Destructin-1 is a collagen-degrading endopeptidase secreted by *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome

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Pseudogymnoascus destructans **is the causative agent of white-nose syndrome, a disease that has caused the deaths of millions of bats in North America. This psychrophilic fungus proliferates at low temperatures and targets hibernating bats, resulting in their premature arousal from stupor with catastrophic consequences. Despite the impact of white-nose syndrome, little is known about the fungus itself or how it infects its mammalian host.** ***P. destructans*** **is not amenable to genetic manipulation, and therefore understanding the proteins involved in infection requires alternative approaches. Here, we identify hydrolytic enzymes secreted by** ***P. destructans*****, and use a novel and unbiased substrate profiling technique to define active peptidases. These experiments revealed that endopeptidases are the major proteolytic activities secreted by** ***P. destructans*****, and that collagen, the major structural protein in mammals, is actively degraded by the secretome. A serine endopeptidase, hereby-named Destructin-1, was subsequently identified, and a recombinant form overexpressed and purified. Biochemical analysis of Destructin-1 showed that it mediated collagen degradation, and a potent inhibitor of peptidase activity was identified. Treatment of** ***P. destructans*****-conditioned media with this antagonist blocked collagen degradation and facilitated the detection of additional secreted proteolytic activities, including aminopeptidases and carboxypeptidases. To our knowledge, these results provide the first molecular insights into the secretome of** ***P. destructans*****, and identify serine endopeptidases that have the clear potential to facilitate tissue invasion and pathogenesis in the mammalian host.**

connective tissue | pathogenesis | peptidase | secretome | fungi

White-nose syndrome (WNS) has caused the deaths of more than 6 million bats in North America since its discovery in a New York cave in 2006 (1, 2). WNS has spread to 26 states in the United States and 5 provinces in Canada, with nearly 100% mortality observed in some locations (3). This spread represents one of the most precipitous declines in North American wildlife seen in the past century (1). If current trends continue, 25 species of hibernating bats in the United States will be threatened, with some previously common species becoming extinct (4). In addition to the devastating impact on bat populations, the disease is an economical threat to the North American agricultural industry, where the loss of bats could cost the industry more than $3 billion a year (5).

The causative agent of WNS is the fungus *Pseudogymnoascus destructans* (formerly *Geomyces destructans*) (6), which grows as a white layer on the muzzle, wings, and ears of bats (7). *P. destructans* is a psychrophilic fungus that belongs to the family *Pseudeurotiaceae*, and appears to be an invasive species with no close relatives in the hibernacula of North America (6). *P. destructans* targets hibernating bats whose normal immune function is reduced and whose body temperatures are lowered. The fungus grows optimally at these lower temperatures, with maximal growth between 12 °C and 16 °C (8). The injuries associated with fungal infections result in increased arousal in hibernating bats and the premature use of fat storage, with the outcome that bats are emaciated and die before the end of hibernation. Infection involves deep penetration of the subcutaneous tissue by fungal hyphae, causing ulcerative necrosis and tissue destruction (7, 9–11). *P. destructans* typically forms more superficial infections in European bat populations, with no evidence for associated mortality (9, 12), although a recent study also found evidence of invasive WNS lesions in European bats (13). Current models suggest that *P. destructans* is an invasive species that originated in Europe, where native bat species may be more resistant to the most debilitating forms of the disease (9).

There is currently little information as to the mechanism by which *P. destructans* causes tissue invasion or infection in bats. To begin to address the properties of *P. destructans* associated with WNS, we focused on secreted enzymes produced by this fungus. Many fungal pathogens secrete a number of important enzymes that promote pathogenesis, of which peptidases have been the most intensively studied (14, 15). Peptidases play diverse roles in fungal disease, as illustrated by the SAP family of aspartyl peptidases produced by pathogenic *Candida* species. In

Significance

To our knowledge, this work is the first to identify molecular factors produced by the fungus *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome in bats. Our study reveals the repertoire of redox enzymes and hydrolytic enzymes secreted by *P. destructans*. We establish that a secreted serine peptidase, Destructin-1, is a major component of the *P. destructans* secretome. This peptidase was purified and shown to degrade collagen, the major structural protein in mammalian connective tissue. Furthermore, chemical inhibition of Destructin-1 blocked collagen degradation in conditioned media from *P. destructans*. We therefore propose that serine endopeptidases aid in invasive growth and tissue destruction by the fungus, and represent potential targets for therapeutic intervention in white-nose syndrome.

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Data deposition: The raw mass spectrometry data files and peak lists are available at the ProteoSAFE resource, massive.ucsd.edu/ProteoSAFe/static/massive.jsp (accession no. MSV000079085).

