19.09 ± 1.51	30.05 ± 1.32	12.85 ± 1.35
Der p 2 (µg/ml)‡	11.02 ± 1.99	13.87 ± 0.52	0.32 ± 0.08

*Specific activities (SA) are expressed as nmol of hydrolysed substrate / min × ml extract, except for alpha-amylase (µg of hydrolysed starch / min × ml extract), glutathione S-transferase (nmol GSH-CDNB conjugate / min × ml extract) and arginine kinase (nmol L-arginine and ATP converted to N^ω-phospho-L-arginine and ADP / min × ml extract). Figures are mean ± standard error of three experimental replicates.

†Protein concentrations were determined by the Bradford method using bovine gamma globulin as standard. Note that, for all the assays, the reference solution of WHO/IUIS 82/518 was obtained by dissolving the content of one ampoule in 0.5 ml of a 0.15 M NaCl, as advised by NIBSC.

‡Major allergen contents were determined by sandwich ELISA.

(26), were suspected to be involved in the digestive physiology of *Dp*. Indeed, these activities could be detected in the highly allergenic faecal pellets. Cathepsin-D activity, although comparatively lower in faecal pellets, was of interest because it has been described as an allergen in other species (24). The consideration of a variety of endoprotease activities in our analysis, not necessarily corresponding to known allergens, is relevant because they can additionally serve as markers of the stability and integrity of mite extracts (as discussed later). In addition, it is not discarded that non-IgE-reactive endoproteases could contribute to the overall allergenicity (1, 13). Interestingly, the described profile of enzymatic activities could be used as a fingerprint of the complexity of AIT products.

Our method offers a step further in terms of accuracy and reliability in contrast to previously applied approaches in HDM research, such as the microbiology-oriented semiquantitative API ZYM test or generic colorimetric assays lacking the adequate prospection of substrate specificity (27–29). As a matter of fact, the optimization of the Der p 1-like cysteine endoprotease activity assay using a recommended substrate (Boc-QAR-AMC) (18, 30) revealed the need to use a serine protease inhibitor (STI) to avoid the unspecific hydrolysis of this substrate. The accumulation of Der p 1, as measured by ELISA, showed a significant correlation with the increase in Der p 1-like protease activity during culture growth. Notably, this correlation was not significant regarding other cysteine endoproteases (cathepsins B and -L) analysed using different substrates, confirming the rather narrow substrate specificity profile expected for Der p 1 (30) and suggesting that the corresponding assay preferentially targets Der p 1 over other cysteine proteases present in the extracts. Tentatively, the

estimation of Der p 1 content of intermediate products during the manufacturing process or between production batches could be performed by measuring specific protease activity. To this end, correlation between ELISA and Der p 1 activity should be specifically established for each production system, making both methods complementary. It is worth noting the unexpected high Der p 1-like activity found in the WHO/IUS standard in relation to its Der p 1 content by ELISA, in comparison with the CBER/FDA standards. This is especially relevant, because the WHO/IUS preparation is currently used as standard to estimate the Der p 1 content. The reason for this discrepancy is unknown, but highlights the utility of combining the two approaches, taking into account that both techniques might introduce bias. On the one hand, estimation of activities in complex extracts is challenging, in spite of reducing risk of nonspecific hydrolysis by using inhibitors. On the other hand, polymorphism in allergen epitopes may alter immunodetection (31), and the use of antibodies might not discriminate intact from partially degraded allergens. This is supported by our results in the stability assays, where the half-life of the Der p 1-like activity was over four-fold shorter than that of the Der p 1 content. The unavailability of commercial specific antibodies or purified proteins for other allergens explored in this study impeded establishing correlations of their actual contents with the analysed activities. Besides, it cannot be discarded that nonallergenic proteins present in the extracts may contribute to the estimated activities. Some enzymatic allergens belong to multi-gene families (e.g. trypsins, GST) (24), and the enzymatic activities may be influenced by the microbiota associated with mite cultures, as reported for exochitinases in other allergenic

