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Ageritin, a ribotoxin from poplar mushroom *(Agrocybe aegerita)* with defensive and antiproliferative activities.

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ABSTRACT

Ribotoxins are a group of extracellular rRNA endoribonucleases produced by ascomycetes that display cytotoxicity toward animal cells, having been proposed as insecticidal agents. Recently, the ribotoxin Ageritin has been isolated from the basidiomycetes *Agrocybe aegerita* (poplar mushroom) suggesting that ribotoxins are widely distributed among fungi. In order to gain insights into the protective properties of Ageritin against pathogens and its putative biotechnological applications, we have tested several biological activities of Ageritin, comparing them with those of the well-known ribotoxin α-sarcin, and we found that Ageritin displayed, in addition to the already reported activities, (i) antibacterial activity against *Micrococcus lysodeikticus*; (ii) activity against the tobacco mosaic virus RNA; (iii) endonuclease activity against a supercoiled plasmid; (iv) nuclease activity against genomic DNA; (v) cytotoxicity to COLO 320, HeLa and Raji cells by promoting apoptosis; and (vi) antifungal activity against the green mould *Penicillium digitatum*. Therefore, Ageritin and α-sarcin can induce resistance not only to insects but also to virus, bacteria and fungi. The multiple biological activities of Ageritin could be exploited to improve resistance to different pathogens by engineering transgenic plants. Furthermore, the induction of cell death by different mechanisms turns these ribotoxins into useful tools for cancer therapy.

Mushrooms and fungi in general, are susceptible to attack by viruses, bacteria, pathogenic and antagonistic fungi, insect, mites and nematodes [1], and to tackle the challenge of diseases and pests, they produce secondary metabolites [2] and proteins [3] as antiviral, antibacterial, antifungal and insecticidal agents. Many of them are of interest for biotechnology because of their applications in medicine and agriculture [4-6].

Ribotoxins are a group of toxic extracellular ribonucleases produced by fungi that display cytotoxic activity toward animal cells [7, 8]. These proteins are highly specific rRNA endonucleases (EC 3.1.27.10) that catalyse the hydrolysis of the phosphodiester bond between the guanosine No. 4325 and the adenosine No. 4326 from the rat 28S rRNA (or the equivalent phosphodiester bond in ribosomes from other organisms) releasing a 460 nt-fragment (α-fragment) at the 3' end of the 28S RNA that is diagnostic of the ribotoxin catalytic action. These nucleotides are located in the Sarcin Ricin Loop (SRL) that is crucial for anchoring the EFG or EF-2 elongation factors on the ribosome during mRNA-tRNA translocation in prokaryotes and eukaryotes respectively [9, 10].

Ribotoxins are produced by a few Ascomycota species, mostly from the genus *Aspergillus*: α-sarcin from *A. giganteus* [11], and restrictocin and mitogillin from *A. restrictus* [12]. The ascomycota entomopathogenic *Hirsutella thompsonii* and *Metarhizium anisopliae* also produce the ribotoxins hirsutellin A [13] and anisoplin [14], respectively. Additionally, some species from the genus *Aspergillus* and *Penicillium* possess genes coding for ribotoxins (for example, accession numbers XP_001276307 from *A. clavatus*, GAO84606 from *A. udagawae*, CAA41217 from *A. fumigatus*, GAQ08839 from *A. lentulus*, XP_001266297 from *A. fischeri*, ACA34736 from *A. clavatonanicus*, ACA34740 from *A. acanthosporus*, ACA34741 from *A. rhizopodus*, ACA34727 from *A. longivesica*, AAB66603 from *P. daleae*, AAB66602 from *P. spinulosum*, AAB66604 from *P. digitatum*, AAB66601 from *P. resedanum* and AAB66606 from *P. chermesinum*).

Due to the translation inhibitory and apoptotic activities of ribotoxins, extensive research has been conducted to investigate their suitability as therapeutic agents. The most promising applications of ribotoxins in experimental medicine, especially in anticancer therapy, are related to their use as components of immunotoxins, in which the enzymatic ribotoxins are linked to antibodies that mediate their binding and internalization by malignant cells [15-18]. Moreover, some of them display antiviral

activity [19] and it has been suggested that they could also be used as specific tools for the study of human ribosomopathies [8] or, in agriculture, as candidates for the design and development of new biopesticides [8, 20].

α-Sarcin, the most significant member of the ribotoxin family, was reported as a new antitumor agent [11, 21] and its mechanism of action has been known since 1983 [22]. It has been reported that α-sarcin is active against ribosomes, cultured cells and larvae from insects [23]. Other fungal ribotoxins such as restrictocin, HtA and anisoplin have also been found to display insecticidal activity therefore, a role for ribotoxins as insecticidal agents has been proposed [8, 20]. However, recently, it has been reported that α-sarcin displays a strong antifungal activity against the green mould *Penicilium digitatum*, a necrotrophic fungus that colonizes the wounds and grows in the inter- and intra-cellular spaces of the tissues of several edible plants and mushrooms [24]. α-Sarcin was able to enter into the cytosol where it inactivated the ribosomes, thus killing the cells and arresting the growth of the fungus [24]. Therefore, this protein could play, besides the already proposed insecticidal function, a role in nature as an antifungal agent.

Recently, a new RNase named Ageritin has been isolated from the basidiomycetes *Agrocybe aegerita* (V. Brig.) Singer (= *Cyclocybe aegerita*) [25-27]. Ageritin was found to inhibit protein synthesis in a rabbit reticulocyte lysate with an IC_{50} value of 133 pM [25] and, although it differs from Ascomycota ribotoxin prototype in the amino acid sequence [27], it was able to release the specific α-fragment from both rabbit and yeast ribosomes. It exerted cytotoxicity and cell death promoting effects toward CNS model cell lines and activated caspase-8, whereas caspase-9 cleavage was not detected, demonstrating the involvement of the extrinsic apoptotic pathway [25]. In order to gain insights into the protective properties of Ageritin against pathogens and its putative biotechnological applications, we have tested other activities of this ribotoxin and we found that Ageritin (i) displayed antibacterial activity against *M. lysodeikticus*; (ii) was active against the tobacco mosaic virus (TMV) RNA; (iii) displayed endonuclease activity against a supercoiled plasmid; (iv) possessed nuclease activity against genomic DNA; (v) was cytotoxic to COLO 320, HeLa and Raji cells by promoting apoptosis; and (vi) displayed antifungal activity against the green mould *P. digitatum*, being able to enter into the cytosol inactivating the fungal ribosomes. The combined effect of these biological activities could result in a broad action against several types of pathogens.