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Candida albicans, the most common fungal pathogen of humans, these enzymes are implicated in multiple processes, including adhesion to epithelial cells, degradation of host proteins, survival and escape from immune cells, and invasion of mucosal tissues (16). Aspartyl and serine peptidases are also associated with dermatophyte fungi, which infect the stratum corneum, nails, and hair of animals; here, they are implicated in promoting adherence to host cells and degradation of keratin during tissue invasion (17, 18). Both *Candida* species and dermatophytes display expanded protein families of peptidases, supporting the idea that these molecules are key virulence factors (15, 18). Given their central role in pathogenesis, there is also now considerable interest in identifying inhibitors of fungal peptidases as potential therapeutic drugs (19). Other virulence factors secreted by mammalian fungal pathogens include lipolytic enzymes (lipases and phospholipases) that can further mediate the destruction of epithelial tissues (20).

In this work, we analyzed the secretome of *P. destructans* and found that the most abundant secreted proteins are predicted to have hydrolytic activity, including a number of peptidases, lipases, and glycosidases, or are redox enzymes, such as catalase peroxidase. The latter is an enzyme that can break down hydrogen peroxide using either catalase activity (hydrogen peroxide is converted to water and oxygen) or peroxidase activity (oxidizes the substrate using peroxide as a donor). Secreted peptidases included those with the ability to degrade collagen, the major component of mammalian connective tissue. To address global proteolytic activity, an unbiased substrate profiling assay was performed, and revealed that endopeptidases are the major proteolytic activities secreted by *P. destructans*. Using conventional chromatography and an internally quenched fluorescence reporter substrate, the major endopeptidase activity was isolated and shown to be associated with a serine endopeptidase, hereby-named Destructin-1. Recombinant Destructin-1 was overexpressed and purified, and shown to actively degrade collagen. Significantly, Destructin-1 activity was potently blocked by the serine peptidase inhibitor chymostatin, and treatment of conditioned media with this inhibitor blocked collagen degradation. Destructin-1 therefore represents a novel virulence factor for *P. destructans*, with the ability to promote tissue damage and invasion in the mammalian host.

Results

Hydrolytic Enzymes Are the Major Proteins Secreted by *P. destructans*. To identify proteins secreted by *P. destructans*, fungal cells were grown in RPMI medium at 13 °C for 7 d. Proteins from the conditioned medium were analyzed by peptide sequencing using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and peptides were searched against the *P. destructans* genome. In total, 44 proteins were identified in the secretome, of which 33 were found in at least two of three independent experiments, and 11 proteins were present at the limit of detection (Table S1 and Datasets S1 and S2). Many of these proteins were predicted to have enzymatic activity based on sequence analysis and were broadly grouped as hydrolytic enzymes, glycosyl transferases, or redox enzymes. The hydrolytic enzymes included 13 glycosidases, 6 peptidases, 2 lipases, and 1 amidase (Fig. 1*A*). The diversity of hydrolytic enzymes present is consistent with previous reports of multiple hydrolytic activities in *P. destructans* cultures, although the proteins responsible for these activities were not determined (21, 22). Many of these enzymes are likely to play a role in saprophytic growth, but peptidases have also been identified in the secretomes of the human pathogens *C. albicans* and *Aspergillus fumigatus* (23, 24), where they mediate host–pathogen interactions (14, 15).

The *P. destructans* secretome included three serine endopeptidases, two serine carboxypeptidases, and an aspartyl endopeptidase (Fig. 1*B*). The aspartyl endopeptidase shared 21–26% sequence identity with the *C. albicans* Sap protein family (25). The two carboxypeptidases were GMDG_06096, which is

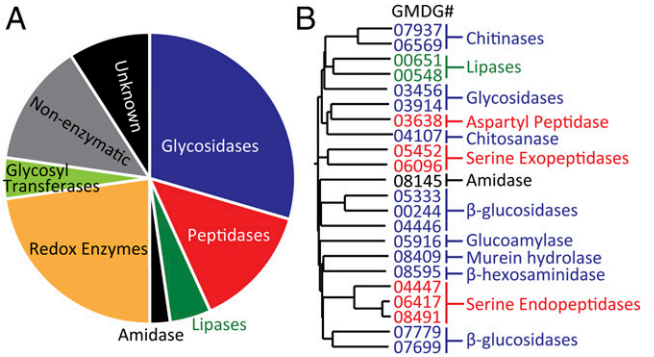

Fig. 1. Analysis of the secretome of *P. destructans*. (*A*) Composition of enzymatic activities present in conditioned medium from *P. destructans*. (*B*) Phylogenetic relationship between hydrolytic activities secreted by *P. destructans*. Secreted activities include three families of peptidases.

closely related to carboxypeptidase Y from *Saccharomyces cerevisiae* (56% sequence identity), and GMDG_05452, which is similar to carboxypeptidase II from *Aspergillus niger* (58% sequence identity). The three serine endopeptidases exhibited similarity to cuticle-degrading enzymes secreted by entomopathogenic fungi that are parasitic to insects (26). These included GMDG_06417 and GMDG_08491, which share 90% amino acid identity and are hereby named Destructin-1 and Destructin-2, respectively. A third serine peptidase, GMDG_04447, showed 56% identity to Destructin-1 and was named Destructin-3 (Fig. S1).