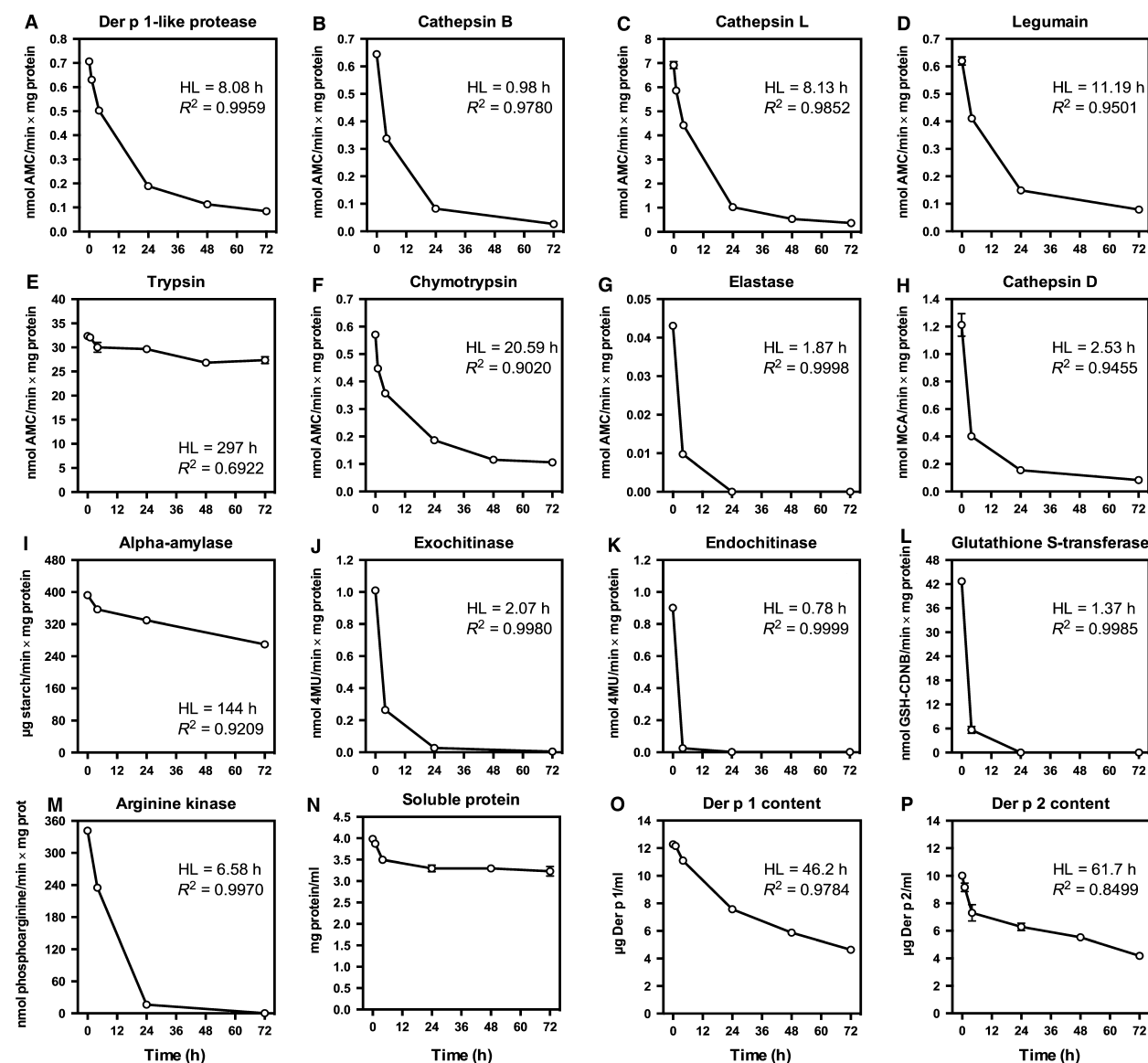


Figure 2 Stability of aqueous mite extracts subjected to accelerated storage conditions. Whole culture extracts were analysed during incubation at 40°C for specific enzymatic activity (panels A to M), protein

content (N), Der p 1 (O) and Der p 2 (P) allergen content. Data are means and SE of three experimental replicates. 'HL' denotes half-life time (h); goodness of fit is indicated according to the R^2 parameter.

mites (32). In any case, the estimation of the whole set of proposed enzymatic activities remains inherently powerful to assess the complexity and stability of mite extracts and thus to assist in their production and standardization.

