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Fungal hemolysins

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Abstract

Hemolysins are a class of proteins defined by their ability to lyse red cells but have been described to exhibit pleiotropic functions. These proteins have been extensively studied in bacteria and more recently in fungi. Within the last decade, a number of studies have characterized fungal hemolysins and revealed a fascinating yet diverse group of proteins. The purpose of this review is to provide a synopsis of the known fungal hemolysins with an emphasis on those belonging to the aegerolysin protein family. New insight and perspective into fungal hemolysins in biotechnology and health are additionally presented.

Keywords

fungi; hemolysins; aegerolysins

Introduction

Hemolysins have been classically defined as exotoxins that are capable of lysing red blood cells as well as nucleated cells. Current knowledge suggests that hemolysins are pore-forming toxins that interact with specific ligands on the surface of various target cells [1]. Although extensively studied in various bacterial species [1,2], hemolysins have also been reported in fungi [3,4], plants [5], invertebrates [6–8], and mammals (perforins) [9]. Bacterial hemolysins have been well characterized due to the role of these proteins in pathogenesis and, their structural details, mechanisms of hemolysis, ligand differences on target cells, and diagnostic potential have been described [2,10–19]. In contrast, less is known about fungal hemolysins. In this review, we aim to describe the structural and biochemical features and the pleiotropic functions of these fungal proteins, with emphasis on aegerolysins. Furthermore, we identify current issues with the isolation and characterization of these proteins and provide perspectives into the possible role for these proteins in fungal

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biology, health, and biotechnology. In addition, we will discuss comparisons to bacterial hemolytic proteins that share sequence homology to fungal hemolysins.

Hemolysins were first reported in higher fungi in 1907 and 1911 by W. W. Ford while studying various basidiomycete genera including *Amanita*, *Entoloma*, *Lactarius*, and *Inocybe* [20,21]. In 1939, Henrici, a microbiologist at University of Minnesota in Minneapolis, reported the first hemolytic activity in filamentous fungi while investigating the pathogenic ascomycete species, *Aspergillus fumigatus* and *Aspergillus flavus* [22]. The authors reported that extracts collected from the mycelium of the fungus grown for 2 – 4 days were heat sensitive, hemolytic, and produced necrosis and edema when introduced in experimental animals. Further studies by Salvin in 1951 at the Rocky Mountain Laboratory in Hamilton, Montana, led to the identification of similar hemolytic activity in the endemic fungal pathogens, *Histoplasma capsulatum* and *Blastomyces dermatidis*, as well as the opportunistic pathogens, including *Candida albicans* and *Cryptococcus neoformans* [23]. This was the first report that suggested the existence of more than one fungal hemolysin. To date, hemolysins have been identified and partially characterized from a wide variety of fungal species and these are summarized in Table 1.

During the last decade, the study of fungal hemolysins has benefitted from efforts to sequence the genomes of medically and biotechnologically important fungal species [24–29]. Using bioinformatic approaches, these databases have enabled investigators to identify homologous hemolytic proteins from other fungal species. One cluster of these proteins, including hemolytically active representatives has been identified as the Aegerolysin family (IPR009413, Pfam: PF06355; Table 1) [30], with Aa-Pri1 (aegerolysin) as the prototype. Aegerolysins form the largest group of fungal hemolysins that have been characterized. They are generally small in size (15 – 20 kDa) with a β -sheet structure, conserved cysteine residues, and characterized by a large number of aromatic and acidic residues. These proteins are stable over a wide range of pH but are heat labile above temperatures of 60 – 65°C. Interestingly, aegerolysins have also been identified in plants such as *Selaginella moellen-dorffi* [31], insects like *Chrysodeixis includens*, and the dsDNA virus Trichoplusia ni ascovirus 2c [32,33].

However, proteins from other families have also been implicated in hemolysis. The genomic data derived from fungi has also aided in the identification of homologous proteins in bacterial species including *Pseudomonas aeruginosa*, *Clostridium bifermentans*, *Burkholderia glumae*, *Vibrio cholerae* as well as some others [34–37].

Mechanism of hemolysis

Fungal hemolysins are typically found in a β -sheet conformation, barring the exception of nigerlysin [30,38–40]. An *in silico* secondary structure analysis of protein sequences suggests that all the proteins belonging to the aegerolysin family are β -sheet proteins. The aegerolysins are pore-forming proteins that aggregate on the cell surface after recognition of distinct membrane components. The process of pore formation is dependent on protein conformation and unfolding as for all pore-forming toxins [1]. Experimentally, ostreolysin was found to bind in a β -sheet conformation and then unfold into an α -helical structure

following permeabilization of target vesicles [41]. Similarly, unfolding of ostreolysin from a β -sheet conformation to α -helical structure prior to interacting with target cells resulted in the loss of lytic activity [38]. These studies demonstrate that the β -sheet conformation is an important prerequisite for initial ligand recognition on target cells that results in eventual permeabilization.

Bacterial β -sheet hemolysins have been characterized as small β -pore forming toxins such as aerolysin (*Aeromonas hydrophila*) and α -toxins (*Staphylococcus aureus* and *Clostridium septicum*), cholesterol dependent cytolysins (CDCs) such as streptolysin O (*Streptococcus pyogenes*) and listeriolysin O (*Listeria monocytogenes*) or AB toxins such as the diphtheria toxin (*Corynebacterium diphtheriae*) [1]. Current knowledge of fungal hemolysin structure does not provide enough information for similar categorization. Fungal hemolysins such as ostreolysin have been reported to form pores 4 nm in size [41], slightly larger than that reported for aerolysin (2 nm) [42]. Elsewhere, in the two-component hemolysin system of *Pleurotus ostreatus*, pleurotolysin A and B form pores of 3.8 – 5 nm in size [43]. Flammutoxin has been reported to form pores of at least two different sizes [44]. Collectively, these sizes reported in these studies are smaller than those typically observed for other β -pore-forming toxins (15 – 30 nm) and CDCs (350 – 500 nm) [45].

