

Manuscript title: antifungal proteins from moulds: analytical tools and potential application to dry-ripened foods

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Abstract Moulds growing on the surface of dry-ripened foods contribute to their sensory qualities, but some of them are able to produce mycotoxins that pose a hazard to consumers. Small cysteine-rich antifungal proteins (AFPs) from moulds are highly stable to pH and proteolysis and exhibit a broad inhibition spectrum against filamentous fungi, providing new chances to control hazardous moulds in fermented foods. The analytical tools for characterizing the cellular targets and affected pathways are reviewed. Strategies currently employed to study these mechanisms of action include ‘omics’ approaches that have come to the forefront in recent years, developing in tandem with genome sequencing of relevant organisms. These techniques contribute to a better understanding of the response of moulds against AFPs, allowing the design of complementary strategies to maximize or overcome the limitations of using AFPs on foods. AFPs alter chitin biosynthesis, and some fungi react inducing cell wall integrity (CWI) pathway. However, moulds able to increase chitin content at the cell wall by increasing proteins in either CWI or calmodulin-calcineurin signalling pathways will resist AFPs. Similarly, AFPs increase the intracellular levels of reactive oxygen species (ROS), and moulds increasing G-protein complex β subunit CpcB and/or enzymes to efficiently produce glutathione may evade apoptosis. Unknown aspects that need to be addressed include the interaction with mycotoxin production by less sensitive toxigenic moulds. However, significant steps have

been taken to encourage the use of AFPs in intermediate-moisture foods, particularly for mould-ripened cheese and meat products.

Keywords Antifungal proteins · Food safety · Proteomics · Toxigenic moulds

Introduction

Ripened foods are spread across the world and represent an important sector in the economy. Fermented foods require the contribution of flavour-producing microorganisms, typically lactic acid bacteria (LAB) and Gram-positive, catalase-positive cocci and yeasts. Filamentous fungi can also contribute to flavour due to their proteolytic and lipolytic activities, particularly in foods of intermediate water activity, such as dry-ripened cheese (Banjara et al. 2015), sausages (Bruna et al. 2003) and hams (Martín et al. 2006).

On the other hand, filamentous fungi can also be responsible for spoilage and health issues in dry-ripened foods. Some moulds have been linked to food allergy after intake of aged mould-coated foods, causing respiratory and digestive disorders in sensitive consumers (Bobolea et al. 2009; González-Olano et al. 2012). Although moulds are not commonly associated with foodborne infections, pneumonitis caused by *Aspergillus fumigatus* is often an occupational disease in grain or sausage handlers (Swan and Crook 1998; Marvisi et al. 2012). However, the most concerning hazard related to mould presence in foods is mycotoxin production, due to their toxic, immunosuppressive, mutagenic and carcinogenic effects (Bezerra da Rocha et al. 2014). Therefore, the control of unwanted moulds in foods is considered a key issue. For this purpose, some efficient physical or chemical treatments, such as modified atmosphere packaging, refrigeration or

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chemical fungicides, are available. However, these treatments are not adequate for mould-ripened foods, since fungal development is essential for their desired sensory characteristics. Moreover, chemical compounds can leave residues, which is against the consumer's demand of residue-free foods. Consequently, biocontrol using natural products could be an alternative to fight against undesirable moulds. Natural antifungal proteins represent an interesting option for biopreservation in foods (Geisen 2000; Delgado et al. 2015a).

Living organisms may defend themselves against fungi by producing antifungal compounds, including proteins. Antifungal proteins (AFPs) are produced by organisms ranging from prokaryotes to plants, insects and mammals (Selitrennikoff 2001). AFPs have been studied as a potential source of new agents to combat opportunistic fungal infections in immunocompromised hosts, as well as fungal plant pathogens. The most extensively studied AFPs are plant defensins (van der Weerden and Anderson 2013), which cause membrane permeabilization in various filamentous fungi (Yan et al. 2015; Zhu et al. 2015) by specifically interacting with host membrane compounds (Vriens et al. 2014).

