

GERMAN COCKROACH FRASS PROTEASES CLEAVE PRO-MATRIX METALLOPROTEINASE-9

Valerie S. Hughes □ *Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center and Cincinnati Children's Research Foundation, Cincinnati, Ohio, USA*

Kristen Page □ *Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center and Cincinnati Children's Research Foundation, Cincinnati, Ohio, USA; and Department of Pediatrics, University of Cincinnati, Cincinnati, Ohio, USA*

□ *Matrix metalloproteinase (MMP)-9, secreted as pro-MMP-9, is cleaved by serine proteases at the N-terminus to generate active MMP-9. Pro-MMP-9 has been found in the bronchoalveolar lavage fluid of patients with asthma. Because many inhaled aeroallergens contain active proteases, the authors sought to determine whether German cockroach (GC) fecal remnants (frass) and house dust mite (HDM) were able to cleave pro-MMP-9. Treatment of recombinant human (rh) pro-MMP-9 with GC frass resulted in a dose- and time-dependent cleavage. This was abrogated by pre-treating frass with an inhibitor of serine, but not cysteine protease activity. GC frass also induced cleavage of pro-MMP-9 from primary human neutrophils dependent on the active serine proteases in GC frass. HDM was less potent at cleaving pro-MMP-9. α_1 -Antitrypsin (A1AT), a naturally occurring protease inhibitor, attenuated GC frass-induced cleavage of pro-MMP-9. A1AT partially inactivated the serine protease activity in GC frass, while GC frass cleaved A1AT in a dose- and time-dependent manner. These data suggest that GC frass-derived serine proteases could regulate the activity of MMP-9 and that A1AT may play an important role in modulating GC frass activity in vivo. These data suggest a mechanism by which inhalation of GC frass could regulate airway remodeling through the activation of pro-MMP-9.*

Keywords α_1 -antitrypsin, asthma, feces, house dust mite, neutrophil, protease

Cockroach exposure has been recognized as an important cause of asthma for over 40 years. Exposure to cockroach is a major risk factor for hospital emergency room visits [1, 2] and morbidity [3] among inner city children

Received 6 February 2007; accepted 16 March 2007.

This work was supported by the National Institutes of Health grant HL075568 (KP).

Address correspondence to Kristen Page, PhD, Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, ML 7006, Cincinnati, OH 45229, USA. E-mail: kristen.page@cchmc.org

with asthma. Studies suggest that exposure to cockroach might be of particular importance relative to other allergens. Indoor concentrations of cockroach allergen, but not house dust mite, were found to be significantly associated with recurrent wheezing and asthma [4]. In a cohort study involving 849 participants, cockroach allergen (Bla g 1), but not dust mite allergen, was associated with an increased risk for wheezing [5]. Children exposed in the first 3 months of life to cockroach, but not house dust mite, develop allergen-specific T-cell proliferative responses and repeated wheezing [6]. Taken together, these studies have led investigators to speculate that cockroach allergens may promote sensitization through direct proinflammatory effects on the airways [7].

Asthma is characterized by airway inflammation and airway hyperresponsiveness. Airway inflammation is characterized by increased activated inflammatory cells, including eosinophils, neutrophils, lymphocytes, and mast cells. Airway hyperresponsiveness is mediated by the inflammation and also remodeling that occurs in the airways. Matrix metalloproteinase-9 (MMP-9) belongs to a family of proteases that function in the degradation of several extracellular matrix (ECM) components, and plays a role in airway remodeling. MMP-9 has been suggested to play a role in the pathogenesis of bronchial asthma. Levels of MMP-9 are significantly increased in bronchoalveolar lavage (BAL) fluid, blood, and sputum of asthmatics [8–11]. Increased MMP-9 levels have been shown in the bronchoalveolar lavage fluid of patients with untreated asthma [8], in patients with severe asthma compared with mild asthma or normal controls [12], and following allergen challenge in allergic individuals [9]. Increased concentrations of MMP-9 were found to be associated with increased concentrations of basement membrane proteins (collagen type III, collagen type V, and tenascin), suggesting the possibility that MMP-9 is playing a role in subepithelial fibrosis [13]. Together these data suggest that MMP-9 may be mediating early remodeling events, which may ultimately contribute to airflow obstruction and airway hyperresponsiveness.

Regulation of MMP-9 is influenced by endogenous activators and inhibitors [14]. MMP-9 is secreted from a cell as an inactive proenzyme (92 kDa), which is then cleaved into an active form (88 kDa). A discrete group of proteases, including trypsin, cathepsin G, α -chymotrypsin, MMP-3, and mast cell chymase [15], have been shown to cleave pro-MMP-9. Proteolytic cleavage of pro-MMP-9 and the enzymatic activity of mature MMP-9 can be inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). MMP-9 complexes with TIMP-1 in a 1:1 ratio and the dynamic balance between MMP and TIMP is thought to be a critical factor in regulating the breakdown of ECM proteins [16]. Pro-MMP-9 is produced by many cells that are involved in allergic airway inflammation, including eosinophils, neutrophils, alveolar macrophages, and

airway epithelium [15, 17]. However, it was shown in allergic patients challenged with allergen that MMP-9 immunoreactivity was associated primarily with neutrophils [9], suggesting neutrophils were the main source of MMP-9 in the airways.

α_1 -Antitrypsin (A1AT) is the main inhibitor of neutrophil elastase and is a key molecule in the defense of the lung. A1AT protects the lower respiratory tract against damage by proteases released during inflammation [18]. However, A1AT is produced mainly in the liver and is found in high concentrations in plasma (90 to 180 mg/dL), which can increase 2- to 4-fold during acute inflammation [19]. A1AT primarily inhibits neutrophil elastase, but may cleave other serine-like proteases. A1AT has been shown to be produced outside the liver by alveolar epithelial cells [20] and monocytes [21]. Sputum from asthmatics has been shown to contain higher levels of A1AT than normal controls [22].