Some fungal hemolysins from the aegerolysin family also appear to act like thiol-activated CDCs in terms of receptor specificities. Like many CDCs, ostreolysin has been reported to require interaction with cholesterol rich domains in the membrane [46]. However, ostreolysin cannot bind pure cholesterol and only interacts with cholesterol when it is in association with sphingomyelin. These observations are similar to those made for the bacterial cytolysin of *Vibrio cholerae* [47].

The two component hemolysin system of pleurotolysin A and B in *P. ostreatus* appears to have involvement of the A and B components, but do not behave like the AB toxins in bacteria [43]. It appears that unlike the B subunit of the AB toxin in bacteria, which does not insert in the membrane of target cells, both pleurotolysin components appear to bind to the membrane directly. Based on these experimental findings, fungal hemolysins likely represent a novel class of β -sheet pore-forming toxins that form pores closer to the size of small β -pore-forming toxins but rely on interactions with cholesterol rich domains like CDCs.

Lectin-based hemolytic activity has also been reported in the parasitic basidiomycete, *Laetiporus sulphureus* [48]. This hemolysin has structural similarities to α toxin from *Clostridium septicum* and the mosquitocidal toxin (MTX2) derived from *Bacillus sphaericus* [49]. The hemolytic protein designated as LSL (*Laetiporus sulphureus* lectin) is composed of two domains with different functions. The N-terminal domain recognizes carbohydrate epitopes, while the C-terminal domain is required for oligomerization. Interestingly, the C-terminal domain is very similar in structure to aerolysin (*A. hydrophila*) [50] and the removal of this domain in mutagenesis studies completely removed hemolytic activity. These findings are consistent with the principle that oligomerization on the surface of target cells is an essential step prior to pore formation. More detailed information on LSL can be found elsewhere [51].

Very few studies have examined the kinetics of fungal hemolysin binding to target cells [52,53]. Colloid-osmotic mechanisms have been proposed as the principle mechanism associated with hemolysis [30,41,54]. This mechanism has been reported in bacteria for δ -toxin of *Bacillus thuringiensis*, θ -toxin of *Clostridium perfringens* and the hemolysins of *Vibrio vulnificus* and *Moraxella bovis* [55–58]. For fungal hemolysins, both ostreolysin and aegerolysin show the typical sigmoidal curve of hemolysis, which is characterized by an initial lag phase followed by rapid lysis of target cells [4]. The lag phase is characteristic of colloid-osmotic mechanism for lysis and likely represents the time required for monomer binding and oligomerization on the surface of target cells followed by the gradual influx of ions and water. This leads to swelling and eventually results in the lysis of the target cells.

Secretion of hemolysins

In contrast to bacterial hemolysins that are secreted, the destination of fungal hemolysins remains unclear and has not been characterized. Initial studies on the secretion of fungal hemolytic factors reported contradicting observations due to the limitations associated with extract preparation [22,59–62].

In a study characterizing asp-hemolysin, polyclonal antibodies were generated to a recombinant asp-hemolysin and used to demonstrate the hemolysin in the supernatant of *A. fumigatus* cultures [63]. Proteomic analysis of *A. fumigatus* and *A. nidulans* demonstrated that asp-hemolysin and its homologue (Accession No. CAK37181) are present in fractions secreted from growing hyphae [64–66]. Furthermore, asp-hemolysin was recently reported as the 4th most abundant protein in the *A. fumigatus* secretome [66].

Studies that utilized *A. fumigatus* glycosylphosphatidylinositol (GPI) mutants showed that asp-hemolysin was absent from the secreted fraction of *apg-a* mutants [67]. This suggests a possible role for GPI proteins in contributing to secretion of hemolysins, however this possibility is currently not well understood.

These findings were recently supported during *in vitro* studies of *A. terreus* putative hemolysin expression by Nayak and colleagues [3,39], in which the authors produced recombinant terrelysin and developed specific monoclonal antibodies that were used to quantify the native protein in hyphal and secreted fractions grown in liquid culture at various temporal intervals. In these studies, it was observed by ELISA that the highest concentrations of terrelysin were found in the culture supernatant during early phases of *A. terreus* hyphal growth [3] when compared to later time points where hyphal growth and apical elongation were reduced. It was further observed using immunohistochemistry that terrelysin was localized to the apical regions of the hyphae. Since terrelysin lacks a signal peptide, it was proposed that terrelysin might either diffuse out or be actively secreted during initial hyphal growth (i.e., apical elongation) through other yet uncharacterized processes. The rapid diffusion of proteins and enzymes from apical regions of hyphal tips is well characterized [68–73].

In contrast to these observations, proteomic studies of *A. flavus* and *A. terreus* revealed that their homologous hemolysins may not be secreted [74,75]. One of the possible reasons for this discrepancy could be that these studies used broad proteomics-based analyses that are

less sensitive than monoclonal antibody-based assays. To date, the kinetics of hemolysin expression from other filamentous fungal species remains to be characterized.

Hemolysins as virulence factors

Based on a number of mechanistic and characterization studies, several fungal hemolysins have been proposed as virulence factors [76,77]. Hemolysins lyse red blood cells resulting in the release of iron, an important growth factor for microbes especially during infection [78]. The requirement of iron in fungal growth is necessary for metabolic processes and as a catalyst for various biochemical processes and has been reviewed in detail elsewhere [79].