Moulds can also produce several types of antifungal peptides and proteins, including some cyclic peptides, such as echinocandins (Emri et al. 2013) or aureobasidin A (Liu et al. 2007), enzymes such as glucose oxidase (Leiter et al. 2004) or chitosanases (Rodríguez-Martín et al. 2010b) and defensin-like proteins (Galgóczy et al. 2013). The latter is a group of small, basic and cysteine-rich AFPs that have been the subject of recent research (Table 1).

AFPs produced by non-toxicogenic moulds normally present on foods can be a valuable means to improve food safety in intermediate-moisture foods, particularly in uncontrolled mould-ripened meats and dairy products.

The present review focuses on AFPs produced by moulds as well as on the analytical tools for characterizing the effects of AFPs to gain insight into their mechanism of action and to analyze their potential application to dry-ripened foods.

Main characteristics of AFPs

The main distinguishing features of AFPs and their effect on sensitive moulds have been extensively reviewed elsewhere (Theis and Stahl 2004; Marx 2004; Marx et al. 2008; Hegedüs and Marx 2013). AFP (Campos-Olivas et al. 1995), PAF (Batta et al. 2009) and BP (Seibold et al. 2011) fold into a compact β -barrel stabilized by disulphide bridges, and it has been assumed that AcAFP, Anafp, NAF and PgAFP also have a similar tertiary structure (Marx 2004; Skouri-Gargouri et al. 2009; Rodríguez-Martín et al. 2010a). This structure confers high stability against adverse biochemical and biophysical conditions, such as low pH, high temperature and some surfactants (Lacadena et al. 1995; Marx 2004; Skouri-Gargouri and Gargouri 2008; Batta et al. 2009; Hajji et al. 2010; Chen et al. 2013; Wen et al. 2014; Delgado et al. 2015a). In addition, AFPs withstand the attack of proteases in vitro (Lacadena et al. 1995; Batta et al. 2009; Delgado et al. 2015a). This raises the possibility of applying AFPs directly onto dry-ripened

Table 1 Antifungal proteins produced by moulds

Antifungal protein	Producer mould	pI	MW (Da)	Reference
AcAFP	<i>Aspergillus clavatus</i>	9.3	5773	Skouri-Gargouri and Gargouri (2008)
AcAMP	<i>Aspergillus clavatus</i>	9.06	6000	Hajji et al. (2010)
AFP	<i>Aspergillus giganteus</i>	9.3	5800	Nakaya et al. (1990)
AfpB	<i>Penicillium digitatum</i>	–	–	Garrigues et al. (2015)
AFP _{NN5353}	<i>Aspergillus giganteus</i>	9.3	–	Binder et al. (2011)
Anafp	<i>Aspergillus niger</i>	7.14	6583	Gun Lee et al. (1999)
BP	<i>Penicillium brevicompactum</i>	7.2	6584	Seibold et al. (2011)
FPAP	<i>Fusarium polyphilaidicum</i>	9.1	6357	Galgóczy et al. (2013)
MAFP1	<i>Monascus pilosus</i>	8.3	6500	Tu et al. (2016)
NAF	<i>Penicillium nalgiovense</i>	8.93	6300	Geisen (2000)
NFAP	<i>Neosartorya fischeri</i>	8.93	6625	Kovács et al. (2011)
PAF	<i>Penicillium chrysogenum</i>	8.93	6250	Marx et al. (1995)
Pc-Arctin	<i>Penicillium chrysogenum</i>	–	7000	Chen et al. (2013)
PcPAF	<i>Penicillium citrinum</i>	–	10,000	Wen et al. (2014)
PgAFP	<i>Penicillium chrysogenum</i>	9.22	6494	Rodríguez-Martín et al. (2010a)

foods to control hazardous moulds, even in foods requiring intensive proteolytic degradation during ripening. These characteristics are seen as an advantage for the use of AFPs in low-acid ripened foods, due to the highly proteolytic microbial population naturally present (Rodríguez et al. 1998; Sousa et al. 2001; Hughes et al. 2002).

According to phylogenetic analysis, AFPs have been classified into three groups, with AFP, PAF, AcAFP, NFAP and FPAP in class A; PgAFP and Anafp in class B; and Pc-Arctin and BP in class C (Garrigues et al. 2015).

