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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.

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

spread onto the casing surface to reach a final concentration of 10^{-4} spores/cm^2 on each piece. In treated samples, PgAFP was added in 200 μL of phosphate buffer to treated samples at two concentrations (5 and 20 μg/cm^2) and was air-dried in the flow cabinet. Control batches received the same volume of phosphate buffer without PgAFP and were inoculated with *A. flavus* or *P. restrictum*. Each treatment was done in three replicates. After inoculation, the samples were incubated at 25 °C for 5 days. The mold load on the sausages was determined from a suspension of the whole piece in 90 mL of peptone water by plating decimal dilutions on Potato Dextrose Agar and incubating at 25 °C for 5 days.

2.8. Statistical analysis

Statistical analyses were performed with the IBM SPSS v.19.0. One way analysis of variance (ANOVA) was carried out to determine significant differences within and between groups. Tukey's test was applied to compare mean values.

3. Results

The concentrations of purified PgAFP obtained from *P. chrysogenum* CECT 20922 were 282 and 617 μg/mL at 7- and 14-day incubations in MEB, respectively. Only a limited increase in PgAFP (up to 678 μg/mL) was obtained by extending incubation for one additional week.

3.1. Growth inhibition of reference organisms

All reference organisms tested showed a fair growth in microtiter plates (OD over 1 absorbance unit). The highest concentration of PgAFP tested (312.7 μg/mL) displayed no inhibition against any bacteria (data not shown) or yeast tested, but efficiently retarded growth of most reference molds, including toxigenic, defect-causing, and desirable species. According to sensitivity, two inhibition levels were established (Table 1). In level H, 4.9 μg/mL of PgAFP significantly ($p < 0.001$) reduced over 50% growth of the reference molds, *A. carbonarius*, *A. flavus*, *A. niger*, *A. ochraceus*, *P. chrysogenum*, and *P. restrictum*, as well as the strain Pc332 of *P. commune*. Level L was characterized by a significant ($p < 0.01$) but moderate inhibition, where at least 4.9 μg/mL was necessary to reduce growth 35% for reference strains, such as *A. parasiticus*, *A. versicolor*, *P. expansum*, *P. griseofulvum*, and strain Pc131 of *P. commune*. Due to the slow growth of *P. nordicum* on MEB incubation was extended up to 120 h. At this incubation time a 40% growth reduction of *P. nordicum* was reached with 19.6 μg/mL. However, there was a group of non-sensitive fungi, including *P. polonicum* and *R. oryzae*, in which growth was not significantly ($p > 0.05$) retarded by any PgAFP concentration tested. Inhibition on sensitive molds showed a remarkable dose–effect relationship (Fig. 1). For the highly sensitive reference strains, a noticeable inhibition was observed even at the lowest PgAFP concentration tested (1.2 μg/mL).

3.2. Sensitivity of PgAFP activity to proteases

None of the enzymes tested lead to any statistically significant decrease ($p > 0.05$) on growth of the reference molds when used as a negative control with no antifungal protein. Preincubation of PgAFP with proteases decreased the antifungal activity on the reference molds, but the resulting effect depended not only on the enzyme, but also on both PgAFP concentration and sensitivity level of the reference mold (Fig. 2). Pronase E efficiently eliminated ($p < 0.001$) any inhibitory effect from PgAFP on every selected reference mold, even at the highest PgAFP concentration (130 μg/mL). Flavourzyme and trypsin lowered PgAFP activity, but the effect was less evident, particularly at the highest PgAFP concentration. These two enzymes efficiently eliminated the inhibition on *A. flavus* and *A. niger* by up to 65 μg/mL PgAFP ($p < 0.001$), but not so much at the highest PgAFP concentration ($p < 0.05$). The inhibition on the two penicillia strains was occasionally altered by flavourzyme and trypsin ($p < 0.05$). The effect of ficin, pepsin, and papain was less obvious with every reference strain, and decreased at higher PgAFP concentrations.