RESULTS AND DISCUSSION

Antibacterial activity

Because there are a number of diseases of mushrooms caused by bacteria [1] and rRNA endonuclease activity might play a role in mushroom defense, we assayed the effect of Ageritin on ribosomes from *Micrococcus lysodeikticus* and *Escherichia coli*, which might be homologous to ribosomes from bacterial mushroom pathogens. As shown in Figure 1a,b, this ribotoxin displayed 23S rRNA ribonuclease activity on prokaryotic ribosomes from both bacteria as indicated by the release of a 243 nt diagnostic fragment, identical to that produced by α-sarcin (Figure 1a, [9]). α-Sarcin cleaves the phosphodiester bond between G2661–A2662 located at the universally conserved SRL, impairing the activity of elongation factor G (EF-G) and leading to complete inactivation of the ribosome [9]. As a consequence of this finding, ageritin was tested on several bacteria (*Micrococcus lysodeikticus*, *Escherichia coli*, *Pectobacterium carotovorum*, *Serratia marcescens* and *Rhizobium leguminosarum*). Among all of the bacteria tested, only *M. lysodeikticus* showed inhibition by Ageritin. Thus, 80 and 40 μg mL^{-1} of Ageritin resulted in 50% and 65% inhibition respectively after 9 h incubation (Figure 1c). Although both, *M. lysodeikticus* and *E. coli* ribosomes were sensitive to Ageritin, only intact *M. lysodeikticus* was susceptible to the toxicity exerted by Ageritin, suggesting that this specificity could be related to differences in the interaction of the ribotoxin with the bacterial plasma membrane or the cell wall and to its ability to cross them. Even though the mechanism by which Ageritin may inhibit certain bacteria remains unknown, the protein might enter into some bacterial cells and inactivate their ribosomes avoiding the propagation of the pathogen.

Ribonuclease activity on tobacco mosaic virus (TMV) RNA

Mushroom cultures are subject to attack by virus, bearing the majority of mycoviruses either linear dsRNA or linear positive sense ssRNA genome [28]. We investigated the endoribonuclease activity of Ageritin and α-sarcin on viral RNAs using the positive strand RNA of TMV as a model. Magnesium ions affect the folding of the RNA and therefore its electrophoretic mobility [29], thus, a small effect on the mobility of control RNA was observed in the presence of 5 mM Mg^{2+} (Figure 1d). Both Ageritin and α-

sarcin displayed RNase activity on TMV genomic RNA, promoting an extensive degradation of the polyphosphate RNA backbone and therefore increasing its mobility, but the activity of Ageritin was much lower than that observed for α-sarcin. Surprisingly, magnesium ions exhibited different effects on these ribotoxins being an activator for Ageritin and an inhibitor for α-sarcin. As expected, the chelating agent EDTA (ethylenediaminetetraacetic acid) reversed the effect (Figure 1d). It is worth mentioning that Ageritin-treated RNA sample, showed a bright trailing in the electrophoresis gel, corresponding to a distribution of different RNA fragment sizes, while in that treated with α-sarcin an intense degradation can also be observed, but in this case several bands appeared, which could indicate the recognition of specific sequences on TMV RNA, most probably the GNRA tetraloop motif [30, 31]. This fact has also been reported for some RIPs that in addition to their main activity, N-glycosylase activity on the SRL, display antiviral activity on RNA from several sources, cultured cells, crops and animals [32, 33]. The reason for such activity could be the presence of the GNRA tetraloop motif that is the target into the SRL for both, ribotoxins and RIPs. This loop is a very common module in all kind of RNA 3D structures [30, 31]. Therefore, both ribotoxins display activity on the viral RNA so they could have a role in the defense of fungi against viruses.

Endonuclease activity on plasmid and genomic DNA

Although not common, a protein from *Helicobacter pylori* having both ribonuclease activity on RNA and nicking endonuclease activity on a plasmid DNA, has been reported [34]. Based on the biological role of some sugar-nonspecific nucleases in prokaryotes that cleave DNA and RNA [35], a defensive role has been proposed for this protein [34]. Therefore, we assayed the nicking endonuclease activity of Ageritin and α-sarcin on the plasmid pCR2.1 in the presence or absence of magnesium ions, since it has been reported that this activity was enhanced by divalent metal ions such as Mg^{2+} [36]. Relaxed and linear form of the plasmid was clearly observed upon incubation with 0.1–1 μg (0.6–5.9 μM) of Ageritin when 5 mM magnesium was present, while EDTA reversed this activation (Figure 2). By contrast, α-sarcin did not promote the conversion of supercoiled plasmid DNA into relaxed and linear forms (Figure 2). Instead, large smears of low mobility appeared upon incubation with 3–5 μg (18–29 μM) of α-sarcin,

most likely due to the binding of α-sarcin molecules to the plasmid. Such effect was not dependent on the presence of either magnesium ions or EDTA (Figure 2).

On the other hand, it has been reported that α-sarcin displays deoxyribonuclease activity and that several antifungal proteins can bind to DNA promoting charge neutralization and condensation of nucleic acids and finally cell death [37]. In order to find out if Ageritin after entering the cell could inhibit cellular functions by hydrolyzing the bound DNA, we tested the endonuclease activity of Ageritin and α-sarcin on genomic DNA from *E. coli*, *P. digitatum* and COLO 320 cells. As shown in Figure 3, the same result was obtained for the three genomic DNAs tested. Both Ageritin and α-sarcin displayed a very high degradative activity on genomic DNA, but this activity was higher for α-sarcin. Just as it happened with the ribonuclease activity of these proteins on TMV RNA, the endonuclease activity of Ageritin on DNA was dependent on magnesium ions and it was reversed with EDTA, while α-sarcin activity was inhibited by magnesium ions. The nuclease activity of ribotoxins on DNA could be, together with the already reported protein synthesis inhibition, one of the mechanisms by which these proteins induce apoptosis in animal cells, that is, the apoptosis could be a consequence of the combination of ribotoxic stress and DNA damage. Therefore, the ability of ribotoxins to act on bacterial, fungal and animal DNA turns these proteins into useful tools for biotechnological applications.