Collagen and Synthetic Peptides Are Degraded by Secreted Peptidases. One of the primary sites of infection by *P. destructans* is the membranous skin of bat wings, where it causes extensive invasion and tissue damage (27). To test whether peptidases in the secretome could contribute to wing damage and tissue invasion, conditioned media was incubated with azo dye-impregnated collagen. We observed a time-dependent release of dye over a 54-h time course (Fig. 2*A*). This finding led us to perform a comprehensive analysis of the proteolytic activity secreted from *P. destructans* with the goal of identifying and characterizing peptidases responsible for collagen degradation. We used a global and unbiased substrate-profiling assay to uncover the secreted proteolytic signature of this fungus. This assay consists of a mixture of 124 physiochemically diverse peptides that are each 14-residues in length. Cleavage at any one of the 1,612 peptide bonds within these peptides can be readily detected by LC-MS/MS sequencing (Fig. 2*B*) (28). Coincubation with the *P. destructans* secretome resulted in 137 cleavage sites detected after 1-h incubation and 308 cleavage events after 20-h incubation. The complexity of these hydrolytic events is illustrated in three example peptides where multiple cleavage sites were often detected within each peptide (Fig. 2*C*). Using iceLogo software (29), a substrate signature was generated corresponding to the global specificity of the peptidases in the media. These peptidases exhibited a preference for hydrophobic residues at P4, Ile and norleucine at P2, Gln, Phe, and Trp at P1, and Ile at P2′ (Fig. 2*D*). In addition, the detected peptidases showed a low tolerance for Glu in almost all positions and Val, Pro, and Gly at P1. Time-dependent trimming of amino acids from the termini of these peptides was not evident, indicating that exopeptidase activity was low compared with that caused by endopeptidases.

Endopeptidase Activity from *P. destructans* Can Be Monitored with Fluorescent Substrates. A diverse set of 15 internally quenched (IQ) fluorescent peptides (Dataset S3) was screened to identify substrates that could be used to monitor endopeptidase activity in *P. destructans*-conditioned media. Two of the 15 peptides were efficiently cleaved (Fig. 3*A*) and the sites of cleavage determined by MALDI-TOF mass spectrometry (Fig. S2). These substrates consisted of tQAS↓SRS (IQ8) and PKRLSAL↓L (IQ12), where t

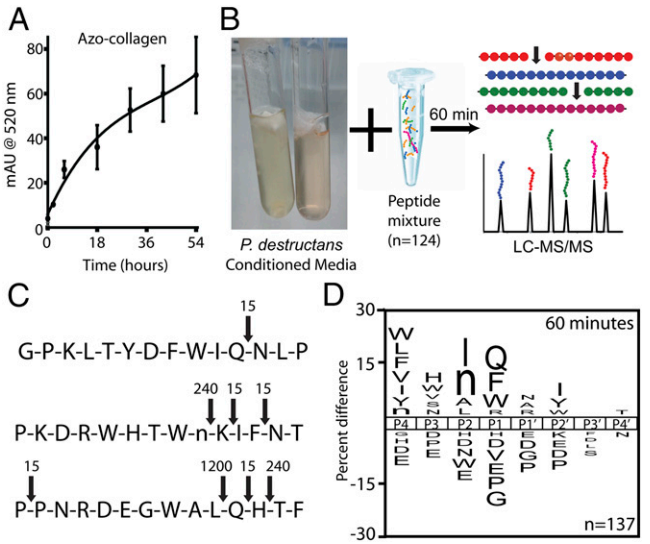

Fig. 2. Peptidase substrate specificity from *P. destructans*-conditioned medium. (*A*) Cleavage of azo-collagen by conditioned medium from *P. destructans*. (*B*) Outline of MSP-MS assay to examine peptidase activities in the *P. destructans* secretome. Conditioned media was incubated with a mixture of 124 peptides and sampled by LC-MS/MS peptide sequencing. (*C*) Cleavage sites are shown for three representative peptides in the MSP-MS assay. Incubation time at which cleavage events were first observed is indicated in minutes. (*D*) iceLogo generated from the pattern of cleavage events at 60 min shows the specificity of peptidase activity. Amino acids that are most frequently observed at each position are shown above the axis, and amino acids least frequently observed below the axis.

represents *tert*-butyl glycine and ↓ the position of cleavage. Analysis of these cleavage sites revealed the presence of a hydrophobic residue at P4 and Ala at P2 in both substrates, consistent with the global iceLogo substrate signature (Fig. 2*D*). However, these initial experiments did not determine whether the endopeptidase activity is derived from one or multiple enzymes.

