Ageritin from Pioppino Mushroom

Subjects: Toxicology

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Ageritin is a specific ribonuclease, extracted from the edible mushroom *Cyclocybe aegerita* (synonym *Agrocybe aegerita*), which cleaves a single phosphodiester bond located within the universally conserved alpha-sarcin loop (SRL) of 23–28S rRNAs.This toxin is the prototype of ribotoxin-like protein family present in edible mushroom and possesses antifungal/antiviral activities and selective cytotoxicity against tumor cells with potential use in biotechnological applications (as bio-insecticides or antitumor agents).

Keywords: Agrocybe aegerita ; Cyclocybe aegerita ; antiviral activity ; antifungal activity ; endonuclease activity ; cytotoxicity ; protein structure function

1. Introduction

Since always, nature inspires medicine for the diversity of biologically active compounds with therapeutic properties and the great attention that researchers paid on mushrooms is one of the most appropriate examples, which deserves to be further investigated. Many fungal species are traditionally used in Chinese medicine or as functional foods in Japan and other Asian countries, representing a great source of nutraceuticals, functional foods, and secondary metabolites. In this context, fungi are useful to discovering new drugs; and many bioactive compounds such as small molecules, polysaccharides, proteins, and polysaccharide–protein complexes have been isolated from them [1][2]. A number of bioactive proteins and peptides, including lectins, fungal immunomodulatory proteins (FIPs), ribosome-inactivating proteins (RIPs), antifungal and antimicrobial proteins, laccases, ribonucleases, and ribotoxins have been isolated and characterized from mushrooms [3]. Among them, fungal ribotoxins are a family of highly specific extracellular rRNA endonucleases (EC 3.1.27.10) well studied since 1960. They are produced by fungi belonging to Ascomycota phylum, mostly from the genus *Aspergillus*, such as the prototype α-sarcin from *Aspergillus giganteus* [4] and restrictocin and mitogillin from *Aspergillus restrictus* [5], while hirsutellin A and anisoplin are isolated by *Hirsutella thompsonii* and *Metarhizium anisopliae*, respectively [6][7]. Specifically, ribotoxins exert their toxicity by entering the cells and cleaving a single phosphodiester bond between G_{4325} and A_{4326} (rat 28S rRNA numbering) located in the Sarcin Ricin Loop (SRL) on the larger RNA subunit (rRNA) of the ribosome [8]. This region is necessary for EF-G or EF-2 elongation factors' ribosome interaction during mRNA–tRNA translocation in prokaryotes and eukaryotes, respectively [9]. The cleavage leads to the release of a 460-nt fragment, known as α-fragment, at the 3′ end of the 28S RNA, causing the inhibition of protein biosynthesis, followed by cellular death through an apoptotic pathway [10][11]. From a structural point of view, ribotoxins are basic proteins of ~150 amino acids residues with a high degree of identity and an ordered secondary structure including two conserved disulphide bridges with a β-sheet, where is located the active site, and a short α-helix [12]. On the other hand, the biological function of ribotoxins is not yet clear, although several studies highlight their defence role as insecticidal and antifungal agents [12][13]. Considering that, until now, no protein receptors exist for ribotoxins, the alteration of cell permeability produced, for example, by tumour transformation or a viral infection facilitates these toxins to cross membranes, making them anticancer or antiviral agents [12].

Recently, a novel family of protein synthesis inhibitors has been discovered in fungi belonging to Basidiomycota phylum. These enzymes, the prototype of which is Ageritin from *Cyclocybe aegerita* (synonym *Agrocybe aegerita*), are specific ribonucleases, named as ribotoxin-like proteins (RL-Ps), that, as ascomycetes ribotoxins, basically act on large rRNA, releasing the specific α-fragment, although the two families differ in structure and mechanism of action [14][15].

