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Contribution of cysteine and serine proteases to proteolytic digestion in an allergy-eliciting house dust mite

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ABSTRACT

The digestive physiology of house dust mites (HDM) is of interest to understand their allergenicity towards humans since many of their allergens are digestive enzymes and/or are excreted into airborne fecal pellets. The aim of this study is to provide insight on the biochemical basis of proteolytic digestion in *Dermatophagoides pteronyssinus*, the most widespread HDM species. First, assays using non-specific protein substrates on purified fecal and body extracts determined that body-associated activity is almost exclusively dependent on cysteine proteases, and specifically on major allergen Der p 1. By contrast, cysteine and serine proteases contributed similarly to the activity estimated on fecal extracts. Second, the screening of group-specific peptide-based protease inhibitors followed by ingestion bioassays revealed that the human skin-derived cysteine protease inhibitor cystatin A produces a significant reduction in mite feeding (i.e. excreted guanine), and triggers the overproduction of Der p 1 (3-fold increase by ELISA). Noteworthy, the inhibition of cysteine proteases by cystatin A also resulted in a reduction in three non-target serine protease activities. Further incubation of these extracts with exogenous Der p 1, but not with other commercial cysteine proteases, restored trypsin (Der p 3) and chymotrypsin (Der p 6) activities, indicating that Der p 1 is responsible for their activation *in vivo*. Finally, the role of serine proteases on the mite's digestive physiology is discussed based on their remarkable activity in fecal extracts and the autocoprophagic behavior reported in mites in this study.

1. Introduction

House dust mites (HDM) such as *Dermatophagoides pteronyssinus*, one of the most widespread species, are the main source of indoor allergy worldwide having huge clinical and socio-economic consequences (Sánchez-Borges et al., 2017). The digestive physiology of HDM is related to their allergenicity in human environments. First, some of the clinically relevant *D. pteronyssinus* allergens are enzymes that could participate in food digestion, namely the proteases Der p 1, 3, 6 and 9, the alpha-amylase Der p 4, and the chitinase Der p 15 (Colloff, 2009; Thomas, 2015; Vidal-Quist et al., 2017a). In addition, other allergens such as Der p 2, 18, 21 and 23 have also been immunolocalized in the mite's digestive tract (Park et al., 2000; Weghofer et al., 2008, 2013; Resch et al., 2016). Finally, many of the known allergens, including the three major serodominant allergens, Der p 1, 2 and 23, get accumulated in fecal pellets (Batard et al., 2016; Bordas-Le Floch et al., 2017; Erban et al., 2017; Waldron et al., 2019), which become easily airborne by their small size, and represent the main route of exposure to HDM allergens for humans (Sánchez-Borges et al., 2017). Noteworthy, growing evidence arises that allergenicity, apart from the canonic immunoglobulin E (IgE) response triggered by allergens, can also be attributed to other intrinsic properties such as proteolytic activity. Exogenous proteases, such Der p 1, 3 and 6, are able to cleave tight junctions of the skin, epithelial and mucosal tissues compromising the integrity of their barrier function and further activating the innate immune system by IgE-independent pathways (Jacquet, 2011; Calderón et al., 2015; Scheurer et al., 2015; Reithofer and Jahn-Schmid, 2017). These findings, although still to be explored clinically in more detail, further stress the interest of broadening the search for immunoactive *D. pteronyssinus* molecules including mite digestive proteases.

Despite being a key aspect of their allergenicity, the exact biological reasons underlying the loss of significant amounts of functional proteolytic enzymes inside fecal pellets as a product of digestion in HDM is still unclear (Colloff, 2009). In contrast to mites, many insects do possess

Abbreviations: CP, cysteine protease; HDM, house dust mite; SP, serine protease; WMC, whole mite culture.

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a countercurrent flux of fluid in the gut that allows a more efficient recycling of digestive enzymes and lowers fecal losses (Terra and Ferreira, 2012). Unfortunately, partly due to their small size and the impossibility to perform gut dissections, our knowledge on the digestive physiology of mites is far away from that of insects. As estimated using a number of methods, including specific fluorometric assays, the extracts of both mite bodies and fecal pellets from *D. pteronyssinus* exhibit a diversity of proteolytic activities belonging to different protease classes (Morgan and Arlian, 2006; Erban and Hubert, 2010a; Morales et al., 2013; Vidal-Quist et al., 2017a). In addition, as revealed by gene annotation of HDM genome assemblies (Randall et al., 2017; Vidal-Quist et al., 2017c), proteolytic allergen genes such as Der p 1 (cysteine protease), Der p 3 (trypsin) or Der p 6 (chymotrypsin) form part of much larger families of protease encoding genes in their genomes. In spite of the high genetic and biochemical diversity reported for proteases in HDM, their participation and relative contribution to the actual digestive process is still unclear. Notably, the high trypsin activity/abundance reported in *D. pteronyssinus* and *Dermatophagoides farinae* fecal pellets (Stewart et al., 1994; Erban et al., 2017; Vidal-Quist et al., 2017a) suggests a possible digestive role. By contrast, trypsin-like activity would have a limited functional importance in *D. pteronyssinus* if we attend to the discrepancy between its alkaline optimal pH (Vidal-Quist et al., 2017a) and the acidic physiological pH reported across the gut in this species (Erban and Hubert, 2010b).

A recent investigation by our group suggested that changes in the nutritional composition of the HDM diet, including the presence of protease inhibitors, can influence the expression of the whole repertoire of allergens and the activity of a number of proteases, which might be expressed to compensate the loss of activity derived from the inhibitor (Vidal-Quist et al., 2017c). The effects recorded in that study were more drastic when the diet was supplemented with beard shavings, as a surrogate of human skin scales shed in domestic environments. Further investigation demonstrated that beard shavings contain protease inhibitors that preferentially inhibit the Der p 1-like cysteine protease activity of mite extracts. The presence of cysteine protease inhibitors in the human skin is well documented (Brocklehurst and Philpott, 2013), especially the inhibitor cystatin A, which is produced by keratinocytes and is known to inhibit Der p 1 (Kato et al., 2005). This is particularly relevant for HDM because shed skin is supposed to be one of their major food sources at homes (Colloff, 2009; Sánchez-Borges et al., 2017). Similarly, the growth media used by the pharmaceutical industry for the mass-rearing of HDM to produce allergy diagnostic and immunotherapy products can also contain ingredients bearing protease inhibitors, such as cereals or industry by-products (Barber et al., 1996; Avula-Poola et al., 2012). Previous studies have stressed the great variability in the allergen composition of *D. pteronyssinus* extracts from different pharmaceutical manufacturers, which lacked relevant allergens in some cases and produced heterogeneous results in allergy diagnostic tests (Casset et al., 2012; Frati et al., 2012). Among other factors linked to the manufacturing process, protease inhibitors contained in industrial media might contribute to this variability, since, as shown in our previous investigation, they have the potential to alter mite performance and allergenicity (Vidal-Quist et al., 2017c).

The fundamental knowledge generated in the present investigation intends to contribute to our understanding of the HDM physiology, which in turn can be of interest to allergy research and help to improve HDM mass-culture methods by the pharmaceutical industry. In a first step, we inspected the contribution of the different protease groups to the digestive physiology of *D. pteronyssinus* by using non-specific protein substrates. In a second step, we provided insight on the biochemical basis of proteolytic digestion by using specific fluorometric methods to screen a number of group-specific peptide-based protease inhibitors, followed by ingestion bioassays and biochemical analysis. Last, by a number of methodological approaches, we explored different biological aspects related to the interaction of *D. pteronyssinus* with its fecal excretions in order to understand the importance of such enzymatically-rich material in the HDM microhabitat.