Many allergens have been shown to contain protease activity, including house dust mite [23], fungus [24], cat [25], grass pollen [26], and cockroach [27, 28]. It is important to note that although none of the cockroach allergens characterized to date are active proteases, protease activity has been detected in whole extracts and fecal remnants (frass) of German cockroaches (GCs; *Blattella germanica*) [27, 28]. Because pro-MMP-9 has been measured in BAL fluid of patients with asthma, and because cockroach frass contains serine protease activity, we hypothesized that these proteases could cleave pro-MMP-9. To this end, we used GC frass and whole body house dust mite (HDM; *Dermatophagoides pteronyssinus*) extract to investigate the role of proteases on activating pro-MMP-9. We also investigated the role of the serine protease inhibitor A1AT on GC frass protease activity.

MATERIALS AND METHODS

Cockroach Frass and House Dust Mite

Fecal remnants (frass) were collected from German cockroaches (*Blattella germanica*) and reconstituted in endotoxin free water for 2 hours at 4°C with continual agitation. Debris was removed by centrifugation, supernatants harvested and protein was determined by using Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA). Protease activity was determined using the Azocoll assay as previously described [27]. To inhibit protease activity in frass, frass was pretreated with either aprotinin (a specific inhibitor of serine proteases; 10 μ g/mL for 30 minutes at 37°C) or E64 (a specific inhibitor of cysteine proteases; 10 ng/mL for 30 minutes at 37°C) or heat-inactivated (55°C for 1 hour). House dust mite was purchased from Greer Laboratories (Lenoir, NC) and reconstituted in endotoxin-free water.

Immunoblot Analysis

Frass was incubated with 80 ng of recombinant human MMP-9 (R&D Systems, Minneapolis, MN) or 80 ng of recombinant human α_1 -antitrypsin (A1AT; Calbiochem, San Diego, CA) at 37°C for 5 to 60 minutes. After incubation, samples were resolved on an 8% to 16% sodium dodecyl sulfate (SDS)-polyacrylamide gel as previously described. After incubation with an anti-human MMP-9 antibody (R&D Systems) or anti-human A1AT (Santa Cruz Biotechnology, Santa Cruz, CA), signals were amplified and visualized using enhanced chemiluminescence.

Cell Culture

Polymorphonuclear leukocytes were isolated from blood collected from healthy volunteers. This protocol was approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. Blood was drawn into Heparin and then subjected to dextran T500 sedimentation, Ficoll-Histopaque density gradient centrifugation, and hypotonic erythrocyte lysis [29]. All steps during the neutrophil isolation were maintained constant and all assays were performed immediately after isolation was complete. Polymorphonuclear neutrophils (PMNs) were resuspended in serum-deprived medium prior to treatment. Cells were treated with German cockroach frass (30 to 300 ng/mL), aprotinin-treated frass, heat-inactivated frass (boiled for 1 hour), or HDM (3 to 30 μ g/mL). Cells were treated for 4 hours with continual rocking and supernatants were harvested for analysis by zymogram.

Gelatin Zymography

Cell supernatants were collected after treatment with frass and were subject to gel electrophoresis on an 8% SDS-PAGE containing 1 mg/mL gelatin (Sigma Chemical, St Louis MO) under nonreducing conditions. The gel was washed for 30 min (2.5% Triton X-100, 3 mM NaN_3), rinsed for 30 minutes (2.5% Triton X-100, 50 mM Tris-HCl, 5 mM CaCl_2 , 1 μ M ZnCl_2 , 3 mM NaN_3 , pH 7.4), and then incubated overnight at 37°C (50 mM Tris-HCl, 5 mM CaCl_2 , 1 μ M ZnCl_2 , 3 mM NaN_3 , pH 7.4). After staining with Coomassie Blue (R-250, 0.125%), gelatin-degrading enzymes are detected as clear zones against a blue background. To confirm the specificity of the bands on the zymogram, 30 μ L of medium from control cells was incubated with a human MMP-9 antibody (Cell Signaling; 10 μ L for 1 hour on ice) prior to running on the gel.

Statistical Analysis

Western blots were scanned and density was determined by ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA). When applicable, statistical significance was assessed by 1-way analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by Student-Newman-Keul's multiple-range test.

RESULTS

Cockroach Frass Contains Active Serine Proteases

Reconstituted GC frass contained active serine proteases (19 $\mu\text{g}/\text{mg}$ frass). Pretreatment of GC frass with the serine protease inhibitor aprotinin inhibited 80% of protease activity as determined by the Azocoll assay [28]. We were unable to detect serine protease activity with the Azocoll assay in the commercially available HDM used in this study.

Cockroach Frass Cleaves Pro-MMP-9

To determine if cockroach frass cleaves MMP-9 from the pro to the active form, we incubated recombinant human pro-MMP-9 (92 kDa) with increasing concentrations (100 pg to 30 ng) of GC frass. In the presence of frass, conversion of the pro (92-kDa) form of MMP-9 to the active (88-kDa) form was demonstrated in a dose- (Figure 1A) and time- (Figure 1B) dependent manner. Following a 30-minute incubation of GC frass with pro-MMP-9, roughly half of the pro-MMP-9 was cleaved into the active form (Figure 1C). To determine if active proteases in frass play a role in cleavage of pro-MMP-9, we incubated the recombinant human pro-MMP-9 with frass that had either been pretreated with aprotinin (serine protease inhibitor) or E64 (cysteine protease inhibitor) or heat-inactivated (55°C for 1 hour). Inhibition of serine protease activity or heat-inactivation of frass abolished the conversion of the 92-kDa form to the 88-kDa form (Figure 1D). However, inhibition of cysteine protease activity using the selective inhibitor E64 did not abolish frass-induced cleavage of pro-MMP-9. A dose-concentration curve of E64 (10 to 300 ng/mL) was tested and no attenuation of frass-induced cleavage was found (data not shown). We attempted to further quantify the cleavage of MMP-9 using the commercially available fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂; however, the serine proteases in GC frass readily cleave that peptide and therefore the determination of GC frass-induced activation of MMP-9 could not be accomplished. Together, these data demonstrate the importance of GC frass serine proteases in mediating pro-MMP-9 cleavage.