The adaptability to the different stages of production of allergenic extracts, from source materials to finished products, is a key feature of the proposed method. Remarkably, knowing the dynamics of enzymatic activities along the culture growth phases and in different source materials, whole mite cultures (WMC), purified mite bodies (PMB) or faecal pellets (PFP), may help obtaining a better controlled final product. Firstly, distinct patterns have been identified for the dynamics of enzymatic activity during the culture, stressing the importance of the collection time and highlighting the

use of this method to fingerprint its status: activities associated with Der p 1, 3 and 6 digestive enzymes are dominant in PFP over PMB and accumulate over time, while other activities remain steady (e.g. alpha-amylase, Der p 4) or significantly decrease at the saturation phase (e.g. GST, Der p 8) or earlier (e.g. cathepsin-D). Secondly, the use of WMC, PMB, PFP or their mixes as source materials may drastically change the final composition of the product, because each fraction remarkably differs in their allergen-related enzymatic profile, that is in their complexity. It is worth noting the variety of HDM products available in this regard; while US commercial extracts, as mandatory, are derived from fractions of PMB, EU-manufactured extracts are typically obtained from WMC (10), or by mixing different proportions of PMB and

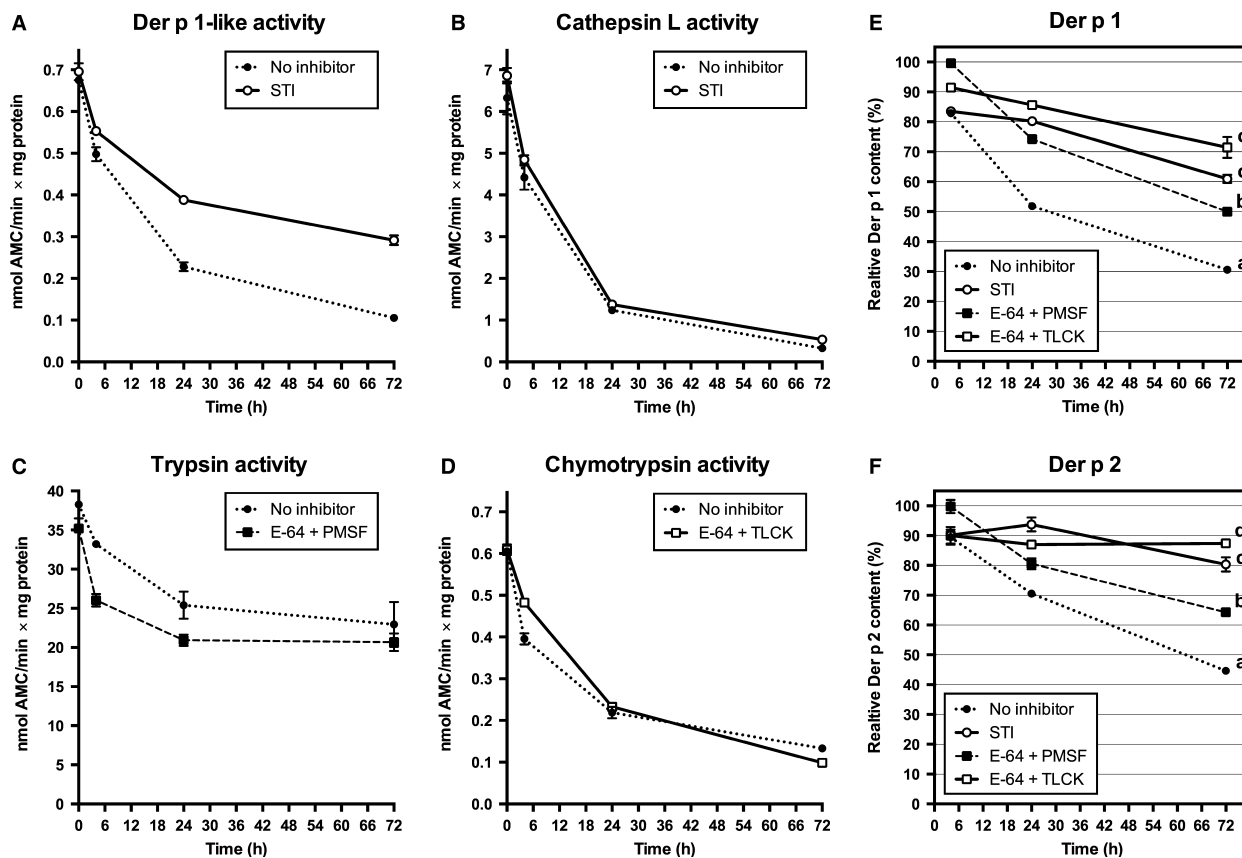


Figure 3 Stability of aqueous mite extracts supplemented with protease inhibitors and subjected to accelerated storage conditions. Whole culture extracts with or without combinations of protease inhibitors (2 μ M STI, 10 μ M E-64, 1 mM PMSF and/or 4 mM TLCK) were subjected to 40°C for 0, 4, 24 and 72 h and analysed for

specific enzymatic activities (panels A to D), Der p 1 (E) and Der p 2 (F) contents by ELISA. Data are means and SE of two experimental replicates; different letters in panels E and F indicate significant differences at 72 h ($P < 0.05$).

PFP fractions (both exhibiting distinct group 1 to group 2 ratios, as confirmed by our results) to ultimately attain a standard major allergen ratio (i.e. that of the IHRP) (33). Our method proved to be compatible with glycerol and phenol, the most common excipients in the formulation of skin prick test and sublingual immunotherapy (SLIT) products (34). In addition, the sensitivity of our method allows the analysis of highly diluted extracts (0.75 μ g Der p 1/ml and 0.63 μ g Der p 2/ml), equivalent to the lowest concentrated products cited in the literature (11, 12, 35–38). Further investigation is needed to