2. Materials and methods

2.1. Mite cultures, purified fractions and extracts

The origin and maintenance of the *D. pteronyssinus* laboratory stock colony used in this study was as previously described (Vidal-Quist et al., 2015). In brief, mites were cultured in 50 ml sterile Erlenmeyer flasks containing 2 g of 1:1 mixture of fish food flakes (Sera Vipan; Sera GmbH, Heinsberg, Germany) and brewer's yeast flakes (Santiveri SI, Barcelona, Spain) as growth medium. Air exchange was allowed through a hydrophilic cotton cap, and flasks were confined into sealed plastic boxes containing a saturated NaCl solution to maintain a constant RH of 76 ± 2%, and kept in a climatic chamber at 24 ± 0.3 °C in the dark. Mixed-stage crawling mites were separated from whole mite cultures (WMC) by the paper disk method (Vidal-Quist et al., 2015). The fecal-enriched fraction (i.e. feces) of a 7-week WMC (1–2 weeks after the population peak) was obtained after pre-drying the culture (2 h at 42 °C) and sieving through a 50 μm test sieve in a sealed container subjected to agitation (1,000 rpm for 30 min). Fine particles were collected onto filter paper and purity was inspected under a stereomicroscope to confirm high content of fecal pellets (>90%) and absence of mites bodies, or eggs (see supplemental Fig. S1); samples were then weighed and stored at −20 °C. Extracts from the different mite sources were prepared as follows: samples were homogenized in ice-cold 0.15 M NaCl solution using a microtube pestle and rotor, and extracted by centrifugation (12,000 rpm, 15 min, 4 °C). Both aqueous soluble and insoluble (centrifugation precipitate) fractions were kept. Prior to storage at −80 °C, soluble fractions from a same experiment were standardized as per protein concentration.

2.2. Bioassays with protease inhibitors

For inhibitor ingestion bioassays, each unit (equivalent to a biological replicate) consisted in a 1.5 ml screw cap conical microtube containing 3 mg of inhibitor pre-treated red fish food flake and 4–6 mg of mixed-instar mites (exact weights were recorded). A schematic diagram of the bioassay method is shown in supplemental Fig. S2. These small-scale cultures were arranged in microtube racks and incubated for 96 h in the standard climatic conditions. Gas exchange was allowed through a Miracloth filter (Merck Millipore, Darmstad, Germany) placed under the microtube cap. The duration of the treatment was adjusted in preliminary tests aiming to maximize ingestion/excretion while avoiding diet depletion and mite stress. Food flakes were pre-treated by applying 6 μl of an aqueous solution of protease inhibitor at 2 mg/ml (or distilled water for controls), and drying at room temperature to a final concentration of 0.4% w/w. The protease inhibitors tested were: three serine protease (SP) inhibitors, SBBI (Soybean Bowman-Birk inhibitor), TEI (Trypsin inhibitor from Turkey Egg White), and LBI (Trypsin Inhibitor from Lima Beans), all purchased from Sigma-Aldrich (St Louis, MO, USA); two cysteine protease (CP) inhibitors, recombinant human cystatin A (Novoprotein, Summit, NJ, USA), and HvCPI-6 barley cystatin, kindly provided by Dr. Manuel Martínez (CBGP, Madrid, Spain) (Martínez et al., 2009); and, finally, an anti-Der p 1 monoclonal antibody showing specific inhibitory potency towards Der p 1 (AcMo; provided by Dr. Manuel Lombardero, ALK, Madrid, Spain). The concentration of protease inhibitors was preliminary tested by visual inspection to avoid mortality or antifeedant effects on mites. Two controls with non-treated food were included to assess possible effects: cultures at the start (time 0) and at the end of the bioassay (96 h from the onset of the experiment). It is to be noted that, for the sake of comparability, all quantitative data derived from the bioassays (e.g. enzymatic activity) was normalized as per the starting weight of bodies included in each bioassay unit.

2.3. Total protein, Der p 1 and guanine contents

Total protein content in soluble mite extracts was measured by the Lowry method using the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin (BSA) as standard. The content of the major allergen Der p 1 was quantified by sandwich ELISA using the Der p 1 ELISA kit (EL-DP1A) as per manufactureŕs instructions (Indoor Biotechnologies, Charlottesville, VA, USA). Prior to the experiments, the impact of protease inhibitors that could eventually interact with Der p 1 and alter ELISA immunodetection was assessed showing minor effects: cystatin A could overestimate Der p 1 content by up to 7%, and the AcMo antibody could underestimate by up to 20%. The guanine content of the aqueous-insoluble fraction of mite extracts was estimated by adapting previous methods (Le Mao et al., 1989; Soto-Quiros et al., 1998). First, the stored precipitates were washed with ice-cold 0.15 M NaCl by thorough mix and centrifugation (7,500 rpm, 4 min at room temperature, RT), and the aqueous supernatant was discarded. Then, in order to dissolve guanine, washed precipitates were resuspended in an alkaline alcoholic solvent (20% Methanol, 0.16 M NaOH, in MilliQ water) at 10% (w/v; sample/solvent) followed by incubation for 10 min at 30 °C with agitation (600 rpm). After centrifugation (10 min, 4,000 rpm, RT), supernatants were recovered whereas precipitates were subjected to a second extraction in alkaline alcoholic solvent, as described above. The supernatants from both steps were pooled and mixed prior to estimating guanine concentration. For that purpose, 80 µl of sample (appropriately diluted in alkaline alcoholic solvent, two duplicates per sample) were mixed with 40 µl of 16% w/v Na_2CO_3 and 80 µl of freshly prepared ice-cold coupling solution (sulfanilic acid at 0.25% w/v in 1 N HCl freshly pre-mixed 1:1 with 5% w/v $NaNO_2$ in ultrapure water, and kept for 10 min in ice) per well in a 96-well microplate. Mixtures were incubated for 20 min at RT to allow the formation of diazoamino compounds, and the absorbance at 490 nm was recorded using a microplate reader. A guanine (Sigma-Aldrich) standard curve was used as reference. Note that the purpose of guanine analysis was to estimate the accumulation of fecal material at the end of the microtube bioassays. Hence, since both mite bodies and the fresh culture medium did also contain measurable amounts of guanine (11.0 ± 0.1 µg guanine/mg, n = 4; 14.5 ± 0.7 µg/mg, n = 4, respectively), in order to estimate guanine as a result of fecal excretion (i.e. fecal-derived guanine), the total guanine contents measured at the end of the bioassay were corrected by deducting the guanine contents estimated for each bioassay unit at the beginning (calculated as per the measured weights of mite bodies and fresh medium in each tube, and the reference guanine contents indicated above).

2.4. Proteolytic activity: hydrolysis of non-specific azo-dye protein substrates

Proteolytic activity was estimated by measuring hydrolysis of sulfanilamide-azocasein and sulfanilic acid-azoalbumin chromogenic substrates (Sigma-Aldrich; freshly pre-diluted in 0.15 M NaCl to a final 0.1% w/v in the reaction volume) by mite-derived extracts (WMC from a 5-week old flask culture at the late exponential growth phase, purified bodies, or purified fecal pellets; all at 166 µg of soluble protein per ml in the reaction volume). Three different pH buffers were tested (0.1 M citrate buffer pH = 5; and 0.1 M phosphate buffer at pH = 6.5 or pH = 8; all including 0.15 M NaCl and 5 mM $MgCl_2$) and in different combinations of presence/absence of protease inhibitors (100 µM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane, E64; and/or 10 µM Soybean Trypsin Inhibitor, STI; both from Sigma-Aldrich) and activators (1 mM dithiothreitol, DTT, freshly prepared). After incubation at 30 °C in a microtube shaker at 600 rpm, non-degraded substrates were precipitated by the addition of ice-cold 5% w/v trichloroacetic acid and centrifugation (12,000 rpm, 5 min, 4 °C). The absorbance at 420 nm was measured on supernatants in triplicate measurements, and compared to their corresponding blanks. Specific activities were expressed as 1,000-fold units of $\triangle Abs_{420nm}$ per minute and mg of soluble protein. The relative contribution of different class-specific protease groups to the total proteolytic activity against non-specific azo-dye substrates was estimated comparing the results obtained with the different conditions of protease inhibitors and activators, and considering their specificity towards *D. pteronyssinus* extracts, as previously determined (Vidal-Quist et al., 2017a). Three categories were established based on the reaction conditions for each protease group: cysteine protease like (CP-like) activity was estimated as being E-64 sensitive and STI insensitive, in the presence of DTT; serine protease like (SP-like) activity was estimated as being STI sensitive and E-64 insensitive, in the absence of DTT that inhibits SP activity; the category "other activities" was defined as being insensitive to both E-64 and STI, in the presence of DTT.