Effect of Ageritin on the growth of *Penicillium digitatum*

Although a role for ribotoxins as insecticidal agents has been proposed [8, 20] it has also been reported recently that α-sarcin displays antifungal activity [24]. Since fungi are the most important group of mushroom pathogens [1], we study the antifungal properties of Ageritin testing the effect of this protein on the growth of the fungus *P. digitatum*. As shown in Figure 4, Ageritin showed a strong growth inhibiting effect on *P. digitatum* and reduced the fungal growth in a concentration-dependent manner. Thus, 50, 30, 10 and 5 μg mL^{-1} of Ageritin (2.9, 1.8, 0.6 and 0.3 μM, respectively) resulted in 79%, 76%, 63% and 49% growth inhibition respectively after 72 h incubation with conidia as starting material. In addition, Ageritin added at 24 h (once conidial germination occurred) inhibited fungal growth to the same extent than Ageritin added from the beginning, and even when it was added at 48 h (the mycelium is at this point actively

growing) Ageritin was able to significantly inhibit fungal growth (Figure 4). On the other hand, experiments to study the fungicidal activity of Ageritin on nongerminated conidia showed that spores were resistant to the action of Ageritin even after 20 h of treatment (data not shown). Taken together, these results strongly suggest that Ageritin acts on fungal mycelium rather than on conidia. The concentrations of Ageritin required to inhibit the growth of *P. digitatum* and therefore to defend the mushroom from the mould infection are easily reached by the ribotoxin when is produced by *A. aegerita* considering that a yield of 1.22 mg of Ageritin from 100 g of fresh mushrooms has been reported [25]. On the other hand, it should be noted that Ageritin was able to inhibit mycelial growth at concentrations similar to those required for toxicity in human culture cells (see later).

The effect of Ageritin on mycelial growth of *P. digitatum* was also visualized microscopically (Figure 4). Corresponding to the growth inhibition, microscopy studies revealed alterations of hyphal morphology after exposure to Ageritin. Untreated mycelia developed regular and homogeneous hyphae, while mycelia treated with Ageritin produced hyper-branching and aborted hyphal branches (Figure 4).

To determine whether the growth inhibiting effect of Ageritin on the mould was a consequence of the endoribonuclease activity of Ageritin on the rRNA, RNAs from Ageritin-treated fungi were studied. Ageritin at concentrations of 25 μg mL^{-1} showed a strong growth inhibitory effect on *P. digitatum* cultures in liquid medium (Figure 5a). After 4 days of growth, the RNAs from these cultures were isolated and analyzed to detect the presence of the RNA fragment diagnostic of rRNA endonuclease activity (Figure 5b). Interestingly, the diagnostic fragment was absent in the RNA from control cultures and present in that from cultures grown in the presence of Ageritin. This indicated that Ageritin, at a concentration of 25 μg mL^{-1}, was able to enter into fungal cells in a way that allowed it to reach the cytosolic ribosomes. The size of the released fragment was around 356 nucleotides in accordance with that expected for the SRL breakdown (359 nucleotides for *Penicillium*) [24]. Therefore this ribotoxin might enter into the fungal cells and inactivate their ribosomes avoiding the propagation of the pathogen as has been reported for α-sarcin [24]. Initially the toxicity of ribotoxins to animal cells was attributed to their ability to inhibit protein synthesis leading to cell death, but there is an increasing body of evidence indicating that the main cause of ribotoxin toxicity in these cells is their ability to induce apoptosis [38]. DNA from *P.*

digitatum, was isolated from the cultures shown in Figure 5a and analyzed to detect the presence of the oligonucleosomal fragments (Figure 5c). Although a smear of degraded DNA was observed in Ageritin-treated fungi compared to the untreated control, no internucleosomal cleavage was visible suggesting that Ageritin intoxication can occur by non-apoptotic mechanisms in the fungal cells. The different ribotoxins seem to display different antifungal capacities and considering that all of them are active against all kind of ribosomes such antifungal ability must be related to their ability to cross cell membranes. Several structures have been suggested to be involved in membrane interaction and cytotoxicity to animal cells [39-41] and similar structural motifs have been proposed to be involved in the antifungal activity of some proteins such as plant defensins [42] or RIPs [36, 43]. Alternatively, differences in the composition, structure and porosity of the different fungal cell walls might be responsible for this disparity in toxicity [44].

Cytotoxic effects in cell cultures

A characteristic that differentiates ribotoxins from other ribonucleases is their cytotoxicity. It has been reported that Ageritin exerts cytotoxicity and cell death promoting effects towards neural and glial human tumour cell lines [25]. To extent this idea to a variety of cancer cells in order to evaluate its anti-tumor potential, we investigated the toxicity of Ageritin toward HeLa, Raji and COLO 320 cells. As shown in Figure 6a the addition of Ageritin decreased the cell viability in a concentration dependent-manner. Cytotoxicity varied in a wide range, with IC_{50} changing from nanomolar to micromolar, depending on the cell lines studied. The IC_{50} values obtained with COLO 320 and Raji cells were remarkably higher than the value obtained with HeLa cells. The IC_{50} was 4 nM for Ageritin-treated HeLa cells whereas for COLO 320 and Raji the IC_{50} was 1.9 and 1 μM, respectively. In addition, when HeLa and COLO 320 cells were treated with α-sarcin, the IC_{50} values obtained were higher than those obtained for Ageritin-treated cells, 13 nM for HeLa cells and 9 μM for COLO 320 cells.