2. Isolation of Ageritin from *Cyclocybe aegerita* (V. Brig.) Singer

At the beginning of 2017, Landi and co-workers reported the isolation of a novel toxin, named Ageritin, from the edible mushroom *C. aegerita* [14]. This is the first documented evidence of the presence in the Basidiomycota phylum of a specific ribonuclease that following ribosomes incubation causes the release of the diagnostic α-fragment, inhibiting protein synthesis in vitro [14]. These activities are similar to those exerted by α-sarcin [16], the prototype of ribotoxins family

from Ascomycetes filamentous fungi [12]. Due to its peculiar structural and functional characteristics, Ageritin differs from Ascomycetes ribotoxins, named by us ‘classic ribotoxins’, and is considered the prototype of a novel family of specific ribonucleases, called ‘ribotoxin-like proteins’ isolated from basidiomycetes [14]. Ageritin can be purified at homogeneity from *C. aegerita* fruiting bodies by using a well-established protocol for the purification of basic proteins [17]. In particular, total proteins extracted in a phosphate-saline buffer were precipitated at pH 4.0 using acetic acid. The obtained soluble proteins were fractionated by cation exchange chromatography on Streamline SP and eluted with 1M NaCl. Subsequently, a gel-filtration chromatography on Sephacryl S-100-HR allowed isolating a pool of active proteins with an elution volume of ~17 k. Subsequently, the pooled fractions were dialyzed and subjected to cation exchange chromatography on an AKTA purifier system. Purified Ageritin was obtained with an estimated amount of about 1.3 mg/100 g of fresh fruiting bodies. Protein synthesis inhibition was monitored by applying the procedure reported previously [14].

In 2019, Ragucci and co-workers optimized the purification procedure of Ageritin by employing a scale-up protocol optimized to shorten purification times and to increase the protein amounts [18]. For this purpose, a gel-filtration step using a Superdex75 Hiload 26/60 column on an AKTA purifier system, followed by a cation exchange chromatography step on a SP-Sepharose fast flow, allowed a significant increase in the protein purification yield (2.5 mg/100 g of fresh fruiting bodies) [18]. By this procedure, we also purify at homogeneity an Ageritin isoform, after named Met-Ageritin, with an additional *N*-terminal methionyl residue, as unique structural difference with respect to Ageritin primary structure. However, this apparently unique inequality dramatically alters the enzymatic features of Met-Ageritin despite, as Ageritin releases the α-fragment and inhibits protein synthesis in vitro with an IC_{50} of 2.8 nM, a value 21-fold higher than that of Ageritin [19].

3. Antiproliferative and Defense Activities of Ageritin

3.1. Cytotoxic Activity

Many studies documented the cytotoxicity of Ageritin toward several tumour cell lines as summarized in Table 1. For instance, Landi et al. [14] describe the effects of Ageritin toward different neural and glial tumour cell lines, while Citores et al. [20] tested the effects of Ageritin on COLO 320, HeLa and Raji cells. Moreover, a novel property of Ageritin has emerged in a recent work by Ragucci et al. investigating the cytotoxic action of Ageritin against SH-SY5Y neuroblastoma cells (undifferentiated or retinoic acid differentiated) showing a selective cell toxicity against undifferentiated cells [18]. The selective toxicity of Ageritin versus malignant cells was also confirmed by Lampitella and co-workers that described the significant effects of the toxin on cancer SVT2 cells viability, while no or slight effects were observed on normal BALB/c 3T3 cells [21]. In the same study, immunofluorescence experiments proved the selective internalization of Ageritin into cancer cells, in association with its selective cytotoxic activity. Therefore, this selective action against tumour cells could make this toxin a novel potential tool for anticancer drugs. Overall, these studies suggest that apoptosis acts as the main mechanism responsible for cell death induced by Ageritin. Cytotoxicity results varied among tested cell lines, with HeLa cells being the most sensitive cells and IC_{50} ranging from nanomolar to micromolar (Table 1). In a pivotal study, Landi and co-workers also highlight the pro-apoptotic effect of Ageritin towards central nervous system (CNS) model cell lines mediated by the activation of caspase-8 (extrinsic pathway) and by the induction of nuclear fragmentation [14].

Table 1. Cytotoxic activity of Ageritin against different cell lines.

Cell Line	Organism	Tissue	Morphology	Disease	Culture Properties	IC_{50} at 48 h		Ref.
						µg/mL	µM	
SH-SY5Y	Human	Bone marrow	Epithelial	Neuroblastoma	Mixed, adherent/suspension	77.30	5.15	[18]
SK-N-BE(2)-C	Human	Bone marrow	Neuroblast	Neuroblastoma	Mixed, adherent/suspension	8.41	0.56	[14]
C6	Rat	Brain	Fibroblast	Glioma	Adherent	4.58	0.30	[14]
U-251	Human	Brain	Pleomorphic/ astrocytoid	Glioblastoma	Adherent	9.46	0.63	[14]
HeLa	Human	Cervix	Epithelial	Carcinoma	Adherent	0.06	0.004	[20]
Colo 320	Human	Colon	rounded and refractile	Adenocarcinoma	Mixed, adherent/suspension	28.50	1.90	[20]
Raji	Human	Lymphoblast	Lymphoblast	Lymphoma	Suspension	15.00	1.00	[20]

