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Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products

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ABSTRACT

Dry-ripened foods favor the development of a superficial fungal population that may include toxigenic molds. To combat unwanted molds, an antifungal protein from *Penicillium chrysogenum* (PgAFP) can be useful. The aim of the present work was to study the antimicrobial activity of PgAFP against microorganisms common in dry-ripened foods, and to evaluate its sensitivity to proteolytic enzymes and heat treatments that may be applied to foods, as well as to different pH values. The inhibitory effect of the purified protein on 38 microbial strains grown in culture medium was determined. PgAFP sensitivity to various proteases, heat treatments, and preincubation at different pH values was tested by means of the residual activity on selected reference strains. Inhibitory activity of PgAFP against unwanted molds was tested in a dry-fermented sausage. This protein exhibited potent inhibitory activity against unwanted molds, including the main mycotoxin-producing species of *Aspergillus* and *Penicillium* of concern for dry-ripened foods. PgAFP withstood most proteases, intense heat and a wide range of pH values. PgAFP efficiently reduced counts of *A. flavus* and *P. restrictum* inoculated on a dry-fermented sausage. This protein can be of interest to control hazardous molds in dry-ripened foods.

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1. Introduction

Most dry-ripened foods favor the development of a superficial fungal population that may include toxigenic molds, mainly *Aspergillus* and *Penicillium* spp., and yeasts such as *Debaryomyces* and *Candida* spp. Surface treatments and packaging may help to control unwanted fungi, but they are not adequate to prevent fungal growth during ripening of mold-ripened cheese, ham, or sausage. Research on new antifungal agents has increased during the recent years due to the increase in human fungal infections, mainly involving immunocompromised patients (Fox, 1993). Many proteins and peptides with antifungal activity from plants, bacteria, arthropods, amphibians, or reptiles have been purified and characterized (Dimarcq et al., 1998; Selitrennikoff, 2001; Wang and Ng, 2003). The number of antifungal proteins described from molds so far is rather limited to AFP from *Aspergillus giganteus* (Nakaya et al., 1990; Lacadena et al., 1995), Anafp from *Aspergillus niger* (Lee et al., 1999), AcAFP from *Aspergillus clavatus* (Skouri-Gargouri and Gargouri, 2008), NFAP from *Neosartorya fischeri* (Kovács et al., 2011), PAF (Marx et al., 1995), PgAFP (Rodríguez-Martín et al., 2010), and Pc-Arctin (Chen et al., 2013) from *Penicillium chrysogenum*. Most of these proteins have some common characteristics, such as small size (5.8–6.6 kDa), high ratio of cysteine residues, and basic character due to the presence of a high content of arginine and lysine residues (Marx, 2004; Skouri-Gargouri et al., 2009; Rodríguez-Martín et al., 2010). The antifungal proteins from molds show potent activity against filamentous fungi, although differences in sensitivity have been reported (Marx, 2004). Only Anafp proved to be active against yeasts (Lee et al., 1999) but none has been shown to inhibit the bacteria tested so far (Marx, 2004).

The amino acid sequence of PgAFP showed only 34% identity with PAF, produced by a different strain of *P. chrysogenum* (Rodríguez-Martín et al., 2010). PgAFP is the only antifungal protein isolated from a foodborne mold, as the *P. chrysogenum* strain which produces it (CECT 20922; formerly *P. chrysogenum* RP42C) was isolated from dry-cured ham (Acosta et al., 2009). Given that PgAFP was active against some toxigenic species of both *Penicillium* and *Aspergillus* (Acosta et al., 2009), the potential inhibition of other unwanted organisms of significance for dry-ripened foods, deserves further investigation.

Microbial proteases are responsible for proteolysis on dry-ripened foods. The addition of enzymes of plant or microbial origin, such as papain or flavourzyme from *Aspergillus oryzae*, has been proposed to accelerate the ripening process of dry-fermented sausages (Fernández et al., 2000). Thus, the antifungal proteins intended for use in foods should be resistant to common proteolytic enzymes. Thus, evaluating the sensitivity of PgAFP to commercial proteases is of interest. The effectiveness of antifungal proteins from molds can also be limited by pH of the food and heat treatments applied.