Mammalian ribosomes seem to be highly sensitive to Ageritin [25], therefore the ribotoxin can promote a strong inhibition of cellular protein synthesis upon reaching the cytoplasm. The lack of specific receptors may result in low internalization and therefore reduced translocation to the cytosol. To see if Ageritin was able to reach the cytosol and

inactivate the ribosomes probably after being endocytosed, we analyzed the ribosomal RNA from COLO 320 and HeLa cells treated with Ageritin for 48 h. Figure 6b showed that the ribotoxin displayed 28S rRNA ribonuclease activity on ribosomes as indicated by the release of the diagnostic fragment, indicating that Ageritin was able to reach the ribosomes to inhibit protein synthesis. α-Sarcin exhibited slightly less rRNA endoribonuclease activity on HeLa cells ribosomes than Ageritin according to a lower cytotoxicity. The different cytotoxicity might be due to different abilities of the ribotoxins to cross the membrane and/or to a different intracellular pathway followed by the ribotoxins. We therefore investigated the intracellular pathway followed by Ageritin studying their toxicity on HeLa cells in the presence of substances interfering with intracellular routing such as the fungal inhibitor Brefeldin A that causes Golgi complex disassembly and the ionophore monensin that at high concentration, have pH-neutralizing effect in endosomes/lysosomes while at low concentration lacks this effect but influence Golgi structure and function [45]. As shown in Figure 6c, Brefeldin A increased the cytotoxicity of Ageritin, indicating that this protein follow a Golgi-dependent pathway to the cytosol. On the other hand, preincubation of HeLa cells with low concentrations of monensin (0.1 μM and 0.5 μM) enhanced the cytotoxicity of Ageritin supporting the fact that Golgi transport is important for translocation. High concentrations of monensin (10 μM) sensitized the cells to Ageritin indicating that the protein does not require a low pH for translocation to the cytosol. A similar behavior was observed for α-sarcin-treated cells in the presence of Brefedin A and monensin (data not shown).

We also investigated the death pathways involved in the cytotoxicity of Ageritin at concentrations close to its IC_{50}. The Ageritin-treated cells exhibited the morphological features characteristic of apoptosis such as cell rounding and blebbing (Figure 7a). Treatment with Ageritin led to the exposure of phosphatidylserine in the cell surface of HeLa cells 48 h after treatment, as revealed by an increase in annexin V-FITC-positive cells (green) demonstrating that significant apoptosis occurred. PI staining (red) was also seen in the cells, indicating late stage apoptosis or necrosis (Figure 7a). Cleavage of genomic DNA into oligonucleosomal fragments is a biochemical hallmark of apoptosis. When COLO and Raji cells were treated with 1 μM Ageritin for 48 h the breakdown of the nuclear DNA into oligonucleosomal fragments was clearly observed (Figure 7b). To demonstrate the involvement of caspase-dependent apoptosis, caspase 3/7 activation

was measured in cells exposed to Ageritin for 48 h. This experiment showed strong dose-dependent activation of effector caspases in both COLO 320 and HeLa cells (Figure 7c). It has been reported that α-sarcin also promotes cell death by apoptosis [38] . As expected, high caspase-3/7 activity could be detected in both COLO 320 and HeLa cells treated with α-sarcin but the activation was lower than in Ageritin-treated cells. To further determine the role of caspase-dependent apoptosis, the pan-caspase inhibitor Z-VAD, which irreversibly binds to the catalytic site of caspases, was used to selectively inhibit the apoptotic pathway. HeLa cells were pretreated and maintained in 100 μM Z-VAD, and the cell viability was determined for different Ageritin concentrations. As shown in Figure 7d, caspase inhibition by Z-VAD largely prevented the cytotoxicity of Ageritin after 48 h. In the presence of the necroptosis inhibitor Necrostatin (Nec-1), cell death induced by Ageritin was also significantly reduced (Figure 7d). However, this protection was lower than that observed with Z-VAD. All together these results suggest that cell death induced by Ageritin occur mainly by apoptosis but in combination with necroptosis.

Whether cell death and inhibition of protein synthesis are linked is not clear yet. Previous studies showed that α-sarcin killed rhabdomyosarcoma cells via apoptosis and suggested that α-sarcin-induced caspase activation is a pathway downstream of the 28S rRNA catalytic cleavage [38]. However, there are some studies showing the ability of α-sarcin to inhibit protein biosynthesis and promote cell death independently of its rRNA cleavage activity and JNK activation [46, 47]. Therefore alternative cell death mechanisms besides protein synthesis inhibition could also be involved in Ageritin toxicity.

In conclusion, ribotoxins might favour the resistance of fungus against a broad variety of pathogens due to having several enzymatic activities, opening new biotechnological applications in agriculture. On the other hand, the induction of cell death by different mechanisms turns these proteins into more useful tools for cancer treatment rendering the selection of ribotoxin-resistant mutants impossible. This could be of particular interest for the construction of immunotoxins and other conjugates for targeting therapy, in particular for cancer therapy. Further work with different pathogens *in vivo* and *in vitro* will be conducted to study the conditions in which the different activities could be more prevalent with respect to the others, since it is probable that the different enzymatic activities have a different significance when the invader pathogen is a virus, a bacterium, a fungus or an invertebrate. *P. digitatum* could be, due to its high sensitivity,

a good model for studying the antifungal properties of ribotoxins. On the other hand, the study of the toxicity of ribotoxin mutants will contribute to a better understanding of the interaction mechanisms between these proteins and the fungal plasma membrane. Finally, the fact that Ageritin seems to behave against ribosomes in a similar way to other ribotoxins and that, conversely to α-sarcin, it is activated by magnesium ions and inhibited by EDTA, suggests that the binding of Ageritin to ribosomes could be similar to that of other ribotoxins but that it might use a different mechanism of catalysis. Further research, including crystallization, if possible, and X-ray diffraction studies will aid to resolve this important question.

METHODS

The sources of the chemicals and the methods have been described previously [24, 43, 48, 49]. Particular experimental details are given in the Supporting Information.

