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Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(24)

ISSN

0027-8424

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Publication Date

2015-06-16

DOI

10.1073/pnas.1507082112

Peer reviewed

Destructin-1 is a Collagen-Degrading Endopeptidase Secreted by *P. destructans,* the Causative Agent of White-Nose Syndrome

Running Title: Collagen-Degrading Peptidase Destructin-1

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Abstract

P. destructans is the causative agent of White-Nose Syndrome (WNS), a devastating disease that has caused the deaths of millions of bats in North America. This psychrophilic fungus targets hibernating bats, resulting in their premature arousal from stupor with catastrophic consequences. Despite the impact of WNS, little is known about the fungus or how it mediates infection of the mammalian host. *P. destructans* is not amenable to genetic manipulation, and therefore understanding the proteins involved in infection requires alternative approaches. Here, we identify a set of proteolytic enzymes that are a part of a broad arsenal of hydrolytic enzymes secreted by *P. destructans*. Collagen, the major structural protein in mammals, was degraded by secreted peptidases from this fungus, and we therefore used a novel and unbiased substrate profiling technique to define active peptidases in the *P. destructans* secretome. These experiments revealed that endopeptidases are the major proteolytic activities secreted by *P. destructans*. 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. These results provide the first molecular insights into the secretome of *P. destructans*, and identify serine endopeptidase(s) that have the clear potential to facilitate tissue invasion and pathogenesis in the mammalian host.

Significance Statement

This work is the first to identify molecular factors produced by the fungus *P. 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 endopeptidase(s) aid in invasive growth and tissue destruction by the fungus, and represent potential targets for therapeutic intervention in WNS.

\body

Introduction

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). It has spread to 22 US states and 5 Canadian provinces, with nearly 100% mortality observed in some locations (3). This 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 US 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 dollars 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 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.*

77 *destructans* is an invasive species that originated in Europe, where native bat species may be
78 more resistant to the most debilitating forms of the disease (9).
79 There is currently little information as to the mechanism by which *P. destructans* causes
80 tissue invasion or infection in bats. To begin to address the properties of *P. destructans*
81 associated with WNS, we focused on secreted enzymes produced by this fungus. Many fungal
82 pathogens secrete a number of important enzymes that promote pathogenesis, of which
83 proteolytic activities have been the most intensively studied (14, 15). Peptidases play diverse
84 roles in fungal disease as illustrated by the SAP family of aspartyl peptidases produced by
85 pathogenic *Candida* species. In *Candida albicans*, the most common human fungal pathogen,
86 these enzymes are implicated in multiple processes including adhesion to epithelial cells,
87 degradation of host proteins, survival and escape from immune cells, and invasion of mucosal
88 tissues (16). Aspartyl and serine peptidases are also associated with dermatophytes that infect
89 the *stratum corneum*, nails, and hair of animals. Here, they are implicated in promoting
90 adherence to host cells and keratin degradation during tissue invasion (17, 18). Both *Candida*
91 species and dermatophytes display expanded protein families of peptidases, supporting the
92 contention that these factors are key virulence factors (15, 18). Given their central role in
93 pathogenesis, there is also now considerable interest in identifying inhibitors of fungal
94 peptidases as potential therapeutic drugs (19). Other virulence factors secreted by mammalian
95 fungal pathogens include lipolytic enzymes (lipases and phospholipases) that can further
96 mediate the destruction of epithelial tissues (20).
97 In this work, we analyzed the secretome of *P. destructans* and found that most proteins
98 are predicted to have hydrolytic activity, including a number of peptidases, lipases and
99 glycosidases, or are redox enzymes such as catalase peroxidase. Secreted peptidases included
100 those with the ability to degrade collagen, the major component of mammalian connective tissue.
101 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*

In order to identify proteins secreted by *P. destructans*, fungal cells were grown in RPMI medium at 13°C for 7 days. Proteins from the conditioned medium were analyzed by peptide sequencing using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and targets searched against the *P. destructans* genome. In total, 44 proteins were identified in the secretome, of which 33 were found in at least 2 of 3 independent experiments (Tables S1-S3). 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. 1A). 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

supporting saprophytic growth, but fungal peptidases can also function in supporting host-pathogen interactions (14, 15).