Expression of a hemolytic protein with capabilities to lyse red blood cells has also been suggested to provide a survival strategy for fungi during opportunistic infections. For example, in *Candida*, the secretion of hemolysin coupled with iron uptake facilitates hyphal invasion during disseminated candidiasis [80]. Fungal hemolysins have been reported as a potential cause of hemorrhage in several investigations [60,81,82]. Ostreolysin has been shown to possess cytolytic and cardiotoxic potential *in vitro* and *in vivo* [83– 86]. Similarly, non-aegerolysin family hemolysins such as phallolysin, rubescenlysin, flammutoxin and others have been identified, purified and extensively characterized for their cytolytic and cardiotoxic activities [87– 100]. Most studies on the role of hemolysins in disease have used purified or partially purified proteins. For many of these studies, concentrations of the proteins used to determine toxic effects have not been studied from a physiologically relevant perspective. A recent research article on *A. fumigatus* that used asp-hemolysin mutants showed that the hemolysin concentrations might have been overestimated and may not be physiologically relevant during infection [66]. Mutation of asp-hemolysin as well as the related asp-hemolysin-like protein did not show any significant reduction in hemolytic and cytotoxic activities of the fungus. In this same study, mutants of asp-hemolysin did not exhibit any attenuation of virulence by *A. fumigatus*. Interestingly, a strain with mutations for both hemolysins was slightly hypervirulent. These data suggest that the previous interpretations of the role of hemolysins in *A. fumigatus* and other fungal virulence may be overestimated. In view of these recent observations, the hemolytic and cytolytic activity appears to be only coincidental to a yet uncharacterized intracellular or extracellular function.

Role in fungal biology

The likelihood that fungi evolved hemolysins for the sole purpose of lysing red blood cells *in vivo* to improve growth is highly unlikely. Most fungi exist in the environment as saprophytes. In some cases, the fungus may grow on or in living tissues especially in an immune suppressed individual. This may provide an opportunity for colonization and infection. For fungi, animal hosts are a rich source of organic material.

As noted above, recent studies of *A. fumigatus*, cause one to question whether fungal hemolysins have any demonstrable role in pathogenesis [66]. A more likely function for this family of proteins would be in the normal physiological processes and raises a valid question regarding the role of these proteins in fungal biology.

Perhaps a clue to understanding hemolysins in relation to fungal biology comes from the basidiomycetes where ostreolysin (*Pleurotus ostreatus*) and aegerolysin (*Agrocybe aegerita*) are expressed during the initiation of fruiting bodies [4,101]. It is believed that these hemolysins have a functional role in hyphal aggregation, although the specific mechanisms remain uncharacterized [4,102]. Ostreolysin transcripts have been detected in the fruiting bodies of *P. ostreatus* [4] and the addition of the protein has been reported to enhance fruiting initiation in *P. ostreatus* [103]. These data suggest that the functional role of the protein is in the promotion of primordial formation, an early stage in the development of the mushroom, especially during hyphal aggregation.

One recent paper does not support this hypothesis [104]. Lakkireddy *et al.* (2011) recently proposed that lectins, aegerolysins and other molecules involved in hyphal aggregation and fruiting body formation are not consistently present in members of closely related mushrooms. Since primordial formation and hyphal aggregation are fundamental processes for growth and development, it is unlikely that a set of proteins with inconsistent distribution would play a significant role. The authors acknowledged that hemolysins interact with specific receptors (most likely lipid rafts) that may indirectly play a role in hyphal aggregation by influencing the frequency of cap formation.

Studies of filamentous fungi have reported a correlation between the kinetics of hemolysin expression and fungal growth and development. In studies of the ascomycete species *A. terreus*, detectable quantities of terrelysin are present extracellularly, especially during the early stages of germination and hyphal development [3]. Asp-hemolysin transcripts were detected from the stages of conidial germination to hyphal extension and branching in *A. fumigatus*; however, deletion of asp-hemolysin in mutant strains of *A. fumigatus* did not alter the phenotype (microscopic and macroscopic) or growth characteristics of the fungus [66]. In yeasts, contradicting observations have been reported on expression of hemolysins. In *B. dermatitidis*, the hemolytic activity was highest in the yeast phase of growth, while in *C. albicans*, it was in hyphal stage of growth accompanied with secretion of the hemolysin [23,105]. Elsewhere, in *C. glabrata*, phase switching in the fungus was associated with changes in the transcript levels of the hemolysin-coding gene *HLP* [106]. Investigating the role of hemolysins during the early stages of growth, especially in filamentous fungi could provide valuable information regarding the functional role of these proteins. It does not appear that these proteins are critical for fungal growth but based on these observations, it is probable that these proteins have a role in regulating fungal growth. In *M. anisopliae*, upregulation of the hemolysin gene was observed with fungal morphological instability or ageing [107]. This suggests a likely function for the product of this gene during fragmentation and apoptosis at least in *M. anisopliae*.

Another interesting aspect related to the role of these proteins in fungal biology is the presence of variable numbers of aegerolysin proteins in unrelated fungal taxa (Table 1). *A. fumigatus* possesses two aegerolysin hemolysins that belong to the aegerolysin family of proteins, and a third hemolysin that has been identified as an asp-hemolysin-like protein. In members of the order Eurotiales, *A. niger* expresses two proteins that belong to the aegerolysin family, while *P. chrysogenum* possesses sequences for five homologous proteins (Table 1). Multiple hemolysins have also been reported in basidiomycetes including *P.*

ostreatus and *M. perniciosus* [4,108–112]. In *M. perniciosus*, one hemolysin peaked during primordial formation, while another peaked during basidiocarp formation. Expression of a homologous protein to pleurotolysin B, peaked in mycelium and primordia, but reduced in the basidiocarp. Similarly, studies in *A. fumigatus* reported that the expression of asp-hemolysin and the asp-hemolysin-like protein especially in the secreted fractions was different at different growth phases [66]. Currently, the biological relevance of having multiple hemolysins remains unclear.