Cell Line	Organism	Tissue	Morphology	Disease	Culture Properties	IC_{50} at 48 h		Ref.
						µg/mL	µM	
SVT2 *	Mouse	Embryo	Fibroblast	-	Adherent	26.00	1.73	[21]
Balb/c 3T3 **	Mouse	Embryo	Fibroblast	-	Adherent	78.00	5.20	[21]
Sf21	Fall armyworm	Ovary	-	-	Suspension	n.r.	n.r.	[22]

* Simian-virus-40-transformed mouse fibroblasts. **, parental non-transformed BALB/c 3T3 cells. n.r., not reported.

The activation of apoptosis pathway induced by Ageritin was further confirmed by the caspase-3 enzymatic activity and protein levels of cleaved PARP in SH-SY5Y cells [18]. Moreover, the irreversible pancaspase inhibitor Z-VAD-FMK was able to prevent the apoptotic pathway activation induced by Ageritin, confirming that the process was completely caspase-dependent [18].

In addition, Citores et al. hypothesized that besides apoptosis, cell death induced by Ageritin was also mediated by necroptosis [20]. Indeed, Ageritin-treated HeLa cells exposed phosphatidylserine on the cell surface, as revealed by an increase in the level of annexin VFITC-positive cells, suggesting the involvement of apoptosis. In addition, the visualization of propidium iodide (PI) staining indicated late stage apoptosis or necrosis. The involvement of caspase-dependent apoptosis was confirmed by the high caspase-3/7 activity in both COLO 320 and HeLa cells, with the cytotoxicity prevented by Z-VAD. However, following the addition of the necroptosis inhibitor Necrostatin (Nec-1), there is a strong decrease of cell death mediated by Ageritin [20]. Then, Citores et al. tested Ageritin toxicity on HeLa cells following the addition of some substances interfering with intracellular routing, such as the fungal inhibitor Brefeldin A and the ionophore monensin in order to study the Ageritin intracellular pathway [20]. In particular, Brefeldin A was shown to cause Golgi complex disassembly associated with an increase in protein cytotoxicity, indicating that Ageritin follows a Golgi-dependent pathway to the cytosol. It was reported that monensin possesses a pH-neutralizing effect in endosomes/lysosomes at high concentrations, lacking this effect at low concentrations, although it influences Golgi structure and function [23]. According to previous findings on the importance of Golgi transport for translocation, it was observed that low concentrations of monensin-pretreated HeLa cells enhanced the cytotoxicity of Ageritin. On the contrary, high ionophore concentrations sensitized the cells to Ageritin, indicating that the protein does not require a low pH for translocation to the cytosol [20]. A study by Lampitella and co-workers [21] suggests that Ageritin is also able to enter malignant cells by altering cell membranes, as occurred in model membranes treated with α-sarcin [24][25], bovine seminal ribonuclease [26][27] and chimeric constructs consisting of an amino-terminal type 1 RIP domain fused to a *C*-terminal protease inhibitor domain [28]. Indeed, studying the changes induced by Ageritin, in thermotropic phase transition of liposomes as models of normal and cancer eukaryotic cell membranes, Lampitella and co-workers demonstrated that Ageritin is unable to interact with normal eukaryotic model membranes (DPPC/Chol liposomes). On the other hand, there is a strong perturbation of cancer cell liposomes (DPPC/DPPS/Chol) due to the preferential interaction of basic Ageritin with anionic DPPS, which mediate protein translocation to the cytosol [21]. It was also reported that Ageritin interacts with bacterial model membranes, albeit only superficially, supporting the finding that Ageritin is not cytotoxic against several bacterial cells [21][20].

3.2. Antifungal Activity

The antifungal activity of Ageritin (see Table 2) represents one of its most interesting features, since it is currently believed that fungal ribonucleases such as ribotoxins have no direct effects on fungi. Nevertheless, in 2018, Citores et al. reported that α-sarcin was endowed with antifungal activity towards the green mold *P. digitatum*, entering the cytosol and inactivating the ribosomes, and killing the cells by arresting the fungal growth [13].

