



Quantitative proteomics reveals new insights into calcium-mediated resistance mechanisms in *Aspergillus flavus* against the antifungal protein PgAFP in cheese



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ABSTRACT

The ability of *Aspergillus flavus* to produce aflatoxins in dairy products presents a potential hazard. The antifungal protein PgAFP from *Penicillium chrysogenum* inhibits various foodborne toxigenic fungi, including *Aspergillus flavus*. However, PgAFP did not inhibit *A. flavus* growth in cheese, which was related to the associated cation content. CaCl₂ increased *A. flavus* permeability and prevented PgAFP-mediated inhibition in potato dextrose broth (PDB). PgAFP did not elicit any additional increase in permeability of CaCl₂-incubated *A. flavus*. Furthermore, PgAFP did not alter metabolic capability, chitin deposition, or hyphal viability of *A. flavus* grown with CaCl₂. Comparative proteomic analysis after PgAFP treatment of *A. flavus* in calcium-enriched PDB revealed increased abundance of 125 proteins, including oxidative stress-related proteins, as determined by label-free mass spectrometry (MS)-based proteomics. Seventy proteins were found at lower abundance, with most involved in metabolic pathways and biosynthesis of secondary metabolites. These changes do not support the blockage of potential PgAFP receptors in *A. flavus* by calcium as the main cause of the protective role. *A. flavus* resistance appears to be mediated by calcineurin, G-protein, and γ -glutamyltranspeptidase that combat oxidative stress and impede apoptosis. These findings could serve to design strategies to improve PgAFP activity against aflatoxigenic moulds in dairy products.

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1. Introduction

Some moulds are able to produce mycotoxins in foods, which causes serious problems throughout the world. *Aspergillus flavus* is one of the most serious mould contaminants, due essentially to aflatoxin production in cereals and nuts. Additionally, *A. flavus* is able to grow and produce aflatoxins and cyclopiazonic acid on intermediate moisture foods, including ripened cheese (Kokkonen et al., 2005; Lie and Marth, 1967; López-Díaz et al., 1996). The strategies to prevent mycotoxin accumulation on ripened foods include taking advantage of antifungal proteins produced by moulds that prevent growth of mycotoxigenic fungi.

The antifungal protein PgAFP is produced by *Penicillium chrysogenum* CECT 20922 (formerly RP42C), that was isolated from dry-cured ham (Rodríguez-Martín et al., 2010). PgAFP belongs to a

group of small, basic, cysteine-rich proteins, which also includes PAF from *P. chrysogenum* (Marx et al., 1995), AFP from *Aspergillus giganteus* (Nakaya et al., 1990), and NFAP from *Neosartorya fischeri* (Kovács et al., 2011). PgAFP can efficiently inhibit various toxigenic moulds in culture medium, even *A. flavus* on a ripened meat product, but not *Penicillium polonicum* (Delgado et al., 2015a). *A. flavus* inhibition co-occurs with increased permeability to SYTOX Green, depressed metabolic capability, compromised cell membrane, apoptosis, and necrosis. The proposed mechanism of action for PgAFP is based on the lower relative abundance of Rho GTPase Rho1 and G protein subunit β CpcB leading to alteration of both cell wall integrity and response to oxidative stress (Delgado et al., 2015b). Conversely, higher levels of Rho GTPase Rho1 are involved in the resistance of *P. polonicum* leading to increased chitin biosynthesis (Delgado et al., 2016).

The presence of cations in the media has been shown to decrease the antifungal capability of these proteins (Galgóczy et al., 2013; Kaiserer et al., 2003; Theis et al., 2003; Thevissen et al., 1999, 1996). High levels of extracellular divalent or monovalent cations

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seem to reduce the ability of the antifungal protein to provoke permeabilization or altered calcium influx, as has been described for PAF (Binder et al., 2010), AFP (Theis et al., 2003), and NFAP (Galgóczy et al., 2013). However, the ultimate mechanism responsible for this effect has not been elucidated. High external calcium levels transiently increase cytosolic calcium levels in *Aspergillus nidulans* (Nelson et al., 2004). Intracellular Ca^{2+} is a secondary messenger regulating various responses to stress signals in fungi, including antifungal proteins (Binder et al., 2011, 2010). Given that divalent cations are present in several foods, particularly calcium in cheeses, the effect of high levels of this cation on PgAFP antifungal activity against sensitive moulds requires urgent evaluation.

Comparative proteomic analysis has been described as a powerful tool to study the unknown effect of substances on moulds and yeasts. It has been used to study the effect of H_2O_2 on moulds (Lessing et al., 2007) and to identify the mechanism of action of antifungal compounds (Cagas et al., 2011; Delgado et al., 2015b; Gautam et al., 2008). Comparative 2D-PAGE is able to distinguish changes in protein abundance, isoforms or post-translational modifications (Görg et al., 2004). However, this technique does not reveal the whole proteome, as it depends on the range of pH chosen (Görg et al., 2009), and it is not able to effectively analyse membrane and hydrophobic proteins (Rabilloud et al., 2009; Zhu et al., 2010). Label-free mass spectrometry-based proteomic analysis is able to identify proteins typically underrepresented in 2D-PAGE studies (Owens et al., 2014), providing deeper proteome coverage. Thus, these two techniques can provide complementary information to study the PgAFP mechanism of action in moulds.

Results obtained from proteomic analysis guides the use of specific assays to elucidate the mechanisms of action. The relative abundance of *A. flavus* proteins can also give information about the differential response of this mould against PgAFP when grown in a calcium-enriched medium.

The objective of the present work was to evaluate the inhibitory activity of PgAFP against *A. flavus* growth in cheese, and to explore the effect of cations, mainly calcium, on PgAFP antifungal potential. The changes induced by high calcium levels, in the proteome profile and in selected metabolic and structural characteristics of *A. flavus*, with and without PgAFP, were assessed.

2. Materials and methods

2.1. Strains

The PgAFP producer *Penicillium chrysogenum* RP42C (Spanish Type Culture Collection, CECT 20922) and *Aspergillus flavus* CECT 2687 were used in this study. The latter produces aflatoxin in intermediate moisture foods (Bernáldez et al., 2014).

2.2. PgAFP purification

P. chrysogenum CECT 20922 was grown in potato dextrose broth (PDB, Scharlab, Barcelona, Spain) pH 4.5, at 25 °C for 21 days without shaking. Mycelia were removed, the culture medium was filtered to obtain cell-free medium and PgAFP was obtained by Fast Protein Liquid Chromatography as previously described (Acosta et al., 2009; Rodríguez-Martín et al., 2010). Briefly, the cell-free medium was separated by chromatography on cationic and gel filtration columns, the sub-fractions containing the highest absorbance peaks were tested against reference sensitive moulds, and the extracts containing purified PgAFP were pooled from several batches. The protein concentration was quantified by the Lowry method (Lowry et al., 1951) and the stock solution was diluted in the elution buffer to the desired active concentration range (1.17–75 µg/mL) for the various assays.

2.3. Proteomics

2.3.1. Protein extraction

A. flavus CECT 2687 was cultured in triplicate in 50 mL PDB supplemented with 0.1 M CaCl_2 , at 25 °C for 24 h with shaking at 200 rpm, either in the presence (10 µg/mL) or absence of PgAFP, as previously described (Delgado et al., 2015b). Mycelia were harvested, washed and lysed as described by Carberry et al. (2006). The lysed mycelia were centrifuged (10,000 g; 30 min), the supernatant was precipitated with trichloroacetic acid/acetone (Carpentier et al., 2005), and analysed by the following two methods as previously described by (Delgado et al., 2015b).

2.3.2. 2D-PAGE

Resuspended extracts containing 250 µg of protein were loaded onto Immobiline Dry strips (IPG strip; Amersham Biosciences, Uppsala, Sweden) in the pH range 4–7, followed by electrofocusing, and electrophoresis using the Protean Xi-II Cell (Bio-Rad Laboratories) as described by Carberry et al. (2006). Resulting gels ($n = 5$) were stained with colloidal Coomassie Blue (Sigma-Aldrich, St. Louis, MO, USA), scanned, normalized and analysed using Progenesis™ SameSpot software (TotalLab, Newcastle, UK).

Protein spots showing differences ($p \leq 0.05$, fold change ≥ 1.5) were excised, destained, sonicated, dehydrated, and trypsin in-gel digested according to Shevchenko et al. (2006). Then, the digest supernatants, containing tryptic peptides, were dried using a DNA Speed Vac Concentrator (Thermo Fischer Scientific, Austin, TX, USA), resuspended in 0.1% formic acid (20 µL), and filtered through 0.22 µm cellulose spin-filters (Agilent Technologies, Ireland).