Interestingly, a report from the USDA laboratories that examined gene expression in the phytopathogen, *Alternaria gaisen*, reported differential expression of the aegerolysin genes in dark and light conditions [113]. Here, significantly higher transcripts were reported from fungi exposed to light than those exposed to dark conditions. Future studies are needed to characterize the underlying mechanisms that govern expression of these proteins during different nutrient, temperature, light, and pH regimens.

Although hemolysins may not be virulence factors, another important function of these proteins may be related to their ecological niche. Fungi exist in the environment in competition with other fungi, bacteria and insects. Expression of a protein that can lyse a competitor's cells could help provide a survival advantage for the fungal species. In nature, especially in basidiomycetes, it has been proposed that hemolysins have a functional role as insecticides [114]. Homologous hemolysins derived from several bacterial species including *Clostridium bifermentans* have been reported to have insecticidal properties [35,115,116]. To date, insecticidal activity has not been identified for ostreolysin nor has it been reported in filamentous fungi. Interestingly, eryngeolysin from *P. eryngii* exhibited antibacterial activity against *Bacillus* species but not with bacteria from other species [110]. Fungi commonly share their local environment with *Bacillus* species in various phyllosphere and rhizosphere ecosystems. Secretion of hemolytic proteins that specifically lyse bacteria may provide an opportunity to outcompete bacteria for available nutrients and resources.

Research studies have also shown that hemolysins bind to membrane lipids on target cells [30,46,84,117–120]. Ostreolysin has been shown to lyse lipid vesicles generated from cholesterol/sphingomyelin and to a lesser extent, ergosterol/sphingomyelin [117]. The specificity of binding may provide the fungus with the ability to lyse target cells from plants or animals without causing damage to the fungal cell wall. Hemolysin specificity for certain lipids must have a relevant biological function. For example, ostreolysin binds to and lyses vesicles containing sitosterol, a phytosterol mainly present in plants [117]. Peanuts contain large amounts of sitosterol and are very susceptible to infection from fungi, especially *Aspergillus* species [121]. The hemolysins may have a functional role in plant pathogenesis; however, this remains largely uncharacterized and is the focus of further study.

Applications of aegerolysins

Enzymes and secondary metabolites of fungi have been utilized in industrial sectors for many years. Secondary metabolites from *Aspergillus* species have been identified as hypolipidemic agents and commercially exploited. Lovastatin, a polyketide-derived metabolite of *A. terreus*, was one of the first statins approved by the FDA for lowering

cholesterol [122]. Binding of fungal hemolysins to lipids and lipoproteins has been extensively studied for asp-hemolysin and ostreolysin [41,123,124]. Asp-hemolysin was characterized for its pharmaceutical potential in binding to low-density lipoprotein (LDL) and one of its derivatives, oxidized LDL (Ox-LDL) [125,126]. Asp-hemolysin was reported to bind specifically to apolipoprotein B in LDL [123,124], and lysophosphatidylcholine in Ox-LDL [126–129]. This specific interaction was reported to interfere with the pathological role of Ox-LDL *in vitro* [130–132]. However, no animal studies have been published that show strong evidence for a pharmaceutical relevance of this hemolysin. Interestingly, terrelysin was not found to have any binding activity with serum lipids (unpublished data). Similarly, ostreolysin and aegerolysin also do not bind LDL [4]. The basis for binding of LDL by asp-hemolysin was suggested based on the positioning of certain acidic amino acids in the homologous repeats in various loops of the LDL receptor [63]. The critical positioning of aspartic acid and glutamic acid is absent for the homologous region in terrelysin and ostreolysin and may be a likely explanation for the lack of LDL binding. However, interactions with LDL or its derivatives are not only limited to fungal hemolysins and have been reported for other aegerolysin family proteins identified in bacteria. PA0122, an aegerolysin from *P. aeruginosa*, has been reported to bind Ox-LDL; however, no pharmaceutical potential has been reported [133].

Binding of fungal hemolysins to lipids has been extensively investigated by Sepcic and her colleagues at the University of Ljubljana in Slovenia [30,41,46,117,118]. They have observed that ostreolysin bound to cholesterol-enriched raft-like microdomains in the cell membrane [46,118,134]. Due to the importance of these microdomains in biological processes such as conidial germination, hyphal extension, signal transduction and pathogen interaction, these hemolysins might be useful tools for characterizing these highly dynamic structures in the cell membrane [135–138].

Mushrooms have been studied extensively for their bio-medical properties. Investigations have identified a possible role for fungal hemolysins on contributing to these properties. This has generated a considerable interest in understanding the contribution made by hemolysins especially as anti-tumor agents [139]. Schizolysin, (*S. commune*), eryngeolysin (*P. eryngii*), nebrodeolysin (*P. nebrodensis*) and phallolysin (*A. phalloides*) have been shown to possess anti-retroviral activity or cytotoxic to tumor cells; however, more research is needed to determine a physiological relevance [110,140–142].

In recent times, considerable interest has grown in use of filamentous fungi and mainly *Aspergillus* species for heterologous protein expression [143,144]. This has been aided by genome sequencing of many filamentous fungi and improvements in DNA purification and transformation technology. Expression of proteins in fungi is a very attractive avenue since they provide an efficient system for secretion of proteins and post-translational modifications in an inexpensive manner. Very recently in *A. oryzae*, the promoter from one of its hemolysin-coding genes (Q2TXT6) was identified to possess high promoter activity [145]. Genes expressed using this promoter overproduced coded proteins efficiently. More importantly, the promoter activity remained high in solid-state fermentations as well as in liquid cultures.