The ribonuclease activity of ribotoxins on tobacco mosaic virus (TMV) RNA was assayed in 25 μl samples containing 5 μg of TMV RNA in 10 mM Tris-HCl buffer (pH 7.6), which were incubated with 1 μg (2.4 μM) of the corresponding protein in the absence or the presence of 5 mM Mg^{2+} or 25 mM EDTA. After treatment, the RNA was extracted and analysed by electrophoresis as described elsewhere [48].

DNA from *E. coli, P. digitatum* and COLO 320 cells was isolated following the instructions of the Genomic Prep Cells and Tissue DNA Isolation Kit. Endonuclease activity of ribotoxins was determined by incubating 200 ng of DNA with 1 μg (5.9 μM) of ribotoxin in 10 μL of a reaction mixture which contained 10 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM NaCl and 50 mM KCl, pH 7.8. Samples were incubated for 1 h at 37 ºC and analysed by electrophoresis on agarose gel.

Antibacterial activity was screened using a turbidimetric method. Bacterial suspensions were incubated at 25 ºC for 9 h in the absence or in the presence of Ageritin ranging 20 to 80 μg mL^{-1} and the absorbance of each tube was determined at 660 nm.

Fungicidal activity of Ageritin toward *P. digitatum* conidia were determined by incubating 1×10^4 conidia mL^{-1} with 180 μg mL^{-1} of Ageritin in distilled water at 26 °C. At 0, 2, 5 and 20 hours, aliquots were removed, diluted, and plated onto PDA plates. Plates were incubated 48 h at 26 °C, and CFU were counted.

To assess the effects of Brefeldin A and monensin, cells were preincubated for 1 h with these substances at a final concentration of 5 μM for Brefeldin A and 0.1, 0.5 and 10 μM for monensin and then ribotoxins were added and after 18 h of further incubation, viability was determined [49].

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ASSOCIATED CONTENT

Supporting Information Available: This material is available free of charge via the Internet.

Materials and Methods

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LEGENDS OF FIGURES

Figure 1. Antibacterial and antiviral activities of Ageritin and α-sarcin **(a)** rRNA endoribonuclease activity of Ageritin and α-sarcin on *E. coli* ribosomes. Each lane contained 3 μg of RNA isolated from untreated (C), 5 μg Ageritin-treated (A) or 3 μg α-sarcin-treated (αS) *E. coli* ribosomes. The arrow indicates the fragment (α-fragment) released as a result of ribotoxin action. Numbers indicate the size of the standards in nucleotides. **(b)** rRNA endoribonuclease activity of Ageritin on *M. lysodeikticus* ribosomes. Each lane contained 3 μg of RNA isolated either from untreated or Ageritin-treated *M. lysodeikticus* ribosomes. The arrow indicates the α-fragment and the numbers indicate the size of the standards in nucleotides **(c)** Antibacterial activity of Ageritin against *M. lysodeikticus*. Bacterial suspensions were incubated with different concentrations of Ageritin at 25 ºC for 9 h and cell viability was evaluated by a turbidimetric assay as indicated in the Methods section. Percentage bacterial survival after 9 h was calculated by considering A_{660} of an untreated control at time 0 as 100%. Data represent the mean ± SD of three experiments performed in duplicate. **(d)** Ribonuclease activity of Ageritin and α-sarcin against TMV RNA. Ribonuclease activity of 1 μg of protein was assayed on TMV RNA in the absence (-) or the presence (+) of 5 mM Mg^{2+} or 25 mM EDTA. Each lane contained 1 μg of RNA. C: untreated TMV RNA.

Figure 2. Nicking endonuclease activity of Ageritin on pCR2.1 DNA. 200 ng of plasmid DNA were incubated with either 0.1–1 μg Ageritin or 3–5 μg α-sarcin in the absence (-) or the presence (+) of 5 mM Mg^{2+} or 25 mM EDTA. L: pCR2.1 DNA was previously linearized using EcoRI. R, L, and S indicate relaxed, linear and supercoiled forms of pCR2.1, respectively and the numbers, the size of the standards (M) (λDNA HindIII/EcoRI) in bp.

Figure 3. Nuclease activity of Ageritin and α-sarcin on genomic DNA from *E. coli*, *P. digitatum* and COLO 320 cell culture. Nuclease activity of 1 μg of protein was assayed on genomic DNA from *E. coli*, *P. digitatum* or COLO 320 cells in the absence (-) or the presence (+) of 5 mM Mg^{2+} or 25 mM EDTA. Each lane contained 100 ng of DNA. C:

control; A: Ageritin; αS: α-sarcin. The numbers indicate the size of the standards (M) in bp.

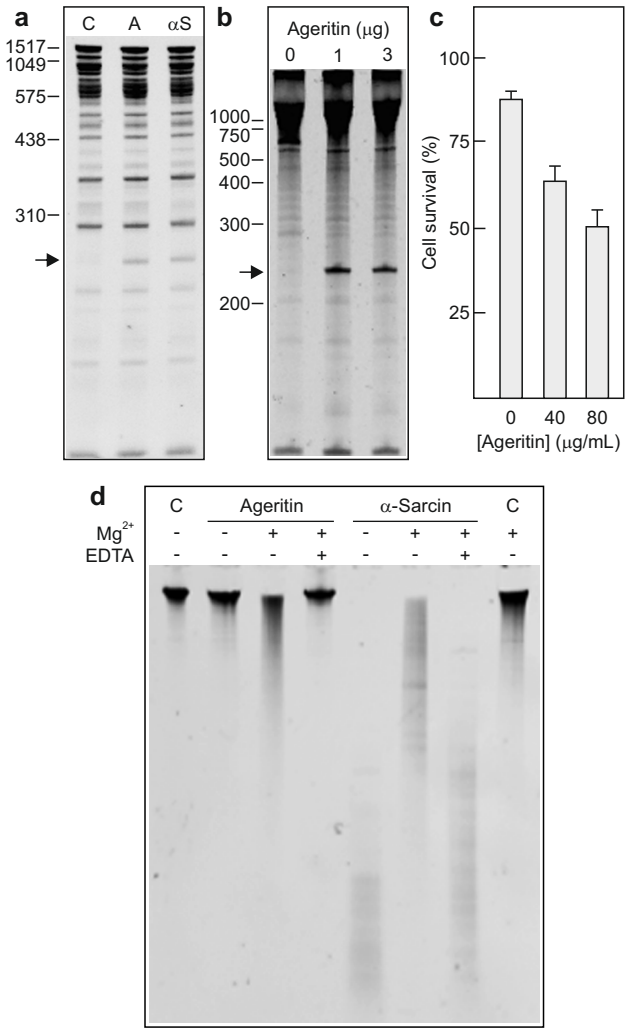
Figure 4. Antifungal activity of Ageritin against *P. digitatum*. Upper panels: Conidia of *P. digitatum* were grown at 26 ºC in PDB medium in the presence of different concentrations of Ageritin added at 0 (left), 24 (center) and 48 h (right). Fungal growth was measured as an increase in absorbance at 620 nm. The curves represent buffer control (●), 5 μg mL^{-1} Ageritin (○), 10 μg mL^{-1} ageritin (■), 30 μg mL^{-1} Ageritin (□) and 50 μg mL^{-1} Ageritin (▲). Lower panels: Morphological changes of *P. digitatum* mycelium exposed to Ageritin. *P. digitatum* mycelium was grown in the absence (control) or in the presence of 30 μg mL^{-1} Ageritin. After 40 h incubation, samples were visualized using light microscopy at 200× magnification.