Table 1
In vitro minimal concentrations of PgAFP achieving over 50% (GR_{50}) or 35% growth reduction (GR_{35}) on fungal strains on malt extract broth at 72 h of incubation at 25 °C.

Inhibition level	Fungal strain	GR50 (μg/mL)	GR35 (μg/mL)
H	*A. carbonarius* CECT 20384	1.2	
	A. flavus CECT 2687	4.9	
	A. fumigatus CBS 192.65	2.4	
	A. niger An261	4.9	
	A. ochraceus CECT 2092	1.2	
	A. oryzae CECT 2095	2.4	
	A. tamarii CBS 109.63	2.4	
	A. westerdijkiae CECT 2948	2.4	
	P. chrysogenum Pg222	2.4	
	P. commune Pc332	4.9	
	P. nalgiovense Pj261	2.4	
	P. restrictum Pr341	2.4	
	P. solitum Ps321	4.9	
L	*A. awamori* CBS 101.702	19.6	9.8
	A. ochraceoroseus CBS 101.887	312.7	19.6
	A. parasiticus CECT 2682	312.7	39.1
	A. tubigensis CECT 20545	19.6	9.8
	A. versicolor CECT 2664	156.4	78.2
	P. aurantiogriseum CECT 2918	>	39.1
	P. commune Pc131	>	78.2
	P. echinulatum Pe321	>	19.6
	P. expansum CECT 2280	9.8	4.9
	P. griseofulvum CECT 2919	>	19.6
	P. nordicum CBS 110.769	312.7	19.6[a]
	P. verrucosum AB11C	>	9.8
Non-sensitive	*P. chrysogenum* CECT 20922	>	—
	P. polonicum Pp51	>	—
	R. oryzae CBS 607.68	>	—
	C. zeylanoides CECT 10048	>	—
	D. hansenii CECT 10360	>	—
	D. hansenii Dh345	>	—
	R. mucilaginosa CECT 10395	>	—
	Y. lipolytica CET 10358	>	—

>: 50% Growth reduction was not reached with 312.7 μg/mL PgAFP.
—: No significant growth reduction was obtained with 312.7 μg/mL PgAFP.
[a] Incubation time extended to 120 h.

3.3. Effect of heat-treatments and different pH values

PgAFP showed a remarkable stability at high temperatures, retaining the antifungal activity against *P. restrictum* and *A. niger* even after being heat-treated at 80 °C for 30 min or 100 °C for 15 min (Fig. 3). More intense heating decreased the antifungal activity, reaching an effective ($p < 0.001$) inactivation with the most severe treatments (100 °C for 30 min or autoclave cycle).

Preincubation in buffers at pH values from 1 to 12 had no deleterious effect ($p > 0.05$) on the activity of PgAFP against the reference molds (Fig. 4).

3.4. Antifungal activity of PgAFP on dry-fermented sausages

To test the potential use in foods, the efficiency of PgAFP against unwanted molds was assayed in dry-ripened sausages. The effect on growth of *A. flavus* and *P. restrictum* was evaluated by plating and CFU counting from both PgAFP treated and non-treated sausages. Lower counts were detected in the batches treated with PgAFP (Table 2, Fig. 5) compared to the non-treated control ($p < 0.05$).

4. Discussion

P. chrysogenum CECT 20922 had shown a strong inhibitory activity against *P. echinulatum*, *P. commune*, and *A. niger* due to production of