Another application of fungal hemolysins has been their use as biomarkers for personal exposure to fungi or species-specific identification of opportunistic fungal disease. Much interest in the adverse health effects of fungal hemolysins was stimulated by initial associations of *Stachybotrys chartarum* with an outbreak of idiopathic pulmonary hemorrhage (IPH) in Cleveland, Ohio, USA [146]. Fungal proteins capable of hemolysis were hypothesized as the causative agent and stachylysin was proposed as a potential biomarker for exposure to *S. chartarum* [81,147,148]. Detection of hemolysins from *A. fumigatus* and *S. chartarum* in sera and tissues of experimentally exposed animals and in some humans that worked in heavily contaminated environments further fueled an interest in utilizing fungal hemolysins as biomarkers for personal fungal exposure [148–150]. Recent reports on the detection of terrelysin in the culture supernatant of *A. terreus* [3] and that asp-hemolysin is the 4th most abundant protein in the secretome of *A. fumigatus* [66] suggest that hemolysins may be promising biomarkers of personal exposure. Collectively, there is considerable interest in the development of diagnostic assays for detecting these proteins as biomarkers of allergic and disseminated fungal exposure.

Limitations of fungal hemolysin research

Initial studies of hemolytic activity from different fungi used crude extracts or partially purified extracts [22,60,61,151–159]. While these studies were performed with the experimental and purification methodologies available at the time, recent studies using recombinant proteins to generate specific antibodies have identified limitations in the previous work. Recently reported studies on the hemolytic activity of *A. terreus*, identified some critical limitations in purification methodologies [3,39,160]. In these studies, hemolytic fractions were purified from *A. terreus* culture supernatant [160] and found to be enriched with the hemolysin based on the functional activity, but on further analysis, the preparations were found to consist of at least two proteases. Using proteomic techniques, sequences for terrelysin could not be identified. Upon further characterization, the kinetics for expression of these proteases and terrelysin were found to be completely different [3,160]. As mentioned above, terrelysin was identified as being produced very early in culture associated with early hyphal growth. The protocols used for isolating hemolysins based on functional activity involved much longer culture times and it is probable that at these later time points (stationary growth phase), little hemolysin was produced and purification of other proteins such as proteases with hemolytic activity was occurring.

When asp-hemolysin was initially investigated from *A. fumigatus*, it was reported as a 30 kDa protein [62]; however, further biochemical characterization by the same group showed that asp-hemolysin is a much smaller protein [39,63,161]. This discrepancy was not reported when asp-hemolysin was sequenced for the first time or when it was expressed as a recombinant protein [63,161]. This raised the question in the early studies on asp-hemolysin [62,82,149,162] whether the protein studied was indeed homogenous. This also questions the interpretation of studies on the characterization of hemolysins as being important for pathogenesis, as later studies clearly provide evidence against their role as virulence factors [66]. Future studies on the characterization of hemolytic proteins from fungi need to identify the hemolysins by immunochemical or proteomic methods rather than relying on hemolysis as a functional assay for purification.

Using recombinant proteins to characterize the functional activity of hemolysins is not without technical concerns. Bacterial aegerolysins in *Clostridium bifermentans* and *Pseudomonas aeruginosa* were expressed in *Escherichia coli* as well as *Bacillus thuringiensis* expression systems; however, no hemolytic activity was observed with the purified proteins [35,133,163]. It is unclear as to why recombinant hemolysins expressed in bacterial expression systems are not functionally active. Several studies have aimed at expression of recombinant fungal hemolysins in *E. coli* also failed to produce functionally active hemolysins [39,161,164]. In the case of asp-hemolysin, the protein was expressed as a fusion protein with maltose binding protein (MBP) [161]. It is possible that MBP, which is almost three times the size of asp-hemolysin, could interfere with the structural conformation of the protein and thus affect the hemolytic activity of the protein. For flammutoxin, the removal a 20-amino acid region in the C-terminal domain was necessary to restore hemolytic activity [164]. In addition, when these proteins are expressed in the cytoplasm of the various expression systems, reducing conditions may inhibit the folding of the protein to its appropriate functional conformation.

Some studies have reported that these hemolysins possibly consist of multiple components and this concept is gaining more credence in recent times [43,111,165]. Previously, two component hemolysins have been reported in bacteria [166,167]. It has been suggested that two individual components interact with each other and this association is essential for hemolysis [165]. Aegerolysin proteins such as asp-hemolysin, ostreolysin, terrelysin, and other homologous proteins may interact with a larger subunit to form a functionally active two-component hemolysin. Based on sequence information available for the larger subunits, we could not identify a homologous protein in *A. terreus*.

The described limitations highlight the need to re-evaluate the term ‘ hemolysin ‘ as it pertains to mycology. A comprehensive review of the literature on fungal hemolysins suggests that the definition of hemolysis is vague and requires refinement based on characterization of mechanisms underlying hemolysis. Initial studies focused on relatively crude or partially purified hemolytic preparations. Functionally, hemolysis was observed but these studies were confounded by the presence of other proteins, more specifically proteases. As mentioned earlier, at least two proteases were identified that were co-purified in these crude preparations [160]. This was further corroborated in a study characterizing *A. fumigatus* strains lacking the protease transcription factor PrtT [168]. PrtT mutant strains showed loss of secreted protease activity and demonstrate very low levels of hemolytic activity. Fungi are known to secrete phospholipases that interact with host cell membranes and result in lysis [77]. In the study of asp-hemolysin mutants with reduced asp-hemolytic activity, extracts did not show a significant reduction in the total hemolytic activity of the fungus [66]. These studies demonstrate that while asp-hemolysin may contribute to hemolysis, specific proteases may be more important to the functional activity that is followed in the hemolysin purification protocols.