Spectrum of activity

AFPs from moulds show a broad inhibition spectrum against filamentous fungi and some zygomycetes, but not against prokaryotes or mammalian cells (Marx 2004; Palicz et al. 2013). Some of the most hazardous moulds, including ochratoxigenic *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Penicillium nordicum* and *Penicillium verrucosum*; aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus*; patulin-producers *Penicillium expansum* and *Penicillium griseofulvum*; and the pathogenic and allergenic *A. fumigatus*, are among the species sensitive to AFPs (Kaiserer et al. 2003; Galgóczy et al. 2008; Delgado et al. 2015a). Only AcAMP exhibited inhibitory activity against bacteria (Hajji et al. 2010) and Anafp and BP against yeasts (Gun Lee et al. 1999; Seibold et al. 2011). The generalized lack of activity against bacteria and yeast can be regarded as an advantage for the potential use of AFPs in dry-ripened foods, given the positive role of these microorganisms on the sensory characteristics of dry-ripened foods.

Analytical tools for characterizing the effects of AFPs

Deciphering the mechanisms of action and the cellular targets of AFPs continues to be an important objective. Enhanced understanding of the target informs on the activity spectrum of the AFPs and the relative susceptibilities of various

organisms. Information can be generated on possible modes of resistance, in addition to guiding the design of combination therapies directed at different targets. Furthermore, potential secondary effects can be elucidated, such as undesirable by-products of treatment (e.g. increased mycotoxin production). Some of the strategies currently employed to study these mechanisms of action include genomics, fluorescence assays, transcriptomics, proteomics and metabolite analysis (Table 2). Many of these ‘omics’ approaches have come to the forefront in recent years, developing in tandem with genome sequencing of relevant organisms.

Use of targeted gene deletions or non-functional gene mutations has been employed to verify pathways affected by antifungal agents in sensitive species. Disruption of genes involved in signalling pathways revealed the involvement of G-protein signal transduction in the antifungal activity of PAF from *Penicillium chrysogenum* and NFAP from *Neosartorya fischeri* (Leiter et al. 2005; Virágh et al. 2015). Further similarities were found among the targets of AFPs, with direct involvement of the GTPase RhoA ruled out in the case of PAF, NFAP and AFP_{NN5353} from *Aspergillus giganteus* (Binder et al. 2010b; Binder et al. 2011; Virágh et al. 2015). Genes responsible for conferring resistance to AFPs can also be identified using screens of deletion strains from resistant species. Ouedraogo et al. (2011) revealed a number of genes (*chs1*, *wsc1*, *vps34* and *tor1*) in the AFP-resistant yeast *Saccharomyces cerevisiae* that give rise to intermediate sensitivity upon deletion. This screen of 100 mutants allowed the authors to ascertain the mechanisms affected in sensitive organisms and directed additional analyses to validate these observations. Recent developments allowing more efficient genetic manipulation of filamentous fungi will enable further large-scale screening using gene disruption libraries (Colot et al. 2006; Park et al. 2011; Liu et al. 2015; Nødvig et al. 2015).

Genome-wide surveys of the pathways affected by antifungal agents have also been achieved through the use of transcriptome analyses. Microarray analysis of the effect of soybean toxin, an AFP, on *Candida albicans* revealed the altered expression of 61 and 51 genes after 16 and 18 h of exposure, respectively (Morais et al. 2013). The observed changes in

Table 2 Potential of selected technologies for advancing the characterization of AFPs