2.5. Proteolytic activity: hydrolysis of specific synthetic peptides

Protease activities were estimated on mite extracts using fluorogenic substrates. Reactions conditions for the estimation of CP activities (Der p 1-like, cathepsin B, cathepsin L, legumain), SP activities (trypsin, chymotrypsin, elastase), and aspartic endoprotease activity (cathepsin D) had been previously optimized for *D. pteronyssinus* based on assays using different pH buffers, group-specific protease inhibitors, and mite-culture fractions (i.e. bodies and feces) (Vidal-Quist et al., 2017a). The leucine aminopeptidase assay (metallo-exoprotease) was optimized for this study, as described in supplementary material Text S1. Reaction conditions, substrates, additives (i.e. protease inhibitors, activators), and buffers are summarized in supplemental Table S1. Activation of mite SP proenzymes by exogenous CP was studied by pre-incubating (1 h at 30 °C, 600 rpm shaking) mite extracts (at 0.2 µg protein/µl) with exogenous enzymes, either purified native Der p 1 allergen (nDer p 1; at 3.2 µM; kindly provided by Dr. Manuel Lombardero, ALK-Madrid, Spain), papain from papaya latex (3.4 µM; Sigma-Aldrich), or cathepsin B from bovine spleen (1.5 µM; Sigma-Aldrich), all in the presence of 0.9 mM DTT. The trypsin and chymotrypsin activities of the resulting mixtures were then analyzed using fluorogenic substrates (as described above). Note that the concentration of exogenous CP enzymes added in the pre-incubation mixtures was intentionally high in order to avoid their inhibition by the residual cystatin A inhibitor that could be present in the mite extract after the cystatin A ingestion bioassay (the molarity of enzymes in the mixture exceeded at least 5-fold the equivalent maximum molarity of residual cystatin A).

2.6. Inactivation of protease inhibitors by mite proteases in vitro

The inactivation of the SP inhibitor SBBI by nDer p 1, and the inactivation of the CP inhibitor cystatin A by mite fecal-derived enzymes were studied by *in vitro* assays. To study the inactivation of the SP inhibitor, SBBI (1 µM) was pre-incubated in 0.1 M phosphate buffer (0.15 M NaCl, 5 mM $MgCl_2$, pH = 7) in the presence/absence of Der p 1 (0.5 M purified native Der p 1), and in the presence/absence of DTT (1 mM). After pre-incubation (2 h at 30 °C, 500 rpm shaking; or freshly prepared in ice), trypsin activity was estimated on a 5 week-old WMC extract supplemented with each of the pre-incubated mixes containing SBBI (equivalent to 50 nM SBBI final concentration) by using a specific fluorogenic substrate (as described above). The eventual loss of inhibitory potency by SBBI after pre-incubation was calculated in relation to a control without inhibitor and DTT (0.15 M NaCl). On the other hand, to study the inactivation of the CP inhibitor, cystatin A (5 µM) was pre-incubated in 0.1 M phosphate buffer (0.15 M NaCl, 5 mM $MgCl_2$, pH = 7) in the presence/absence of an extract of *D. pteronyssinus* fecal pellets (0.2 µg soluble protein/µl). Controls without extract were included to take into account the activity of fecal extracts in the final reaction. After pre-incubation (2 h at 30 °C, 500 rpm shaking; or freshly prepared in ice), three CP activities (Der p 1-like; cathepsin B, and cathepsin L) were estimated on a 5 week-old WMC extract supplemented with each of the pre-incubated mixes containing the cystatin A inhibitor

(equivalent to 0.25 μM cystatin A final concentration) by using specific fluorogenic substrates (as described above). The eventual loss of inhibitory potency by cystatin A after pre-incubation was calculated in relation to the corresponding control for each pre-treatment (identical conditions in the absence of cystatin A).

2.7. Interactions of D. pteronyssinus with its fecal pellets: small-scale bioassays on slides

To study autocoprophagia in *D. pteronyssinus*, glass concave microscope slides were used by adapting a previous methodology (Vidal-Quist et al., 2017b). Mixed-stage mites were isolated under starvation for 48 h prior to transfer 70 ± 20 motile mites into the cavity of a slide containing a small fish food flake (approximately 1 mg, pre-stained with red food coloring dye E122) side by side with 0.5 mg of blue fecal pellets, so that both components were accessible to the mites *ad libitum* during the duration of the assay. Blue fecal pellets were obtained by sieving a saturated culture (as described above) of mites fed on a pre-stained blue medium (supplemented with blue food coloring dye E133, and prepared as described previously (Vidal-Quist et al., 2017c)). The slide cavities were sealed with a glass cover and incubated under the standard climatic conditions. After 3, 8, 24, and 96 h the slides were inspected under stereomicroscope to determine the exact numbers of mites having ingested the different materials, or nothing. The color of the food bolus was clearly visible through the *exo*-cuticle: red color indicated ingestion of fish food, greenish blue color indicated ingestion of fecal pellets, and purple tones ingestion of both. This parameter was used to estimate the feeding preference of mites.

To study the attraction of mites to fecal pellets in their food, a choice assay was conducted using custom-made Plexiglas slides (25 × 75 mm) containing three excavated arenas arranged in line and connected by two corridors (Fig. S3). Mixed-stage mites were isolated under starvation for 24 h prior to transfer 75 ± 25 motile mites into the central arena, which was empty. Slides were closed with a cover slip (22 × 66 mm) to avoid mite escape, while permitting gas exchange. The experimental set-up offered the opportunity for mites to access the two remaining arenas, one containing a 2 mg flake of fish food, and the opposite with a similar flake supplemented with purified fecal pellets. For that, prior to the addition of mites, 5 μl of water or 5 μl of a suspension of fecal pellets in distilled water at 10% w/v (approximately 0.5 mg of fecal pellets) were applied onto each flake and let dry for 2 h. The experimental units were incubated in the standard climatic conditions and inspected under the stereomicroscope after 3, 24 and 96 h from the introduction of the mites. Based on their location, live mites were classified in three categories: fish food arena, fish food & fecal pellets arena, or no choice (mites in the central arena or corridors).

To assess *D. pteronyssinus* growth on fecal pellets as the only nutritional source, glass concave microscope slides were used as described above. Mite protonymphs (10 ± 1; six legs instead of 8 legs as in subsequent instars) were exposed to *ad libitum* amounts of either purified fecal pellets (>1 mg), or fish food (a flake of at least 2 mg). Mites were incubated for 20 days under the standard climatic conditions, and development data were recorded by stereomicroscope observation.

2.8. Statistical analysis

Data were statistically compared using parametric tests, either unpaired *t*-test, one-way ANOVA coupled with Dunnetťs post-test, or standard two-way ANOVA, as indicated, together with replication parameters, for each experiment in the results section, tables and/or figures. For the choice experiment specifically, percentage values were transformed by the angular transformation method (arcsin of square root of *p*; where *p* is the proportion), and means of the two alternative choices at the three time points were compared by repeated measures two-way ANOVA coupled with Bonferronís post-test (repeated measures considered by both factors, choice and time). Statistical analyses were computed using GraphPad PRISM v7.0a (GraphPad Software, La Jolla, USA).

3. Results

3.1. Degradation of non-specific protein substrates by class-specific proteases

In order to assess the contribution of different protease groups to the digestion by *D. pteronyssinus* we have studied the hydrolytic activity of extracts from whole mite cultures (WMC), purified bodies, purified feces, and native Der p 1 (nDer p 1) over azo-dye full protein substrates (azocasein, azoalbumin), in the presence of group-specific protease inhibitors (E-64, STI) and activators (DTT), and across a range of pH values (see methods) (Fig. 1A, B). Overall, the profile of degradation of azocasein and azoalbumin by WMC extracts was fairly similar to that of fecal extracts, with maximum total specific activity in the range of pH = 6.5 to 8. Both the CP-like (cysteine protease) and the SP-like (serine protease) groups contributed significantly to proteolysis, collectively accounting for over 84% of the total specific activity in any of the tested conditions. As expected, the relative contribution of each class of proteases was pH-dependent, with higher SP activity at increasing pH, and maximum CP activity at pH = 5 to 6.5, depending on the substrate. Remarkably, the proteolytic activity associated to mite bodies showed a distinct profile, being almost exclusively CP-like and with very low total specific activity at pH = 8. Additionally, the contribution of Der p 1 to the total CP activity of a WMC extract was studied by comparing the CP-like hydrolysis of azo-dye substrates by both nDer p 1 and the extract. After normalizing their amounts in the reaction, nDer p 1 degraded as much as 34% of azocasein and 66% of azoalbumin compared to the WMC extract (Fig. 1C).