The samples were analysed by a 6340 Model Ion Trap LC-Mass Spectrometer (Agilent Technologies, Ireland) using electrospray ionisation on a Zorbax 300 SB C-18 Nano-HPLC Chip (150 mm × 75 µm). The eluted peptides were ionized and analysed by mass spectrometry. MS^n analysis was carried out on the three most abundant peptide precursor ions at each time point, as selected automatically by the mass spectrometer. MASCOT MS/MS Ion search, NCBI (National Centre for Biotechnology Information) database, FungiFun (Priebe et al., 2011) and KEGG (Kyoto Encyclopedia of Genes and Genome, www.genome.jp/kegg/) were used for protein identification and functional characterisation.

2.3.3. Label-free comparative quantitative proteomic analysis

The proteins precipitated from mycelial lysates were resuspended in 8 M urea, reduced with dithiothreitol and alkylated with iodoacetamide (Owens et al., 2014). Samples ($n = 3$) were digested with trypsin combined with ProteaseMax surfactant, and desalted by application to C18 ZipTips® (Millipore, Darmstadt, Germany). One µg of each peptide mixture was analysed via a Thermo Scientific Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 4 to 35% B (A: 0.1%(v/v) formic acid, B: 80%(v/v) acetonitrile, 0.1%(v/v) formic acid) over 2 h, and data was collected using a Top15 method for MS/MS scans (Dolan et al., 2014; O'Keefe et al., 2014; Owens et al., 2015). Comparative proteome abundance and data analysis was performed using MaxQuant software (Version 1.3.0.5) (Cox and Mann, 2008), with Andromeda used for database searching and Perseus (Version 1.4.1.3) used to organise the data. Carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines and acetylation of N-terminals were set as variable modifications. The maximum peptide/protein false discovery rates were set to 1%. The LFQ algorithm was used to generate normalized spectral intensities and infer relative protein abundance (Luber et al., 2010). Proteins that matched to a contaminants database or the reverse database were removed and proteins were only retained in final analysis if detected in at least two replicates from at least one treatment

group. Quantitative analysis was performed using a *t*-test to compare pairs of samples, and proteins with significant change in abundance (p value ≤ 0.05 ; fold change ≥ 2) were included in the quantitative results. Qualitative analysis was also performed, to detect proteins that were found in at least 2 replicates of a particular treatment, but undetectable in the comparison sample group.

2.4. Effect of cations on PgAFP activity

The growth of PgAFP-treated *A. flavus* was evaluated in microtiter plate wells (DeltaLab, Rubí, Spain), containing 200 μ L of PDB supplemented with either 0.1 M or 0.01 M CaCl_2 and 10^5 conidia per well incubated at 25 °C without shaking. An additional plate containing only PDB was used as a control. Seven different two-fold dilutions of PgAFP, (75, 37.5, 18.75, 9.38, 4.69, 2.34, and to 1.17 μ g/mL), and a non-treated control were assayed. Six sample replicates for each combination of PgAFP treatment and cation concentration were processed. Optical density was measured at 550 nm every 24 h during 96 h in a microplate reader (TECAN Infinite M2000, Männedorf, Switzerland). Additionally, to test the effect of the monovalent KCl and the divalent MgCl_2 , two different plates were evaluated following the same procedure described herein, and containing 0.01 and 0.1 M of KCl or MgCl_2 . These cation concentrations were chosen as they are representative of the levels found in cheese (Chekri et al., 2012). Given that 0.1 M CaCl_2 was the concentration that abolished PgAFP antifungal ability, and this concentration is well within the levels present in cheese, the following assays were carried out using this concentration. Additionally, all of the following tests were also carried out with *A. flavus* grown in PDB with no calcium added as controls. All the micrographs from the same assay were taken with the same exposure.

2.5. Metabolic tests

To study the effect of CaCl_2 on the metabolism of PgAFP-treated *A. flavus*, the following tests were carried out according to Delgado et al. (2015b). *A. flavus* was cultured in three replicate test tubes containing 200 μ L PDB supplemented with 0.1 M CaCl_2 in either presence (75 μ g/mL, to maximise the potential effect of this anti-fungal protein) or absence of PgAFP at 25 °C for 24 h without shaking. Additionally, to test the effect on membrane permeability on a whole range of PgAFP and calcium concentrations, seven PgAFP two-fold dilutions (75, 37.5, 18.75, 9.38, 4.69, 2.34, and 1.17 μ g/mL) as well as 0.1 M and 0.01 M CaCl_2 were assayed in six sample replicate wells. To assess membrane permeability, *A. flavus* grown in microtiter plates with different CaCl_2 and PgAFP concentrations were supplemented with 0.2 μ M SYTOX Green (Molecular Probes, Eugene, OR, USA). The emitted fluorescence was measured after 10, 30, and 210 min.

To study the metabolic capability through viability staining, triplicate samples of mycelia were washed with 10 mM HEPES pH 7.5 before staining with 100 μ L FUN-1 (Molecular Probes) for 30 min at 25 °C according to Kaiserer et al. (2003). Stained hyphae were examined and photographed by fluorescence microscopy.

Membrane integrity was assessed with acridine orange/ethidium bromide (AO/EB) double staining (Sigma-Aldrich). Triplicate samples of *A. flavus* cultures were stained with 4 μ g/mL of AO/EB, incubated 30 min and washed twice with phosphate-buffered saline (PBS) to be observed by fluorescence microscopy.

To detect apoptotic events, triplicate samples of grown mycelia were stained with Apoptosis Detection Kit (Sigma-Aldrich) composed of Annexin V-fluorescein isothiocyanate/propidium iodide (AnV-FITC/PI), according to manufacturer's instructions.

2.6. Chitin staining

Conidia of *A. flavus* were inoculated in 10 mL PDB in a Petri dish containing a coverglass (Harris et al., 1994) and incubated at 25 °C for 24 h in the presence (75 μ g/mL) and absence of PgAFP. Mycelia were washed, fixed and stained with fluorescent brightener 28 (Sigma-Aldrich) to visualize chitin by fluorescence microscopy as previously described (Delgado et al., 2015b). Additional batches with no CaCl_2 added were also run as controls. All batches were prepared in triplicate.

2.7. Localization of FITC-labelled PgAFP

PgAFP was labelled with fluorescein isothiocyanate (FITC) by DareBio S.L. (Elche, Spain), as previously described (Delgado et al., 2015b). *A. flavus* was grown in triplicate PDB cultures, both with and without 0.1 M CaCl_2 , containing 20 μ g/mL PgAFP-FITC for 24 h at 25 °C. Hyphae were washed twice with PBS and visualized by fluorescence microscopy.

2.8. Effect of PgAFP on *A. flavus* in cheese

One cm thick wedges of commercial Gouda type cheese of 10 cm² were dipped in ethanol and dried in a laminar flow cabinet (Bio Flow II, Telstar, Tarrasa, Spain). Three sample wedges were separately placed in pre-sterilized containers after vapour-liquid equilibrium with a saturated KCl solution, to reach a_w values of 0.84, similar to those found in the rind at the final stage of cheese ripening (Rüegg and Blanc, 1981). Next, PgAFP was added in 200 μ L PBS to treated samples at two concentrations (18 and 35 μ g/cm²) and left to dry in a flow cabinet. The non-treated batch received the same volume of PBS but with no PgAFP. Then, a volume of 100 μ L of a suspension of *A. flavus* spores was spread onto the upper surface of cheese wedges to reach *c.a.* 10^5 conidia/cm². The containers were incubated for 96 h and growth of the resulting mycelia was assessed. This test was performed in triplicate. Water activity (a_w) was determined in cheese wedges by a LabMASTER- a_w instrument (Novasina AG, Lachen, Switzerland) both before inoculation and after 96 h incubation. This test was run in three independent trials.

2.9. Statistical analysis

Statistical analyses were performed with IBM SPSS v.22 (www-03.ibm.com/software/products/es/spss-stats-standard). Growth inhibition and membrane permeability data were tested for normality (Kolmogorov–Smirnov with Lilliefors correction) and homoscedasticity (Levene's test). These data were non-normally distributed, then mean values were compared using non-parametric Kruskal–Wallis test. To compare treatments in pairs, Mann–Whitney *U* test was applied ($p \leq 0.05$).

3. Results

3.1. Antifungal activity of PgAFP on cheese

To study the potential application of PgAFP against *A. flavus* in the dairy industry, the antifungal activity of 18 and 35 μ g/cm² PgAFP was tested on cheese wedges simulating common ripening conditions. The a_w value of cheese samples was 0.940 (± 0.004 SD) at the time of inoculation and decreased to 0.910 (± 0.003 SD), 0.883 (± 0.003 SD), and 0.844 (± 0.002 SD) after 4, 8, and 15 days of incubation, respectively. Growth of *A. flavus* on cheese treated with any of the two PgAFP concentrations was extensive (Fig. 1), indicating that PgAFP did not affect *A. flavus* growth on cheese.