Selected technologies	Application to antifungal protein characterization
Gene-disruption strategies	<ul style="list-style-type: none"> Targeted gene deletion for confirmation of proteins/pathways targeted by AFPs Deletion libraries to screen for genes associated with sensitivity or resistance to AFPs
Transcriptome analyses	<ul style="list-style-type: none"> Microarrays and RNA-seq utilized to identify the mechanism of action of AFPs and identification of potential secondary effects of treatment
Proteomics	<ul style="list-style-type: none"> Gel-based methods (e.g. 2D-PAGE) for the characterization of pathways affected by treatment with AFPs; some limitations associated with constraints in proteome coverage MS-based methods: enable large-scale survey of the proteome for identification of direct targets of AFPs, identification of potential synergistic drug targets, characterizing mechanisms of resistance and sensitivity to AFPs

gene expression led the authors to propose a preliminary model for the mechanism of action of this AFP, whereby nutrient uptake is likely blocked and filamentous growth is suppressed (Morais et al. 2013). The effect of an antimicrobial compound resveratrol on *A. flavus* was also investigated, using a highly sensitive transcriptomic technique, RNA-seq (Wang et al. 2015). Differential expression of 453 genes was detected in response to resveratrol treatment, including genes involved in mycelial and conidial development, and also secondary metabolite-associated genes. Expression of aflatoxin biosynthetic genes, *aflA* and *aflB*, was significantly reduced, corresponding with significantly decreased levels of aflatoxin detected following resveratrol treatment (Wang et al. 2015). Here, transcriptomics not only informed on the possible molecular mechanisms of the antifungal treatment, but also informed on the secondary effects (e.g. altered mycotoxin production) that could prove relevant in application of these agents in food microbiology. Employing time-course experiments has the potential to greatly expand these surveys, informing on the dynamics of the molecular response to antifungal treatments (Lopez-Moya et al. 2016).

Proteomic analyses have provided fundamental information on the systems altered in response to antifungal treatments. Until more recently, the majority of these studies involved gel-based comparative proteomics using SDS-PAGE alone or in combination with isoelectric separation (2D-PAGE). 2D-PAGE has been used to evaluate the responses of sensitive fungi to established antifungal therapies, including azoles, polyenes and echinocandins (Gautam et al. 2008; Hoehamer et al. 2010). This technique has also been used to characterize the mechanisms of action of novel antifungal agents (Gautam et al. 2011; Singh et al. 2012; Silva et al. 2013). Gel-based proteomic methods do present some limitations, including difficulties resolving proteins with extremes of pI or mass, hydrophobic proteins and constraints in the dynamic range visible (Owens et al. 2014). These gel-based techniques can be used in combination with an additional analysis strategy to gain a more comprehensive overview of the systems affected by the antifungal agent. Combining subcellular fractionation with comparative proteomics extends the limits of detection (Komatsu et al. 2011; Cagas et al. 2011; Moloney et al. 2016), while including genomics or alternative proteomic analyses (e.g. gel-free methods) serves to increase sensitivity and validate results (Gautam et al. 2008; Cagas et al. 2011; Gautam et al. 2011; Ene et al. 2012; Delgado et al. 2015b, 2016).

Advances in mass spectrometry (MS)-based proteomic methods have enabled in-depth analysis of the protein levels altered in response to an antifungal challenge. Label-free methods have been utilized to profile the AFPs response within both resistant and sensitive fungi, enabling the identification of resistance mechanisms as well as susceptible targets (Delgado et al. 2015b; Prado et al. 2015; Delgado et al. 2016).

MS has the potential not only just to decipher these modes of action, but also to distinguish between sensitive and resistant strains of a clinically relevant organism using MS-based antifungal susceptibility testing (msAFST) (Vella et al. 2013). In addition to the capacity for proteomics to provide insight into the pathways affected by antifungals, it can also inform on promising targets for antifungal activity. Evaluation of proteomic changes in response to a time-course with the antifungal drug fluconazole revealed potential synergistic drug targets in *Cryptococcus gattii* following compilation of protein interaction networks (Chong et al. 2012). An MS-based comparative proteomic strategy involving the use of labels has been used to elucidate the mechanism of action of the human AFP histatin 5 (Komatsu et al. 2011). Employing subcellular fractionation to purify mitochondria and two distinct labelling approaches, the authors determined that histatin 5 exerts its activity by decreasing mitochondrial ATP synthesis leading to loss of cellular energy (Komatsu et al. 2011).