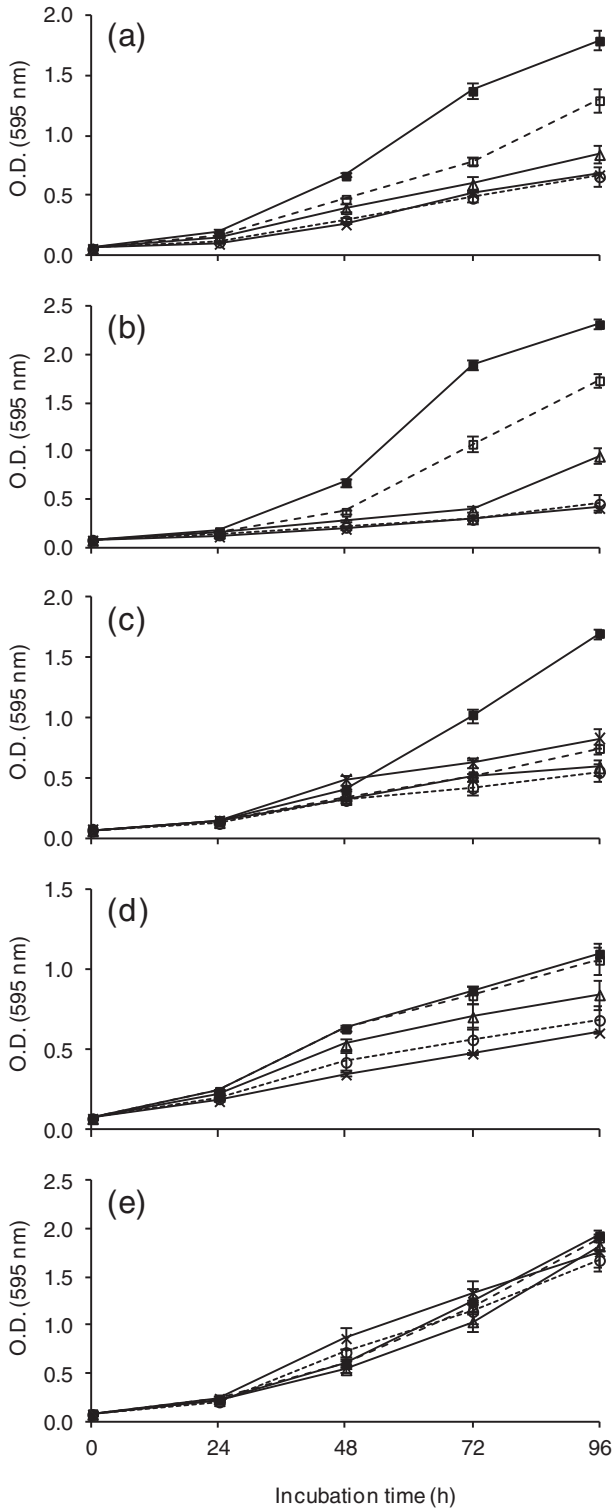

Fig. 1. Effect of PgAFP on growth of selected molds incubated with 0 (■), 1.2 (□), 2.4 (Δ), 18.8 (○), and 37.5 (×) μg/mL PgAFP. (a) *Aspergillus flavus* CECT 2687; (b) *Aspergillus niger* An261; (c) *Penicillium restrictum* Pr341; (d) *Penicillium griseofulvum* CECT2919; (e) *Penicillium polonicum* Pp51. Values are means with standard deviation of optical density of reference molds.