confirm compatibility with other AIT products (e.g. SLIT tables, adsorbed SCIT). Indeed, a method to quantify Der p 1 in adsorbed SCIT products by estimating cysteine protease activity has already been patented (International publication number, WO 2007/059776). Notably, our method could also be implemented for the quality control of processes such as chemical modification of native allergens in the manufacture of allergoid vaccines, or production of recombinant allergens of use in standardization, diagnosis and/or immunotherapy. As learned from the CREATE project, physicochemical and biological resemblance of recombinant allergens to their natural counterparts

is crucial although technically challenging (39). Validation of the biochemical activity of recombinant enzymatic allergens could therefore be instrumental.

Upon registration of a new product, regulatory bodies impose precise guidelines on stability testing of both APIs and finished products (7, 40). We conducted a stability experiment that exposed aqueous extracts to an accelerated storage condition (40), revealing very low stability of most enzymes, except for trypsin and alpha-amylase, and a substantial degradation of Der p 1 and Der p 2 allergens (70% and 55% reduction in three days, respectively). The degradation levels reported here were considerably higher than a similar study using aqueous extracts at 25°C under long-term storage (41). On the other hand, contrarily to a previous report using an inhibitor cocktail on HDM-glycerinated extracts (42), co-incubation of aqueous allergen extracts with different combinations of group-specific protease inhibitors revealed the crucial role of proteases, particularly trypsin, on the degradation of Der p 1 (as reported by both ELISA and Der p 1-like protease activity) and Der p 2. It is worth noting that in the redox conditions of the extracts, in the absence of exogenous reducing agents, cysteine proteases are only partially

activated and this could contribute to their lower impact on allergen degradation (e.g. Der p 1-like activity was 14% compared to extracts supplemented with DTT in our conditions). The observed prominence of trypsins over other proteases in the degradation of major allergens, coupled with their high stability, points out the interest of this activity as control parameter in stability assessment of APIs or commercial HDM extracts. Notably, source materials would not only be critical for extract composition but might also determine stability: the higher the proportion of trypsin-enriched faecal material (over 10-fold higher trypsin activity than PMB), the higher the risk of instability.