The *P. destructans* secretome included three serine endopeptidases, two serine carboxypeptidases, and an aspartyl endopeptidase (Fig. 1B). The aspartyl endopeptidase shared 21% to 26% sequence identity with the *C. albicans* Sap protein family (23). The two carboxypeptidases were GMDG_06096, which is 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 (24). 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 bats' wings, where it causes extensive invasion and tissue damage (25). 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 hour time course (Fig. 2A). This finding led us to perform a comprehensive analysis of the proteolytic activity secreted from *P. destructans* with the goal of identifying and characterizing peptidase(s) 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 1612 peptide bonds within these peptides can be readily

detected by LC-MS/MS sequencing (Fig. 2B) (26). Co-incubation with the *P. destructans* secretome resulted in 137 cleavage sites detected after 1-hour incubation and 308 cleavage events after 20 hours incubation. The complexity of these hydrolytic events is illustrated in three example peptides where multiple cleavage sites were often detected within each peptide (Fig. 2C). Using iceLogo software (27), 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. 2D). 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 rare and that the major activity was due to one or more endopeptidases.

Endopeptidase activity from *P. destructans* can be monitored with fluorescent substrates

A diverse set of 15 internally quenched (IQ) fluorescent peptides (Table S5) 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. 3A) 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 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. 2D). 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 peptidase(s) 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. 3B). Fractions with activity were pooled, applied to a Phenyl sepharose column, and eluted fractions assayed again using IQ8 and IQ12 (Fig. 3C). Proteolytic activity on these substrates was found to co-purify, 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. 3D). Analysis of protein from the active fractions showed two major bands on a silver-stained SDS-PAGE gel (Fig. 3D, 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 domain. A number of unique peptides support the specific identification of Destructin-1 (Fig. S1 and Table S4); however, due to the high sequence conservation with Destructin-2 it is not possible to exclude its presence at lower abundance. Indeed, analysis of individual protein bands excised after SDS-PAGE analysis of the Phenyl sepharose eluate showed the presence of Destructin-2-specific peptides (Table S4).

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) (28, 29). 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 utilize a catalytic triad of aspartic acid, histidine, and serine residues (30), which are conserved in Destructin-1 at positions 160, 192, and 345, respectively (Fig. S1).

Destructin-1, -2, and -3 contain an *N*-terminal signal sequence and a pro-domain that are predicted to be removed during secretion and catalytic maturation, respectively. Analysis of the N-terminus of Destructin-1 using SignalP 4.0 (31) 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 auto-catalytic processing of the Destructin-1 pro-domain occurs after Asn^{119} to yield a mature peptidase of 27.7 kDa, which correlates with its elution size from gel filtration (Fig. 3D). Peptide sequencing showed coverage exclusively within the mature peptidase domain (highlighted in Fig. S1) and the absence of tryptic peptides corresponding to the pro-domain (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 pro-enzyme was expressed with a C-terminal hexahistidine tag and purified from *Pichia pastoris* (Fig. S3A). 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 pro-enzyme is auto-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, and no activity was evident below pH 4.2 (Fig. S3B).

Degradation of collagen by Destructin-1

Destructin-1 was assayed with azo dye-impregnated collagen for 72 hours and shown to release dye in a time-dependent manner (Fig. 4A). 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. 4B, collagen consists of several major protein bands; the lower

molecular weight α-bands at ~120 kDa consist only of triple helical protein while the higher molecular weight β-bands contain additional non-helical regions. Destructin-1 rapidly degraded the β-bands but did not cleave the alpha bands, even after extended incubation. These experiments reveal that Destructin-1 readily degrades the non-helical 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 minutes, 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. 4C).

The MSP-MS assay was validated as a tool for defining the substrate specificity of recombinant PdSP1 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 (32). This assay consists of 80 sub-libraries 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 due to an inability to detect aminopeptidase and carboxypeptidases activity and a requirement for >5 μg of each peptidase. As was observed in the MSP-MS assay, PdSP1 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. 4E). Both assays showed a strong positive correlation of 0.86, 0.93, 0.54 and 0.73 (Pearson chi-squared

test) at positions P4, P3, P3 and P1, respectively (Table S6). This substrate signature represents the most detailed specificity profile of a peptidase from a fungal species to date.