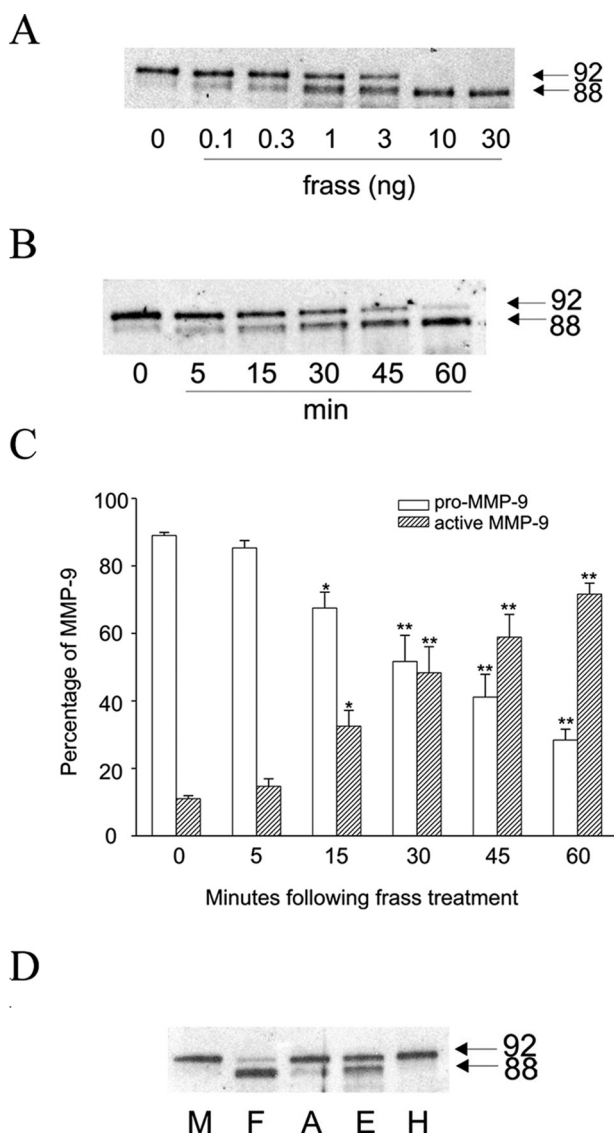


FIGURE 1 GC frass cleaves recombinant human pro-MMP-9. *A*, Various doses of frass (0.1 to 30 ng) were incubated with rh pro-MMP-9 (80 ng) for 1 hour at 37°C. MMP-9 was detected by Western blot analysis. The top arrow depicts pro-MMP-9 (92 kDa), the bottom arrow depicts active MMP-9 (88 kDa). The lower bands are further degradation products of MMP-9. This experiment was repeated 3 times. *B*, Frass (10 ng) was incubated with rh pro-MMP-9 for 0 to 60 minutes. This experiment was repeated 3 times. *C*, Quantification of the percentage of pro-MMP-9 and active MMP-9 from the Western blots detailed in *B*. Bars represent percent of pro-MMP-9 (*open bar*) and active MMP-9 (*hatched bar*) as mean $\pm$ SEM (compared to the untreated sample: * $P = .014$, ** $P < .001$; ANOVA). *D*, Frass (10 ng) was pretreated with either aprotinin (10 μ g/mL at 37°C for 1 hour), E64 (10 ng/mL at 37°C for 1 hour), or heat-inactivated (55°C for 1 hour) prior to its addition to rh pro-MMP-9. Rh pro-MMP-9 alone (M), or following treatment with frass (F), aprotinin-treated frass (A), E64-treated frass (E), and heat-inactivated frass (H) are shown. This experiment was performed 3 times.

House Dust Mite Cleaves Pro-MMP-9 but with Less Potency

Incubation of recombinant human pro-MMP-9 (92 kDa) with increasing concentrations of HDM (1 to 30 μ g) resulted in a dose-dependent cleavage of pro-MMP-9 into the active 88-kDa form (Figure 2). The highest concentration of HDM resulted in only a 27% cleavage of pro-MMP-9 (Figure 2B). Compare this to the cleavage of pro-MMP-9 using 10,000-fold less GC frass (30 ng resulting in 50% cleavage of pro-MMP-9; see Figure 1A). These data suggest that GC frass is a more potent activator of pro-MMP-9.

Cockroach Frass Cleaves Neutrophil-Derived Pro-MMP-9

Next, we sought to determine if cockroach frass could cleave neutrophil-derived pro-MMP-9. Primary human neutrophils were isolated and treated with increasing concentrations of frass. Isolation and culture of