Our method was applied to study three *Dp* reference standards, each one differing in terms of formulation (see methods) and origin [CBER/FDA extracts, obtained from PMB; WHO/IUIS 82/518, obtained from WMC (43)]. Interestingly, the allergen-related enzymatic profile of both CBER/FDA preparations was fairly similar but remarkably different to the WHO/IUIS International Standard, and this was most likely related to the source materials used in each case. As a matter of fact, it is this general divergence among types of extracts which partly explains the historical difficulty to find a widely adopted international reference standard extract (31). Hence, the differences reported here were consistent with the profiles determined for PMB, PFP and WMC: CBER/FDA PMB-derived standards showed remarkably lower serine protease activity (trypsin, chymotrypsin), but higher cathepsin-D, alpha-amylase, chitinase and GST activities. The negligible arginine kinase, chitinase and GST activities in the WHO/IUIS standard could be related to its high trypsin activity that, together with the low stability reported here for these enzymes, could have promoted the degradation.

Here, we present a wide-range biochemical method to monitor the complete allergen-related enzymatic profile of HDM allergen extracts that, as supported by a variety of experimental approaches, could be of interest for the quality control and optimization of multiple manufacturing steps, from source materials to the stability of finished products. The method has been optimized in base of the known allergenic enzymes of *D. pteronyssinus* but it could eventually be extended, and adapted when needed, to other house dust and storage mites, with particular interest in those species for which no standardization methodology exists. Thus, we suggest that the development and inclusion of this approach in

current industry-based procedures would help improving allergen extract standardization and quality and thus the efficacy and safety of HDM allergy diagnosis and AIT.

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Authors' contributions

JCVQ performed the experiments. JCVQ, FO, PC and PHC participated in the design of the experiments, their analysis and the writing of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

Pedro Hernández-Crespo has led research contracts between CSIC and ALK-Abelló. The rest of authors declare that they have no relevant conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Extended materials and methods (including sections S1.1, S1.2 and S1.3).

Data S2. Development of an allergen-related enzymatic method. Extended results.

Figure S1. Hydrolysis of fluorogenic protease substrates by mite extracts: pH activity curves.

Table S1. Summary of analytical methods for the allergen-related enzymatic activity profiling of *D. pteronyssinus* extracts.

Table S2. Inhibition profiles of the hydrolysis of fluorogenic substrates by *D. pteronyssinus* proteolytic enzymes.

Table S3. Compatibility of glycerol, phenol and dilution with the enzymatic analysis of mite extracts.

References

- Calderón MA, Linneberg A, Kleine-Tebbe J, De Blay F, Fernández H, de Rojas D et al. , et al. Respiratory allergy caused by house dust mites: what do we really know? *J Allergy Clin Immunol* 2015;**136**:38–48.
- Jutel M, Agache I, Bonini S, Burks AW, Calderón MA, Canonica W et al. International consensus on allergy immunotherapy. *J Allergy Clin Immunol* 2015;**136**:556–568.
- Frati F, Incorvaia C, David M, Scurati S, Seta S, Padua G et al. Requirements for acquiring a high-quality house dust mite extract for allergen immunotherapy. *Drug Des Devel Ther* 2012;**6**:117–123.
- Avula-Poola S, Morgan MS, Arlian LG. Diet influences growth rates and allergen and endotoxin contents of cultured *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* house dust mites. *Int Arch Allergy Immunol* 2012;**159**:226–234.
- Yella L, Morgan MS, Arlian LG. Population growth and allergen accumulation of *Dermatophagoides pteronyssinus* cultured at 20 and 25 degrees C. *Exp Appl Acarol* 2011;**53**:103–119.
- Vidal-Quist JC, Ortego F, Lombardero M, Castañera P, Hernández-Crespo P. Allergen expression in the European house dust mite *Dermatophagoides pteronyssinus* throughout development and response to environmental conditions. *Med Vet Entomol* 2015;**29**:137–146.