Finally, non-protein components of fungi with hemolytic activity have also been reported [169,170]. Preliminary characterization of hemolysis in *Wallemia sebi*, a xerotolerant basidiomycete, identified unsaturated fatty acids responsible for hemolytic activity, while the hemolysin of *R. nigricans* was characterized as a lipoprotein. These observations suggest

that the role of other macro-molecules and secondary metabolites may show hemolysis during initial characterization and further highlights issues with extraction and purification.

Summary

Fungal hemolysins belonging to the aegerolysin protein family are characterized by pleiotropic functions. These proteins were first identified for their role in pathogenesis as virulence factors; however, biochemical characterization studies highlight their function in other aspects of fungal biology. The ability of these proteins to bind to unique microdomains in the cell membrane opens a new area for research and serves as an essential tool in the characterization of the membrane lipids. Additionally, early studies on the detection of hemolysins during fungal infections and recent studies on secretion *in vitro* demonstrate their utility as putative biomarkers for fungal exposures. Finally, the wealth of information on the function of these proteins in fungal biology suggests a more important role for these proteins in fungal growth and regulation. The continued research in this area should provide additional insights into these unique proteins and provides an impetus for future research.

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Table 1

List of fungi with identified and described hemolysins

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
Ascomycetes				
<i>Aspergillus clavatus</i>	YES	A1CGD5	Putative uncharacterized protein	
<i>Aspergillus flavus</i>	YES	B8N206, B8N7M1, B8NLV7, B8NXA7	Putative uncharacterized proteins	
	NO	N/A	<ul style="list-style-type: none"> Initial report on hemolysis in <i>A. flavus</i> Crude analysis of hemolytic toxins 	[22] [151]
<i>Aspergillus fumigatus</i>	YES	Q00050 (Strain FGSCA1100)	Asp-hemolysin <ul style="list-style-type: none"> Initial report on hemolysis in <i>A. fumigatus</i> No correlation between hemolytic activity and toxicity in crude preparations Hemolytic component can be purified only during a limited period Initial attempts for purification of the hemolysin Hemolysin detected in tissues of experimental animals Toxic effects in animals inject with asp-hemolysin First report of nucleotide sequence of asp-hemolysin Derivation of hypothesis for interaction between asp-hemolysin and LDL Asp-hemolysin and LDL and Ox-LDL interactions Expression of recombinant asp-hemolysin 4th most abundant protein in the secretome Deletion of the gene did not affect hemolytic, cytotoxic and pathological properties of the fungus 	[22] [60] [61,62] [149] [82] [63] [123–132] [161] [66]
	NO	Q4WA30 (Strain FGSCA1100)	Asp-hemolysin-like protein <ul style="list-style-type: none"> Not detected in the secretome Deletion of the gene did not affect hemolytic, cytotoxic and pathological properties of the fungus 	[66]
	YES	A4DA65 (Strain FGSCA1100)	Putative uncharacterized protein	
	YES	B0XX60, B0YET6 (Strain FGSC A1163)	Putative uncharacterized proteins	
<i>Aspergillus nidulans</i>	YES	Q5BD27	Putative uncharacterized protein	
<i>Aspergillus niger</i>	YES	A2QA29 (Strain FGSCA1513)	<ul style="list-style-type: none"> Sequence reported from genome database 	[28] [64]

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
			<ul style="list-style-type: none"> Detected as a part of the secretome based on <i>in silico</i> analysis 	
	YES	A2RBK6 (Strain FGSCA1513)	Putative uncharacterized protein	
	YES	G3XTV2, G3Y3Z1 (Strain ATCC 1015)	Putative uncharacterized proteins	
	NO	N/A	Nigerlysin <ul style="list-style-type: none"> 72 kDa secreted hemolysin Heat labile at 75°C α-helical secondary structure Toxicity to neuronal cells 	[40]
<i>Aspergillus oryzae</i>	YES	Q2TXT6	HlyA encoded protein <ul style="list-style-type: none"> First report of sequence in the genome database of <i>A. oryzae</i> Novel promoter for expression of heterologous proteins Stronger production of expressed proteins compared to other promoter systems 	[25] [145]
	YES	Q2U8×3, Q2ULJ5	Putative uncharacterized proteins	
<i>Aspergillus terreus</i>	YES	Q0CRX8	Terrelysin <ul style="list-style-type: none"> Expression of recombinant terrelysin 16.5 kDa β-sheet protein Presence of terrelysin during early stages of growth Detection of terrelysin in culture supernatant Development of monoclonal antibodies for the first aegerolysin family protein 	[39] [3]
<i>Altemaria tenuis</i>	NO	N/A	<ul style="list-style-type: none"> First report on hemolytic activity in <i>A. tenuis</i> 	[152]
<i>Blastomyces dermatitidis</i>	YES	C5GE86 (Strain ER-3)	Putative uncharacterized protein	
	YES	C5JIZ7 (Strain SLH14081)	Putative uncharacterized protein	
	YES	F2T7L8 (Strain ATCC 18188)	Putative uncharacterized protein	
	NO	N/A	<ul style="list-style-type: none"> Identification of hemolytic activity associated in the yeast stage 	[23]
<i>Candida albicans</i>	NO	N/A	<ul style="list-style-type: none"> Hemolysin secretion leads to iron uptake which induces phase switching 	[80]
	NO	N/A	<ul style="list-style-type: none"> Identification of hemolytic activity associated in the yeast stage 	[23]
	NO	N/A	<ul style="list-style-type: none"> Enhanced hemolytic activity in hyphae and secretion of hemolysin 	[105]
<i>Candida glabrata</i>	NO	N/A	HLP encoded protein	[106]