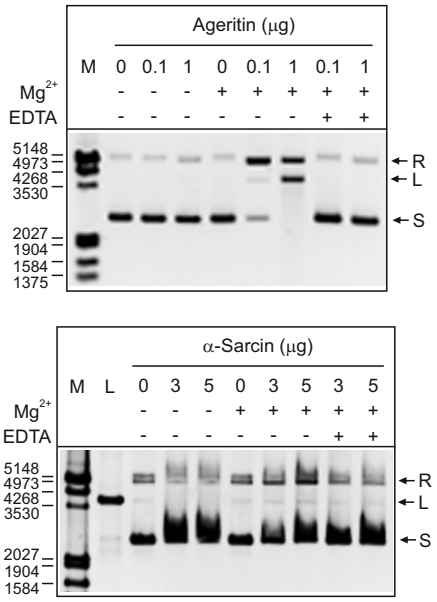
Figure 5. Antifungal and rRNA endonuclease activity of Ageritin against *P. digitatum*. **(a)** *P. digitatum* was grown at 26 ºC in PDB in the absence (control) or in the presence of 25 μg mL^{-1} Ageritin. After 48 h incubation, samples were visualized using light microscopy at 40× magnification. After 96 h incubation, the mycelium was extensively washed with sterile water and harvested to extract the RNA and the DNA. **(b)** rRNA endonuclease activity. Each lane contained 3 μg of RNA isolated from either untreated (C) or Ageritin-treated (A) cultures from *P. digitatum*. The arrow indicates the RNA fragment released as a consequence of Ageritin action. Numbers indicate the size of the standards in nucleotides. (**c)** The DNA was isolated from untreated (C) or Ageritin-treated (A) cultures from *P. digitatum* and 4 μg was electrophoresed. The numbers indicate the corresponding size of the standards (M) in bp.

Figure 6. Cytotoxicity of Ageritin and α-sarcin on HeLa, Raji and COLO 320 cells. **(a)** Effect of ribotoxins on viability of HeLa (squares), Raji (triangles) and COLO 320 (circles) cells. Cells were incubated with different concentrations of Ageritin (filled symbols) and α-sarcin (open symbols) for 48 h and cell viability was evaluated by a colorimetric assay. Data represent the mean ± SD of three experiments performed in triplicate. (**b)** rRNA endoribonuclease activity of Ageritin and α-sarcin on RNA from

COLO 320 and HeLa cells. Each lane contained 3 μg of RNA isolated from either untreated cells or cells incubated with the ribotoxins for 48 h (HeLa cells with 5.3 and 53 nM Ageritin and 5.8 and 58 nM α-sarcin and COLO 320 with 5300 nM Ageritin). The arrow indicates the RNA fragment released as a result of ribotoxin action (**c)** Effect of Brefeldin A and monensin on viability of HeLa cells treated with Ageritin. Cells were preincubated with Brefeldin A and monensin for 3 h and then incubated with different concentrations of Ageritin for 18 h and cell viability was evaluated by a colorimetric assay. Symbols: (●), untreated; (○), Brefeldin A; (■), 0.1 μM monensin; (△), 0.5 μM monensin; (▲), 10 μM monensin.

Figure 7. Induction of apoptosis by Ageritin and α-sarcin on cultured cells. **(a)** Phase contrast microscopy and double staining with AnnexinV(green)/PI(red), followed by fluorescence microscopy at 48 h after treatment of HeLa cells with 1 nM Ageritin. Bar, 100 μm. **(b)** Effect of Ageritin on internucleosomal DNA fragmentation. COLO 320 and Raji cells were incubated in the absence (C) or presence (A) of 1 μM of Ageritin for 48 h. The DNA was isolated and 4 μg was electrophoresed. The numbers indicate the corresponding size of the standards (M) in bp. **(c)** Caspase-3/7 activation in COLO 320 and HeLa cells treated with different concentrations of Ageritin (filled bars) and α-sarcin (open bars) for 48 h. Activity is expressed as the percentage of control values obtained from cultures grown in the absence of the ribotoxins (dashed line). Data represent the mean ± SD of two experiments performed in duplicate. **(d)** Effect of Z-VAD and Nec-1 on cytotoxicity of Ageritin on HeLa cells. Cells were left untreated (○) or preincubated with Z-VAD (●) or Nec-1 (■) for 3 h and then incubated with different concentrations of Ageritin for 48 h and cell viability was evaluated by a colorimetric assay. Data represent the mean ± SD of three experiments performed in triplicate.


a
C
A
αS
1517
1049
575
438
310
b
Ageritin (μg)
0
1
3
1000
750
500
400
300
200
c
100
75
50
25
Cell survival (%)
0
40
80
[Ageritin] (μg/mL)
d
C
Ageritin
α-Sarcin
C
Mg2+
EDTA