Direct identification of antifungal targets is also possible using pull-down strategies combined with MS for identification of protein binding partners. Proteins interacting with the histatin 5 protein described above were detected following co-immunoprecipitation (Co-IP) and a pull-down scheme (Moffa et al. 2015). Among these proteins, one was detected that interfered with histatin 5 activity once complexed, thus identifying potential antagonists of antifungal activity (Moffa et al. 2015). Ghosh et al. (2015) identified potential interactors of the plant AFP mASAL using a different technique involving ligand blots probed with the protein of interest. Collectively, these methods highlight the array of tools that can be utilized to give a system-wide perspective on the molecular targets and biological pathways affected by antifungal agents. These global approaches can facilitate the subsequent focusing of complementary techniques, such as biochemical assays, targeted gene disruption or metabolite analysis.

Mode of action

The mode of action of these cationic AFPs has been described as multifactorial, but it is hypothesized to involve a direct interaction with anionic phospholipids in the fungal membrane (Lacadena et al. 1995). Thus far, two different mechanisms of action have been elucidated. Some class A AFPs, typically AFP, AFP_{NN5353} and AcAFP, bind chitin and are localized to the outer layer of sensitive fungi (Theis et al. 2003, 2005). Then, they alter the cell wall by inhibiting chitin synthesis and permeabilize the cell membrane (Liu et al. 2002; Moreno et al. 2006; Hagen et al. 2007; Skouri-Gargouri et al. 2009). This leads to the disintegration of intracellular structures and cell death (Theis et al. 2005), without relevant morphological changes in sensitive moulds (Theis et al. 2003). Sensitive fungi react to AFP, though

unsuccessfully, inducing cell wall integrity (CWI) pathway to counteract the AFP (Hagen et al. 2007).

Alternatively, class A PAF interacts with specific components of the plasma membrane, being actively internalized (Oberparleiter et al. 2003). This protein induces severe changes in cell morphology (Kaiserer et al. 2003) that are related to modulation of specific ion channels rather than to chitin or DNA binding (Batta et al. 2009). Class B PgAFP also binds to the outer layer of treated hyphae but, similarly to AFP, leads to no morphological change (Delgado et al. 2015b). Furthermore, class A, B and C AFPs, including PAF, NFAP and PgAFP, lead to reduced chitin biosynthesis at the cell wall and increased levels of intracellular reactive oxygen species (ROS) that trigger programmed cell death (PCD) and apoptosis (Leiter et al. 2005; Galgóczy et al. 2013; Delgado et al. 2015b) (Fig. 1). The onset of apoptotic cell death programs can also be initiated by glutathione/glutathione disulphide redox imbalance (Hegedus et al. 2011). The increased relative abundance of glutathione synthetase and Cys-Gly metalloprotease Dug1 in a PgAFP-treated sensitive mould (Delgado et al. 2015b) reveals an unsuccessful reaction to counteract the higher ROS levels, given that these two enzymes would lead to glutathione overproduction. The reduced chitin content associated to this mechanism of action has been related to the fact that PAF fails to activate Pkc/Mpk signalling cascade to induce CWI pathway (Binder et al. 2010b), whereas PgAFP even represses CWI pathway (Delgado et al. 2015b). Therefore, the mechanism of action of AFPs includes alteration or inability to trigger basic defence mechanisms such as CWI pathway, which is aggravated by cell damage due to ROS (Fig. 1). PAF-initiated apoptotic cell death signals may be transmitted by heterotrimeric G protein signalling via cAMP/protein kinase A pathway (Leiter et al. 2005; Binder et al. 2010a; Virágh et al. 2015), where both FadA and GanB G protein α -subunits may be involved (Hegedus et al. 2011), as well as G protein complex β subunit CpcB (Delgado et al. 2015b).

PAF also perturbs Ca^{2+} homeostasis, increasing the resting level of cytosolic-free Ca^{2+} , and the disruption of Ca^{2+} signalling may also lead to PCD (Binder et al. 2010a). Interestingly, high external concentrations of Ca^{2+} or other cations have been shown to reduce the inhibitory effect of AFPs, as will be discussed later.