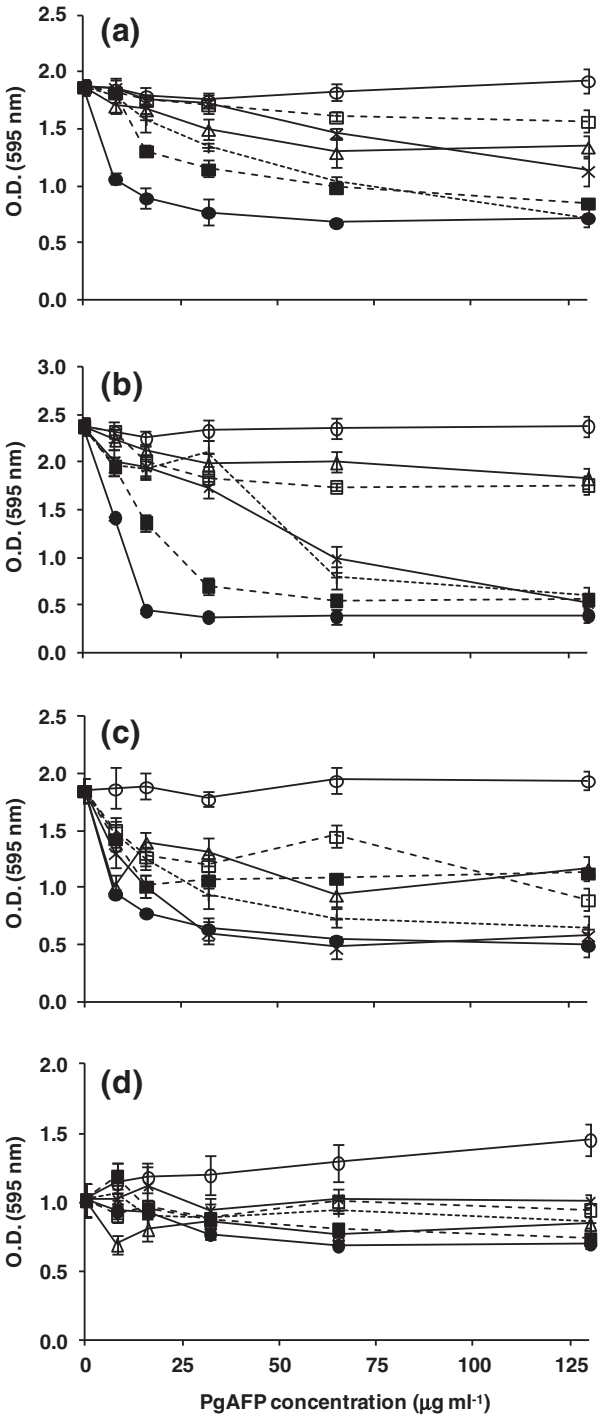

Fig. 2. Effect of proteases on the antifungal activity of different concentrations of PgAFP against selected reference molds. Treatments: (■) pepsin, (×) ficin, (+) papain, (Δ) flavourzyme, (○) pronase E, (□) trypsin, and (●) untreated. (a) *Aspergillus flavus* CECT 2687; (b) *Aspergillus niger* An261; (c) *Penicillium restrictum* Pr341; (d) *Penicillium griseofulvum* CECT2919. Values are means with standard deviation of optical density of reference molds grown for 96 h.

the cationic protein PgAFP (Acosta et al., 2009). The strain CECT 20922 of *P. chrysogenum* has been demonstrated to limit growth of the aflatoxin-producing *A. flavus* and ochratoxigenic molds on dry-cured ham (Bernáldez et al., 2014; Rodríguez et al., 2015). However, no mold growth, including that of protective cultures, is wanted in some types of dry-ripened sausage. Thus, the use of purified PgAFP could be of interest. To evaluate the activity of the purified protein, its effect on growth of penicillia, aspergilli, yeasts, and bacteria was assessed. No effect on growth of the tested yeasts and bacteria was observed, even at the highest concentration of PgAFP used (312.7 μg/mL). The lack of activity against yeasts and prokaryotes has also been reported for other small, basic antifungal proteins (Marx, 2004). In fact, the only activity reported is limited to Anafp on *Candida albicans*, *Saccharomyces*

