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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. $CaCl_2$ increased *A. flavus* permeability and prevented PgAFP-mediated inhibition in potato dextrose broth (PDB). PgAFP did not elicit any additional increase in permeability of $CaCl_2$-incubated *A. flavus*. Furthermore, PgAFP did not alter metabolic capability, chitin deposition, or hyphal viability of *A. flavus* grown with $CaCl_2$. 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.

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-incides 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, filtered, 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'Keeffe 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