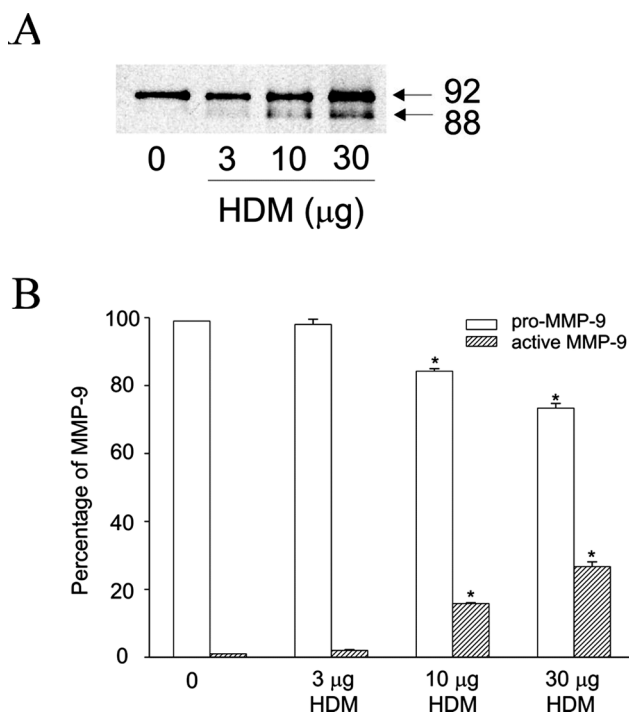


FIGURE 2 HDM cleaves recombinant human pro-MMP-9. *A*, Various doses of HDM (1 to 30 μ g) were incubated with rh pro-MMP-9 (80 ng) for 1 hour at 37°C. MMP-9 was detected by Western blot analysis. The top arrow depicts pro-MMP-9 (92 kDa), the bottom arrow depicts active MMP-9 (88 kDa). The lower bands are further degradation products of MMP-9. This experiment was repeated 3 times. *B*, Quantification of the percentage of pro-MMP-9 and active MMP-9 from the Western blots detailed in *A*. Bars represent percent of pro-MMP-9 (open bar) and active MMP-9 (hatched bar) as mean $\pm$ SEM (compared to control: * P < .001, ANOVA).

primary neutrophils resulted in the release of the biologically inactive proenzyme (92 kDa), the covalently bound complex of MMP-9 and neutrophil gelatinase-B-associated lipocalin (120 kDa), and MMP-9 homodimers (220 kDa) [30]. Conversion of the 92-kDa form to the active 88-kDa form was detected in a dose-dependent manner from a variety of donors (Figure 3A). Inhibition of the serine proteases in GC frass resulted in the lack of cleavage of pro-MMP-9 into active MMP-9 (Figure 3B). Frass alone did not cleave gelatin (data not shown). To confirm that the 92-kDa band was indeed pro-MMP-9, we treated cell culture medium from control cells with an antibody against MMP-9. This treatment resulted in the shifting of the 92-kDa band (data not shown). GC frass contains endotoxin (92.3 ng/mg frass); however, lipopolysaccharide (LPS) added at the same concentration as contaminating endotoxin did not cleave pro-MMP-9 (data not shown). In addition, HDM was ineffective at cleaving neutrophil derived pro-MMP-9, even with concentrations up to 100 µg/mL (data not shown).

α₁-Antitrypsin (A1AT) Inhibits GC Frass Protease Activity

We asked whether A1AT could inhibit GC frass proteases and abolish GC frass-derived pro-MMP-9 cleavage. To this end, we incubated GC frass with increasing concentrations of A1AT and quantified the serine protease activity by Azocoll. A1AT inhibited roughly 32% to 54% of the serine protease activity in GC frass (Figure 4A). Because A1AT can inhibit the protease activity in GC frass, we asked whether incubation of GC frass with A1AT would attenuate GC frass-induced cleavage of pro-MMP-9. Indeed, treating GC frass with increasing concentrations of A1AT resulted in a lack of cleavage of pro-MMP-9 in a dose-dependent manner (Figure 4B). Quantification of the partial reversal of frass-induced pro-MMP-9 cleavage by A1AT (10 µg/mL) pretreatment is shown in Figure 4C.

GC Frass Cleaves A1AT

Finally, we were interested in the role of GC frass on cleaving A1AT. To this end, we incubated recombinant human A1AT with increasing concentrations of GC frass and detected A1AT by Western blot. GC frass (100 ng) cleaved A1AT in a dose-dependent manner. The highest dose of GC frass (100 ng) resulted in the cleavage of 84% of A1AT (Figure 5A and B). Although we did not measure the activity of A1AT, we suspect that cleavage of A1AT resulted in its loss of activity. Lastly, we determined that GC frass mediated the cleavage of A1AT rapidly, i.e., within the first 5 minutes GC frass cleaves roughly 63% of the A1AT, and by 15 minutes 75% is cleaved (Figure 5C). Cleavage of A1AT is dependent on the serine proteases in frass