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 peptidases based on the auto-activation site of the enzyme (33) or on the optimal sequences found in the substrate specificity profile (34). An IQ substrate was therefore synthesized corresponding to the P4 to P4' residues at the pro-Destructin-1 auto-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 kcat/Km of 14.3 x 10^6 M^{-1} s^{-1}, which is a 10-fold improvement over IQ8 and 6-fold more efficient than IQ-Pro (Fig. 4D). Both IQ-Pro and IQ-Opt could be accommodated into a homology model for the destructin-1 structure (Fig. 4F 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 F,Y,Q and n,I,V, respectively, consistent with the substrate recognition motif shown in Fig. 4C. These data highlight the use of specificity profiling to develop optimized peptide substrates that can serve as highly sensitive biochemical probes, even when compared to natural peptide substrates.

Contribution of Destructin-1 to global proteolytic activity in the *P. destructans* secretome

In order 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 IC50 values of 46.1 μM, 85 nM, and 7.5 nM, respectively (Fig. 5A). Addition of the potent agonist chymostatin to *P. destructans*

conditioned media resulted in a 77% reduction in collagen degradation at 54 hours (Fig. 5B). This confirms 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 minutes and 1, 4 and 20 hours. Media that was treated with chymostatin resulted in a loss of 74% or more of the cleavage sites that were detected in the DMSO control (Fig. 5C-D). This indicated that Destructin-1 and its homologs are the source of 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. 5E). 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). Together, this data indicates that chymostatin-resistant aminopeptidases and carboxypeptidases are present in the conditioned media, and are revealed upon inhibition of the dominant serine endopeptidases.

Discussion

White-Nose Syndrome is a devastating disease that has targeted bat populations in North America over the last decade. The disease is caused by *P. destructans*, a fungus that infects hibernating bats and causes extensive tissue damage, particularly to the fragile membranous wings (1). Connective tissue, vascular structures, and muscle fibers are degraded during

299 infection, suggesting that hydrolytic enzymes are used by the invading pathogen (25). Secreted
300 hydrolytic activities have been described by monitoring growth of *P. destructans* on a wide range
301 of *in vitro* substrates (21, 22), but the fungal proteins responsible for these activities have not
302 been elucidated.

303 In this work, we analyzed the secretome of *P. destructans*, and identified a number of
304 prevalent hydrolytic and redox enzymes. The array of secreted proteins shows similarities to
305 those described in other fungal species, including the human pathogens *C. albicans* and *A.*
306 *fumigatus* (35, 36). These fungi produce multiple hydrolytic enzymes that target host cells,
307 including peptidases that function in tissue degradation, nutrient acquisition and host invasion
308 (37). *P. destructans* secretes two serine carboxypeptidases (S10 family), an aspartyl peptidase
309 (A1 family) and three serine endopeptidases (S8 family). Our functional studies determined that
310 one or more of these peptidases degrades collagen, the major structural protein in mammalian
311 tissue (38). Therefore, we surmised that uncovering the peptidase(s) responsible for
312 degradation of this protein would be a valuable step towards understanding bat tissue invasion
313 by *P. destructans*.

314 A global and unbiased substrate profiling technology (26) was used to determine that
315 endopeptidase-type activities dominate the *P. destructans* secretome. Using a set of fluorescent
316 reporter substrates, a serine endopeptidase, Destructin-1, was identified as the principal
317 proteolytic activity present in *P. destructans* cultures. A recombinant form of the enzyme was
318 purified and shown to be capable of degrading collagen. In contrast, no cleavage was observed
319 by Destructin-1 on keratin and only very weak activity on elastin (data not shown). Collagen
320 consists of a core triple helix structure linked together by non-helical cross-links to form a
321 collagen fiber (38). Collagenases such as those produced by *Clostridium* species readily degrade
322 the helical regions of collagen (39). In contrast, however, Destructin-1 specifically cleaved the
323 non-helical cross-links between alpha 1 and 2 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 was shown to readily cleave 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 us to design a synthetic peptide that was a more efficient substrate than one corresponding to the pro-Destructin-1 auto-activation site.