Purification and Identification of Endopeptidases from *P. destructans*. To isolate the peptidases responsible for cleavage of IQ8 and IQ12 peptides, conditioned *P. destructans* medium was applied to a DEAE Sepharose column and eluted fractions assayed for proteolytic activity (Fig. 3*B*). Fractions with activity were pooled, applied to a Phenyl Sepharose column, and eluted fractions assayed again using IQ8 and IQ12 (Fig. 3*C*). Proteolytic activity on each of these substrates was found to copurify, and active fractions pooled and subjected to gel-filtration chromatography. Activity from the gel-filtration column identified a peptidase with a molecular weight of ~25 kDa (Fig. 3*D*). Analysis of protein from the active fractions showed two major bands on a silver-stained SDS/PAGE gel (Fig. 3*D*, *Inset*). These bands were excised and analyzed by LC-MS/MS, and the upper band shown to represent Destructin-1 (GMDG_06417). The lower, minor band was GMDG_08104, a highly abundant protein in the secretome that contains a WSC (cell wall integrity and stress response component) domain, and is not likely to contribute to the proteolytic activity of the purified fraction (see below). Although a number of unique peptides support the identification of Destructin-1 (Fig. S1 and Dataset S4), we cannot exclude the presence at lower abundance of the closely related protein, Destructin-2.

These results suggest that Destructin-1 encodes the major proteolytic activity responsible for cleavage of both IQ8 and IQ12 substrates. This enzyme shares 50–52% amino acid identity with secreted cuticle-degrading peptidases from nematode-trapping fungi, such as *Dactylella varietas* and *Arthrobotrys conoides* (DvS8 and AcAC1) (Fig. S1) (30, 31). In addition, Destructin-1 shares 46% identity with EaS8 (Fig. S1), a broad-spectrum endopeptidase from *Engyodontium album* that is stable in SDS, urea, chelating agents, and sulfhydryl reagents, and is commercially marketed as "Proteinase K." These enzymes use a catalytic triad of aspartic acid, histidine, and serine residues (32), which are conserved in Destructin-1 at positions 160, 192, and 345, respectively (Fig. S1).

Destructin-1 (along with Destructin-2 and -3) contains an N-terminal signal sequence and a prodomain that are predicted to be removed during secretion and catalytic maturation, respectively. Analysis of the N terminus of Destructin-1 using SignalP 4.0 (33) identified a signal peptide (residues 2–20) that was highly conserved with Destructin-2 and Destructin-3 (Fig. S1). Protein alignment with other fungal enzymes predicted that processing of the Destructin-1 prodomain occurs after Asn^{119} to yield a mature peptidase of 27.7 kDa, which correlates with its elution size from gel filtration (Fig. 3*D*). Peptide sequencing showed coverage exclusively within the mature peptidase domain (highlighted in Fig. S1) and the absence of tryptic peptides corresponding to the prodomain (Ala^{21}-Asn^{119}). This establishes that the protein species detected here is the activated form.

Expression and Characterization of Recombinant Destructin-1. To further characterize the activity of Destructin-1, a recombinant form of the proenzyme was expressed with a C-terminal hexahistidine tag and purified from *Pichia pastoris* (Fig. S3*A*). The resulting major band on a SDS/PAGE gel was excised and analyzed by MS sequencing and Edman degradation. These results established the identity of recombinant Destructin-1 and confirmed that the proenzyme is processed between Asn^{119} and Ala^{120} (Fig. S1). The recombinant Destructin-1 hydrolyzed IQ8 and IQ12 substrates with optimal activity between pH 9 and 10 (Fig. S3*B*). The temperature optimum was between 20 °C and 30 °C, with >50% of optimum activity retained at lower temperatures (4–15 °C) (Fig. S3*C*). As a control, a second his-tagged protein was purified from *P. pastoris*; this protein did not show cleavage activity on IQ8 or IQ12, supporting the specific degradation of these substrates by Destructin-1.