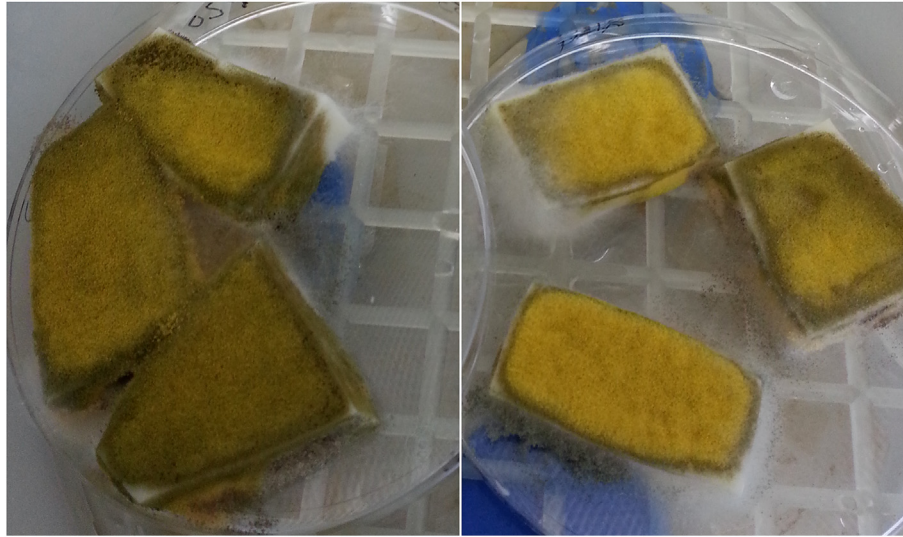


Fig. 1. Exuberant growth of *A. flavus* in cheese wedges treated with 18 $\mu\text{g}/\text{cm}^2$ (left) and 35 $\mu\text{g}/\text{cm}^2$ PgAFP (right).

3.2. Effect of cations on PgAFP antifungal activity

The effect of calcium, magnesium and potassium on the antifungal activity of PgAFP against *A. flavus* was studied in PDB cultures (Fig. 2). The maximum O.D. reached at 96 h in control cultures, without PgAFP, was affected by KCl and CaCl_2 , with lower levels of growth observed with KCl ($p \leq 0.05$) and higher with CaCl_2 ($p \leq 0.05$). Control cultures, with no cations added, confirmed that 18.75 $\mu\text{g}/\text{mL}$ PgAFP reduced *A. flavus* growth over 50% at 96 h. With either 0.01 M (data not shown) or 0.1 M KCl, PgAFP antifungal activity was essentially unaffected (Fig. 2). Similarly, 0.01 M MgCl_2 did not have a substantial impact on PgAFP antifungal activity, whereas 0.1 M MgCl_2 lowered PgAFP effect, so that 50% growth inhibition

was not reached at 96 h incubation even at the highest PgAFP concentration tested ($p > 0.05$). The strongest effect was obtained with CaCl_2 ; with 0.01 M CaCl_2 , 37.5 $\mu\text{g}/\text{mL}$ PgAFP was required to reach 50% *A. flavus* growth inhibition at 96 h, but with 0.1 M CaCl_2 no growth inhibition was obtained even with 75 $\mu\text{g}/\text{mL}$ PgAFP ($p > 0.05$).

3.3. PgAFP localization and membrane permeability

Given that 0.1 M CaCl_2 abolished PgAFP antifungal activity, and this concentration is well within the range of calcium levels in ripened cheese (Chekri et al., 2012), only CaCl_2 was tested for the remaining assays. To determine whether calcium influences PgAFP

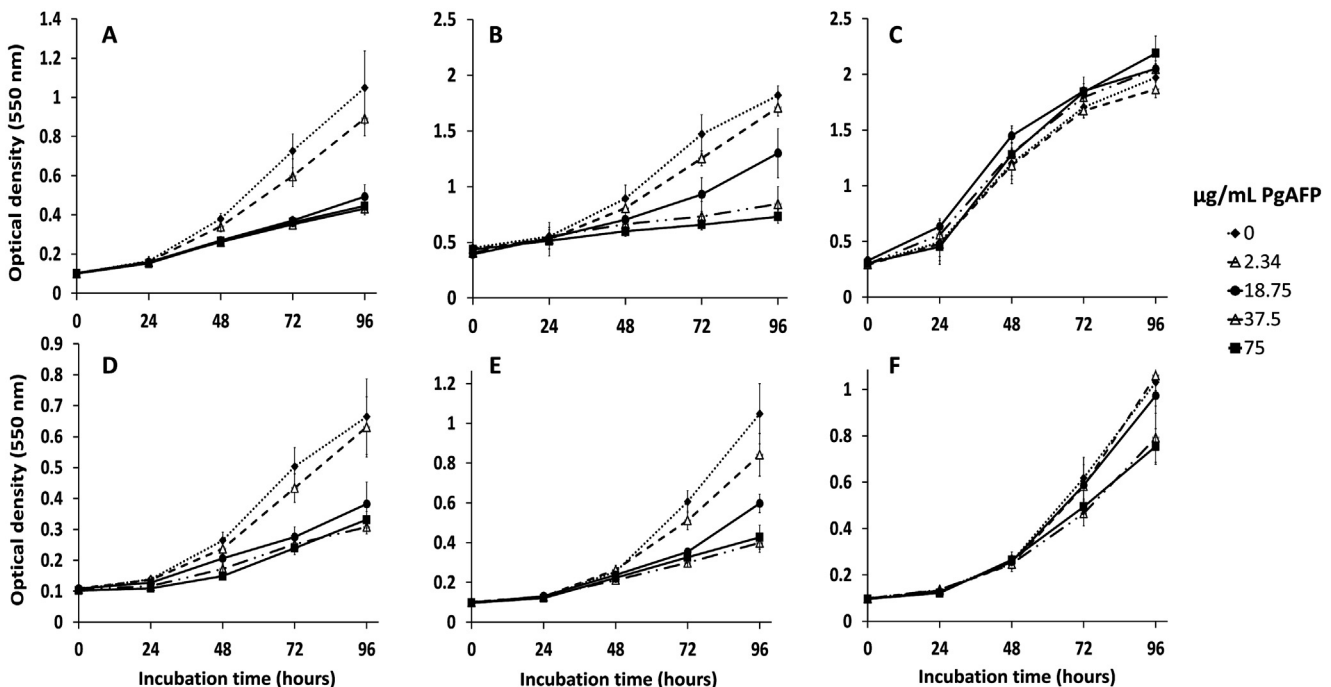


Fig. 2. Effect of selected cations on the antifungal activity of different PgAFP concentrations against *A. flavus*. Growth development in potato dextrose broth for 96 h supplemented with (A) no cations, (B) 0.01 M CaCl_2 , (C) 0.1 M CaCl_2 , (D) 0.1 M KCl, (E) 0.01 M MgCl_2 and (F) 0.1 M MgCl_2 . Vertical bars represent standard deviation of the mean.

localization, *A. flavus* grown in PDB supplemented with 0.1 M CaCl₂ was treated with FITC-labelled PgAFP. The FITC-labelled PgAFP was visualized bound to the outer layer of *A. flavus* hyphae, but unlike the CaCl₂-free control, it was unable to penetrate into the cytoplasm (Fig. 3). The antifungal activity of FITC-labelled PgAFP was evaluated against *A. flavus* in CaCl₂-free PDB, showing similar levels to those of non FITC-labelled PgAFP (data not shown).

To assess the influence of CaCl₂ on *A. flavus* permeabilization by PgAFP, the SYTOX Green uptake test was used. With no calcium added, fluorescence values increased as PgAFP concentration increased from 0 to 4.69 µg/mL ($p \leq 0.05$) and then progressively decreased from 9.38 to 75 µg/mL (Fig. 4), as shown previously (Delgado et al., 2015b). With 0.01 M CaCl₂ a slightly higher PgAFP concentration (18.75 µg/mL) was required to reach the highest fluorescence values, as opposed to 4.69–9.38 µg/mL PgAFP in the control batch without CaCl₂. In addition, the decrease of fluorescence obtained at the highest PgAFP concentrations (37.5–75 µg/mL) with 0.01 M CaCl₂ was even more pronounced than that obtained with 0 or 0.1 M CaCl₂. On the other hand, high fluorescence values were obtained when *A. flavus* was cultured in 0.1 M CaCl₂ PDB, even when no PgAFP was present. Therefore, *A. flavus* permeability was strongly increased by either PgAFP or 0.1 M CaCl₂. However, PgAFP at any concentration barely increased *A. flavus* permeability in 0.1 M CaCl₂ PDB (Fig. 4).