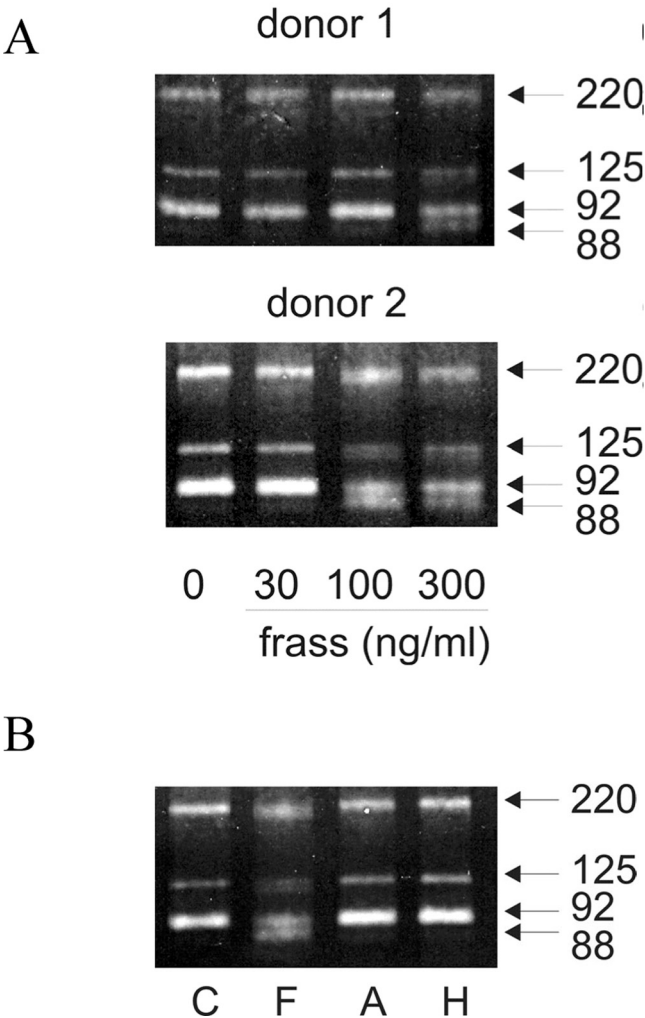


FIGURE 3 GC frass cleaves pro-MMP-9 in primary human neutrophils. *A*, Human neutrophils were isolated and incubated with increasing concentrations of frass (4 hours at 37°C). The supernatants were run on a gelatin zymogram. Shown here are neutrophils from 2 different individuals; however, this experiment has been performed on 4 other individuals with similar results. *B*, CR frass was preincubated with aprotinin (37°C for 1 hour) or heat-inactivated (50°C for 1 hour) prior to treatment of neutrophils. Cells were untreated (C) or treated with frass (F), aprotinin-treated frass (A), or heat-inactivated frass (H). Cell supernatants were subject to SDS-PAGE/gelatin electrophoresis under nonreducing conditions. Gelatinolytic activity is identified as clear zones on a blue background. This experiment was performed 3 times.

as preincubation of GC frass with the serine protease aprotinin abolished frass-dependent cleavage of A1AT, whereas frass pretreated with E64 had no effect (Figure 5D).

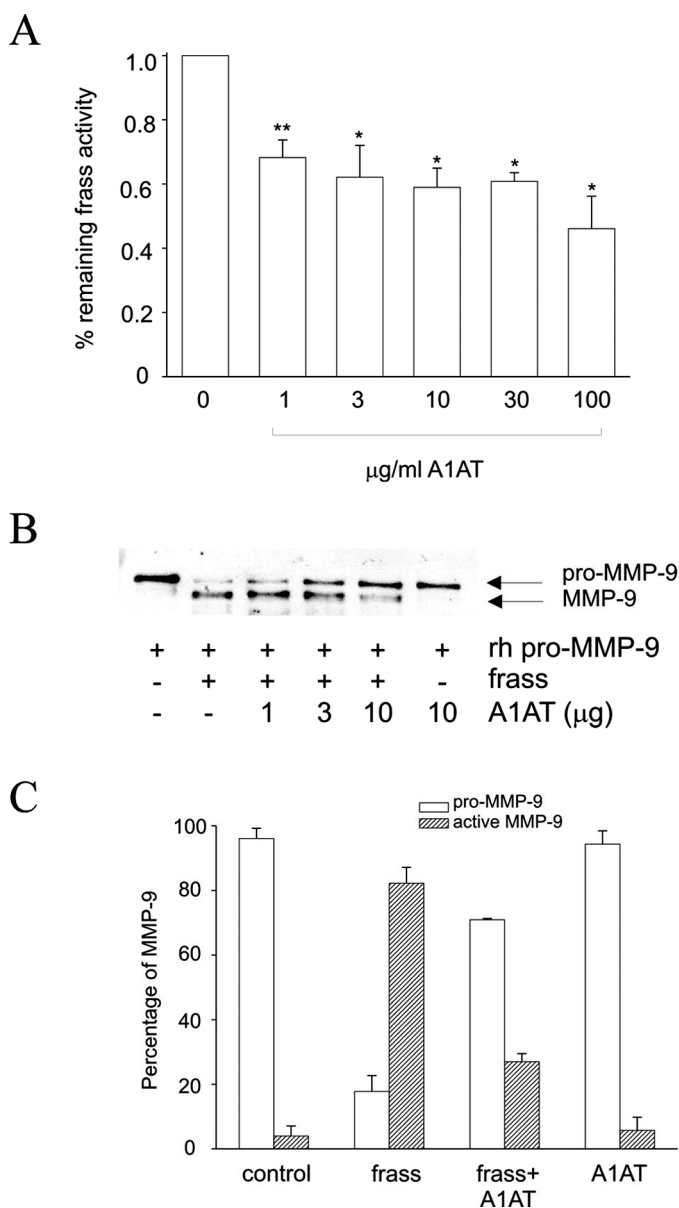


FIGURE 4 A1AT inactivates GC frass. *A*, GC frass was pretreated with increasing concentrations of A1AT (1 to 100 µg/mL for 1 hour at 37°C) prior to running the Azocoll assay. Data are presented as percentage of GC frass protease activity remaining following treatment with A1AT. Data represent means $\pm$ SEM for 4 experiments (compared to untreated: * $P < .001$, ** $P = 0.003$, ANOVA). *B*, GC frass (10 ng) was pretreated with A1AT (1 to 10 µg, 1 hour at 37°C) prior to the addition of pro-MMP-9 (80 ng) for 1 hour at 37°C. MMP-9 was detected by Western blot analysis. The top arrow depicts pro-MMP-9 (92 kDa), the bottom arrow depicts active MMP-9 (88 kDa). The lower bands are further degradation products of MMP-9. This experiment was repeated 3 times. *C*, Quantification of the percentage of pro-MMP-9 and active MMP-9 from the Western blots showing only the 10 µg concentration of A1AT as detailed in *B*. Bars represent percent of pro-MMP-9 (open bar) and active MMP-9 (hatched bar) as mean $\pm$ SEM.