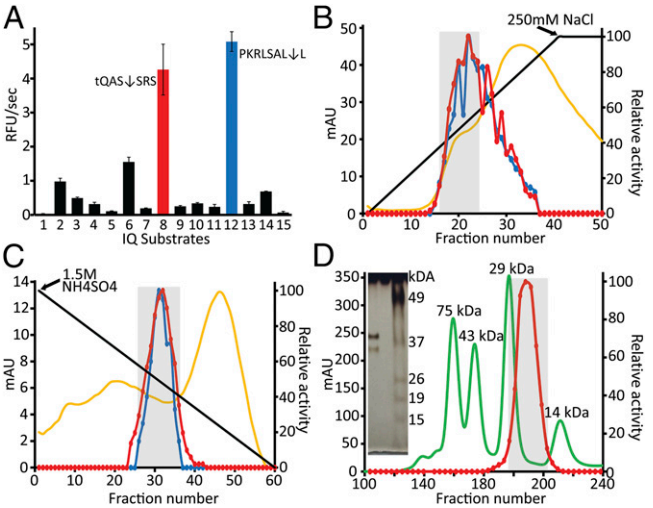

Fig. 3. Purification of a serine S8 peptidase, Destructin-1, from *P. destructans* conditioned medium. (*A*) Relative cleavage rates by *P. destructans* conditioned media on 15 different IQ substrates. Conditioned medium was purified using (*B*) DEAE sepharose, (*C*) Phenyl Sepharose, and (*D*) gel filtration. Peptidase activity was monitored using cleavage of IQ8 (red line) and IQ12 (blue line) substrates. Yellow line indicates milli absorbance units (mAU) at 280 nm and gray box shows fractions that were pooled for subsequent analysis. Green line indicates protein standards on gel filtration column. The most purified fraction was analyzed on a silver-stained SDS/PAGE gel (*D*, *Inset*).

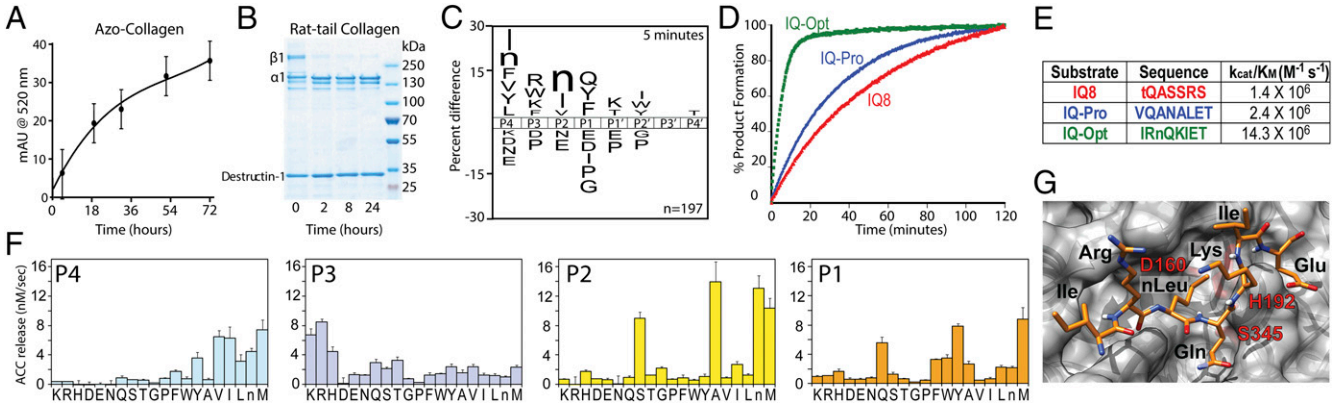

Fig. 4. Characterization of recombinant Destructin-1 activity. (*A*) Destructin-1 was incubated with Azo-collagen for 54 h at 20 °C and release of Azo dye measured at 520 nm. (*B*) Analysis of collagen degradation by Destructin-1 by SDS/PAGE. The α1 and β1 bands indicate the major protein components of collagen. (*C*) iceLogo analysis of the recombinant Destructin-1 activity in the MSP-MS assay. (*D*) Comparison of kinetics of cleavage between IQ8, IQ-Pro, and IQ-Opt substrates. (*E*) k_{cat}/K_m values are shown for IQ8, IQ-Pro and IQ-Opt substrates. (*F*) PS-SCL profiling of the recombinant Destructin-1 protein to determine cleavage specificity at P1–P4 positions. (*G*) Homology model of the Destructin-1 substrate-binding pocket (gray ribbons and semitransparent surface) with the IQ-Opt sequence IRnQKIE shown in orange, and the catalytic triad Asp160, His192, and Ser345 in red.

Degradation of Collagen by Destructin-1. Destructin-1 was assayed with azo dye-impregnated collagen for 72 h and shown to release dye in a time-dependent manner (Fig. 4*A*). The recombinant enzyme was also incubated with soluble rat-tail collagen and the hydrolytic products assessed by SDS/PAGE and Coomassie staining. As shown in Fig. 4*B*, collagen consists of several major protein bands; the lower molecular weight α-bands at ∼120 kDa consist only of triple helical protein, whereas the higher molecular weight β-bands contain additional nonhelical regions. Destructin-1 rapidly degraded the β-bands but did not cleave the α-bands, even after extended incubation. These experiments reveal that Destructin-1 readily degrades the nonhelical regions of collagen that function in the cross-linking of the helical components.