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
			<ul style="list-style-type: none"> Hemolysin gene is involved in the phenotype switching 	
<i>Coccidioides posadasii</i>	YES	C5P7J6	Putative uncharacterized protein	
<i>Cordyceps militaris</i>	YES	G3JFK8	Putative uncharacterized protein	
<i>Cryptococcus neoformans</i>	NO	N/A	<ul style="list-style-type: none"> Identification of hemolytic activity associated in the yeast stage 	[23]
<i>Fusarium oxysporum</i>	YES	F9GD59	Putative uncharacterized protein	
<i>Histoplasma capsulatum</i>	YES	A6R7E8 (Strain WU24)	Putative uncharacterized protein	
	YES	C0NGB1 (Strain G18AR)	Putative uncharacterized protein	
	YES	C6H8A0 (Strain H143)	Putative uncharacterized protein	
	YES	F0U5M1 (Strain H88)	Putative uncharacterized protein	
	NO	N/A	<ul style="list-style-type: none"> Identification of hemolytic activity associated in the yeast stage 	[23]
<i>Metarhizium acridum</i>	YES	E9E2I6	Putative uncharacterized protein	
<i>Metarhizium anisopliae</i>	YES	E9EKK1, E9F766	Putative uncharacterized proteins	
	UNCLEAR	N/A	<ul style="list-style-type: none"> Hemolysin associated with ageing of the fungus 	[107]
<i>Nectria haematococca</i>	YES	C7YXA1	Putative uncharacterized protein	
<i>Neurospora crassa</i>	YES	Q1K511	Putative uncharacterized protein	
<i>Neurospora tetrasperma</i>	YES	F8MNX9 (Strain FGSC 2508)	Putative uncharacterized protein	
	YES	G4UTV2 (Strain FGSC 2509)	Putative uncharacterized protein	
<i>Paracoccidioides brasiliensis</i>	YES	C0S9D2 (Strain Pb03)	Putative uncharacterized protein	
	YES	C1GBU4 (Strain Pb18)	Putative uncharacterized protein	
	YES	C1HAI8 (Strain Pb01)	Putative uncharacterized protein	
<i>Penicillium chrysogenum</i>	YES	B6GXR4, B6H164, B6H690, B6HE31, B6HIT7	Putative uncharacterized proteins	
	NO	N/A	<p>Chrysolysin</p> <ul style="list-style-type: none"> 2 kDa monomer hemolysin Increased production of MIP-2 in murine macrophages 	[153]
<i>Sordaria macrospora</i>	YES	F7W167	Putative uncharacterized protein	
<i>Stachybotrys chartarum</i>	NO	N/A	<p>Stachylysin</p> <ul style="list-style-type: none"> Initial reports on hemolytic activity in <i>S. chartarum</i> + Identification of a 11.9 kDa protein Heat inactivated Slow hemolytic process May cause hemorrhaging based on an earthworm model All strains produce stachylysin in presence of blood in growth medium 	[154,155] [147] [81] [150] [148]

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
			<ul style="list-style-type: none"> Stachylysin is localized in inner wall of conidia/hyphae In tissues from exposed animals stachylysin staining was observed immediately surrounding the conidia, suggesting diffusion from it Detection of stachylysin in sera of patients and experimental animals Stachylysin as a measurable indicator of <i>S. chartarum</i> exposure 	
<i>Trichophyton sp.</i>	NO	N/A	<ul style="list-style-type: none"> <i>T. rubrum</i>, <i>T. verrucosum</i>, <i>T. mentagrophytes</i> and <i>T. equinum</i> show complete hemolysis 	[156]
Basidiomycetes <i>Moniliophthora perniciosa</i>	YES	E2LG72, E2LMN6 E2LQH3	Putative uncharacterized proteins	
	YES	E2LVY5	MpPRIA1 <ul style="list-style-type: none"> First report of aegerolysins in <i>M. perniciosa</i> Decreased levels of transcripts in yellow and reddish pink mycelium stage and prior to stress Increased levels of transcripts in mycelia and primordia 	[112] [109]
	YES	E2LE80	MpPRIA2 <ul style="list-style-type: none"> First report of aegerolysins in <i>M. perniciosa</i> Increased levels of transcripts in the basidiomata Decreased levels of transcripts in mycelia and primordia 	[112] [109]
	YES	E2LNF8	Putative uncharacterized protein <ul style="list-style-type: none"> The sequence of this protein is very similar to E2LVY5, but is truncated to 104 amino acids in the database 	[112]
	NO	N/A	Deuterolysins <ul style="list-style-type: none"> Identification of sequences similar to deuterolysins 	[112]
<i>Agrocybe aegerita</i>	YES	042717	Aegerolysin <ul style="list-style-type: none"> Cloning and sequencing of aegerolysin (Aa-Pril) Expressed during fruiting initiation Isolation of a 16 kDa hemolytic protein 	[102] [4]
<i>Pleurotus eryngii</i>	YES	D0FZZ2	Erylysin <ul style="list-style-type: none"> Part of a two-component hemolysin system and erylysin A belongs aegerolysin family 	[111]