As it has been shown for PgAFP (Delgado et al. 2015b), some AFPs lead to an increased abundance of proteins involved in energy-hungry ribogenesis and translation processes, whereas key enzymes involved in energy metabolism decrease. These changes could explain the fungistatic effect due to depletion of the cell energy reserves. In addition, the morphological changes caused by AFPs in sensitive fungi (Kaiserer et al. 2003) could be related to the lower abundance of proteins involved in hyphae structure, such as septins and actin (Delgado et al. 2015b).

Mechanisms of resistance

Different defensive strategies in response to treatment with AFPs have been described for AFPs-producing moulds, naturally resistant fungi or sensitive moulds grown at high CaCl_2 concentrations. For some antimicrobial peptides, the resistance has been related to a constitutive lack of electrostatic affinity or receptors for these proteins in cell outer layer (Yeaman and Yount 2003). PgAFP, AFP_{NN5353} and PAF do not bind to the cell surface of their respective insensitive producer strain (Oberparleiter et al. 2003; Binder et al. 2011; Delgado et al. 2015b).

In naturally resistant fungi, interactions with AFPs have been reported. In the non-sensitive *P. chrysogenum*, AFP is internalized and used as a nutritional source, but it binds neither to any specific organelle, nor to the outer layer (Theis et al. 2005). Alternatively, resistant fungi respond against cellular stress caused by AFPs with adaptive mechanisms aimed to maintain the integrity of the cell wall. Echinocandins induce compensatory increases in chitin synthesis, which confers resistance in *Candida* spp. (Cota et al. 2008; Lee et al. 2012). In the yeast *S. cerevisiae*, the chitin fortification in the presence of AFP is largely dependent on transcriptional stimulation via the calcium/calciuretin/Crz1p pathway (Ouedraogo et al. 2011). Stress signals caused by echinocandins can also be transmitted in *S. cerevisiae* from the cell surface integrity sensor Wsc1 to the protein kinase C (PKC) cell integrity signalling pathway, which is controlled by Rho1 protein (Reinoso-Martín et al. 2003; Levin 2005). Rho1 binds and activates Pkc, which through Mpk signalling, activates genes involved in cell wall synthesis, resulting in an elevated chitin content (Munro et al. 2007; Delgado et al. 2016).

The successful response of PgAFP-treated *Penicillium polonicum* is attributed to an efficient CWI pathway activation by Rho1 that promotes increased chitin deposition and, as a consequence, lower cell permeability (Delgado et al. 2016) (Fig. 1). Conversely, sensitive *A. flavus* treated with PgAFP showed lower relative amount of Rho1 and chitin deposition (Delgado et al. 2015b). Therefore, chitin deposition mediated by Rho1 seems to play a key role in the sensitivity or resistance of moulds to PgAFP. In addition, *P. polonicum* resistance to PgAFP was weakened by a combined treatment with chitinase, and this combination has been proposed as a means to increase the efficiency of AFPs (Delgado et al. 2016).

On the other hand, the presence of high levels of extracellular cations in the environment provokes a decrease of the antifungal activity of AFPs (Theis et al. 2003; Kaiserer et al. 2003; Martín-Urdiroz et al. 2009; Binder et al. 2010a; Galgóczy et al. 2013). The saturation of the cellular target with cations could potentially be the cause of this reduction in antifungal activity. Given that AFPs have a positive net charge, cations could compete for the putative binding sites at the fungal cell surface (Marx 2004; Martín-Urdiroz et al.