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 resulted in a loss of 85% of the peptide cleavage sites in the MSP-MS assay compared to a vehicle-treated control. Interestingly, because inhibitor treatment prevented the breakdown of many substrates in the MSP-MS assay, proteolytic activities derived from other peptidases could now be detected. Analysis of the proteolytic activities uncovered by chymostatin treatment revealed that aminopeptidases and carboxypeptidases were present in the media. 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 are then substrates for trimming by exopeptidases.

The closest homologs of Destructin-1 are cuticle-degrading subtilisin peptidases found in nematophagous 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 (24). The

subtilisin-type peptidases promote penetration and digestion of nematode cuticles, and are key enzymes in nematophagous species for killing of their prey (24, 29, 40-42). Interestingly, 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 anti-microbial 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 due to its role in processing of proproteins (44). This suggests that the family of subtilisin-type peptidases can 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 further address the potential role of Destructin-1 and its homologs as novel virulence factors, and will 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, limiting these hydrolytic activities is predicted to be a successful approach for the prevention or treatment of WNS in bats.

Materials and Methods

Proteome analysis, biochemical assays, protein expression and protein purification were performed as described in *SI Materials and Methods*.

Acknowledgements

This research was funded in part by the UCSF Program for Breakthrough Biomedical Research, and by the Sandler Foundation (to C.S.C.). Mass spectrometry analysis was performed in the Bio-Organic Biomedical Mass Spectrometry Resource at UCSF (A.L. Burlingame, Director) supported

374 by the Biomedical Technology Research Centers program of the NIH National Institute of General
375 Medical Sciences, NIH NIGMS 8P41GM103481. Work was also supported by the NIH
376 T32GM007601 (to C.B.) and P50GM082250 (to C.S.C). The authors also thank Jeremy A. Horst
377 for structural modeling assistance with the Destructin-1 homology model.
378

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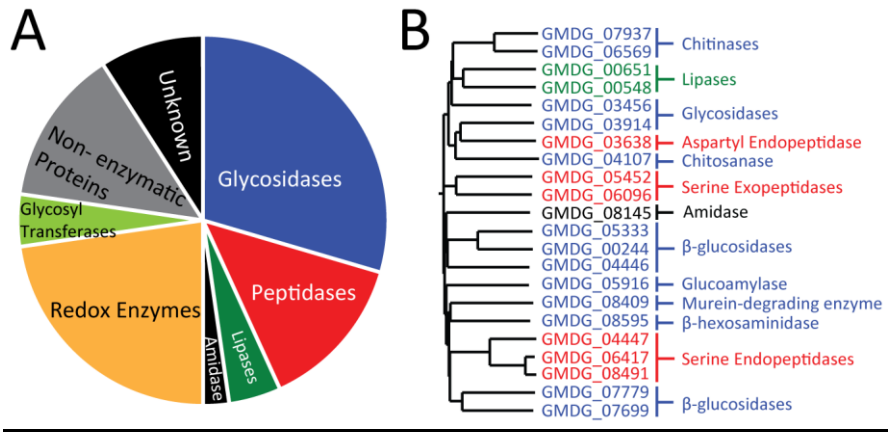
483

Figure Legends



<u>Figure 1</u>. 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*. Note that secreted activities include three families of peptidases.