7. European Medicines Agency. Guideline on Allergen Products: Production and Quality Issues. Committee for Medicinal Products for Human Use (CHMP) and Biologics Working Party (BWP). 2008. EMEA/CHMP/BWP/304831/2007.
8. Monograph on Allergen Products. European Pharmacopoeia vol. 8. 2014. 07/2014:1063.
9. Code of Federal Regulations Title 21, Part 680: Additional Standards for Miscellaneous Products. Food and Drug Administration of Health and Human Services. 2015. 21CFR680.
10. Cox L, Jacobsen L. Comparison of allergen immunotherapy practice patterns in the United States and Europe. *Ann Allergy Asthma Immunol* 2009;**103**:451–459.
11. Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R et al. Varying allergen composition and content affects the in vivo allergenic activity of commercial *Dermatophagoides pteronyssinus* extracts. *Int Arch Allergy Immunol* 2012;**159**:253–262.
12. Brunetto B, Tinghino R, Braschi MC, Antonicelli L, Pini C, Iacovacci P. Characterization and comparison of commercially available mite extracts for in vivo diagnosis. *Allergy* 2010;**65**:184–190.
13. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ et al. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* 2012;**67**:488–495.
14. Weghofer M, Thomas WR, Kronqvist M, Mari A, Purohit A, Pauli G et al. Variability of IgE reactivity profiles among European mite allergic patients. *Eur J Clin Invest* 2008;**38**:959–965.
15. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. *Trends Mol Med* 2010;**16**:321–328.
16. Batard T, Baron-Bodo V, Martelet A, Le Mignon M, Lemoine P, Jain K et al. Patterns of IgE sensitization in house dust mite-allergic patients: implications for allergen immunotherapy. *Allergy* 2016;**71**:220–229.
17. Chapman MD, Briza P. Molecular approaches to allergen standardization. *Curr Allergy Asthma Rep* 2012;**12**:478–484.
18. Takai T, Kato T, Sakata Y, Yasueda H, Izuhara K, Okumura K et al. Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity. *Biochem Biophys Res Commun* 2005;**328**:944–952.
19. Stewart GA, Ward LD, Simpson RJ, Thompson PJ. The group III allergen from the house dust mite *Dermatophagoides pteronyssinus* is a trypsin-like enzyme. *Immunology* 1992;**75**:29–35.
20. Yasueda H, Mita H, Akiyama K, Shida T, Ando T, Sugiyama S et al. Allergens from *Dermatophagoides* mites with chymotryptic activity. *Clin Exp Allergy* 1993;**23**:384–390.
21. Stewart GA, Kollinger MR, King CM, Thompson PJ. A comparative study of three serine proteases from *Dermatophagoides pteronyssinus* and *D. farinae*. *Allergy* 1994;**49**:553–560.
22. King C, Simpson RJ, Moritz RL, Reed GE, Thompson PJ, Stewart GA. The isolation and characterization of a novel collagenolytic serine protease allergen (Der p 9) from the dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1996;**98**:739–747.
23. Lake FR, Ward LD, Simpson RJ, Thompson PJ, Stewart GA. House dust mite-derived amylase: allergenicity and physicochemical characterization. *J Allergy Clin Immunol* 1991;**87**:1035–1042.
24. Colloff MJ. *Dust Mites*. Dordrecht: Springer; 2009.
25. Jacquet A. Interactions of airway epithelium with protease allergens in the allergic response. *Clin Exp Allergy* 2011;**41**:305–311.
26. Santamaría ME, González-Cabrera J, Martínez M, Grbic V, Castañera P, Diaz L et al. Digestive proteases in bodies and faeces of the two-spotted spider mite, *Tetranychus urticae*. *J Insect Physiol* 2015;**78**:69–77.