Rational Design of Optimal Fluorescent Substrates for Destructin-1. The substrate specificity of recombinant Destructin-1 was further investigated using an expanded MSP-MS assay containing 228 tetradecapeptides. Using 10 nM of enzyme, 197 peptide bonds were cleaved within 5 min, with a preference for Phe, Gln, and Tyr at P1. Hydrophobic residues were preferred at P4 and P2, with positively charged or bulky residues at P3. On the prime side of the scissile bond Lys and Thr were preferred at P1′ and Ile, Trp, and Tyr at P2′ (Fig. 4*C*). The P4 to P2′ specificity profile with the purified protein had a strong positive correlation (Pearson χ^2 test score > 0.7) to the specificity profile of the conditioned media, indicating that Destructin-1 was likely to be the major contributor to overall peptidase activity in the conditioned media (Dataset S5), as further discussed below.

The MSP-MS assay was validated as a tool for defining the substrate specificity of recombinant Destructin-1 by direct comparison with specificity data generated using a positional scanning synthetic combinatorial library (PS-SCL). The PS-SCL assay has been used to profile the P1 to P4 substrate specificity of more than 90 endopeptidases, most of which are serine and cysteine peptidases (34). This assay consists of 80 sublibraries, each containing 8,000 unique tetrapeptides linked to a fluorogenic 7-amino-4-carbamoylmethylcoumarin group on the C terminus. This assay cannot be used to characterize complex protease mixtures, such as conditioned media, because of an inability to detect aminopeptidase and carboxypeptidase activities and a requirement for >5 μg of each peptidase. As was observed in the MSP-MS assay, Destructin-1 preferentially cleaved substrates containing hydrophobic residues at P4, positively charged residues at P3, small or flexible residues at P2, and large bulky residues at P1 (Fig. 4*F*). Both assays showed a strong positive correlation of 0.86, 0.63, 0.54, and 0.73 (Pearson χ^2 test) at positions P4, P3, P2, and P1, respectively (Dataset S6).

Based on the substrate specificity data, we predicted that IQ8 and IQ12 were suboptimal substrates for Destructin-1. We have previously synthesized improved substrates for other peptidases based on the auto-activation site of the enzyme (35) or on the optimal sequences found in the substrate specificity profile (36). An IQ substrate was therefore synthesized corresponding to the P4 to P4′ residues at the pro–Destructin-1 activation site (VQAN-SLET) with flanking methylcoumarin and dinitrophenol groups (IQ-Pro). An additional IQ substrate was synthesized corresponding to the preferred residues in the P4 to P4′ positions from the MSP-MS assay (IQ-Opt). IQ-Opt was the most efficiently cleaved substrate with a k_{cat}/K_m of 14.3×10^6 M^{-1}/s^{-1}, which is a 10-fold improvement over IQ8 and 6-fold more efficient than IQ-Pro (Fig. 4 *D* and *E*). Cleavage of IQ-Pro indicates that the proprotein is likely to undergo auto-activation, and that activation should not require a transactivating peptidase when expressed in either *P. destructans* or *P. pastoris*. Both IQ-Pro and IQ-Opt could be accommodated into a homology model for Destructin-1, which was based on the structures of related subtilisin-like enzymes (Fig. 4*G* and Fig. S4). In the homology model, P3′ and P4′ positions of the peptide do not significantly interact with the enzyme, but there are deep hydrophobic S1 and S2 pockets on the enzyme that could bind to FYQ and nIV, respectively, consistent with the substrate recognition motif shown in Fig. 4*C*. These data highlight the value of specificity profiling to develop optimized peptide substrates that can serve as highly sensitive biochemical probes, even compared with natural peptide substrates.

Contribution of Destructin-1 to Global Proteolytic Activity in the *P. destructans* Secretome. To determine the contribution of Destructin-1 and related serine peptidases to global proteolytic activity, we tested known protease inhibitors for inhibition of Destructin-1 activity. Using the IQ8 substrate, we found that the serine inhibitors PMSF, antipain, and chymostatin were antagonists of Destructin-1 activity with IC_{50} values of 46.1 μM, 85 nM, and 7.5 nM, respectively (Fig. 5*A*). Addition of the potent agonist chymostatin to *P. destructans*-conditioned media resulted in a 77% reduction in collagen degradation at 54 h (Fig. 5*B*). This result indicates that Destructin-1, together with its close homologs, is the dominant collagen-degrading activity secreted by *P. destructans*.