3.2. Effects of the ingestion of serine protease inhibitors

At a preliminary stage we assessed the *in vitro* inhibitory effect of six peptide-based SP inhibitors over seven proteolytic activities of a WMC extract using specific fluorometric assays (Table S2, A). Three of these inhibitors were selected for a further inhibitor ingestion bioassay based on their characteristic inhibition profile: SBBI, a SP inhibitor towards both trypsin and chymotrypsin; TEI, a SP inhibitor preferentially inhibiting chymotrypsin; and LBI, a SP inhibitor with a preference towards trypsin. During the bioassays, mites were actively eating as demonstrated by both the accumulation of fecal-derived guanine (average 2.4-fold increment of total guanine in control, from an initial level of 15.2 ± 0.8 μg guanine/mg bodies) and the increase of Der p 1 allergen after four days (average 1.7-fold increment in control) (Fig. 2A). In addition, no significant differences were detected on the accumulation of either guanine or Der p 1 when comparing the SP inhibitor treatments and control at the end of the bioassay. Changes in the proteolytic profile of the cultures (four CP, three SP, one aspartic protease, and one metalloprotease) were also considered. The comparison of control extracts at the beginning and at the end of the bioassay revealed a significant increase of most of these enzymatic activities after four days (ranging from 1.57-fold, for legumain, to 10.75-fold, for chymotrypsin), except for cathepsin D, that slightly decreased, and leucine aminopeptidase, that did not change significantly (Fig. 3A). However, none of the assayed SP inhibitors had significant impact over the proteolytic activities under study besides their expected targeted activities (i.e. trypsin and chymotrypsin) when compared to controls at the end of the bioassay. It is to be noted that any residual inhibitor remaining after the extraction of an inhibitor-treated culture will affect subsequent enzymatic assays of activities targeted by that inhibitor. Thus, SP activity estimates in cultures treated with SP inhibitors must be considered with caution since they do not reflect the actual activity *in vivo* of the corresponding enzymes or their contents.

Finally, the proteolytic inactivation of the ingested SP inhibitors by

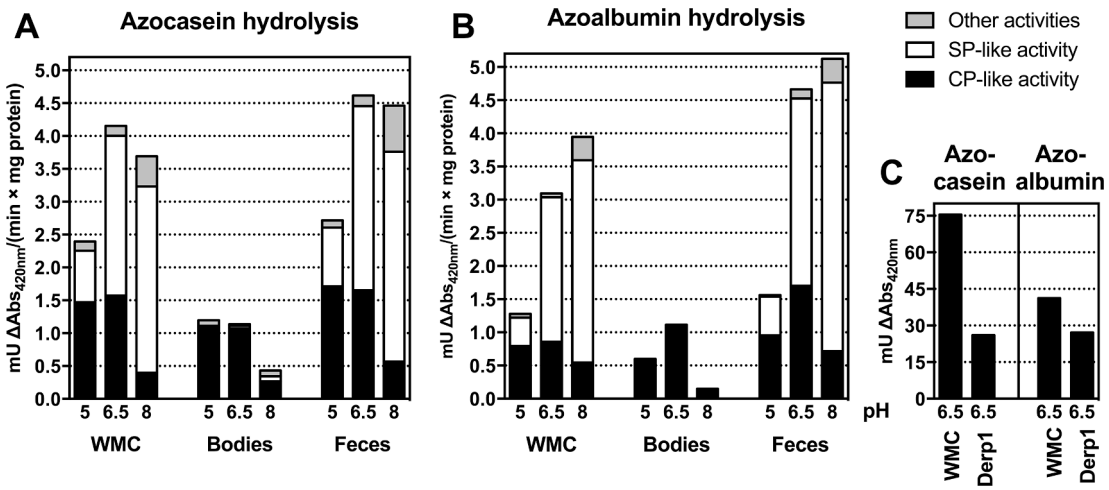

Fig. 1. Hydrolysis of non-specific protein substrates by class-specific proteases from whole mite cultures, purified bodies, feces and purified native Der p 1. Data were obtained from three technical absorbance measurements comparing activities with/without the protease inhibitors E-64, STI and DTT as activator at three different pH values (5, 6.5, or 8). CP denotes cysteine protease-like activity (E-64 sensitive, STI insensitive, with DTT); SP denotes serine protease-like activity (STI sensitive, E-64 insensitive, without DTT); "other activities" refers to activity that is insensitive to both E-64 and STI in the presence of DTT; WMC denotes whole mite culture. Panels A and B indicate specific hydrolytic activities for azocasein and azoalbumin, respectively. Panel C shows the contribution of Der p 1 to the total CP activity of a WMC extract. Total substrate hydrolysis (ΔAbs_{420nm}) by both the WMC extract and purified native Der p 1 (Derp1) was compared after adjusting the amount of Derp1 in the reaction to equal the specific Der p 1-like activity in both sources (fluorometric method).

Der p 1, as a predominant CP protease in the mite's digestive tract, was inspected *in vitro*. The inhibitor SBBI was pre-incubated with nDer p 1 in neutral pH and reducing conditions. A moderate reduction of its inhibition potency towards WMC trypsin activity was observed when compared to the same pre-treatment in the absence of nDer p 1 (64.2% and 77.3% inhibition, respectively) (Table S3). Noteworthy, the inhibitory potency of SBBI was remarkably higher (92.1%) in the absence of pre-incubation with DTT, meaning that SBBI stability and/or folding appears to be also compromised by reducing conditions.

3.3. Effects of the ingestion of cysteine protease inhibitors

At a preliminary stage, the *in vitro* inhibitory effects of seven peptide-based CP inhibitors over seven proteolytic activities of a WMC extract were evaluated (Table S2, B). Two of these inhibitors were selected for further bioassays based on their inhibition profile: cystatin A, a rather general CP inhibitor with a preference towards Der p 1-like and cathepsin L activities; and barley HvCPI-6 cystatin, with a preference towards cathepsin L activity. In addition, the monoclonal anti-Der p 1 antibody AcMo was selected for bioassay based on its specific inhibition of Der p 1-like activity, in both WMC extracts and purified nDer p 1 (33% and 80% inhibition at 67 nM AcMo, respectively; Table S4). As opposed to our observations after the ingestion of SP inhibitors, CP inhibitors did produce significant effects over the mite performance, in particular cystatin A. Hence, the accumulation of fecal-derived guanine after four days was reduced approximately by half in the cystatin A treatment compared to control, whist a significant slight increase was found in the treatment with HvCPI-6 (Fig. 2B). Consistently, visual inspection of cystatin A cultures revealed a remarkably slower consumption of diet compared to controls and other treatments (data not shown). In addition, despite the reduced feeding, the total Der p 1 content in WMC after ingestion of cystatin A was 3.1-fold higher than in controls at day 4, and 6.8-fold higher than at the initiation of the experiment (T_0). The ingestion of AcMo antibody also induced a significant, but lower, increase of Der p 1 in the culture (Fig. 2B). Similarly to previous results with SP inhibitors (see comments on residual inhibition in Section 3.2), the ingestion of CP inhibitors resulted in a reduction of their target CP activities after analysis of WMC extracts (for cystatin A, Der p 1-like, cathepsin B/L, and legumain activities; for HvCPI-6, cathepsin B/L activities), with the exception of the antibody AcMo treatment that did not affect protease activities (Fig. 3B). The latter is likely related to the moderate inhibitory potency of this antibody over WMC extracts (Table S4), and the overproduction of Der p 1 reported by ELISA (Fig. 2B) that could have compensated inhibition.

Noteworthy, whereas cathepsin D and leucine aminopeptidase activities were not altered by any of the treatments, the ingestion of cystatin A led to a significantly high reduction of all SP activities, which are not its enzyme targets: trypsin, chymotrypsin and elastase (3.93, 6.58 and 2.2-fold in relation to control, respectively; Fig. 3B). This reduction could not (only) be attributed to a lower excretion of fecal pellets (which concentrate most of the SP activity) in the cystatin A treatment, since estimates were still lower in the cystatin A treatment than in control when SP activities (increment in 4 days) were corrected by the amount of fecal-derived guanine (nmol substrate/min × mg excreted guanine; 3.75, 5.13 and 3.0-fold lower, for trypsin, chymotrypsin and elastase activity, respectively). Other tested CP inhibitors, HvCPI-6 (that shows low potency towards Der p 1-like activity; Table S2) and antibody AcMo, did not significantly affect SP activities in the culture.