3.4. Effect of PgAFP in presence of calcium on protein abundance

No differences ($p > 0.05$) in *A. flavus* mycelia weight were noted between PgAFP-treated and untreated samples grown in PDB supplemented with 0.1 M CaCl₂. 2D-PAGE comparative proteomic analysis revealed 19 unique proteins from 24 spots, whose relative abundance was altered in treated samples (Supplementary Material Table S1). In the PgAFP-treated batch, the relative abundance was from 1.5 to 1.6 fold higher for 7 proteins including G-protein complex β subunit CpcB, whilst 12 proteins, identified from 17 spots were found between 1.6 and 2.5 fold lower. Among proteins found in reduced quantities, three isoforms of elongation factor 3, and two isoforms each of aminopeptidase, glycogen branching enzyme GbeA, and aldehyde dehydrogenase AldA were identified. The two spots found from AldA were observed with different molecular masses and isoelectric points, possibly due to breakdown and side-chain deamination.

The label-free quantitative analysis in PDB supplemented with 0.1 M CaCl₂ revealed a total of 1651 proteins, 195 of them showed significantly altered abundance due to PgAFP-treatment (Supplementary Material Table S2). A total of 125 proteins were detected at elevated levels in PgAFP-treated *A. flavus*. Among them, 90 proteins were found in a higher abundance, from 2 to 53 fold

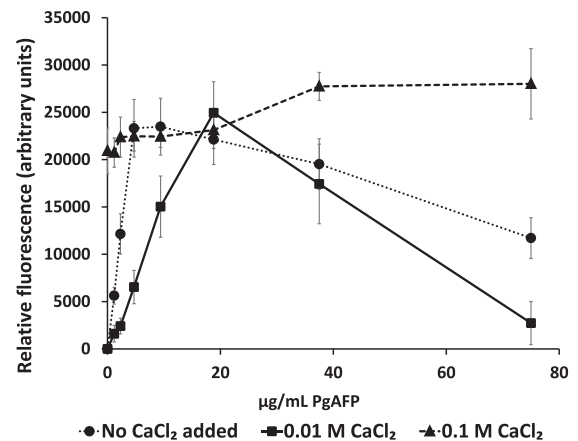


Fig. 4. Effect of 0.01 M and 0.1 M CaCl₂ on membrane permeability of *A. flavus* treated with PgAFP (0–75 µg/mL) at 210 min after SYTOX Green addition. Vertical bars represent standard deviation of the mean.

(quantitative results), and 35 proteins were exclusively detected in the treated batch (qualitative results). In addition, 70 proteins were detected at lower abundance in PgAFP-treated samples, 32 of which were found in lower abundance, from 2.1 to 703 fold, and the 38 remaining were not detected in the treated batch.

According to KEGG, 13 out of the 70 proteins found in lower amount following PgAFP treatment were involved in metabolic pathways and biosynthesis of secondary metabolites. In contrast, among the proteins whose abundance was higher in PgAFP-treated *A. flavus*, 23 of them were related with ribosome and 11 with spliceosome. From the proteins of the oxidative stress-related glutathione metabolism, glutathione S-transferase and γ-glutamyltranspeptidase (γ-GT) showed higher relative abundance in PgAFP-treated samples (Supplementary Material Table S2). In addition, G-protein complex β subunit CpcB, recently described as related to the mechanism of action of antifungal proteins (Delgado et al., 2015b), was also increased in PgAFP treated samples (Supplementary Material Table S1). All 19 proteins showing relative abundance altered in 2D-PAGE were found in the label-free proteomic assay, but showing different fold change in both methods.

3.5. Effect on metabolic capability and chitin deposition

The metabolic capability of *A. flavus* grown with 0.1 M of CaCl₂ was not influenced by PgAFP, as shown by FUN-1 staining. Intravacuolar red spots were present in both PgAFP-treated and non-treated batches, revealing a normal metabolic capability (Fig. 5).

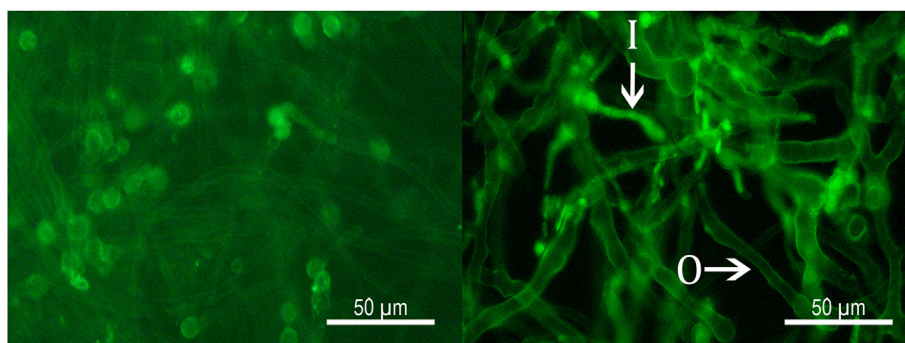


Fig. 3. FITC-labelled PgAFP localization in *A. flavus* grown in presence of 0.1 M CaCl₂ (left). PgAFP was only visualized bound to the outer layer of *A. flavus* hyphae, but not in the cytoplasm. FITC-labelled PgAFP localization in *A. flavus* without CaCl₂ (right). PgAFP was visualized both in the cytoplasm (I) and bound to the outer layer (O) of *A. flavus* hyphae.

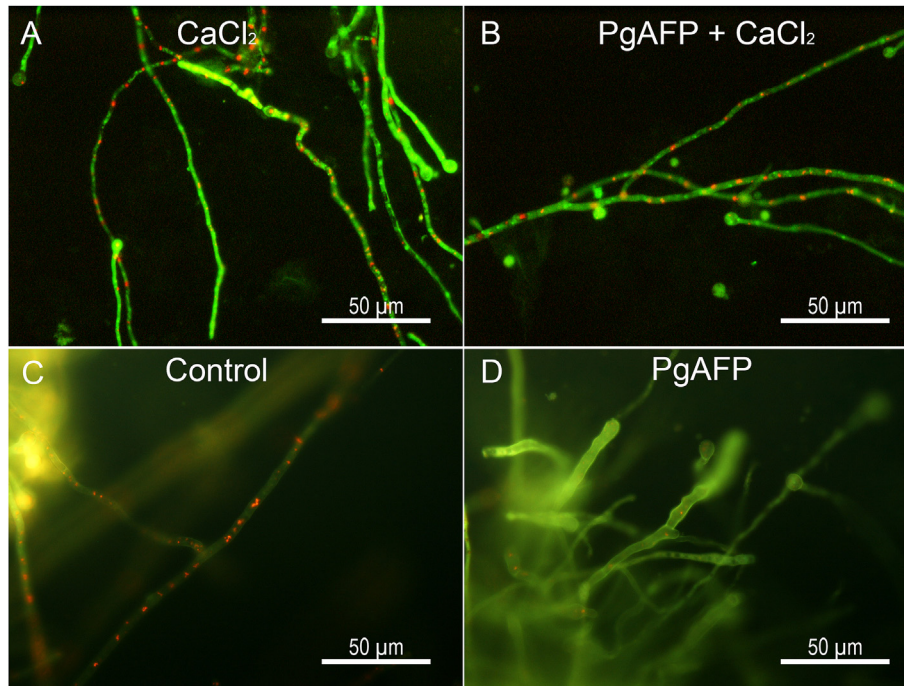


Fig. 5. Metabolic capability of *A. flavus* grown with 0.1 M CaCl_2 (A and B) or without calcium (C and D) revealed by FUN-1 staining. Non-treated controls (A and C) and hyphae treated with 75 $\mu\text{g}/\text{mL}$ PgAFP (B and D). Red vacuoles indicate metabolic activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the absence of added calcium, non-treated control also displayed intra-vacuolar red spots, whereas *A. flavus* grown with 75 $\mu\text{g}/\text{mL}$ PgAFP scarcely showed red spots.