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
			<ul style="list-style-type: none"> 15 kDa protein β-sheet protein 	
	UNCLEAR	N/A	<p>Eryngeolysin</p> <ul style="list-style-type: none"> Not identified as an aegerolysin family protein, however, N-terminal sequence suggests homology Heat labile 17 kDa hemolysin Specific cytotoxicity to L1 210 leukemia cell line Antibacterial activity against <i>Bacillus</i> species 	[110]
<i>Pleurotus ostreatus</i>	YES	Q56QW9	<p>Ostreolysin</p> <ul style="list-style-type: none"> First report of cytolytic activity in <i>P. ostreatus</i> Isolation of ostreolysin, a 16 kDa hemolytic protein High expression during fruiting initiation and primordial formation Interactions with membrane lipids Mechanism of hemolysis action and identification of 4 nm pore size Interaction of ostreolysin with cholesterol-rich domains in cell membranes First report on β-sheet secondary structure of ostreolysin Deviation from optimal pH and temperature results in unfolding of ostreolysin to α-helical structure Unfolding prior to interaction with target cells results in loss of hemolytic activity Immunolocalization of ostreolysin in primordia and fruiting bodies Interactions of ostreolysin with lipid membranes composed of different steroids Toxicity of ostreolysin in experimental animals External addition of ostreolysin enhances fruiting initiation Putative role for ostreolysin in pathogenesis 	[108] [4] [41] [46,118] [38] [101] [117] [83,85,86] [103] [84,85]
	YES	Q8X1M9	<p>Pleurotolysin</p> <ul style="list-style-type: none"> Part of a two-component hemolysin system and pleurotolysin A belongs to aegerolysin family 17 kDa protein The two-component hemolysin specifically interacts with cholesterol/sphingomyelin liposomes 	[165] [43]
<i>Pleurotus nebrodensis</i>	NO	N/A	<p>Nebrodeolysin</p>	[140]

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
			<ul style="list-style-type: none"> • 27 kDa protein • Cytotoxic to different cell lines • Induces apoptosis in tumor cells • Anti-HIV activity 	
<i>Amanita phalloides</i>	NO	N/A	<p>Phallolysin</p> <ul style="list-style-type: none"> • Initial characterization of cytolytic activities of phallolysin • Purification of phallolysin and reporting of its cytotoxic activities • Biochemical characterization of phallolysin • Extensive characterization of hemolysin activity • Electron microscopy-based characterization of cytolytic activity • Mast cell degranulation • Mechanism of hemolysis by phallolysin • Phallolysin lyses liposomes containing phosphatidylcholine as well as sphingomyelin • Specifically damages liposomes containing negatively charged phospholipids • Lectin based hemolytic activity • Cytotoxic towards tumor cells 	[87] [88] [89] [91] [90] [92] [54] [119] [141]
<i>Amanita rubescens</i>	NO	N/A	<p>Rubescenlysin</p> <ul style="list-style-type: none"> • First report on identification of rubescenlysin • Further characterization of hemolytic and cytolytic activities • Mast cell degranulation • Toxic effects in experimental animals • Intravascular hemolysis and alveolar obstruction, hemorrhagic pulmonary edema in experimental animals • Further reporting of pathology in experimental animals 	[93] [94] [92] [95] [96]
<i>Hypholoma fasciculare</i>	NO	N/A	<p>Fascicularelysin</p> <ul style="list-style-type: none"> • Mast cell degranulation 	[92]
<i>Laetiporus sulphureus</i>	NO	N/A	<p>LSL</p> <ul style="list-style-type: none"> • Lectin-based hemolytic activity • 35 kDa protein • N-terminal domain recognizes carbohydrates • C-terminal domain is responsible for oligomerization and its removal abolished hemolytic activity 	[48] [49] [50]

Fungal division/Fungi	Aegerolysin	Accession number (UniProtKB)	Important notes	Reference
			<ul style="list-style-type: none"> Structural similarity to MTX2 (<i>B. sphaericus</i>) and a toxin (<i>C. septicum</i>) N-terminal domain structure has a β-trefoil scaffold similar to that of ricin C-terminal domain structure is similar to that aerolysin (<i>Aeromonas hydrophila</i>) 	
<i>Rhodophyllus rhodopolius</i>	NO	N/A	<ul style="list-style-type: none"> Purification of hemolysin from <i>R. rhodopolius</i> 40 kDa heat labile hemolysin 	[157]
<i>Flammulina velutipes</i>	NO	N/A	<p>Flammutoxin</p> <ul style="list-style-type: none"> Isolation of flammutoxin and identification of cardiotoxicity Characterization of cardiotoxicity of flammutoxin Characterization of hemolytic activity of flammutoxin Purification of a 31 kDa protein and aggregates to form a 180 kDa protein on erythrocytes Electrophysiological properties of flammutoxin and identification of two different forms of pores on target cells Cloning and expression of recombinant flammutoxin 	[97] [98] [99] [158] [44] [164]
<i>Volvariella volvacea</i>	NO	N/A	<ul style="list-style-type: none"> Cardiotoxic hemolytic protein 	[100]
<i>Termitomyces clypeatus</i>	NO	N/A	<ul style="list-style-type: none"> Purification of ~64 kDa hemolysin Suggestion of interactions with phospholipids 	[120]
<i>Schizophyllum commune</i>	NO	N/A	<p>Schizolysin</p> <ul style="list-style-type: none"> 29 kDa hemolysin isolated from fruiting bodies Heat labile above 60°C Inhibits HIV-1 reverse transcriptase activity 	[142]
<i>Wallemia sebi</i>	NO	N/A	<ul style="list-style-type: none"> Non-protein hemolytic activity caused by unsaturated fatty acids 	[169]
Zygomycetes <i>Rhizopus nigricans</i>	NO	N/A	<ul style="list-style-type: none"> Initial characterization of hemolytic activity in <i>R. nigricans</i> Heat stable hemolysin and resistant to proteolytic activity Hemolysin may be a lipoprotein 	[159] [170]
<i>Rhizopus arrhizus</i>	NO	N/A	<ul style="list-style-type: none"> Initial characterization of hemolytic activity in <i>R. arrhizus</i> 	[159]