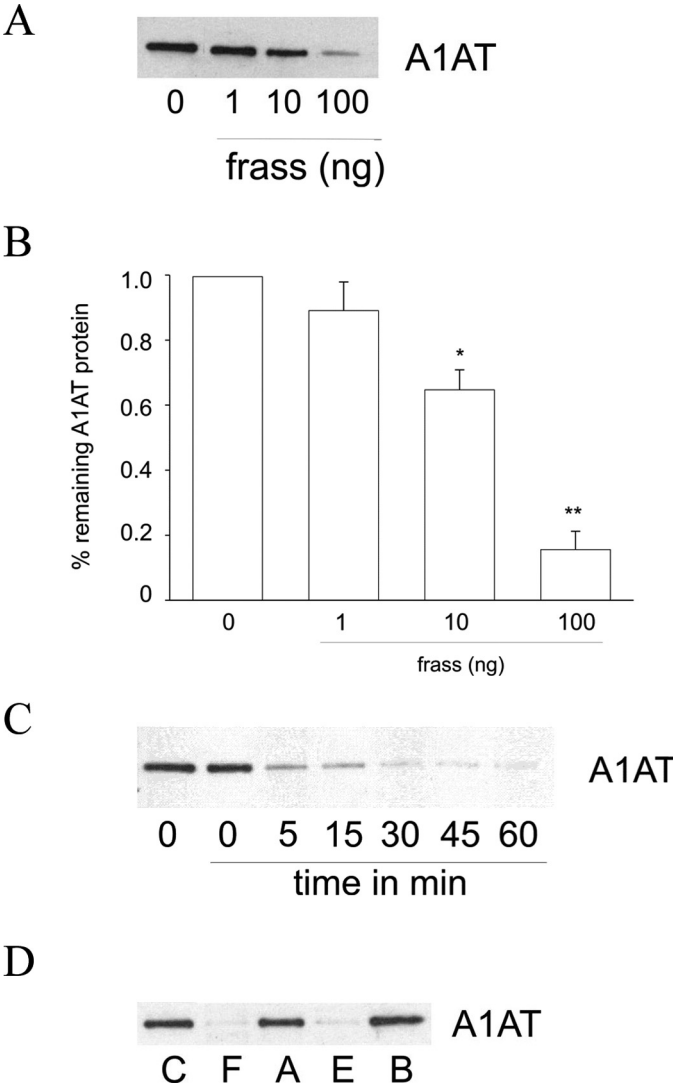


FIGURE 5 GC frass serine protease cleaves A1AT protein in a dose- and time-dependent manner. **A**, GC frass (1 to 100 ng) was incubated with rh A1AT protein (80 ng) for 1 hour at 37°C. A1AT was detected by Western blot analysis. **B**, Quantification of the percentage of remaining A1AT from the Western blot detailed in **A**. Bars represent percent of remaining A1AT protein as mean $\pm$ SEM (compared to control: * $P = .008$, ** $P < .001$; ANOVA). **C**, A1AT (80 ng) was treated with frass (10 ng) for 0 to 60 minutes. Western blot analysis was performed using an antibody against A1AT. This experiment was repeated 3 times with similar results. **D**, A1AT was untreated (**C**), or treated with frass (**F**), or frass pretreated with either aprotinin (**A**), E64 (**E**), or boiling (**B**). This experiment was repeated 3 times with similar results.

DISCUSSION

In this report, we have shown for the first time that GC frass serine proteases cleavage pro-MMP-9 (92 kDa) into its active form (88 kDa). HDM,

which using the Azocoll assay, did not contain serine protease activity, was only able to partially cleave recombinant human pro-MMP-9 at very high concentrations, but was unable to cleave neutrophil-derived pro-MMP-9. This, and the fact that inhibition of serine, but not cysteine proteases in GC frass inhibited cleavage, suggests the importance of serine protease activity in cleaving pro-MMP-9. During the preparation of our manuscript, a paper was published reporting that *Aspergillus fumigatus* and storage mite extracts cleaved pro-MMP-9 [31]. However, the concentrations of allergen used were 0.5 mg/mL for *Aspergillus* and 1 mg/mL for storage mite. Our data show that cleavage of recombinant human pro-MMP-9 occurred with as little as 100 pg frass, a concentration that conceivably could reach the airways. Treatment with 1 ng frass resulted in roughly 50% cleavage of recombinant human pro-MMP-9 into its active form, and no active form remained following treatment with 10 ng frass. GC frass also cleaved the physiological source of pro-MMP-9, namely neutrophil-derived pro-MMP-9; however, this cleavage required higher concentrations of GC frass. One possible explanation is that this source of pro-MMP-9 may be bound to TIMP-1, which may mask the pro-MMP-9 binding site. Together, these data suggest the potency of GC frass in cleaving pro-MMP-9 and the possibility of this occurring in vivo.

Frass is a likely source of allergen exposure because fecal extracts have been shown to contain 3 to 6 times more of the cockroach allergens Bla g 2 and Bla g 1 than CR whole body extracts [32]. This group also estimated the release of 3 µg dry weight of feces per cockroach per day. We hypothesize that the protease is either a cockroach-derived digestive enzyme or an enzyme derived from symbiotic bacteria that normally reside in the cockroach gut. Digestive enzymes, including amylases, invertase, maltase, β-glucosidase, esterases, and lipases, have been found in the gut of the American cockroach [33]. In addition, significant numbers of bacteria have been isolated from the midgut of the American cockroach, *Periplaneta Americana* [33]. Studies are currently underway to determine the source of the serine protease in GC frass. Importantly, it is thought that desiccated fecal remnants may easily incorporate into house dust. In fact, it has been shown that after disturbance, CR-carrying dust particles greater than 10 µm in diameter may be deposited in the airways [34]. The very low concentration of GC frass used in this study speaks for the physiological relevance of GC frass in the airways. In addition, we have preliminary data to suggest that proteases remain active in dust collected from carpeting in homes (K. Page and B. Lanphear, unpublished observations). This would suggest that the proteases may continue to be active even upon lying dormant for a period of time and may provide a possible therapeutic intervention in the future for those with asthma. It is likely that inhalation of desiccated particles into the moist airway would allow for solubilization and activation of these proteases.

HDM is known to contain both serine and cysteine proteases; however, in this study we were unable to determine serine protease activity using the Azocoll assay. The commercially available HDM was processed and lyophilized, which may have altered the serine protease activity. We did not test house dust mite feces, and it is possible that the proteases responsible for this effect are highly concentrated in the fecal remnants. It is important to note that the same preparation of HDM used in this study induced experimental asthma (i.e., increased airway hyperresponsiveness to acetylcholine in Balb/c mice similar to GC frass (K. Page, unpublished observations). These data suggests that GC frass serine protease is a more potent activator of pro-MMP-9 but further studies need to be performed to confirm this suspicion.