Chitin deposition has been described as a successful response against PgAFP in resistant *P. polonicum* (Delgado et al., 2016). Staining with fluorescent brightener 28 showed that 0.1 M CaCl_2

made no obvious difference to chitin deposition in non PgAFP-treated *A. flavus*, as compared to the altered chitin deposition in PgAFP-treated samples (Fig. 6). However, 0.1 M CaCl_2 allowed maintenance of similar chitin levels to those observed in the non PgAFP-treated control. As expected, PgAFP-treated controls with no calcium added displayed alterations (Figs. 5 and 6), but non-treated

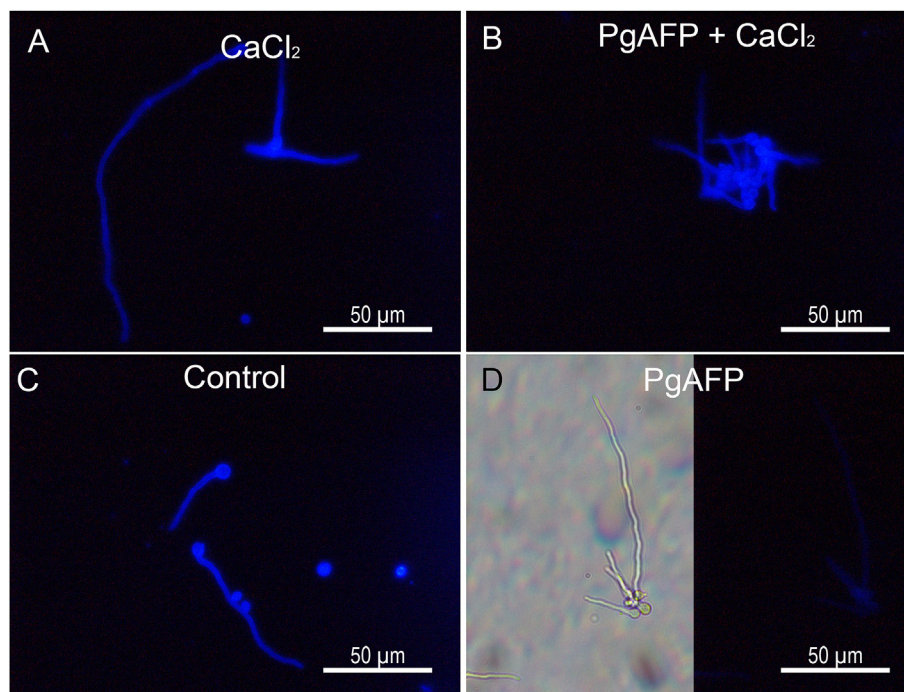


Fig. 6. Effect of calcium on chitin deposition PgAFP-treated *A. flavus* grown in potato dextrose broth and stained with fluorescent brightener 28. A: non-PgAFP treated supplemented with 0.1 M CaCl_2 ; B: PgAFP treated (75 $\mu\text{g}/\text{mL}$) supplemented with 0.1 M CaCl_2 ; C: non-PgAFP treated with no CaCl_2 added; D: PgAFP treated (75 $\mu\text{g}/\text{mL}$) with no CaCl_2 added, bright field (left) and fluorescence observation (right).

A. flavus remaining unaltered.

3.6. Effect on oxidative status and viability

Hyphal viability and apoptotic or necrotic processes of *A. flavus* grown in 0.1 M CaCl₂ PDB were studied with AO/EB double staining and AnV-FITC/PI staining, respectively. The AO/EB double staining showed a predominant green colour both in PgAFP-treated and untreated samples due to AO but not EB staining (Fig. 7). This implies that 0.1 M CaCl₂ maintains the viability of PgAFP-treated *A. flavus*. Moreover, both treated and non-treated batches were not stained by AnV-FITC/PI (Fig. 8), implying that PgAFP did not induce apoptosis or necrosis in cultures containing 0.1 M CaCl₂. Relevantly, PgAFP-treated controls with no calcium added displayed alterations (Figs. 7 and 8), whilst non-treated *A. flavus* remained unaltered.

4. Discussion

The capacity of just 5 µg/cm² PgAFP to reduce *A. flavus* counts was demonstrated on dry-fermented sausage (Delgado et al., 2015a). Such effect would also be useful in ripened cheese, but the high calcium levels could interfere with PgAFP activity. The treatment with PgAFP was not effective in reducing *A. flavus* growth on cheese (Fig. 1). Both dry-fermented sausage and cheese assays were performed under identical environmental conditions of 25 °C, with constant relative humidity maintained by means of a saturated KCl solution (Delgado et al., 2015a). Environmental factors such as pH or NaCl concentration may affect PgAFP antifungal activity. However this activity appears not to be affected by monovalent cations, and withstands a broad range of pH values (Delgado et al., 2015a). Given that the PgAFP concentrations tested on cheese were up to 7-fold higher than that effective on the sausage, the lack of PgAFP activity has to be related to cheese composition. Other

researchers have proven that concentrations of mono- and divalent cations over 20–100 mM suppress the inhibitory activity of other antifungal proteins in culture media (Galgóczy et al., 2013; Kaiserer et al., 2003; Theis et al., 2003; Thevissen et al., 1999, 1996). Average calcium, magnesium, and potassium concentrations in cheese are c.a. 0.14, 0.013, and 0.03 M, respectively (Chekri et al., 2012). Given that these concentrations are higher than those suppressing the activity of other antifungal proteins, the effect of each of these minerals on PgAFP activity against *A. flavus* was investigated.

The antifungal capability of all concentrations of PgAFP tested against *A. flavus* in PDB was strongly reduced with 0.1 M CaCl₂ or MgCl₂ (Fig. 2), whereas only the antifungal effect of low PgAFP concentrations was prevented with 0.01 M concentrations of these divalent cations. For the monovalent potassium salt, 0.01 and 0.1 M only slightly reduced PgAFP antifungal activity. These results infer that the lack of activity of PgAFP on *A. flavus* observed in cheese could be explained solely by the calcium content.

Similar results have been reported for AFP, PAF, NFAP, and plant defensins, where the antifungal activity was significantly counteracted by the presence of high concentrations of monovalent cations or MgCl₂ (Galgóczy et al., 2013; Kaiserer et al., 2003; Theis et al., 2003; Thevissen et al., 1999). This loss of antifungal activity has been explained by a saturation of the cellular target of antifungal proteins with cations, making the target site no longer accessible for the antifungal protein (Theis et al., 2003). Small, basic, and cysteine-rich antifungal proteins from moulds have a positive net charge, and cations could interfere by competing for putative binding sites at the fungal cell surface (Martín-Urdiroz et al., 2009; Marx, 2004).

FITC-labelled PgAFP was located both bound to outer layer and intracellularly in *A. flavus* grown in PDB with no CaCl₂ added (Delgado et al., 2015b). However, when *A. flavus* was grown in 0.1 M CaCl₂ PDB, FITC-labelled PgAFP was only found at the outer layer and not internalized (Fig. 3), implying that membrane integrity was

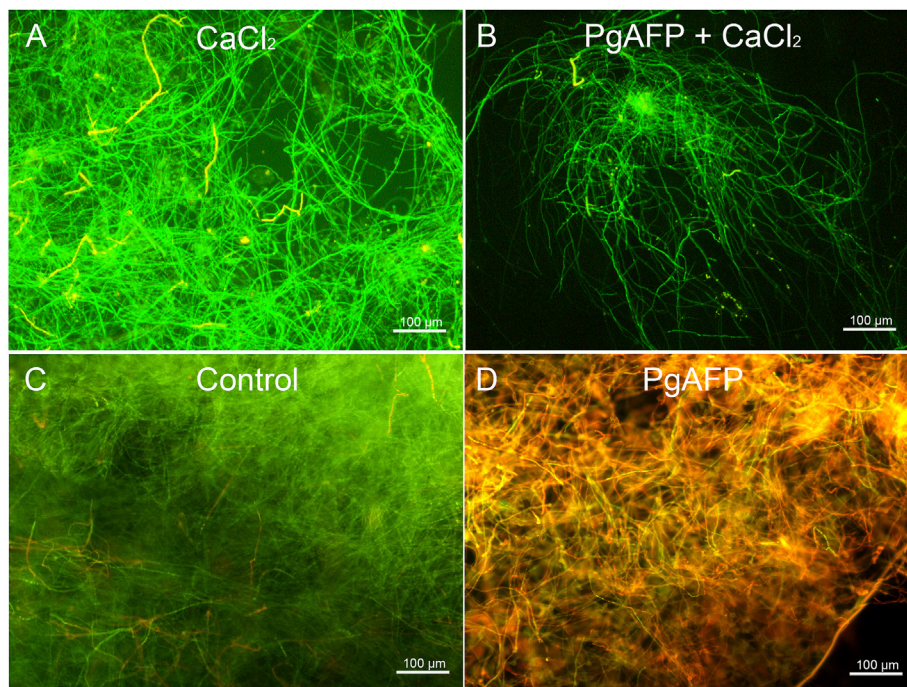


Fig. 7. Effect of PgAFP on hyphae viability revealed by acridine orange/ethidium bromide (AO/EB) double staining of *A. flavus* grown in potato dextrose broth supplemented with 0.1 M CaCl₂ (A and B) and without added CaCl₂ (C and D). Merged pictures of green (AO) and red colour (EB) from samples non-treated (A and C) and treated with 75 µg/mL PgAFP (B) showing scarce orange colour due to the lack of EB penetration, meaning they remained viable. *A. flavus* treated with 75 µg/mL PgAFP grown with no added calcium (D) showed intense orange colour due to compromised membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