The contribution of the chymostatin-sensitive serine endopeptidases to the global secreted proteolytic activity of *P. destructans* was

evaluated using the MSP-MS assay. Conditioned media was treated with either DMSO or chymostatin and incubated with the peptide library. The appearance of cleavage products was assessed after 15 min and 1, 4, and 20 h. Media that was treated with chymostatin resulted in a loss of 74–83% of the cleavage sites that were detected in the DMSO control at the equivalent time points (Fig. 5 *C* and *D*). In agreement with the specificity profiling experiments, this indicates that Destructin-1 and its homologs contribute most of the peptidase activity secreted from *P. destructans*. Interestingly, many of the cleavage sites that were resistant to chymostatin were located at the amino and carboxyl terminus. In fact, treatment with the inhibitor resulted in the appearance of additional cleavage sites at each termini (Fig. 5*E*). These sites were not detected in the control assay because the 14-mer substrates were rapidly degraded into short oligopeptides by the serine endopeptidases. The enzymes responsible for generation of cleavage sites at the termini are likely to be the exopeptidases detected in the proteomic study (Fig. 1). Taken together, these data indicate that chymostatin-resistant aminopeptidases and carboxypeptidases are present in the conditioned media, and are revealed upon inhibition of the dominant serine endopeptidases.

Discussion

WNS is a disease that has devastated bat populations in North America over the last decade. The causative agent is *P. destructans*, a fungus that results in extensive tissue damage to the bats, particularly to the fragile membranous wings (1). Connective tissue, vascular structures, and muscle fibers are degraded during infection, suggesting that hydrolytic enzymes are used by the invading pathogen (27). Secreted hydrolytic activities have been described by monitoring growth of *P. destructans* on a wide range of in vitro substrates (21, 22), but the fungal proteins responsible for these activities have not been elucidated.

In this work, we analyzed the secretome of *P. destructans* and identified a number of prevalent hydrolytic and redox enzymes. The array of secreted proteins shows similarities to those described in other fungal species, including the human pathogens *C. albicans* and *A. fumigatus* (23, 24, 37). We detected six peptidases secreted by *P. destructans* and determined that one or more of these enzymes degrades collagen, the major structural protein in mammalian tissue (38). We surmised that uncovering the peptidases responsible for collagen degradation would therefore be a valuable step toward understanding bat tissue invasion by *P. destructans*.

Destructin-1, a serine endopeptidase, was identified as the principal proteolytic activity present at neutral pH in *P. destructans* cultures. This enzyme was capable of degrading collagen, which consists of a core triple helix structure linked together by nonhelical cross-links to form a fiber (38). Collagenases, such as those produced by *Clostridium* species, readily degrade the helical regions of collagen (39). In contrast, Destructin-1 specifically cleaved the nonhelical cross-links between α1 and α2 proteins; this disrupts the integrity of collagen and may allow the fungus to penetrate further into the host tissue, possibly in combination with other peptidase activities.

An in-depth study of recombinant Destructin-1 activity was performed using an expanded MSP-MS assay containing 228 tetradecapeptides and a fluorescent library of 160,000 tetrapeptides. Destructin-1 readily cleaved on the C-terminal side of Gln, Tyr, and Phe residues, particularly when hydrophobic residues were present at the P4 position and Nle, Ile, or Val were present at the P2 position. This study represents the most detailed substrate specificity profile performed on a fungal peptidase to date, and allowed the design of a synthetic peptide that was a highly efficient substrate for Destructin-1.

The recombinant enzyme was potently inhibited by the serine peptidase antagonist chymostatin with an IC_{50} of 7.5 nM. Treatment of *P. destructans*-conditioned media with chymostatin established that Destructin-1 and its close homologs were responsible for collagen degradation. Inhibition of these endopeptidases