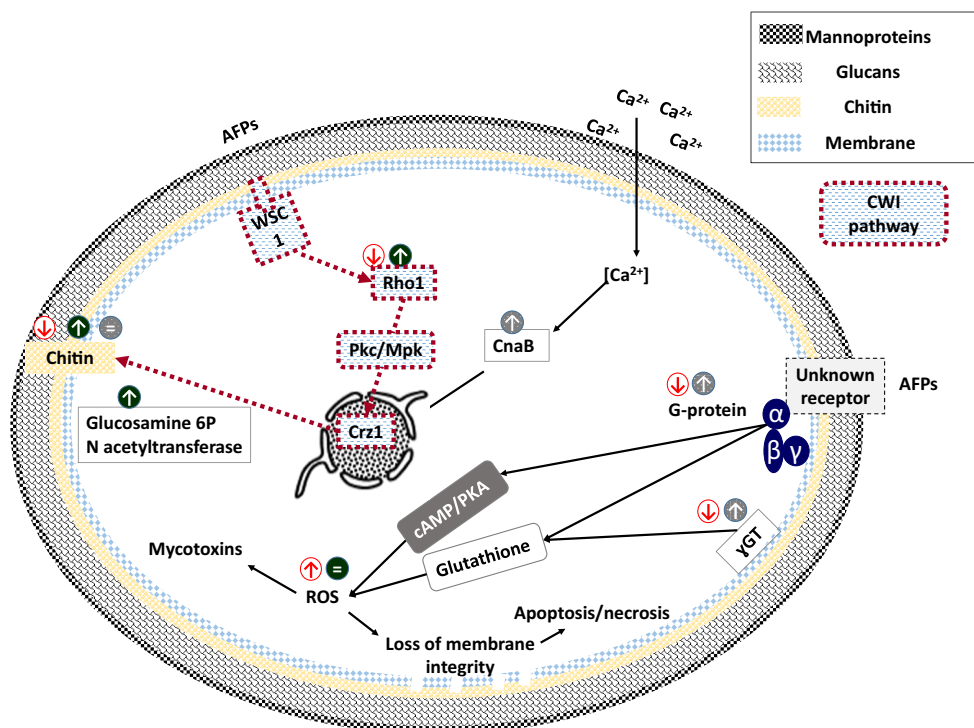


Fig. 1 Role of key proteins in the mechanism of action of AFPs on moulds. Cell wall integrity (CWI) pathway activation: Wsc1 activates Rho1 (Philip and Levin 2001) that plays a key role in the mould response to AFPs (Delgado et al. 2015b; Delgado et al. 2016). Extracellular signals are transmitted via Pkc/Mpk (Binder et al. 2010b), increasing chitin content in the cell wall through Crz1 (Ouedraogo et al. 2011). Calcium effect: A high cytosolic calcium concentration activates calcineurin (Thewes 2014). Calcineurin induces post-translational changes through activation of Crz1 (Fortwendel et al. 2010), increasing chitin content in the cell wall. Role of G-protein: G-protein transmits apoptotic cell death signals through cAMP/PKA (Leiter et al. 2005) and modulates glutathione pathway (Hegedus et al. 2011), together with γ GT. cAMP/PKA pathway and glutathione modulate the intracellular ROS levels. High ROS levels may increase mycotoxin production (Jayashree and Subramanyan 2000) and loss of membrane integrity that leads to

apoptosis and necrosis phenomena (Leiter et al. 2005; Delgado et al. 2015b). γ T gamma glutamyl transpeptidase, Pkc/Mpk protein kinase C/mitogen-activated protein kinase, CWI cell wall integrity, cAMP/PKA cyclic adenosine monophosphate/protein kinase A, Rho1 small GTP binding protein, Crz1 calcineurin-responsive zinc finger 1, CnaB calcineurin Ca^{2+} -binding regulatory subunit CnaB, Wsc1 wall stress component sensor, ROS reactive oxygen species. \uparrow Higher quantity, \downarrow lower quantity, \equiv unaltered quantity. \odot Variation in a sensitive mould treated with an AFP (Delgado et al. 2015b). \bullet Variation in a wild-resistant mould treated with an AFP (Delgado et al. 2016). \odot Variation in a sensitive mould grown in a calcium-enriched medium and treated with an AFP (unpublished data)