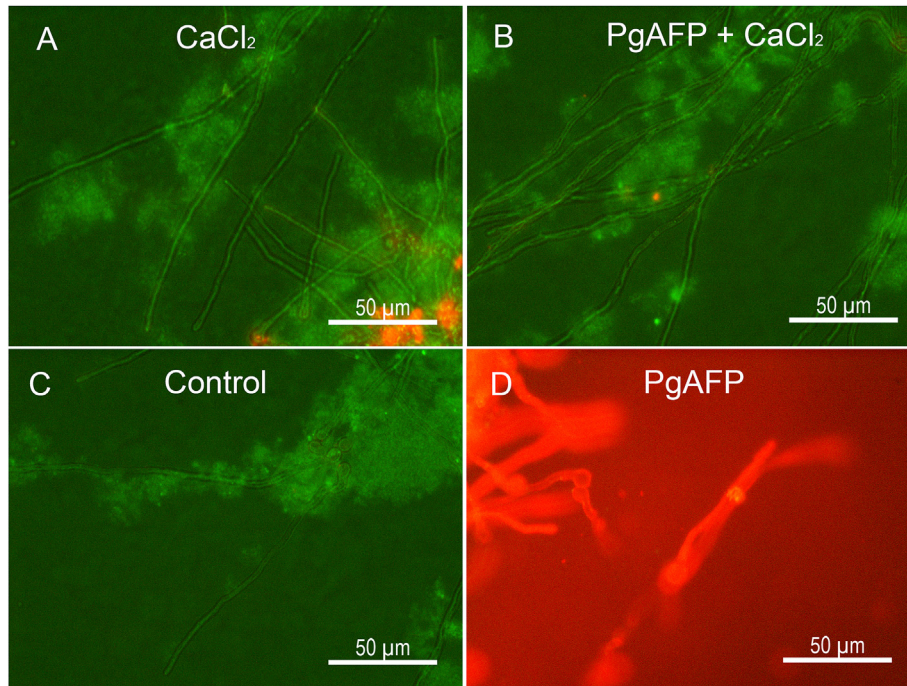


Fig. 8. Induction of apoptosis or necrosis by PgAFP on *A. flavus* grown in potato dextrose broth supplemented with 0.1 M CaCl_2 (A and B) and with no calcium added (C and D), evaluated with Annexin V-fluorescein isothiocyanate/propidium iodide (AnV-FITC/PI). Merged pictures of green (AnV-FITC) and red colour (PI) from non-treated hyphae (A and C) or treated with 75 $\mu\text{g}/\text{mL}$ PgAFP (B) showing no green or red intense fluorescence due to no apoptotic or necrotic events. *A. flavus* grown in PDB with no calcium added and treated with 75 $\mu\text{g}/\text{mL}$ PgAFP (D) displayed intense red colour due to AnV-FITC and PI penetration as a consequence of necrotic events. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not compromised. When grown in 0.1 M CaCl_2 PDB, *A. flavus* permeability to SYTOX Green increased to the maximum level provoked by PgAFP with no calcium added (Fig. 4), similarly to the pattern obtained in *Aspergillus niger* with 1 M CaCl_2 (Gomaa et al., 2013). When *A. flavus* was grown with both PgAFP and 0.1 M CaCl_2 , no further increase in permeability was observed. Thus, all the above results support that calcium ions did not prevent PgAFP from binding to the outer layer. Moreover, no other detrimental effect was observed in *A. flavus* at any PgAFP concentration through metabolic capability or viability, as evaluated by FUN-1, AO/EB, and AnV-FITC/PI staining. Therefore, calcium may have rendered the putative membrane targets non-accessible to PgAFP. Nonetheless, comparative proteomics disclosed remarkable changes in fungal metabolism triggered by PgAFP when grown in 0.1 M CaCl_2 PDB.

A total of 19 and 195 proteins showed altered relative abundances by CaCl_2 according to 2D-PAGE and label-free proteomics, respectively. The difference between the number of proteins obtained by these two methods have been explained as a consequence of 2D-PAGE being able to separate isoforms and post-translational modifications but only within a limited pH range, whereby the whole proteome is not represented (Görg et al., 2009, 2004). In contrast, label-free proteomics is not able to separate isoforms and compares the total quantity of the protein, but has a higher coverage with regard to pH and molecular mass range. Hence, the differences observed in relation to the fold change for any given protein seem to be related to the involvement of isoforms or post-translational modifications, as well as the different threshold used by both methods (Delgado et al., 2015b, 2016).

The comparative proteomic assays for *A. flavus* in the presence of CaCl_2 revealed a large number of proteins affected by PgAFP exposure. According to KEGG, proteins related to ribosome and spliceosome accounted for 27% of the proteins with altered relative amount. The relative quantities for most proteins of these two

groups were higher in PgAFP-treated samples. Similar results were obtained for ribosomal and spliceosomal proteins when PgAFP was applied to cultures grown in absence of CaCl_2 of both *A. flavus* (Delgado et al., 2015b) and the PgAFP-resistant *P. polonicum* (Delgado et al., 2016). Given that the increase of these two groups of proteins is a common response to PgAFP in both sensitive and resistant moulds, it does not seem to be crucial to discriminate when inhibition is overcome.

The mechanism of action of antifungal proteins has been described through different inter-related metabolic pathways, including oxidative stress, cell wall integrity (CWI) pathway, and chitin synthesis (Delgado et al., 2015b; Hagen et al., 2007; Marx et al., 2008). Some proteins involved in these pathways showed higher relative abundance in response to PgAFP in *A. flavus* grown in 0.1 M CaCl_2 PDB, including G-protein complex β subunit CpcB, and proteins of the oxidative stress-related glutathione pathway, namely glutathione S-transferase and γ -GT. These proteome changes would rule out the blockage of PgAFP receptors as the only protective mechanism of Ca^{2+} in *A. flavus*, suggesting a survival reaction of *A. flavus* to the antifungal protein as discussed below.

No significant differences were found in the protein Rho GTPase Rho1 between PgAFP-treated and untreated *A. flavus* grown with 0.1 M CaCl_2 (Supplementary Material Table S2). Lower Rho1 levels have been identified among the main factors involved in the inhibition of *A. flavus* by PgAFP in PDB with no CaCl_2 added (Delgado et al., 2015b), whereas higher levels of Rho1 play a key role in increasing chitin deposition in PgAFP-treated *P. polonicum*, likely activating Pkc/Mpk pathway (Delgado et al., 2016). The unaltered chitin deposition in PgAFP-treated *A. flavus* grown with CaCl_2 (Fig. 6) can be related by the substantially unaffected quantity of Rho1 and any other protein related to the CWI pathway.

Extracellular calcium can act as a stress signal in moulds that increases the intracellular Ca^{2+} concentration and lead to the

activation of calmodulin, which in turn can activate calcineurin (Thewes, 2014). Calcineurin is a heterodimer comprised of subunits A and B. Calcineurin Ca^{2+} -binding regulatory subunit CnaB is essential for activation of the calcineurin A (calmodulin-binding catalytic subunit CnaA) that is important in stress adaptation (Juvvadi et al., 2003). Calcineurin regulates the transcription factor CRZ1A, that induces transcription of chitin synthases *chsA* and *chsC* in filamentous fungi, but can also control chitin synthases themselves (Fortwendel et al., 2010) or interact genetically with the endogenous calcineurin regulators, calcipressins (Soriani et al., 2008). Activation of calmodulin-calcineurin signalling by high cytosolic Ca^{2+} concentration leads to *A. fumigatus* growth in presence of antifungal drugs (Juvvadi et al., 2015). The 0.1 M CaCl_2 level specifically led to the higher relative abundance of CnaB in both PgAFP-treated and untreated *A. flavus* (Supplementary Material Table S1) (Delgado et al., 2015b). Thus, the protective effect of high calcium levels in *A. flavus* can be mediated by increased CnaB.

G protein CpcB may also play a central role in vegetative growth and development, as its deficiency severely impaired cellular growth in Aspergilli (Kong et al., 2013). The lower quantity of CpcB has been related to apoptosis signalling in PgAFP-treated *A. flavus* (Delgado et al., 2015b). In the present work, the higher relative abundance of CpcB subunit was not accompanied by apoptotic or necrotic signals in PgAFP-treated *A. flavus* grown in 0.1 M CaCl_2 . Therefore, CpcB can be considered a key factor in promoting or arresting apoptotic events by PgAFP, leading to sensitivity or resistance, respectively. The protective mechanism mediated by CpcB in *A. flavus* grown with calcium seems to be based on the modulation of glutathione that would impact on reactive oxygen species (ROS) levels.