Pro-MMP-9 is synthesized by a variety of inflammatory cells, including neutrophils, macrophages, eosinophils, and lymphocytes, as well as airway epithelial cells. It is plausible that inhaled CR frass would come in contact with any of these cell types in the airways of an allergic/asthmatic individual. We tested frass on primary human neutrophils because they contain an abundance of pro-MMP-9; however, we would expect that pro-MMP-9 isolated from any of these cells would be cleaved.

A1AT, the naturally occurring protease inhibitor in serum, inhibited GC frass protease activity by about 30% to 50%. Interestingly, GC frass was able to cleave A1AT within a matter of 5 minutes, suggesting that the lack of A1AT inhibition on GC frass may be due to its own inactivation by the protease it is attempting to inactivate. This could explain why A1AT did not cause a dose-dependent decrease in GC frass activity. It has been shown that protease activity in one of the HDM proteins, Der p1, cleaved A1AT [35]. The same report showed that antiproteases in human bronchoalveolar lavage fluid were markedly lost following treatment with Der p1, suggesting the high susceptibility of A1AT to mite proteases. The importance of A1AT in modulating the asthma phenotype is highlighted by the fact that A1AT can inhibit antigen-dependent airway hyperresponsiveness in sheep [36]. These studies were performed by sensitizing the sheep to antigen and then 30 minutes following antigen challenge, the sheep were treated with aerosol A1AT. In humans, a relative decrease in A1AT activity resulted in the increase in elastase in the sputum, which was associated with increased bronchoconstriction [22]. This information highlights the need for better understanding of the regulation of A1AT in the airways and the importance of the protease/antiprotease balance.

Proteolytic cleavage of pro-MMP-9 and the enzymatic activity of mature MMP-9 can be inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). MMP-9 complexes to TIMP-1 in a 1:1 ratio and the balance between MMP and TIMP are thought to be critical factors in regulating the breakdown of ECM proteins [16]. The ratio of MMP/TIMP is important in a number of disease states including asthma and chronic obstructive pul-

monary disease (COPD). A recent study of COPD patients suggested that prior to an exacerbation the ratio of MMP-9/TIMP-1 was decreased and during the exacerbation, the ratio increased due to a significant increase in MMP-9 levels with a corresponding decrease in TIMP-1 levels [37]. A similar finding was reported in asthmatics, where upon exacerbation an increase in MMP-9 levels were detected [38]. The stoichiometric ratio of MMP-9/TIMP-1, when unbalanced in favor of MMP-9, has been shown to correlate with an increase in subepithelial fibrosis and a decrease in a fall in forced expiratory volume in 1 second (FEV₁) [13]. Recently, a polymorphism in TIMP-1 in the region responsible for TIMP-1 binding to and preventing activation of MMP-9 has been shown to correlate with asthma in Australian women [39]. These data suggest the importance of maintaining the protease/antiprotease balance in the airways.

Future work will be needed to establish the role of activation of pro-MMP-9 on airway remodeling *in vivo*. In addition, the role of A1AT in combating the inhaled GC frass serine protease will be an important area of investigation. It is also possible that when GC frass is inhaled, the proteases cause damage to the airways leading to leakiness and an increase in plasma-derived A1AT. Once in the airways, A1AT and GC frass are able to cleave each other and the concentration of each protein may be important in determining the protease/antiprotease state and regulation of airway remodeling.

REFERENCES

- [1] Pollart SM, Chapman MD, Fiocco GP, Rose G, Platts-Mills TA: Epidemiology of acute asthma: IgE antibodies to common inhalant allergens as a risk factor for emergency room visits. *J Allergy Clin Immunol.* 1989;83:875–882.
- [2] Gelber LE, Seltzer LH, Bouzoukis JK, Pollart SM, Chapman MD, Platts-Mills TA: Sensitization and exposure to indoor allergens as risk factors for asthma patients presenting to hospital. *Am Rev Respir Dis.* 1993;147:573–587.
- [3] Rosenstreich DL, Eggleston P, Kattan M, Baker D, Slavin RG, Gergen P: The role of cockroach allergy and exposure to cockroach allergen is causing morbidity among inner city children with asthma. *New Engl J Med.* 1997;336:1356–1363.
- [4] Litonjua AA, Carey VJ, Burge HA, Weiss ST, Gold DR: Exposure to cockroach allergen in the home is associated with incident doctor-diagnosed asthma and recurrent wheezing. *J Allergy Clin Immunol.* 2001;107:41–47.
- [5] Belanger K, Beckett W, Triche E, Bracken MB, Holford T, McSharry JE: Symptoms of wheeze and persistent cough in the first year of life: associations with indoor allergens, air contaminants, and maternal history of asthma. *Am J Epidemiol.* 2003;158:195–202.
- [6] Finn PW, Boudreau JO, He H, Wang Y, Chapman MD, Vincent C: Children at risk for asthma: home allergen levels, lymphocyte proliferation, and wheeze. *Allergy Clin Immunol.* 2000;105: 933–942.
- [7] Arruda LK, Vailes LD, Ferriani VPL, Santos ABR, Pomes A, Chapman MD: Cockroach allergens and asthma. *Curr Rev Allergy Clin Immunol.* 2001;107:419–428.
- [8] Mautino G, Oliver N, Chanez P, Bousquet J, Capony F: Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol.* 1997;17:583–591.
- [9] Kelly EAB, Busse WW, Jarjour NN: Increased matrix metalloproteinase-9 in the airway after allergen challenge. *Am J Respir Crit Care Med.* 2000;162:1157–1161.