Table 2. Antifungal activity of Ageritin against different fungal species.

Organism	Strain	Observation in Petri Dish	(°C) **	Medium	Growth Conditions	Toxin Final Concentration (μM)	Growth Inhibition (%)	Ref.
Filamentous fungus	*Penicillium digitatum* *		26	PDB [a]; 150 μL	100 spores/well	2.9	79.0	[20]
						1.8	76.0	
						0.6	63.0	
						0.3	49.0	
Filamentous fungus	*Trichoderma asperellum* (TC74)		26	PDB [a]; 150 μL	100 spores/well	13.3	46.6	[19]
						6.7	29.2	
						3.3	23.7	
Unicellular fungus	*Saccharomyces cerevisiae* (BY4741)		30	YPD [b]; 5 mL	0.1 O. D. [c]	13.3	~10	[19]

* Strain typified from Spanish Type Culture Collection (CECT), ** Optimal Growth Temperature; Valencia, Spain; [a] Potato dextrose broth; [b] Yeast extract peptone dextrose; [c] Initial optical absorbance at 600 nm.

As described for α-sarcin, Ageritin inhibited the growth of *P. digitatum* by acting on major rRNA and irreversibly inactivating fungal ribosomes, thus inhibiting protein synthesis. Indeed, Ageritin was less active than α-sarcin and a concentration 60-fold higher was required to inhibit fungal growth. This difference of activity could be possibly related to their diverse ability to cross cell membranes [20]. Subsequently, Ragucci et al. tested the antifungal activity of Ageritin against the filamentous fungus *Trichoderma asperellum* and the single-celled eukaryote fungus *Saccharomyces cerevisiae.* This study demonstrated that Ageritin is able to exert inhibitory action only against the *T. asperellum* although the protein concentration able to inhibit 50% of fungal growth was about 40-fold higher than that required for *P. digitatum* growth inhibition [18]. These findings are in agreement with the defense role hypothesized for Ageritin, also considering that during cultivation, edible mushrooms could be contaminated by several ascomycetes fungi, such as *Trichoderma* species, that cause fungal fruiting bodies damage by inhibiting its growth [29]. RNAs from Ageritin-treated *P. digitatum* were also isolated and analyzed confirming the release of α-fragment mediated by Ageritin in vivo [20].

3.3. Entomotoxic and Nematotoxic Activity

Entomotoxic activity is a common feature of classic ribotoxins from ascomycetes [30], such as anisoplin [7] and hirsutellin A [31], both toxic towards insect cells. In light of this, Tayyrov and coworkers recently demonstrated that, although less active than α-sarcin, Ageritin also exerts strong toxicity against *Aedes aegypti* larvae and *Spodoptera frugiperda* Sf21 cells [22]. These findings suggest a defense role for Ageritin in *C. aegerita* mushroom towards insect antagonists, like fungus gnats. On the other hand, since nematodes are important predators of fungi, Tayyrov and co-workers tested the nematotoxicity of Ageritin, considering that it structurally differs from other ribotoxins, which not display this activity [22][32]. However, as already reported for classic ribotoxins, Ageritin is inactive, indicating that nematode ribosomes are either resistant or not accessible to Ageritin [22].

3.4. Antibacterial Activity

Bacteria can cause significant yield losses in mushrooms crops, being responsible for a wide range of mushroom diseases [33]. Since bacterial ribosomes are sensitive to Ageritin, the antifungal activity of this ribonuclease (native form) was tested on several species such as *Micrococcus lysodeikticus*, *Escherichia coli*, *Pectobacterium carotovorum*, *Serratia marcescens*, and *Rhizobium leguminosarum* [20]. In another work, both native and alkylated Ageritin were tested on a panel of selected Gram-negative (*E. coli* ATCC 25922, *Salmonella enterica* 706 RIVM and *Pseudomonas aeruginosa* 01) and Gram-positive (*Bacillus globigii* TNO BMO13, *Staphylococcus aureus* ATCC 12,600 and MRSA WKZ-2) strains [21]. Among all tested bacteria, native Ageritin is toxic only against *M. lysodeikticus* with a concentration of 5.3 μM leading to 50% of growth inhibition. Moreover, a very limited susceptibility against the alkylated protein was only observed for the MRSA WKZ-2 strain, likely due to a different interaction of the ribotoxin with the bacterial plasma membrane and/or cell wall and its ability to cross them [21][20].

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