The efficacy of PgAFP could be affected also by other characteristics of the food, such as water activity, temperature, chemical composition, and microbial population; thus the antifungal activity of PgAFP should be tested on dry-fermented meat products.

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The objective of this work was to study the antimicrobial activity of PgAFP against some of the main microorganisms of significance for dry-ripened foods, to evaluate the sensitivity of the protein to proteolytic enzymes and heat treatments that may be applied to foods, as well as to different pH values. Finally, the inhibitory activity of PgAFP was checked against unwanted molds on dry-fermented sausages.

2. Materials and methods

2.1. Reference organisms

Inhibition tests were carried out against 38 microbial strains from 26 fungal species, five yeasts, and five bacteria. The following strains were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain), the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), or the microbial collection of Food Hygiene and Safety, University of Extremadura (Cáceres, Spain): *Aspergillus awamori* CBS 101.702, *Aspergillus carbonarius* CECT 20384, *Aspergillus flavus* CECT 2687, *Aspergillus fumigatus* CBS 192.65, *Aspergillus niger* An261, *Aspergillus ochraceoroseus* CBS 101.887, *Aspergillus ochraceus* CECT 2092, *Aspergillus oryzae* CECT 2095, *Aspergillus parasiticus* CECT 2682, *Aspergillus tamarii* CBS 109.63, *Aspergillus tubingensis* CECT 20545, *Aspergillus versicolor* CECT 2664, *Aspergillus westerdijkiae* CECT 2948, *Penicillium aurantiogriseum* CECT 2918, *P. chrysogenum* CECT 20922 (formerly *P. chrysogenum* RP42C), *P. chrysogenum* Pg222, *Penicillium commune* Pc131, *Penicillium commune* Pc332, *Penicillium echinulatum* Pe321, *Penicillium expansum* CECT 2280, *Penicillium griseofulvum* CECT 2919, *Penicillium nalgiovense* Pj261, *Penicillium nordicum* CBS 110.769, *Penicillium polonicum* Pp51, *Penicillium restrictum* Pr341, *Penicillium solitum* Ps321, *Penicillium verrucosum* AB11C, *Rhizopus oryzae* CBS 607.68; *Candida zeylanoides* CECT 10048, *Debaryomyces hansenii* CECT 10360, *Debaryomyces hansenii* Dh345, *Rhodotorula mucilaginosa* CECT 10359, *Yarrowia lipolytica* CECT 10358; *Brochothrix thermosphacta* PA7B2, *Escherichia coli* CECT 4267, *Listeria monocytogenes* CECT 4032, *Salmonella enterica* subsp. *enterica* CECT 4374, and *Serratia liquefaciens* PA7VRBG.

2.2. Protein purification

The PgAFP producing *P. chrysogenum* CECT 20922 was inoculated into malt extract broth (20 g/L malt extract, 20 g/L glucose, and 1 g/L peptone; MEB), pH 4.5, and incubated up to 21 days at 25 °C without shaking. PgAFP was obtained from the cell free medium by fast protein liquid chromatography with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Uppsala, Sweden), further purified with a HiLoad 26/60 Superdex 75 gel filtration column for FPLC (Amersham Biosciences), and concentrated as previously described (Acosta et al., 2009; Rodríguez-Martín et al., 2010).

Protein concentration in the purified extract was estimated by protein nitrogen determination according to the Johnson method (Johnson, 1941). To calculate the concentration of protein, the nitrogen percentage of 19.17% deduced from the amino acid composition of PgAFP (Rodríguez-Martín et al., 2010) was used.

2.3. Growth inhibition of reference organisms

Quantitative assay for microbial growth inhibition was carried out by a microspectroscopic method (Acosta et al., 2009) adapted from Broekaert et al. (1990). The inhibition test was performed in 96-well microtiter plates, with 100 μL of the purified protein mixed with 100 μL of culture media containing ca. 10^6 CFU/mL of the reference organism per well. Molds and yeasts were grown in double-strength (82 g/L) MEB, whereas bacteria were cultivated in double-strength (74 g/L) brain heart infusion broth (BHI, Scharlab, Barcelona, Spain). The final concentration of PgAFP in the wells was set from 1.2 to 312.7 μg/mL (0.2 to 48.2 μM). The assay was run in sextuplicate wells, using separate plates for each reference strain. The corresponding fraction from uninoculated medium, also purified by gel filtration, was used as negative control. Fungal cultures were incubated for up to 120 h at 25 °C and bacterial cultures were incubated for 48 h at 20 °C. Growth was monitored by measuring the optical density variation at 595 nm every 24 h for fungi or 12 h for bacteria. Growth reduction (GR) was defined as the lowest PgAFP concentration that produced prominent growth reduction of 50% (GR50) or 35% (GR35) compared to control plates, according to Espinel-Ingroff et al. (1997).