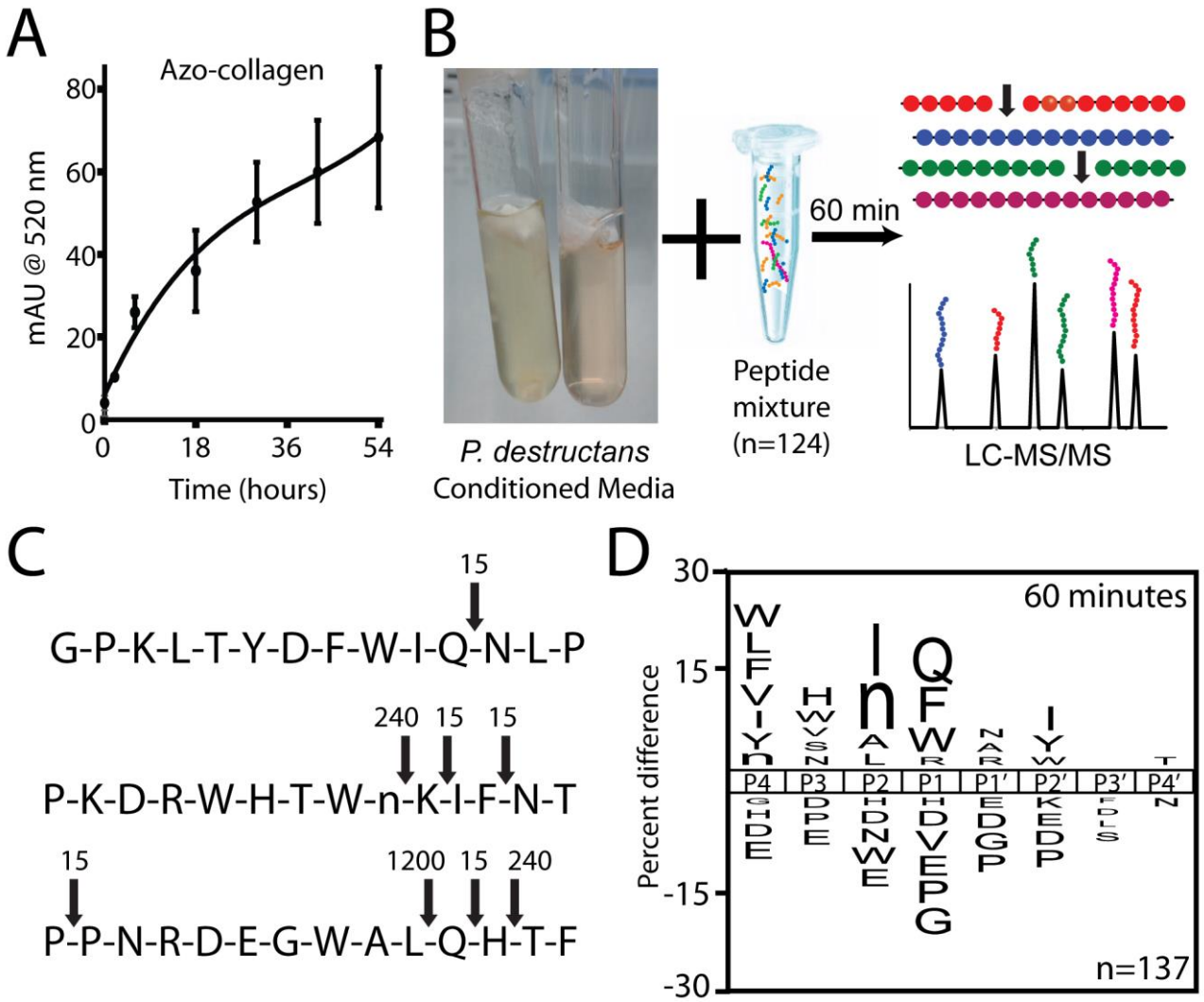