27. Morales M, Iraola V, Leonor JR, Carnés J. Enzymatic activity of allergenic house dust and storage mite extracts. *J Med Entomol* 2013;**50**:147–154.
28. Martínez J, Eraso E, Palacios R, Guisantes JA. Enzymatic analyses of house dust mite extracts from *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (Acari: Pyroglyphidae) during different phases of culture growth. *J Med Entomol* 1999;**36**:370–375.
29. Morgan MS, Arlian LG. Enzymatic activity in extracts of allergy-causing astigmatid mites. *J Med Entomol* 2006;**43**:1200–1207.
30. Schulz O, Sewell HF, Shakib F. A sensitive fluorescent assay for measuring the cysteine protease activity of Der p 1, a major allergen from the dust mite *Dermatophagoides pteronyssinus*. *Mol Pathol* 1998;**51**:222–224.
31. van Ree R. Indoor allergens: relevance of major allergen measurements and standardization. *J Allergy Clin Immunol* 2007;**119**:270–277.
32. Erban T, Rybanska D, Harant K, Hortova B, Hubert J. Faeces derived allergens of *Tyrophagus putrescentiae* reared on dried dog food and evidence of the strong nutritional interaction between the mite and *Bacillus cereus* producing protease bacillolysins and exo-chitinases. *Front Physiol* 2016;**7**:53.
33. Fernández-Caldas E. Towards a more complete standardization of mite allergen extracts. *Int Arch Allergy Immunol* 2013;**160**:1–3.
34. Nelson H. Chapter 24. Preparing and mixing allergen vaccines. In: Lockett RF, Bukantz SC, Bousquet J, editors. *Allergens and Allergen Immunotherapy*, 3rd edn. New York: Marcel Dekker, 2004: 457–480.
35. Larenas-Linnemann D, Esch RE, Guidos-Fogelbach G, Rodríguez-Pérez N. A comparison of in vitro potency between European and Mexican allergen extracts and US (CBER/FDA) reference extracts. *Allergol Immunopathol* 2010;**38**:170–173.
36. Larenas-Linnemann D, Esch R, Plunkett G, Brown S, Maddox D, Barnes C et al. Maintenance dosing for sublingual immunotherapy by prominent European allergen manufacturers expressed in bioequivalent allergy units. *Ann Allergy Asthma Immunol* 2011;**107**:448–458.
37. Park KH, Son M, Choi SY, Park HJ, Lee JH, Jeong KY et al. In vitro evaluation of allergen potencies of commercial house dust mite sublingual immunotherapy reagents. *Allergy Asthma Immunol Res* 2015;**7**:124–129.
38. Moreno-Benítez F, Espinazo RM, Letrán CA, Mas S, García-Cozar FJ, Tabar AI. Variation in allergen content in sublingual allergen immunotherapy with house dust mites. *Allergy* 2015;**70**:1413–1420.
39. van Ree R, Chapman MD, Ferreira F, Vieths S, Bryan D, Cromwell O et al. The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. *Allergy* 2008;**63**:310–326.
40. CH Topic Q 1 A (R2) Stability Testing of new Drug Substances and Products. European Medicines Agency. 2003. CPMP/ICH/2736/99.
41. Jeong KY, Choi SY, Han IS, Lee JH, Lee JS, Hong CS et al. The effects of storage conditions on the stability of house dust mite extracts. *Allergy Asthma Immunol Res* 2013;**5**:397–401.
42. Soldatova LN, Paupore EJ, Burk SH, Pastor RW, Slater JE. The stability of house dust mite allergens in glycerinated extracts. *J Allergy Clin Immunol* 2000;**105**:482–488.
43. Ford AW, Rawle FC, Lind P, Spiessma FT, Lowenstein H, Platts-Mills TA. Standardization of *Dermatophagoides pteronyssinus*: assessment of potency and allergen content in ten coded extracts. *Int Arch Allergy Appl Immunol* 1985;**76**:58–67.