Ageritin (μg)
M 0 0.1 1 0 0.1 1 0.1 1
Mg2+ - - - + + + + +
EDTA - - - - - - + +
5148
4973
4268
3530
2027
1904
1584
1375
R
L
S
α-Sarcin (μg)
M L 0 3 5 0 3 5 3 5
Mg2+ - - - + + + + +
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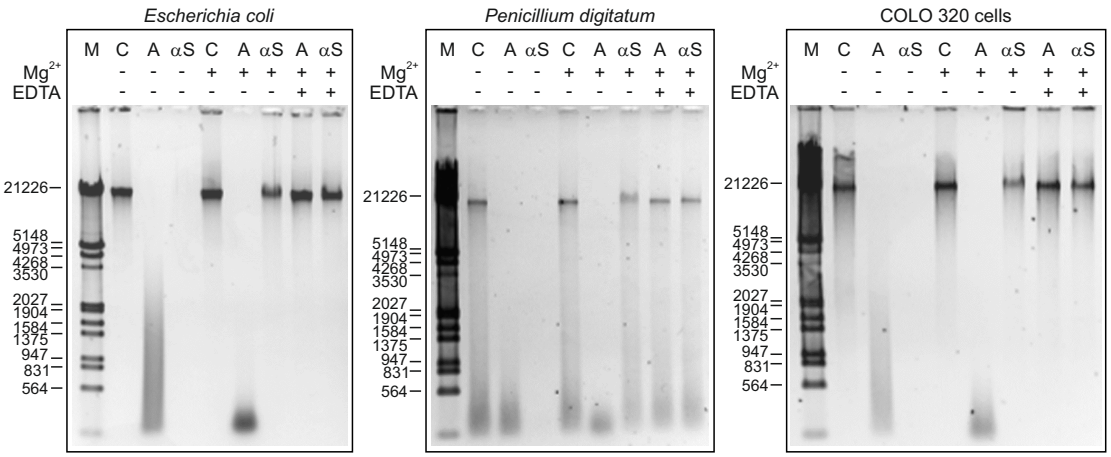
Escherichia coli
Penicillium digitatum
COLO 320 cells
M C A αS C A αS A αS
Mg2+
EDTA
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4973
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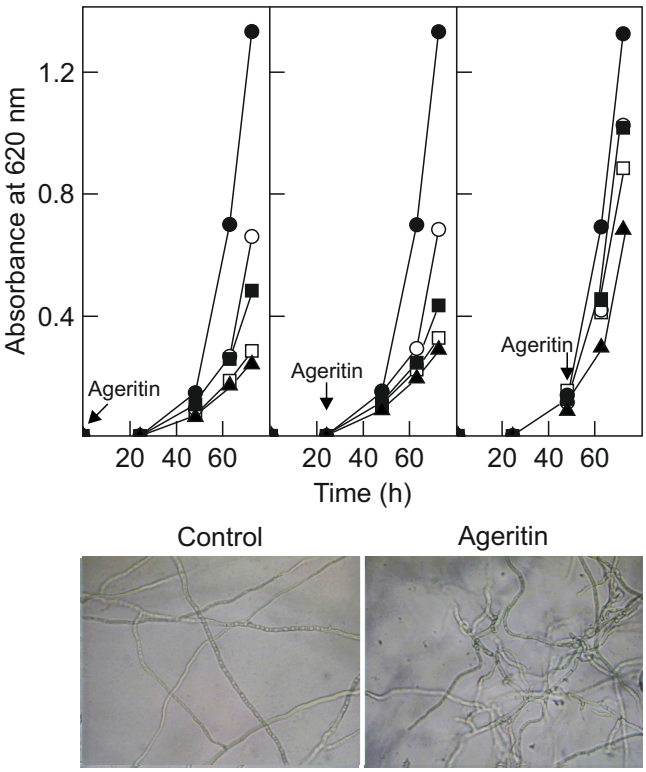
3530
2027
1904
1584
1375
947
831
564


Absorbance at 620 nm
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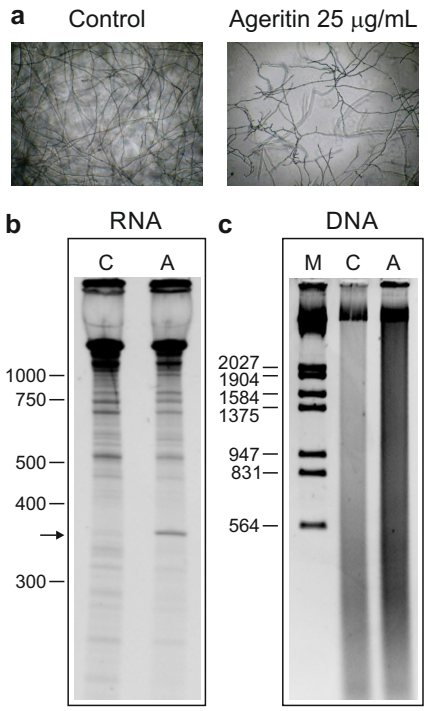
0.4
Ageritin
Ageritin
Ageritin
20 40 60
20 40 60
20 40 60
Time (h)
Control
Ageritin