2.4. Susceptibility to enzymes

The sensitivity of PgAFP to digestion by enzymes was tested using the following commercial enzymes dissolved individually as indicated: pepsin, in 0.3 M KCl, 0.15 M NaCl, pH 2; lysozyme and papain, in 0.3 M PO_4Na_2, 0.15 M NaCl, pH 6.2; ficin, in 0.3 M PO_4Na_2, 0.15 M NaCl, pH 7; flavourzyme, in 0.3 M PO_4Na_2, 0.15 M NaCl, pH 7.5; pronase E and trypsin, in 0.3 M PO_4Na_2, 0.15 M NaCl, pH 7.5. All enzymes were obtained from Sigma Chemical Co. (St. Louis, USA) and used at a final concentration of 500 μg/mL. PgAFP was added at different concentrations (8 to 130 μg/mL, 1.2 to 20 μM) to enzyme preparations, and the mixtures were incubated 12 h at the following optimal temperatures recommended for each enzyme by manufacturer: 25 °C for lysozyme, papain, and trypsin, and 37 °C for the remaining enzymes. Then, the residual antifungal activity against *A. flavus* CECT 2687, *A. niger* An261, *P. griseofulvum* CECT 2919, and *P. restrictum* Pr341 was tested by the microspectroscopic method. Aliquots of the prepared PgAFP concentrations without enzyme treatment were used as positive control. Additional tests with 500 μg/mL of each enzyme and no PgAFP were run to discount interferences with mold growth.

2.5. Stability to heat treatments

The study of the protein stability after heat treatment was carried out according to Okkers et al. (1999). Aliquots of PgAFP protein (300 μg/mL) were exposed to different combinations of temperatures (60, 80, and 100 °C) and times (10, 20, and 30 min) in a dry block heater Termobloc (JP Selecta, Barcelona, Spain), as well as to 121 °C for 15 min in an autoclave cycle. After heat treatment, the samples were cooled on ice and tested for antifungal activity in microtiter plates. The reference strains tested were *Aspergillus niger* An261 and *Penicillium restrictum* Pr341.

2.6. pH stability

PgAFP (300 μg/mL) was retained in microcon centrifugal filter units YM-3 (Millipore) and then dissolved in different buffers at the following values: pH 1, 2 (HCl/KCl); pH 3 (glycine/HCl); pH 4, 5, 6 (citric acid/sodium phosphate); pH 7 (sodium phosphate/NaCl); pH 8 (Tris/HCl); pH 9, 10 (Tris/NaOH); and pH 12 (KCl/NaOH). Samples were incubated at 25 °C for 2 h and the pH adjusted to 4.5 before testing the antifungal activity. Negative controls with no PgAFP added were prepared following the same procedure. The reference strains tested were *Aspergillus niger* An261 and *Penicillium restrictum* Pr341.

2.7. Antifungal activity of PgAFP on dry-fermented sausages

The effect of PgAFP on growth of toxigenic molds was tested using a commercial raw dry-fermented sausage "salchichón" (pH 5.4, 0.95 a_w) shortly after filling into natural casing from beef. The sausage was surface-sterilized by dipping in ethanol and longitudinally cut into ca. 10 cm^2 over 1 cm thick pieces in a laminar flow cabinet (Bio Flow II, Telstar, Tarrasa, Spain). To simulate the evolution of a_w during industrial sausage processing, the pieces were separately placed in pre-sterilized receptacles with humidity kept constant at 84% after vapor–liquid equilibrium by a saturated potassium chloride solution. A volume of 100 μL of *A. flavus* CECT 2687 or *P. restrictum* Pr341 spore suspensions was

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