The proteins related to glutathione metabolism have been shown to be important in the maintenance of the redox status upon oxidative stress in fungi (Kang et al., 2005; Park et al., 2005, 2004; Springael and Penninckx, 2003). Thus, the higher relative abundance of these proteins could be related to the successful response of *A. flavus* to PgAFP in the calcium-enriched culture medium. No direct link between increased calcium levels and induction of proteins involved in glutathione metabolism can be established at present. However, it is plausible that a primary adaptive response mediated by CnaB or CpcB allow the time for *A. flavus* to develop an effective response to oxidative stress, leading to increased levels of proteins involved in the glutathione pathway. To overcome the protective role of divalent cations against antifungal proteins, PgAFP treatment could be combined with other preservatives showing complementary mechanisms of action. Future studies with extended incubation time will also help to assess the effect of PgAFP on mould growth and mycotoxin production in ripened cheese.

5. Conclusion

This work does not support that occlusion of specific receptors is responsible for the observed protective role of divalent cations in preventing the antifungal effect of PgAFP on *A. flavus*. Conversely, the data indicate that *A. flavus* resistance to PgAFP in 0.1 M CaCl_2 is primarily mediated by an increased CnaB, CpcB and γ -GT, which would enhance the response to oxidative stress and impede apoptosis. Understanding the mechanism of resistance against PgAFP can be useful to design strategies aiming to prevent growth of mycotoxigenic moulds in foods with high levels of divalent cations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fm.2017.03.015>.

References

- Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, F., Asensio, M.A., 2009. Selection of antifungal protein-producing molds from dry-cured meat products. *Int. J. Food Microbiol.* 135, 39–46. <http://dx.doi.org/10.1016/j.ijfoodmicro.2009.07.020>.
- Bernaldez, V., Rodríguez, A., Martín, A., Lozano, D., Córdoba, J.J., 2014. Development of a multiplex qPCR method for simultaneous quantification in dry-cured ham of an antifungal-peptide *Penicillium chrysogenum* strain used as protective culture and aflatoxin-producing moulds. *Food Control* 36, 257–265. <http://dx.doi.org/10.1016/j.foodcont.2013.08.020>.
- Binder, U., Bencina, M., Eigentler, A., Meyer, V., Marx, F., 2011. The *Aspergillus giganteus* antifungal protein AFPNNS5353 activates the cell wall integrity pathway and perturbs calcium homeostasis. *BMC Microbiol.* 11, 209. <http://dx.doi.org/10.1186/1471-2180-11-209>.
- Binder, U., Chu, M., Read, N.D., Marx, F., 2010. The antifungal activity of the *Penicillium chrysogenum* protein PAF disrupts calcium homeostasis in *Neurospora crassa*. *Eukaryot. Cell* 9, 1374–1382. <http://dx.doi.org/10.1128/EC.00050-10>.
- Cagas, S.E., Jain, M.R., Li, H., Perlin, D.S., 2011. Profiling the *Aspergillus fumigatus* proteome in response to caspofungin. *Antimicrob. Agents Chemother.* 55, 146–154. <http://dx.doi.org/10.1128/AAC.00884-10>.
- Carberry, S., Neville, C.M., Kavanagh, K.A., Doyle, S., 2006. Analysis of major intracellular proteins of *Aspergillus fumigatus* by MALDI mass spectrometry: identification and characterisation of an elongation factor 1B protein with glutathione transferase activity. *Biochem. Biophys. Res. Commun.* 341, 1096–1104. <http://dx.doi.org/10.1016/j.bbrc.2006.01.078>.
- Carpentier, S.C., Witters, E., Laukens, K., Deckers, P., Swennen, R., Panis, B., 2005. Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics* 5, 2497–2507. <http://dx.doi.org/10.1002/pmic.200401222>.
- Chekri, R., Noël, L., Millour, S., Vastel, C., Kadar, A., Sirot, V., Leblanc, J.C., Guérin, T., 2012. Calcium, magnesium, sodium and potassium levels in foodstuffs from the second French Total Diet Study. *J. Food Compos. Anal.* 25, 97–107. <http://dx.doi.org/10.1016/j.jfca.2011.10.005>.
- Cox, J., Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. <http://dx.doi.org/10.1038/nbt.1511>.
- Delgado, J., Acosta, R., Rodríguez-Martín, A., Bermúdez, E., Núñez, F., Asensio, M.A., 2015a. Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. *Int. J. Food Microbiol.* 205, 23–29. <http://dx.doi.org/10.1016/j.ijfoodmicro.2015.03.029>.
- Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., Núñez, F., 2015b. Impact of the antifungal protein PgAFP from *Penicillium chrysogenum* on the protein profile in *Aspergillus flavus*. *Appl. Microbiol. Biotechnol.* 99, 8701–8715. <http://dx.doi.org/10.1007/s00253-015-6731-x>.
- Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., Núñez, F., 2016. Increased chitin biosynthesis contributes to the resistance of *Penicillium polonicum* against the antifungal protein PgAFP. *Appl. Microbiol. Biotechnol.* 100, 371–383. <http://dx.doi.org/10.1007/s00253-015-7020-4>.
- Dolan, S.K., Owens, R.A., O'Keefe, G., Hammel, S., Fitzpatrick, D.A., Jones, G.W., Doyle, S., 2014. Regulation of nonribosomal peptide synthesis: bi-thiomethylation attenuates gliotoxin biosynthesis in *Aspergillus fumigatus*. *Chem. Biol.* 21, 999–1012. <http://dx.doi.org/10.1016/j.chembiol.2014.07.006>.
- Fortwendel, J.R., Juvvadi, P.R., Perfect, B.Z., Rogg, L.E., Perfect, J.R., Steinbach, W.J., 2010. Transcriptional regulation of chitin synthases by calcineurin controls paradoxical growth of *Aspergillus fumigatus* in response to caspofungin. *Antimicrob. Agents Chemother.* 54, 1555–1563. <http://dx.doi.org/10.1128/AAC.00854-09>.
- Galgóczy, L., Kovács, L., Karácsony, Z., Virágh, M., Hamari, Z., Vágvolgyi, C., 2013. Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*. *Microbiology* 159, 411–419. <http://dx.doi.org/10.1099/mic.0.061119-0>.
- Gautam, P., Shankar, J., Madan, T., Sirdeshmukh, R., Sundaram, C.S., Gade, W.N.,