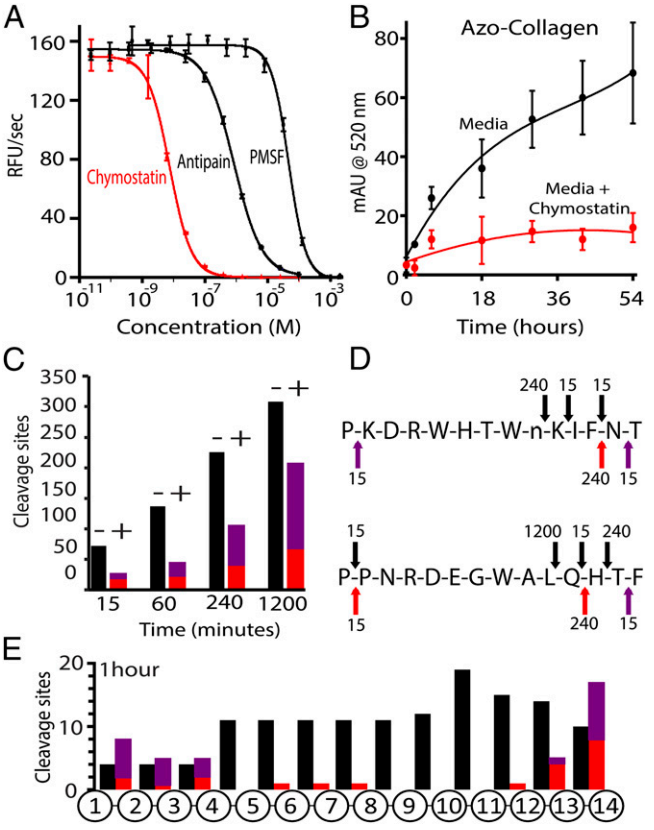

Fig. 5. Inhibition of Destructin-1 reveals the presence of other peptidases in the *P. destructans* secretome. (*A*) Inhibition of Destructin-1 activity using chymostatin, antipain, or PMSF. Assays were performed using the IQ8 substrate. (*B*) Cleavage of azo-collagen by conditioned media in the presence/absence of chymostatin. (*C*) Total number of cleavage sites in the MSP-MS assay using conditioned media in the presence (red) or absence (black) of chymostatin. Cleavage sites that are only present with chymostatin-treated media are colored purple. (*D*) Examples of two peptide substrates from the MSP-MS assay that are differentially hydrolyzed in the presence (red/purple arrows) or absence (black arrows) of chymostatin. The time in minutes at which cleavage events were first detected is indicated. (*E*) Positional analysis of peptide cleavage by conditioned media after 1-h incubation in the MSP-MS assay in the presence/absence of chymostatin. Color scheme the same as in *D*.

resulted in an overall loss of 74–83% of the peptide cleavage sites in the MSP-MS assay compared with the equivalent time points in vehicle-treated controls. Because inhibitor treatment prevented the breakdown of many substrates in the MSP-MS assay, additional proteolytic cleavage sites were detected that were derived from aminopeptidases and carboxypeptidases. The potential synergy between endopeptidases and exopeptidases is intriguing, as Destructin-1 may cleave intact proteins in the bat tissue, resulting in the appearance of neo-termini that could be substrates for exopeptidases.

The closest homologs of Destructin-1 are cuticle-degrading subtilisin peptidases found in nematode-infecting fungi, such as *A. conoides* and *D. varietas*. Nematophagous fungi use a variety of methods to capture and kill nematodes, which are subsequently digested by the fungi (26). The subtilisin-type peptidases promote penetration and digestion of nematode cuticles, and are key enzymes in nematophagous species for killing of their prey (26, 31, 40–42). A subtilisin-like serine peptidase was also recently identified in *Batrachochytrium dendrobatidis*, a chytrid fungus responsible for a global decline in amphibian species. This peptidase was shown to cleave antimicrobial peptides produced by frog skin, and is thus implicated in fungal survival and pathogenesis (43). Furthermore, the kexin gene in *C. albicans* encodes a

subtilisin-type protease that is necessary for virulence because of its role in processing of proproteins (44). These observations suggest that the family of subtilisin-type peptidases play diverse roles as fungal virulence factors.

In summary, this work details the composition of the *P. destructans* secretome and identifies the serine peptidase Destructin-1 as the major extracellular, collagen-degrading endopeptidase. Future studies will need to address the potential role of Destructin-1 and its homologs as novel virulence factors, and determine the role of other secreted proteins in promoting infection of epithelial tissues. It is expected that a combination of hydrolytic activities are used by *P. destructans* to invade and destroy bat tissues. As such, inhibiting these hydrolytic activities may well be a successful approach for the prevention or treatment of WNS in bats.

Materials and Methods

P. destructans was grown in RPMI medium at 13 °C for 7 d and conditioned medium used for LC-MS/MS analysis. Protein hits were identified from the sequenced genome at the Broad Institute of Harvard and Massachusetts Institute of Technology. Peptide degradation assays were performed at room temperature in PBS. Cleavage of internally quenched substrates was detected in a microplate reader using λ_{ex} 328 nm and λ_{em} 393 nm. Cleavage of the tetradecapeptide mixture was detected using LC-MS/MS after 15- to 1,200-min incubation. Purification of native Destructin-1 was achieved using DEAE, Phenyl Sepharose, and gel-filtration chromatography. Recombinant Destructin-1 was expressed and purified from *P. pastoris*. Peptide synthesis was performed using standard Fmoc chemistry. For full experimental details, see *SI Materials and Methods*.

While this paper was under review, an independent study by Pannkuk et al. (45) identified the same set of three subtilisin-like proteins as being the predominant peptidases secreted by *P. destructans* (in this case when *P. destructans* was grown on minimal nutrient broth supplemented with protein substrates). These results support subtilisin-like peptidases as being the major proteolytic activities produced by *P. destructans* under two culture conditions.

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