- [10] Lee YC, Lee HB, Rhee YK, Song CH: The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. *Clin Exper Allergy*. 2001;31:1623–1630.
- [11] Cataldo DD, Bettiol J, Noel A, Bartsch P, Foidare JM, Louis R: Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. *Chest*. 2002;122:1553–1559.
- [12] Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ: Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. *Chest*. 2002;122:1543–1552.
- [13] Hoshino M, Nakamura Y, Sim JJ, Shimojo J, Isogai S: Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol*. 1998;102:783–788.
- [14] Das S, Mandal M, Chakraborti T, Mandal A, Chakraborti S: Structure and evolutionary aspects of matrix metalloproteinases: a brief overview. *Mol Cell Biochem*. 2003;253:31–40.
- [15] Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T: Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem*. 2003;253:269–285.
- [16] Nagase H: Activation mechanisms of matrix metalloproteinases. *Biol Chem*. 1997;378:151–160.
- [17] Ohbayashi H, Shimokata K: Matrix metalloproteinase-9 and airway remodeling in asthma. *Current Drug Targets Inflamm Allergy*. 2005;4:177–181.
- [18] Crystal RG: Alpha 1-antitrypsin deficiency, emphysema, and liver disease. Genetic basis and strategies for therapy. *J Clin Invest*. 1990;85:1343–1352.
- [19] Patterson SD: Mammalian alpha 1 antitrypsins: comparative biochemistry and genetics of the major plasma serpin. *Comp Biochem Physiol*. 1991;100:439–454.
- [20] Cichy J, Potempa J, Travis J: Biosynthesis of alpha 1-proteinase inhibitor by human lung derived epithelial cells. *J Biol Chem*. 1997;272:8250–8255.
- [21] Knoell DL, Ralston DR, Coulter KR, Wewers MD: Alpha 1-antitrypsin and protease complexation is induced by lipopolysaccharide, interleukin 1-beta, and tumor necrosis factor-alpha in monocytes. *Am J Resp Crit Med*. 1998;157:246–255.
- [22] Vignola AM, Bonanno A, Mirabella A, Riccobono L, Mirabella F, Profita M: Increased levels of elastase and alpha 1 antitrypsin in sputum of asthmatic patients. *Am J Resp Crit Care Med*. 1998;157:505–511.
- [23] King C, Brennan S, Thompson PJ, Stewart GA: Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol*. 1998;161:3645–3651.
- [24] Borger P, Koeter GH, Timmerman AB, Vellenga E, Tomee JFC, Kauffman HF: Proteases from *Aspergillus fumigatus* induced interleukin (IL)-6 and IL-8 production in airway epithelial cell lines by transcriptional mechanisms. *J Infect Dis*. 1999;180:1267–1274.
- [25] Ring PC, Wan H, Schou C, Kristensen AK, Roepstorff P, Robinson C: The 18-kDa form of cat allergen *Felis domesticus* 1 (Fel d 1) is associated with gelatin- and fibronectin-degrading activity. *Clin Exp Allergy*. 2000;30:1085–1096.
- [26] Grobe K, Poppelmann M, Becker WM, Petersen A: Properties of group I allergens from grass pollen and their relation to cathepsin B, a member of the C1 family of cysteine proteinases. *Eur J Biochem*. 2002;269:2083–2092.
- [27] Bhat RK, Page K, Tan A, Hershenson MB: German cockroach extract increases bronchial epithelial cell interleukin-8 expression. *Clin Exp Allergy*. 2003;33:35–42.
- [28] Page K, Hughes VS, Bennett GW, Wong HR: German cockroach proteases regulate matrix metalloproteinase-9 in human bronchial epithelial cells. *Allergy*. 2006;61:988–995.
- [29] Tennenberg SD, Zemlan FP, Solomkin JS: Characterization of *N*-formyl-methionyl-leucyl-phenylalanine receptors on human neutrophils. *J Immunol*. 1988;141:3937–3944.
- [30] Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE: Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. *J Allergy Clin Immunol*. 2003;112:1064–1071.
- [31] Simonen-Jokinen T, Maisi P, Tervahartiala T, McGorum B, Pirie S, Sorsa T: Direct activation of gelatinase B (MMP-9) by hay dust suspension and different components of organic dust. *Vet Immunol Immunopathol*. 2006;109:289–295.
- [32] Yun YY, Ko SH, Park JW, Lee IY, H.I. R, Hong CS: Comparison of allergic components between german cockroach whole body and fecal extracts. *Ann Allergy Asthma Immunol*. 2001;86:551–556.

- [33] Cruden DL, Markovetz AJ: Microbial ecology of the cockroach gut. *Ann Rev Microbiol.* 1987;41:617–643.
- [34] De Lucca SD, Taylor DJ, O'Meara TJ, Jones AS, Tovey ER: Measurement and characterization of cockroach allergens detected during normal domestic activity. *J Allergy Clin Immunol.* 1999;104:672–680.
- [35] Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C: House dust mite Der p1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Resp Cell Mol Biol.* 2003;29:381–389.
- [36] Forteza R, Botvinnikova Y, Ahmed A, Cortes A, Gundel RH, Wanner A: The interaction of alpha 1-proteinase inhibitor and tissue kallikrein in controlling allergic ovine airway hyperresponsiveness. *Am J Resp Crit Care Med.* 1996;154:36–42.
- [37] Mercer PF, Shute JK, Bhowmik A, Donaldson GC, Wedzicha JA, Warner JA: MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res.* 2005;6:151–160.
- [38] Oshita Y, Koga T, Matsuo K, Rikimaru T, Aizawa H: Increased circulating 92 kDa matrix metalloproteinase (MMP-9) activity in exacerbations of asthma. *Thorax.* 2003;58:757–760.
- [39] Lose F, Thompson PJ, Duffy D, Stewart GA, Kedda MA: A novel tissue inhibitor of metalloproteinase-1 (TIMP-1) polymorphism associated with asthma in Australian women. *Thorax.* 2006;60:623–628.