- Basir, S.F., Sarma, P.U., 2008. Proteomic and transcriptomic analysis of *Aspergillus fumigatus* on exposure to amphotericin B. *Antimicrob. Agents Chemother.* 52, 4220–4227. <http://dx.doi.org/10.1128/AAC.01431-07>.
- Gomaa, O.M., Selim, N.S., Linz, J.E., 2013. Biochemical and biophysical response to calcium chloride stress in *Aspergillus niger* and its role in malachite green degradation. *Cell Biochem. Biophys.* 65, 413–423. <http://dx.doi.org/10.1007/s12013-012-9444-0>.
- Görg, A., Drews, O., Lück, C., Weiland, F., Weiss, W., 2009. 2-DE with IPGs. *Electrophoresis* 30 (Suppl. 1), S122–S132. <http://dx.doi.org/10.1002/elps.200900051>.
- Görg, A., Weiss, W., Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 4, 3665–3685. <http://dx.doi.org/10.1002/pmic.200401031>.
- Hagen, S., Marx, F., Ram, A.F., Meyer, V., 2007. The antifungal protein AFP from *Aspergillus giganteus* inhibits chitin synthesis in sensitive fungi. *Appl. Environ. Microbiol.* 73, 2128–2134. <http://dx.doi.org/10.1128/AEM.02497-06>.
- Harris, S.D., Morrell, J.L., Hamer, J., 1994. Identification and characterization of *Aspergillus nidulans* mutants defective in cytokinesis. *Genetics* 532, 517–532.
- Juvvadi, P.R., Kuroki, Y., Arioka, M., Nakajima, H., Kitamoto, K., 2003. Functional analysis of the calcineurin-encoding gene *cnaA* from *Aspergillus oryzae*: evidence for its putative role in stress adaptation. *Arch. Microbiol.* 179, 416–422. <http://dx.doi.org/10.1007/s00203-003-0546-3>.
- Juvvadi, P.R., Muñoz, A., Lamoth, F., Soderblom, E.J., Moseley, M.A., Read, N.D., Steinbach, W.J., 2015. Calcium-mediated induction of paradoxical growth following caspofungin treatment is associated with calcineurin activation and phosphorylation in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 59, 4946–4955. <http://dx.doi.org/10.1128/AAC.00263-15>.
- Kaiserer, L., Oberparleiter, C., Weiler-Görz, R., Burgstaller, W., Leiter, E., Marx, F., 2003. Characterization of the *Penicillium chrysogenum* antifungal protein PAF. *Arch. Microbiol.* 180, 204–210. <http://dx.doi.org/10.1007/s00203-003-0578-8>.
- Kang, H.-J., Kim, B.-C., Park, E.-H., Ahn, K., Lim, C.-J., 2005. The gene encoding gamma-glutamyl transpeptidase II in the fission yeast is regulated by oxidative and metabolic stress. *J. Biochem. Mol. Biol.* 38, 609–618. <http://dx.doi.org/10.5483/BMBRep.2005.38.5.609>.
- Kokkonen, M., Jestoi, M., Rizzo, A., 2005. Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry. *Food Addit. Contam.* 22, 449–456. <http://dx.doi.org/10.1080/02652030500089861>.
- Kong, Q., Wang, L., Liu, Z., Kwon, N.-J., Kim, S.C., Yu, J.-H., 2013. Gβ-like CpcB plays a crucial role for growth and development of *Aspergillus nidulans* and *Aspergillus fumigatus*. *PLoS One* 8, e70355. <http://dx.doi.org/10.1371/journal.pone.0070355>.
- Kovács, L., Virágh, M., Takó, M., Papp, T., Vágvolgyi, C., Galgóczi, L., 2011. Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). *Peptides* 32, 1724–1731. <http://dx.doi.org/10.1016/j.peptides.2011.06.022>.
- Lessing, F., Kniemeyer, O., Wozniak, L., Loeffler, J., Kurzai, O., Haertl, A., Brakhage, A.A., 2007. The *Aspergillus fumigatus* transcriptional regulator AfYap1 represents the major regulator for defense against reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. *Eukaryot. Cell* 6, 2290–2302. <http://dx.doi.org/10.1128/EC.00267-07>.
- Lie, J.L., Marth, E.H., 1967. Formation of aflatoxin in cheddar cheese by *Aspergillus flavus* and *Aspergillus parasiticus*. *J. Dairy Sci.* 50, 1708–1710 doi:S0022-0302(67)87698-7 [pii] r10.3168/jds.S0022-0302(67)87698-7.
- López-Díaz, T.M., Román-Blanco, C., García-Arias, M.T., García-Fernández, M.C., García-López, M.L., 1996. Mycotoxins in two Spanish cheese varieties. *Int. J. Food Microbiol.* 30, 391–395.
- Lowry, O.H., Rosebrough, N.J., Farr, L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Luber, C.A., Cox, J., Lauterbach, H., Fancke, B., Selbach, M., Tschopp, J., Akira, S., Wiegand, M., Hochrein, H., O’Keefe, M., Mann, M., 2010. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32, 279–289. <http://dx.doi.org/10.1016/j.immuni.2010.01.013>.
- Martín-Urdiroz, M., Martínez-Rocha, A.L., Pietro, A. Di, Martínez-del-Pozo, Á., Roncero, M.I.G., 2009. Differential toxicity of antifungal protein AFP against mutants of *Fusarium oxysporum*. *Int. Microbiol.* 12, 115–121. <http://dx.doi.org/10.2436/20.1501.01.88>.
- Marx, F., 2004. Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. *Appl. Microbiol. Biotechnol.* 65, 133–142. <http://dx.doi.org/10.1007/s00253-004-1600-z>.
- Marx, F., Binder, U., Leiter, E., Pöcsi, I., 2008. The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. *Cell. Mol. Life Sci.* 65, 445–454. <http://dx.doi.org/10.1007/s00018-007-7364-8>.
- Marx, F., Haas, H., Reindl, M., Stöffler, G., Lottspeich, F., Redl, B., 1995. Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum paf* gene encoding an abundantly secreted protein with antifungal activity. *Gene* 167, 167–171.
- Nakaya, K., Omata, K., Okahashi, I., Nakamura, Y., Kolkenbrock, H., Ulbrich, N., 1990. Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. *Eur. J. Biochem.* 38, 32–38.
- Nelson, G., Kozlova-Zwinderman, O., Collis, A.J., Knight, M.R., Fincham, J.R.S., Stanger, C.P., Renwick, A., Hensing, J.G.M., Punt, P.J., Van Den Hondel, C.A.M.J.J., Read, N.D., 2004. Calcium measurements in living filamentous fungi expressing codon-optimized aequorin. *Mol. Microbiol.* 52, 1437–1450. <http://dx.doi.org/10.1111/j.1365-2958.2004.04066.x>.
- O’Keefe, G., Hammel, S., Owens, R.A., Keane, T.M., Fitzpatrick, D.A., Jones, G.W., Doyle, S., 2014. RNA-seq reveals the pan-transcriptomic impact of attenuating the gliotoxin self-protection mechanism in *Aspergillus fumigatus*. *BMC Genomics* 25, 1–26. <http://dx.doi.org/10.1186/1471-2164-15-894>.
- Owens, R.A., Hammel, S., Sheridan, K.J., Jones, G.W., Doyle, S., 2014. A proteomic approach to investigating gene cluster expression and secondary metabolite functionality in *Aspergillus fumigatus*. *PLoS One* 9, e106942. <http://dx.doi.org/10.1371/journal.pone.0106942.g001>.
- Owens, R.A., O’Keefe, G., Smith, E.B., Dolan, S.K., Hammel, S., Sheridan, K.J., Fitzpatrick, D.A., Keane, T.M., Jones, G.W., Doyle, S., 2015. Interplay between gliotoxin resistance, secretion, and the methyl/methionine cycle in *Aspergillus fumigatus*. *Eukaryot. Cell* 14, 941–957. <http://dx.doi.org/10.1128/EC.00055-15>.
- Park, H.-J., Lim, H.-W., Kim, K., Kim, I.-H., Park, E.-H., Lim, C.-J., 2004. Characterization and regulation of the γ-glutamyl transpeptidase gene from the fission yeast *Schizosaccharomyces pombe*. *Can. J. Microbiol.* 50, 61–67. <http://dx.doi.org/10.1139/w03-106>.
- Park, H.-J., Moon, J.-S., Kim, H.-G., Kim, I.-H., Kim, K., Park, E.-H., Lim, C.-J., 2005. Characterization of a second gene encoding γ-glutamyl transpeptidase from *Schizosaccharomyces pombe*. *Can. J. Microbiol.* 51, 269–275. <http://dx.doi.org/10.1139/w04-137>.
- Priebe, S., Linde, J., Albrecht, D., Guthke, R., Brakhage, A.A., 2011. FungiFun: a web-based application for functional categorization of fungal genes and proteins. *Fungal Genet. Biol.* 48, 353–358. <http://dx.doi.org/10.1016/j.fgb.2010.11.001>.
- Rabilloud, T., Vaezzadeh, A.R., Potier, N., 2009. Power and limitations of electrophoretic Mass Spectrom. *Rev.* 28, 816–843. <http://dx.doi.org/10.1002/mas>.
- Rodríguez-Martín, A., Acosta, R., Liddell, S., Núñez, F., Benito, M.J., Asensio, M.A., 2010. Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. *Peptides* 31, 541–547. <http://dx.doi.org/10.1016/j.peptides.2009.11.002>.
- Rüegg, M., Blanc, B., 1981. Influence of water activity on the manufacture and aging of cheese. In: Rockland, L.B., Stewart, G.F. (Eds.), *Water Activity: Influences on Food Quality*. Academic Press, New York, pp. 791–811.
- Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M., 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 1, 2856–2860. <http://dx.doi.org/10.1038/nprot.2006.468>.
- Soriani, F.M., Malavazi, I., Da Silva Ferreira, M.E., Savoldi, M., Von Zeska Kress, M.R., De Souza Goldman, M.H., Loss, O., Bignelli, E., Goldman, G.H., 2008. Functional characterization of the *Aspergillus fumigatus* CRZ1 homologue, CrzA. *Mol. Microbiol.* 67, 1274–1291. <http://dx.doi.org/10.1111/j.1365-2958.2008.06122.x>.
- Springael, J.-Y., Penninckx, M.J., 2003. Nitrogen-source regulation of yeast gamma-glutamyl transpeptidase synthesis involves the regulatory network including the GATA zinc-finger factors Gln3, Nil1/Gat1 and Gzf3. *Biochem. J.* 371, 589–595. <http://dx.doi.org/10.1042/BJ20021893>.
- Theis, T., Wedde, M., Meyer, V., Stahl, U., 2003. The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. *Antimicrob. Agents Chemother.* 47, 588–593. <http://dx.doi.org/10.1128/AAC.47.2.588>.
- Thevissen, K., Ghazi, A., De Samblanx, G.W., Brownlee, C., Osborn, R.W., Broekaert, W.F., 1996. Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem.* 271, 15018–15025.
- Thevissen, K., Terras, F.R., Broekaert, W.F., 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl. Environ. Microbiol.* 65, 5451–5458.
- Thewes, S., 2014. Calcineurin-Crz1 signaling in lower eukaryotes. *Eukaryot. Cell* 13, 694–705. <http://dx.doi.org/10.1128/EC.00038-14>.
- Zhu, W., Smith, J.W., Huang, C.-M., 2010. Mass spectrometry-based label-free quantitative proteomics. *J. Biomed. Biotechnol.* 2010, 1–6. <http://dx.doi.org/10.1155/2010/840518>.