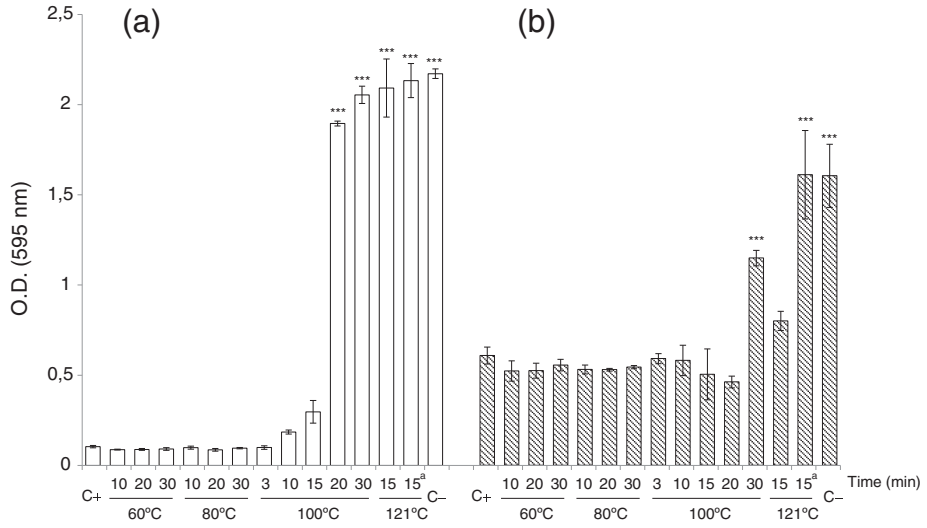

Fig. 3. Antifungal activity of 300 μg/mL heat-treated PgAFP expressed as mean and standard deviation of optical density of reference molds grown for 96 h. (a) *Aspergillus niger* An261; (b) *Penicillium restrictum* Pr341. Asterisks indicate statistically significant differences to growth of the positive control reference mold: *** ($p < 0.001$). C+: positive control with non-heated PgAFP; C−: negative control without PgAFP. [a]: Autoclave cycle.

cerevisiae, and *Trichosporon beigelii* (Lee et al., 1999). The lack of inhibitory activity on yeasts can be regarded as a positive property. First, because yeasts such as *D. hansenii* contribute to develop the desirable characteristics of dry-ripened meats with no detrimental effects. Second, a limited number of yeasts, including *D. hansenii*, also may combat toxigenic molds (Andrade et al., 2014; Simoncini et al, 2014; Núñez et al., 2015).

PgAFP retarded growth of most molds tested, including the selected aspergilli and most penicillia. Interestingly, the main toxigenic molds tested, such as those producing aflatoxin (*A. flavus* and *A. parasiticus*), ochratoxin A (*A. carbonarius, A. ochraceus*, and *P. nordicum*), sterigmatocistin (*A. versicolor*), and patulin (*P. expansum* and *P. griseofulvum*) were in the PgAFP-sensitive group of fungi. The observed effect is fungistatic, not achieving total inhibition of the sensitive mold at the highest concentration tested. Nonetheless, this effect would contribute to reduction of the hazard due to mycotoxins in dry-ripened foods because many secondary metabolites are produced mainly in the idiophase. Despite reports that mycotoxin production may increase in stressed toxigenic fungi, the strong growth inhibition shown in the sausage could be useful to control mycotoxin production, as discussed later. The fact that some molds, such as *P. polonicum* and *R. oryzae*, were not sensitive should not be regarded as a drawback, given that none of them is linked to foodborne mycotoxicosis (Richard, 2007). The minimal inhibitory concentration to reach over 50% growth reduction was in the range of 1.2–4.9 μg/mL (0.2–0.75 μM) PgAFP for the high sensitive strains (level H), whereas low sensitive strains (level L) required 4.9–78.2 μg/mL (0.75–12 μM) for a 35% growth reduction (Table 1). The inhibition rate for highly sensitive strains can be compared to that of 6 to 25 μM required for complete inhibition of sensitive molds by AFP (Lacadena et al., 1995). Growth inhibition differs greatly not only between species of the same genus, but also within the same species. Accordingly, *P. commune* Pc332 was highly sensitive to PgAFP, with 4.9 μg/mL GR_{50}, whereas *P. commune* Pc131 required 78.2 μg/mL for just a 35% growth reduction. GR_{50} for *P. chrysogenum* Pg222 was 2.4 μg/mL, but the PgAFP-producing strain *P. chrysogenum* CECT 20922 withstands 312.7 μg/mL, the maximum level tested. Similar differences in sensitivity to antifungal proteins have been reported for AFP, Anafp, PAF, and AcAFP, showing both sensitive and non-sensitive species in a limited number of strains from genera *Aspergillus*, *Penicillium*, or *Fusarium* (Lee et al., 1999; Kaiserer et al., 2003; Theis et al., 2003; Skouri-Gargouri and Gargouri, 2008).