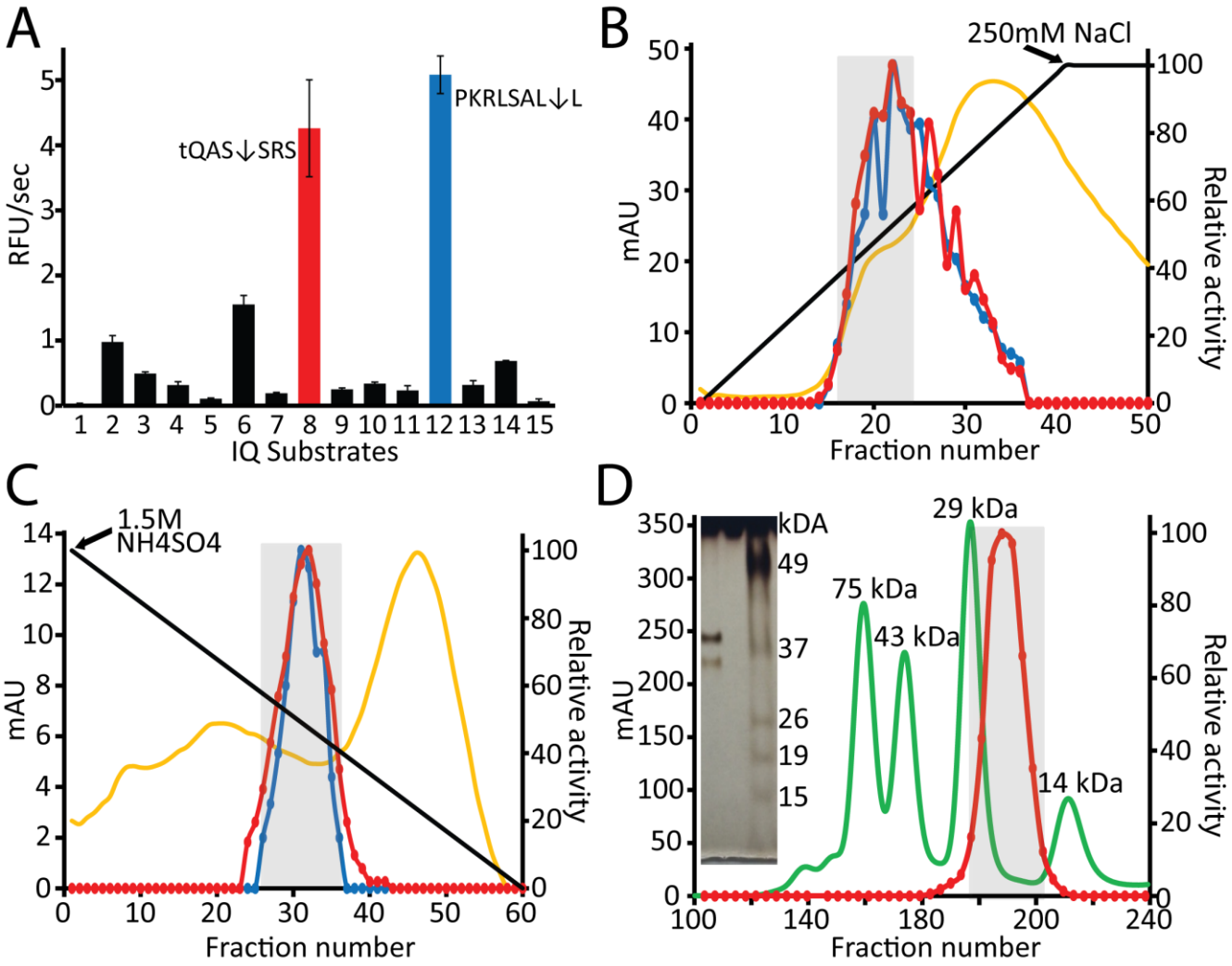