3.4. Activation of serine proteases in mite extracts

Since the reduction of trypsin and chymotrypsin SP activities in mite cultures treated with cystatin A (see Section 3.3) could be associated to a lack of activation of the SP proenzymes by the inhibited Der p 1 enzyme, as has been previously proposed (Dumez et al., 2014), we inspected whether these SP activities changed after adding excess amounts of purified nDer p 1 and other commercial CP to WMC extracts of the cystatin A treatment. Results are shown in Fig. 4A and 4B. Following our expectations, the pre-incubation of cystatin A treated extracts with exogenous nDer p 1 led to a significant increase on their trypsin and chymotrypsin activities in relation to the same extracts subjected to pre-incubation with only DTT (3.0-fold and 2.1-fold, respectively). By contrast, the pre-incubation with commercial papain or cathepsin B did not result in an increase of trypsin activity. These results suggest that the ingestion of cystatin A had direct consequences on the activation of SP by reducing Der p 1 activity. In addition, in order to better understand the normal activation status of SP in mites (low basal SP activity in bodies and high in feces), we followed the same methodology to pre-incubate extracts from purified mite bodies and feces from the stock culture with nDer p 1. As seen in Fig. 4C, the body-associated trypsin

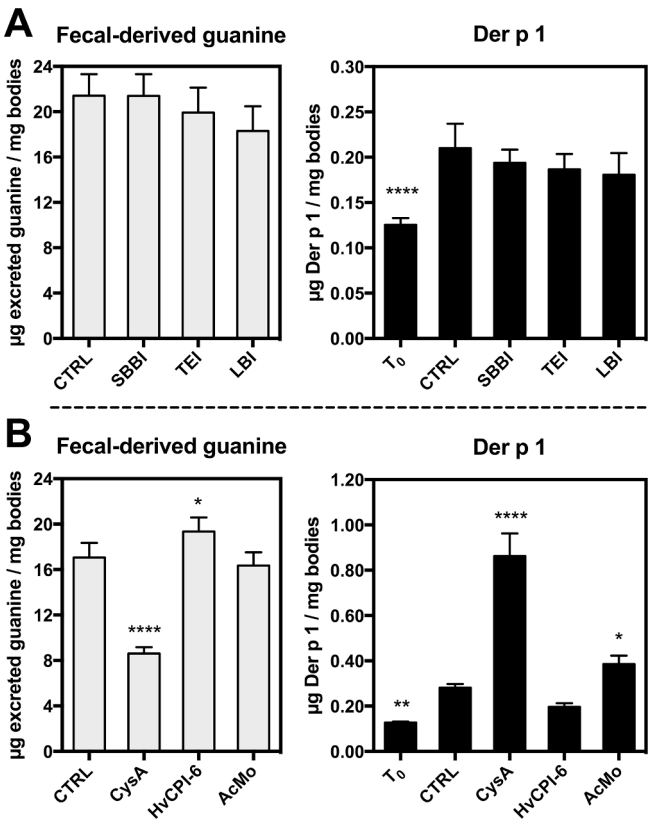

Fig. 2. Contents of fecal-derived guanine and major allergen Der p 1 in *D. pteronyssinus* cultures supplemented with protease inhibitors. Two independent experiments were conducted: A) ingestion of serine protease inhibitors (SBBI; TEI; LBI); B) ingestion of cysteine protease inhibitors (cystatin A, i.e. CysA; HvCPI-6; antibody AcMo). CTRL denotes control cultures after four days; T_0 denotes control cultures at the beginning of the experiment. Data are means and SEM of four biological replicates. Fecal-derived guanine was estimated as the increment of guanine after four days (i.e. for each bioassay unit, the total measured guanine was corrected by deducting the estimated guanine derived from mites and diet at the beginning). Asterisks indicate significant differences compared to CTRL (four days), as assessed by one-way ANOVA coupled with Dunnett's post-test (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

activity was not affected by this treatment, and only a slight 1.17-fold increase was observed in fecal extracts after pre-incubation, meaning that trypsins in control cultures are regularly found in the activated form. Notably, possibly due to the lower fecal accumulation in the cystatin A cultures, the absolute trypsin and chymotrypsin specific activities after activation with nDer p 1 in the cystatin A treatment were still significantly lower than in the corresponding control. However, when activities were normalized to the fecal-derived guanine, to account for differences in excretion, estimates were indeed higher in the cystatin A treatment (nmol substrate/min × mg excreted guanine; 1.54 and 1.34-fold higher for trypsin and chymotrypsin, respectively). Thus, although the excretion was lower and these SP were mostly inactive, their total concentration in feces of the cystatin A treatment was higher.

3.5. Interaction of *D. pteronyssinus* with its fecal excretions

As a considerably high enzymatic activity was detected in mite fecal extracts, we intended to further explore the interaction of *D. pteronyssinus* with their own fecal excretions. With this aim, we studied the mite's behavior regarding autocoprophagy, their attraction to fecal pellets in their food, as well as the capacity of feces to sustain mite development as the only food source. Additionally, the potential of excreted fecal enzymes to inactivate cystatin A protease inhibitor was also assessed. By placing mites in an arena with *ad libitum* amounts of fish food (red stained) and purified fecal pellets (blue stained), our first experiment evaluated whether *D. pteronyssinus* ingests fecal pellets as part of its digestive strategy. Notably, our results confirmed autocoprophagy, since 19.7% of the mites containing a visible food bolus in the digestive tract 3 h after the initiation of the experiment had ingested fecal pellets (showing a distinct bluish to purple color), and this percentage raised to 28.3% after 96 h (Fig. 5). However, our following choice behavioral assay showed that mites were not attracted to a growth medium pre-inoculated with fecal pellets when an alternative fresh growth medium was available (Fig. S3). The overall migration was dependent on the time of analysis ($P < 0.0001$, as assessed by repeated measures two-way ANOVA), with an increasing proportion of mites leaving the initial central arena along the duration of the assay. The choice of the food-containing arena (with/without fecal pellets) was time-depended, choice and time factors showing a significant interaction ($P = 0.0301$). At the end of the bioassay (96 h) and not before, a moderate but significant preference for the arenas containing only fish food was observed, receiving average 45% of the mites compared to 28% in arenas with food supplemented with fecal pellets ($P = 0.0185$, as assessed by Bonferroni post-hoc test; n = 26). In view of the autocoprophagic behavior observed in *D. pteronyssinus*, an additional experiment was conducted to assess whether mite growth was possible using fecal pellets as the only nutritional source. The average mortality attained 20 days after the introduction of the initial protonymphs in a diet based on fecal pellets was approximately 40%, being significantly higher to that of mites reared on a control fish food flake ($P = 0.0042$; Table S5). Stereomicroscope observations revealed that, to a certain extent, mites were ingesting fecal pellets, thus most of the surviving protonymphs in the fecal-based diet were able to develop to the tritonymphal instar. However, none of them reached adulthood at the end of the assay, whilst nearly 80% of adults were recorded in the control treatment. Finally, since the excretion of fecal enzymes could also exhibit external (i.e. extracorporeal) effects, an assay was conducted to evaluate the ability of mite fecal enzymes to inactivate exogenous peptide-based protease inhibitors that, such as cystatin A, could eventually be present in the microhabitat. Our results showed that the *in vitro* pre-incubation of commercial cystatin A with mite-derived fecal extracts did not substantially impair its inhibitory potency towards the CP activities of a WMC extract. Average inhibition levels attained after different pre-treatments of cystatin A in the presence or absence of fecal extract, and with or without pre-incubation, ranged from 69% to 96% depending on the CP activity and were very similar when comparing each activity separately (Der p 1-like, cathepsin B-like or cathepsin L-like) (Table S6).

4. Discussion

Four medically relevant *D. pteronyssinus* allergens are proteolytic enzymes that are believed to participate in the digestion process: the major allergen Der p 1 is a papain-like cysteine protease (CP) of the C1A subfamily; Der p 3, Der p 6 and Der p 9 are serine proteases (SP) of the S1A subfamily showing trypsin-like, chymotrypsin-like and elastase-like activity, respectively (Vidal-Quist et al., 2017a). All of them are synthetized as proenzymes, and a model for their activation cascade has been recently proposed (Dumez et al., 2014). This model suggests that Der p 1 is synthesized and auto-activated at the anterior midgut, where conditions are acidic, and that the other SP allergens are activated by Der p 1 at the posterior end of the digestive tract (i.e. hindgut), where the pH is closer to Der p 1 maximum activity (i.e. pH = 6–6.5). The present work confirms the main role of CP in the proteolytic digestion of *D. pteronyssinus*. However, our results raise questions on the involvement on SP and their possible roles after fecal excretion, as discussed below.