2009), making the target site no longer accessible for the AFP (Theis et al. 2003). On the other hand, extracellular calcium may enter fungal cells increasing intracellular Ca^{2+} concentration and leading to the activation of calcineurin (Thewes 2014). Calcineurin signalling pathway induces transcription of chitin synthases in filamentous fungi and also controls chitin synthases themselves (Fortwendel et al. 2010). In fact, the fortification of the cell wall following the Ca^{2+} -mediated induction of chitin synthase *chsD* expression seems to protect *Aspergillus niger* from AFP (Binder et al. 2011; Ouedraogo et al. 2011). PgAFP-treated *A. flavus* cultured in a CaCl_2 -enriched culture medium showed no decrease in chitin deposition but increased abundance of calcineurin Ca^{2+} -binding regulatory subunit CnaB, G-protein complex β subunit CpcB and γ -glutamyl transpeptidase (γ GT) (unpublished data) (Fig. 1). The compensatory increase of chitin synthesis through calmodulin-calcineurin signalling pathway has been

also related to resistance of *A. fumigatus* and *Candida* spp. to echinocandins (Juvvadi et al. 2015; Perlin 2015).

The high calcium levels present in some dry-ripened foods could prevent a successful antifungal effect of AFPs, particularly in cheeses. Given that both LAB (Mandal et al. 2013) and yeasts (Andrade et al. 2014; Núñez et al. 2015) commonly found in ripened foods have shown antifungal capability against toxigenic moulds, the combined action of the AFPs with protective cultures is an alternative that deserves to be investigated.

Impact of AFPs on mycotoxin production

AFP have fungistatic effect on sensitive moulds (Theis et al. 2005; Skouri-Gargouri et al. 2009). Retarded growth of treated moulds may result in lower mycotoxin production, as it has been shown for PgAFP on *A. flavus* at 24-h incubation

(Delgado et al. 2015b). However, the effect of long-term treatments with AFPs on mycotoxin production is not known. ROS are among the main factors that activate mycotoxin production (Reverberi et al. 2012; Schmidt-Heydt et al. 2014). In fact, oxidative stress is considered a prerequisite for aflatoxin production in toxigenic moulds (Jayashree and Subramanyan 2000). Therefore, mycotoxin production in dry-ripened foods treated with AFPs poses a potential health hazard and deserves to be studied. On the other hand, mycotoxin production can be effectively reduced by antioxidants, such as butylated hydroxyanisole (BHA) (Reverberi et al. 2006), butylated hydroxytoluene (BHT) (Fanelli and Fabbri 1989), β -glucans from *Lentinula edodes* (Reverberi et al. 2005) or caffeic acid (Kim et al. 2008). Therefore, antioxidants could be used to minimize mycotoxin production, but their impact on the antifungal effect of AFPs is subject to further investigation.

The use of AFPs may raise additional concerns about public health due to allergic reactions in atopic patients and consumers. On one hand, fungi can cause sensitization and develop asthma, with *Aspergillus* and *Penicillium* spp. among the major sources of indoor mould allergies. In fact, allergenic proteins from AFPs-producing species include seven demonstrated allergens from *A. niger* (Asp n 14, 18, 25, 30, glucoamylase, hemicellulose and pectinase), three from *N. fischeri* (Neo fi 6, transaldolase and Cu/Zn superoxide dismutase), two from *Penicillium brevicompactum* (Pen b 13 and 26), six from *P. chrysogenum* (Pen ch 13, 18, 20, 31, 33 and 35), ten from *Penicillium citrinum* (Pen c 1, 2, 3, 13, 18, 19, 22, 24, 30 and 32) and one from *Penicillium nalgiovense* (Pen na 13) (Allergome 2016). On the other hand, the molecular weight of all characterized allergenic proteins range from 11 to 105 kDa, being much larger than AFPs, and the route of exposure described for these allergens is inhalation, with none identified as food allergens so far.

In conclusion, the stability and inhibitory capability of AFPs on toxigenic moulds make these proteins a valuable strategy to prevent mould development and mycotoxin contamination on foods. AFPs-producing moulds, such as *P. chrysogenum*, have a long record of industrial use and the toxicological information on AFPs is quite encouraging (Szappanos et al. 2005; Palicz et al. 2013). To fully understand the mechanism of action and resistance to AFPs, MS-based proteomic analyses are providing insight into the pathways affected and can inform on new promising targets for antifungal activity. There is still work to be done to find adequate combined treatments for calcium-rich products, but significant steps have been taken to encourage the use of AFPs in dry-ripened foods.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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