a
Control
Ageritin 25 μg/mL
b
RNA
C
A
1000
750
500
400
300
c
DNA
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A
2027
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1375
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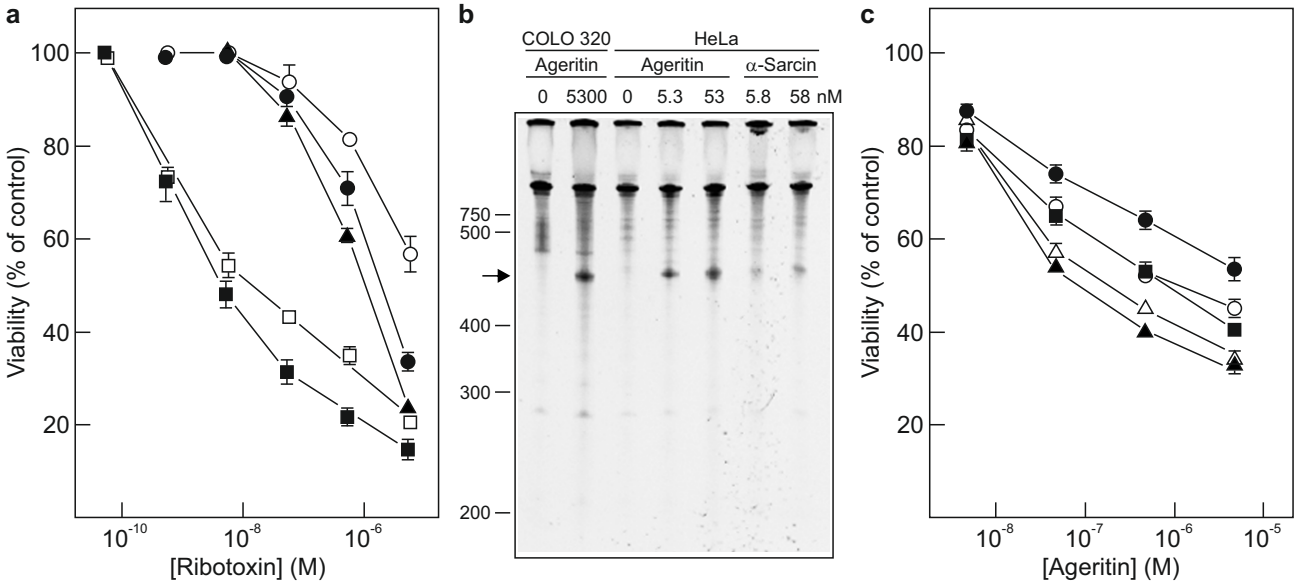
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Viability (% of control)
100
80
60
40
20
10^{-10}
10^{-8}
10^{-6}
[Ribotoxin] (M)
b
COLO 320
HeLa
Ageritin
Ageritin
α-Sarcin
0 5300 0 5.3 53 5.8 58 nM
750
500
400
300
200
c
Viability (% of control)
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80
60
40
20
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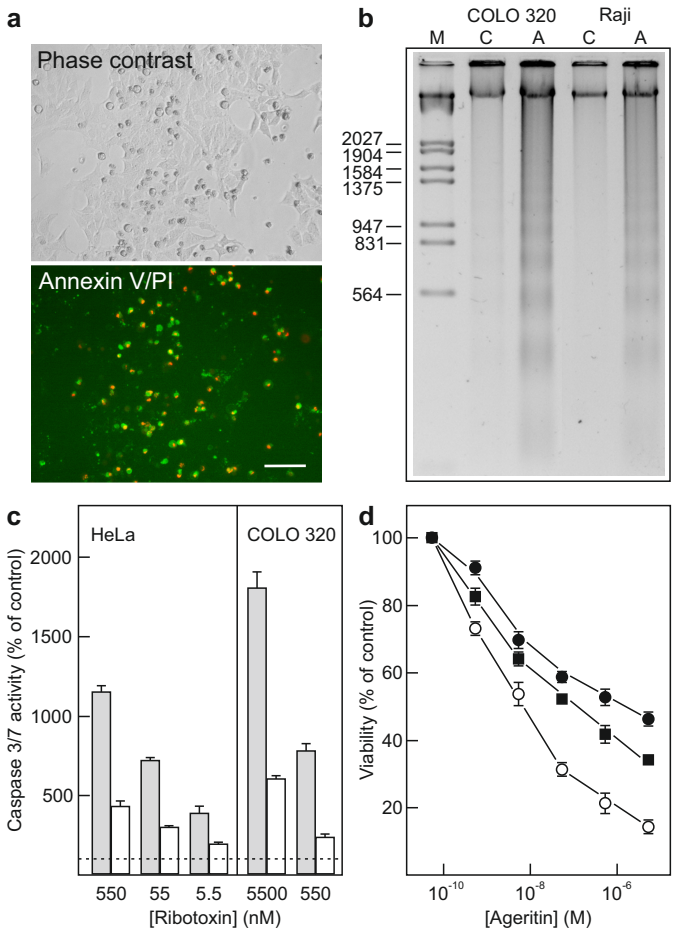
10^{-7}
10^{-6}
10^{-5}
[Ageritin] (M)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Phase contrast
Annexin V/PI
b
COLO 320
Raji
M
C
A
C
A
2027
1904
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1375
947
831
564
c
HeLa
COLO 320
Caspase 3/7 activity (% of control)
2000
1500
1000
500
550
55
5.5
5500
550
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Viability (% of control)
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80
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40
20
10-10
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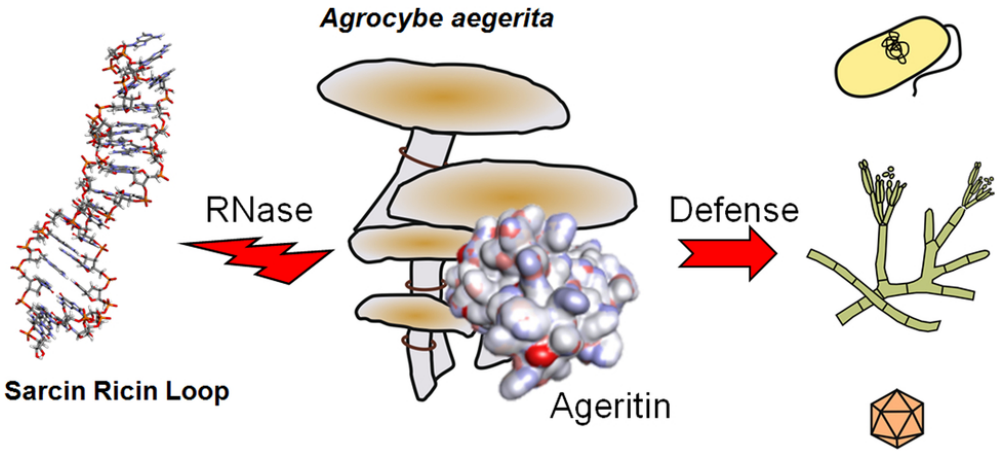
10-6
[Ageritin] (M)



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