The use of general azo-dye full protein substrates, in combination with class-specific protease inhibitors and activators, revealed that the

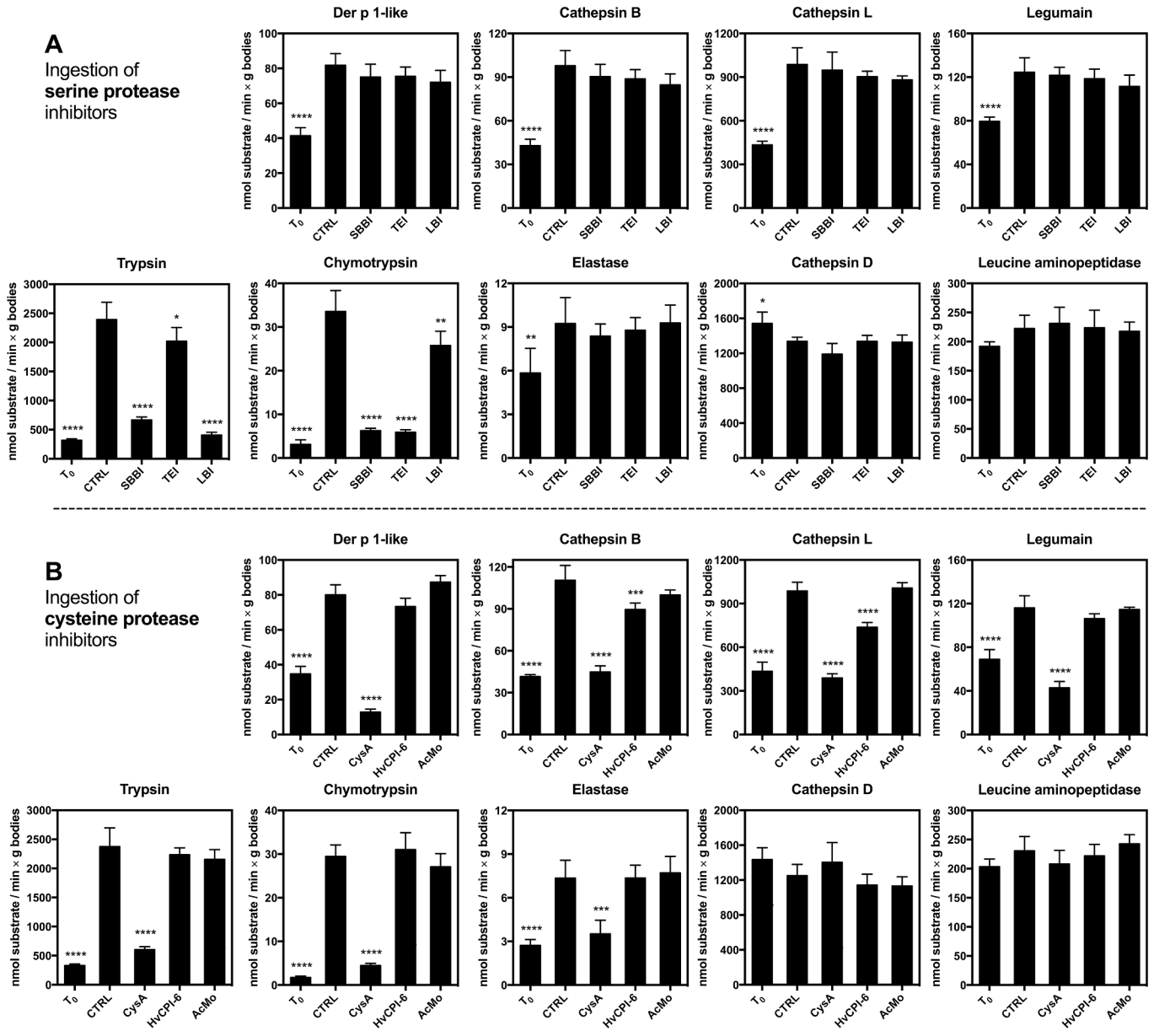

Fig. 3. Protease activities in *D. pteronyssinus* cultures supplemented with protease inhibitors. Two independent experiments were conducted: A) ingestion of serine protease inhibitors (SBBI; TEI; LBI); B) ingestion of cysteine protease inhibitors (cystatin A, i.e. CysA; HvCPI-6; antibody AcMo). CTRL denotes control cultures after four days; T_0 denotes control cultures at the beginning of the experiment. Data are means and SEM of four biological replicates. Asterisks indicate significant differences compared to CTRL as assessed by one-way ANOVA coupled with Dunnett's post-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

proteolytic activity detected in mite bodies is almost exclusively dependent on CP, other protease families playing a minor role. Proteolysis digestion models based on cysteine proteases have also been described in other Acari, such as the phytophagous mite *Tetranichus urticae* (Grbić et al., 2011; Santamaría et al., 2015), hematophagous ticks (Sojka et al., 2013), and also in coleopteran insects (Zhu-Salzman and Zeng, 2015). In accordance with the main digestive function of CP, ingestion bioassays using a number of SP and CP inhibitors coupled with specific biochemical assays revealed that the CP inhibitor cystatin A was the only treatment showing substantial short-term effects over mites. Specifically, the ingestion of cystatin A produced a significant reduction in mite feeding estimated by the excreted guanine, the main nitrogenous waste product of chelicerates (Le Mao et al., 1989). Additionally, it triggered the overproduction of Der p 1 allergen (estimated by ELISA), and affected the proteolytic profile of the culture. Notably, the inhibition of CP by the ingested cystatin A did not induce any alternative class-specific protease activity, including aspartic protease cathepsin D, metalloprotease leucine aminopeptidase, and SP (trypsin, chymotrypsin and elastase; which were even reduced as discussed later). Enzymatic compensation, despite not being detected in our conditions, is common in herbivory and predatory insects and form part of their adaptation and defense mechanisms to feed on diets bearing protease inhibitors (Ortego, 2012; Santamaría et al., 2015; Zhu-Salzman and Zeng, 2015). The lack of an equivalent short-term response to cystatin A challenge in *D. pteronyssinus* coupled with the remarkable induction of Der p 1 further support the high dependence of the species on digestive CP. Yet, it cannot be discarded that longer-term exposures to cystatin A could have produced different adaptive responses. As a matter of fact, in a previous work following a different methodology, the growth of mites on a diet supplemented with beard shavings (rich in keratin, and containing CP inhibitors) during at least six mite generations led to a significant decrease of both content and activity of Der p 1 in mite bodies,