Figure 2. Peptidase substrate specificity from *P. destructans* conditioned medium.

(**A**) Cleavage of azo-collagen by conditioned medium from *P. destructans*. (**B**) Outline of the MSP-MS assay used to examine peptidase activities in the secretome of *P. destructans*. Conditioned media was incubated with a mixture of 124 peptides and sampled at subsequent time points 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 are shown below the axis.



501

502 **Figure 3. Purification of a serine S8 peptidase, Destructin-1, from *P. destructans***

503 **conditioned medium.**

504 (**A**) Analysis of relative cleavage rates by *P. destructans* conditioned media on 15 different IQ

505 substrates. Conditioned medium was purified using a 3-step process using (**B**) DEAE sepharose,

506 (**C**) Phenyl sepharose, and (**D**) gel filtration. Peptidase activity was monitored using cleavage of

507 IQ8 (red line) and IQ12 (blue line) substrates. Yellow line indicates total protein by absorbance

508 at 280 nm and the grey box shows the fractions that were pooled for subsequent separation or

509 characterization. Green line indicates protein standards on gel filtration column. The most

510 purified fraction was also analyzed on a silver-stained SDS-PAGE gel (inset, part D).

511

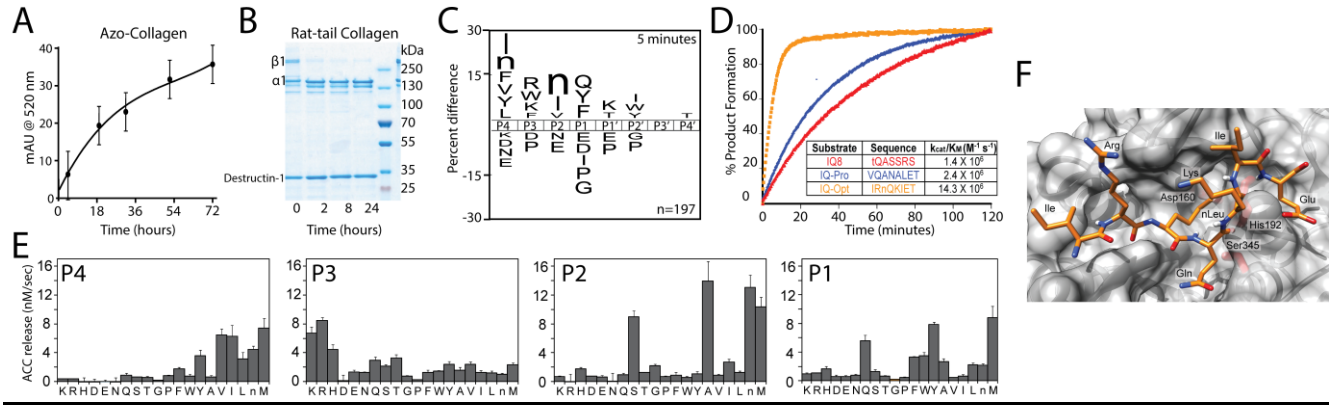
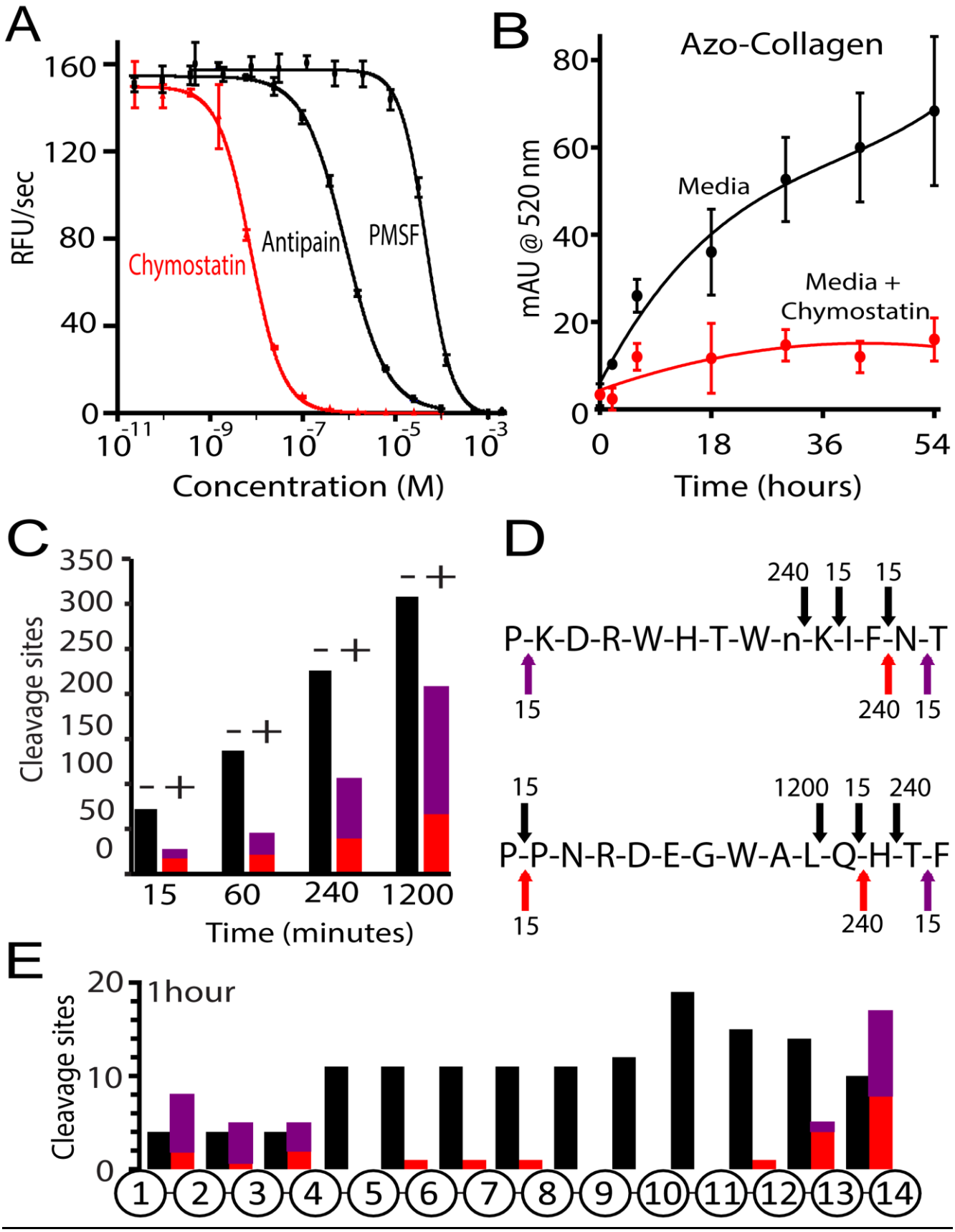