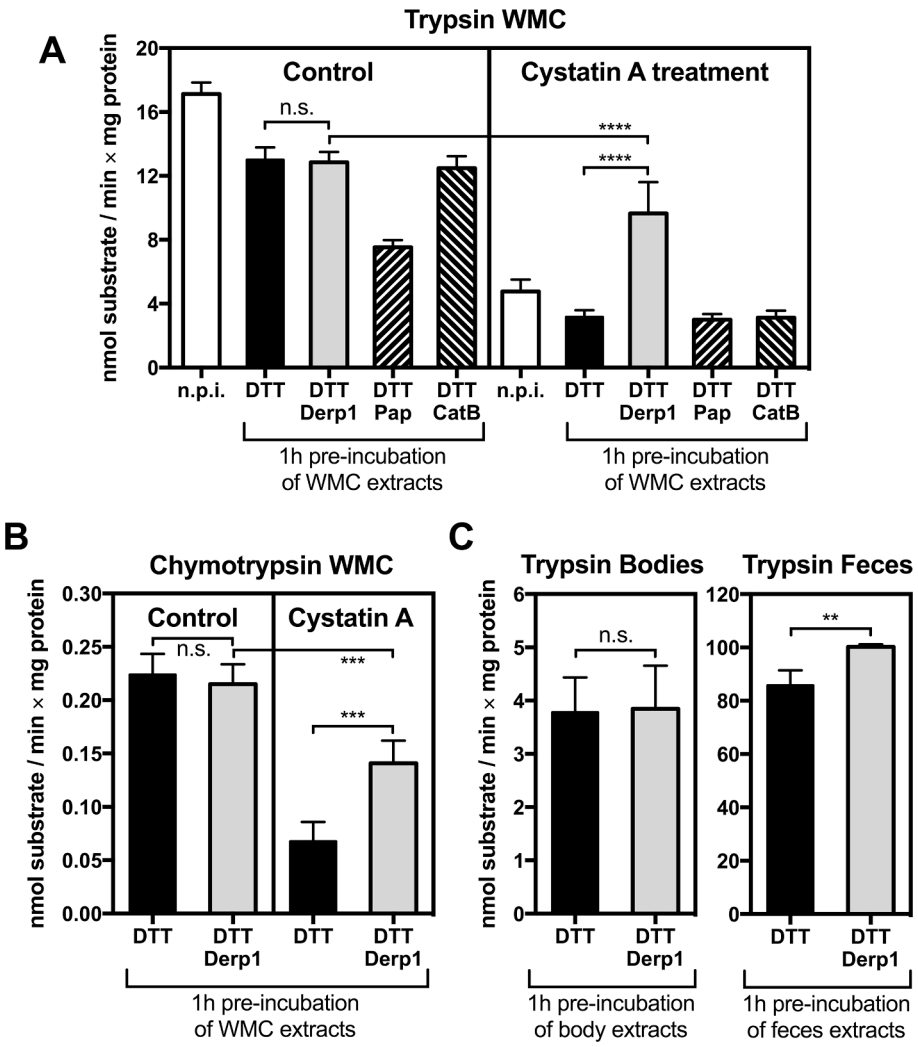

Fig. 4. *In vitro* activation of serine proteases from mite extracts by pre-incubation with exogenous cysteine proteases. The activation of trypsin (panel A) and chymotrypsin (panel B) activities from whole mite culture (WMC) extracts of the cystatin A ingestion bioassay and control, and the activation of trypsin from extracts of purified mite bodies and feces from the stock culture (panel C) were analyzed. "n.p.i." denotes not pre-incubated; "Derp1", purified native Der p 1; "Pap", commercial papain; "CatB" commercial cathepsin B. Data in panels A and B are means and SEM of four biological replicates; asterisks in these panels indicate significant differences as assessed by one-way ANOVA coupled with Dunnett's post-test (***$P < 0.001$; ****$P < 0.0001$; n.s. denotes "not significant"), only selected statistical comparisons are shown. Data in panel C are means and SEM from two replicated assays, each with three experimental replicates; asterisks in this panel indicate significant differences between treatments as assessed by two-way ANOVA (treatment/replicated assay) (**$P < 0.01$).

but a 40% increase in the body-associated trypsin activity (Vidal-Quist et al., 2017c).

Der p 1 appears to exhibit a prominent digestive role in *D. pteronyssinus*. A recent study estimated that about 50% of the protease-like transcripts in a *D. pteronyssinus* transcriptome belonged to CP, and that Der p 1 alone accounted for approximately 22% of the total protease transcripts (Randall et al., 2017). Likewise, proteomic surveys on laboratory *D. pteronyssinus* cultures have identified Der p 1 as the most abundant protease in feces and fecal-rich spent media (Erban et al., 2017; Waldron et al., 2019). In our study, by comparing the hydrolysis of azo-dye substrates by nDer p 1 and WMC at equivalent Der p 1-like specific activities, we showed that 34 to 66% of the CP-like activity of a WMC extract could be attributed to Der p 1, depending on the affinity to the protein substrate (azocasein or azoalbumin, respectively). Also, as opposed to cystatin A, the CP inhibitor HvCPI-6, which preferentially targets cathepsin L-like activity, did not produce any measurable effect in our ingestion bioassay suggesting a lower contribution of cathepsin L-like CP in digestion. In fact, a recent proteogenomic survey on *D. farinae* has revealed that the content of at least three cathepsin L1-like proteins was sex-dependent, thus presumably holding a non-digestive function (Erban et al., 2020). Altogether, our results indicate that Der p 1 would play a central role among other digestive CP that may also be important. In addition, the digestive function of Der p 1 is also supported by immunolocalization methods, since this allergen has been detected along most of the gut including the anterior midgut, where it is presumably synthesized and where it is believed that the larger part of digestion takes place (Thomas et al., 1991; Hamilton et al., 2003; Colloff, 2009; Herman et al., 2014). Furthermore, as introduced earlier, an additional role for Der p 1 on the maturation of SP allergens Der p 3 (trypsin) and Der p 6 (chymotrypsin) has also been highlighted based on *in vitro* experiments with recombinant proteins (Dumez et al., 2014; Herman et al., 2014). This kind of orchestration of the activation of SP by a CP is rather uncommon, and depends on specific sequences at the C-terminus of these SP's pro-peptides that match cleavage specificity by Der p 1. Herein, by further analyzing WMC extracts from the cystatin A ingestion bioassay that exhibited an unexpected low SP activity despite not being their enzyme targets, we demonstrate for the first time *in vivo* that the activation of trypsin(s) and chymotrypsin(s) is tightly regulated by Der p 1. Specifically, pre-incubation of these extracts with exogenous nDer p 1 to surpass residual cystatin A inhibition, allowing SP activation, produced a remarkable increase in their trypsin and chymotrypsin activities in relation to controls. Notably, other exogenous CP (commercial papain and cathepsin B) were unable to activate SP activities, suggesting a Der p 1-specific activation.

Despite their relevance for the mite's allergenicity (Thomas, 2015; Reithofer and Jahn-Schmid, 2017), the actual physiological role of Der p 3, Der p 6 and other SP in the *D. pteronyssinus* digestive system is still intriguing. On the one hand, proteolytic profiling using azo-dye protein substrates revealed that fecal pellets and mature WMC, which are rich in feces, exhibit high SP activity together with CP (pH-dependent fashion). The analysis of activation of SP in cystatin A treated extracts also suggested that Der p 3 and Der p 6, and eventually other SP with similar

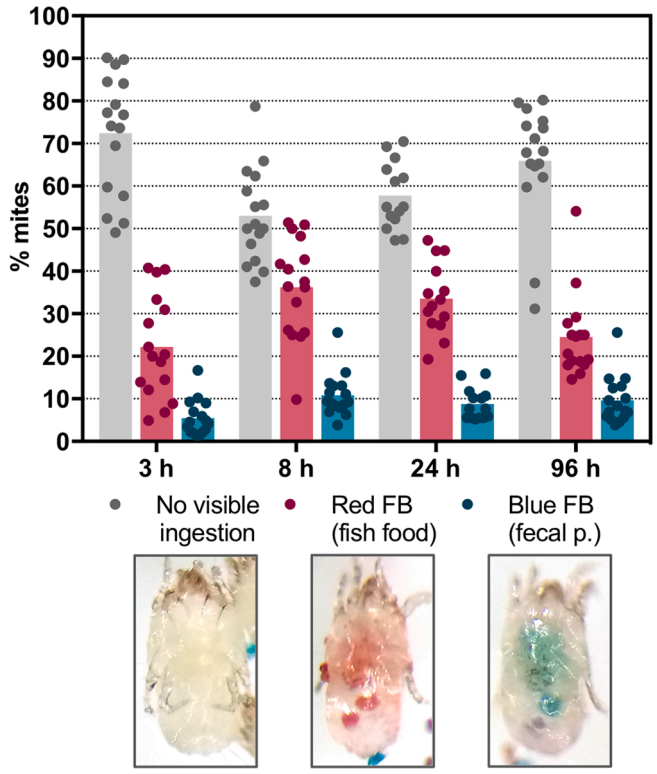

Fig. 5. Ingestion of fecal pellets by *D. pteronyssinus*. Mites (n = 70 ± 20) were exposed to two possible food sources, fish food (red stained) and fecal pellets (blue stained). The food ingestion preference was tracked at four time points (3, 8, 24 and 96 h) by the color of the food bolus (FB), which was visible through the mite *exo*-cuticle: no color indicates no ingestion; red color indicates fish food only; bluish color, including purple tones, indicates ingestion of fecal pellets only or together with fish food. Each dot represents the percentage of mites showing a specific choice of ingestion at each arena (i.e. biological replicate), bars indicate the average of sixteen biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

unusual pro-peptides activated by Der p 1 (Dumez et al., 2014), are the main sources of SP activity in *D. pteronyssinus* WMC, since total trypsin and chymotrypsin-like activities tripled and doubled after activation, respectively. In addition, previous works have shown that fecal-derived trypsin and chymotrypsin activities reach remarkable levels (Stewart et al., 1994; Vidal-Quist et al., 2017a), and SP allergens are generally highly abundant by proteomics in fecal extracts of the species, although this may vary among laboratory culture systems (Batard et al., 2016; Erban et al., 2017; Waldron et al., 2019). Fecal accumulation is a trait normally associated to digestive enzymes (Ortego et al., 2000; Hamilton et al., 2003). However, the low contribution of SP to the total protein hydrolysis in body extracts, and the absence of clear effects after ingestion of SP inhibitors shown in our study, raise questions on their precise digestive role. This discrepancy, low SP activity in crude body homogenates, but high SP activity in fecal extracts, has already been pointed out for *Dermatophagoides* spp. (Stewart et al., 1994), yet, so far no conclusive explanation has been proposed. A possible reason for the low SP activity in mite bodies could be that these enzymes were mainly in the proenzyme inactive state prior to excretion. Nevertheless, our *in vitro* activation tests of SP from body extracts with nDer p 1 showed that, in normal conditions, body-associated SP are mostly active. In addition, a tentative reason for the low impact of SP inhibitors in our experiments could be that they were eventually hydrolyzed/inactivated prior to binding their SP targets in the gut. Yet, our *in vitro* SBBI degradation tests indicate that, at the high dose of our bioassay, most of the inhibitor should have been functional *in vivo*. The pre-treatment of SBBI with nDer p 1 and/or its incubation in reducing conditions (i.e. DTT), as those expected in the mite's gut (Colloff, 2009), showed only a moderate reduction in trypsin inhibition potency. Interestingly, a previous report assessing the effect of protease inhibitors on *D. farinae* also showed that SBBI had no effect on mite survival, while CP inhibitors dramatically triggered mortality (Pernas et al., 2000). Immunolocalization of the SP allergens group 3 (trypsin) or group 6 (chymotrypsin) in *Dermatophagoides* spp., and visualization of fluorescent SP substrates on the storage mite *Lepidoglyphus destructor* have shown that these SP enzymes are synthesized and active mainly at the posterior end of the gut (posterior midgut and hindgut) (Zhan et al., 2010; Erban and Hubert, 2011; Herman et al., 2014). This is the region where SP would be concentrated and voided into fecal pellets, but its overall contribution to the proteolytic processing of foods is expected to be low compared to the anterior midgut (Hamilton et al., 2003; Colloff, 2009). In addition, the slightly acidic physiological conditions in this region are suboptimal for SP (Erban and Hubert, 2010b; Vidal-Quist et al., 2017a). Overall, our results support that such late-stage SP activity would be of secondary importance in digestion. However, since we used a laboratory diet of easy digestibility, we cannot discard that SP, which are tightly regulated by Der p 1, could be more relevant in natural conditions, where foods are more complex and contain skin scales rich in structural proteins (e.g. keratin, collagen, elastin) or fungi, and digestion is expected to be slower (Colloff, 2009).

Herein, we explored additional possible roles for the accumulation of functional proteolytic enzymes inside fecal pellets. By using color dyes and visualizing the food bolus of mites exposed to both normal diet and fecal pellets, we could demonstrate and quantify for the first time that *D. pteronyssinus* does indeed ingest fecal pellets on a regular basis, suggesting that autocoprophagia may be part of its routine feeding. This behavior has previously been suggested in HDM (Sporik and Platts-Mills, 1992; Colloff, 2009), but it had never been tested empirically. Our finding points out a possible participation of fecal proteases in an eventual extracorporeal digestion. According to this mechanism, the continued digestion of proteins *ex vivo* within the excreted fecal pellets by SP, or other enzymes, and their further re-ingestion and second passage through the gut could allow a more efficient digestion of foods, especially those of low digestibility. In our laboratory experiments, mite feces, as expected, were of insufficient nutritional value to sustain mite cultures on their own, yet they may have an added value when ingested in combination with food, or even before ingestion by facilitating the pre-digestion of fresh food by fecal enzymes released to the environment. It is to be noted that HDM do permanently spend their life cycle within their food, either in natural or commercial cultures, and, as the population evolves the accumulation of enzymatically-rich feces does modify the physical–chemical microhabitat. Interestingly, the supplementation of fresh laboratory HDM cultures with purified fecal fractions has shown to promote population growth in a previous study (McGregor and Peterson, 2000), yet, as revealed by our choice experiment, fecal pellets added to the diet do not appear to hold especial attraction for mites. Finally, autocoprophagy in astigmatic mites may favor the dissemination and maintenance of mite associated microbiota (Hubert et al., 2018).

The deployment of resources by the excretion of enzymatically-rich fecal pellets could also be related to other biological processes besides food digestion, such as the interaction of mites with microorganisms, the host, or its chemical environment. Regarding the latter, one of the defense mechanisms of arthropod herbivores against plant peptide-based protease inhibitors is to directly inactivate them by proteolysis (Zhu-Salzman and Zeng, 2015). Here we explored the possibility a similar mechanism in *D. peronyssinus* by which fecal enzymes excreted to the environment would degrade the antinutritional cystatin A present in the natural skin diet. However, *in vitro* pre-treatment of cystatin A with mite fecal proteases failed to affect its inhibitory potency, thus rejecting this hypothesis. Also, out of the scope of this study, fecal proteases released to the environment could putatively contribute in shaping bacterial and

fungal communities associated to HDM, which can be remarkably complex as the ecological interactions established in the HDM microhabitat (Hubert et al., 2019). Proteases can exhibit antimicrobial properties as reported in plants (Díaz et al., 2018) or, at the contrary, they may facilitate the *ex vivo* degradation of detritus on which microbes feed. Besides this interaction, HDM are detritivorous and have evolved in association to vertebrate hosts by dwelling nests of birds and mammals, including humans (Colloff, 2009; Klimov and OConnor, 2013). It could be speculated that, by delivering allergenic proteases into fecal pellets that exacerbate the hosts immune response (Reithofer and Jahn-Schmid, 2017) and eventually lead to dermatitis and skin exfoliation (Yamada et al., 2018), HDM could have evolved a mechanism to increase the provision of skin products on which they feed.

Finally, regarding the use of HDM extracts for the diagnosis and treatment of allergy, it is significant that the only inhibitor that substantially affected the mite's performance was cystatin A, the one to which HDM would naturally be exposed by consuming skin-derived products. For the sake of efficacy, it is believed that human exposure to HDM allergens by immunotherapy extracts should mimic as much as possible natural exposure to wild-type HDM (Waldron et al., 2019). Pharmaceutical HDM cultures are often reared using media that contain ingredients with protease inhibitors such as cereals, leguminous seeds, or by-products (Barber et al., 1996; Avula-Poola et al., 2012). Based on our results, it would be interesting to consider protease inhibitors when selecting new ingredients for HDM industrial media. Hence, ingredients exhibiting SP inhibitory potency would be less likely to affect HDM cultures and allergen production than ingredients inhibiting CP, particularly Der p 1.

5. Conclusions

This work contributes to the understanding of the HDM physiology, specifically regarding the role of the different protease groups to the digestive physiology of *D. pteronyssinus*. We have shown that digestion is mostly dependent on cysteine proteases, particularly on major allergen Der p 1. However, both cysteine and serine protease activities are found in HDM feces, raising questions on the involvement of the later in digestion and their possible roles *ex vivo*, particularly taking into account the species autocoprophagic behavior revealed in this work. We additionally demonstrate for the first time *in vivo* that the cysteine protease Der p 1 is necessary for the activation of at least two important *D. pteronyssinus* serine proteases. Future application of transcriptomic and proteomic approaches to the study of the digestive physiology of *D. pteronyssinus* using protease inhibitors would be useful to assess broader effects on both the mite's allergome and its proteolytic degradome.

6. Authors' contribution statement

J. Cristian Vidal Quist (JCVQ), Félix Ortego and Pedro Hernández Crespo participated in the conceptualization, design of experiments, and writing of the manuscript. JCVQ carried out bench experiments and data analysis. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2021.104285.

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