Figure 4. Characterization of recombinant Destructin-1 activity.

(**A**) Destructin-1 was co-incubated with Azo-collagen for 54 hours at 20°C and the release of Azo dye measured photometrically at 520 nm. (**B**) Cleavage and analysis of collagen degradation by Destructin-1 by SDS-PAGE. α1 and β1 bands indicate the major protein components of collagen. (**C**) iceLogo analysis of the recombinant Destructin-1 protein in the MSP-MS assay. (**D**) Comparison of kinetics of cleavage between IQ8, IQ-Pro and IQ-Opt substrates. kcat/Km values are shown for each IQ substrate. (**E**) PS-SCL profiling of the recombinant Destructin-1 protein to determine cleavage specificity at P1-P4 positions. (**F**) Homology model of the Destructin-1 substrate-binding pocket (grey ribbons and semitransparent surface) with the IQ-Opt sequence IRnQKIE shown in orange, and the catalytic triad residues Asp160, His192, and Ser345 in red.


A
160
120
80
40
0
RFU/sec
Chymostatin
Antipain
PMSF
10-11
10-9
10-7
10-5
10-3
Concentration (M)
B
Azo-Collagen
80
60
40
20
0
mAU @ 520 nm
Media
Media +
Chymostatin
0
18
36
54
Time (hours)
C
350
300
250
200
150
100
50
0
Cleavage sites
- +
- +
- +
- +
15
60
240
1200
Time (minutes)
D
240 15 15
P-K-D-R-W-H-T-W-n-K-I-F-N-T
15
240 15
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1200 15 240
P-P-N-R-D-E-G-W-A-L-Q-H-T-F
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240 15
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10
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Cleavage sites
1hour
1 2 3 4 5 6 7 8 9 10 11 12 13 14

524

Figure 5. Inhibition of Destructin-1 reveals the presence of other peptidases in the *P. destructans* secretome.

(**A**) Inhibition of Destructin-1 peptidase activity using chymostatin, antipain or PMSF inhibitors. Activity assays were performed using the IQ8 substrate. (**B**) Cleavage of azo-collagen by Destructin-1 in the presence or absence of chymostatin. (**C**) Total number of Destructin-1 cleavage sites in the MSP-MS assay in the presence (red) or absence (black) of chymostatin. Cleavage sites that are only present in the presence of chymostatin are colored purple. (**D**) Examples of two peptides from the MSP-MS assay cleaved by recombinant Destructin-1 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 Destructin-1 after 1 hour incubation in the MSP-MS assay in the presence or absence of chymostatin. Color scheme is the same as in D.