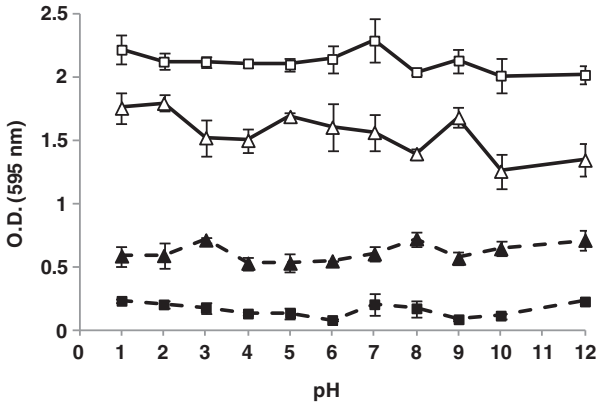

Fig. 4. Antifungal activity of PgAFP after incubation at different pH values, expressed as mean and standard deviation of optical density of reference molds grown for 96 h. *Aspergillus niger* An261 (■) and *Penicillium restrictum* Pr341 (▲) treated with 300 μg/mL PgAFP (closed symbols) or untreated negative controls (open symbols).

To study the effect of proteases on the antifungal activity of PgAFP the reference species selected included both sensitivity levels and fungal genera. The enzymes chosen are among the most active proteases that can be added to processed foods or, for flavourzyme and pronase E, have been proposed to enhance flavor development in dry fermented sausages (Fernández et al., 2000). No enzyme tested showed any effect on growth of reference strains (Fig. 2). The activity of the proteases against PgAFP was evidenced by the loss of activity at low PgAFP levels. At higher PgAFP concentrations, the more active proteases decreased the antifungal effect. The less active proteases were less efficient, and the residual amount of antifungal protein seems to be enough to limit growth of the reference molds. This effect was not observed with the low-sensitive *P. griseofulvum*, partially because the short extent

Table 2
Effect of PgAFP on growth of *Aspergillus flavus* and *Penicillium restrictum* on a dry-fermented sausage after 5 days at 25 °C. Mold growth is given as mean ± SD.

	Fungal species	
PgAFP (μg/cm^2)	*A. flavus* CECT 2687 (log CFU/cm^2)	*P. restrictum* Pr341 (log CFU/cm^2)
0	5.0 ± 0.21	4.7 ± 0.17
5	2.6 ± 0.24[a]	3.6 ± 0.19[a]
20	2.1 ± 0.15[a]	3.1 ± 0.25[a]

[a] Different ($p < 0.05$) from the non-treated control.

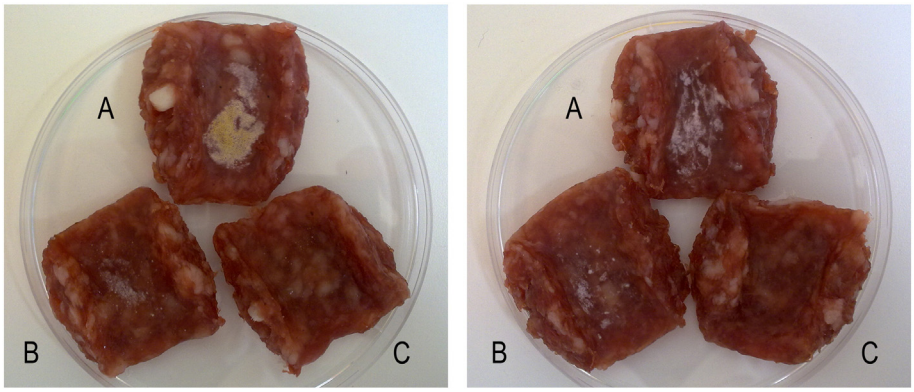

Fig. 5. Effect of 0 (A), 5 (B), and 20 (C) μg/cm^2 PgAFP on growth of *Aspergillus flavus* (left) and *Penicillium restrictum* (right) on dry-fermented sausage after 5 days at 25 °C.

between growth of PgAFP-treated and non-treated samples did not allow detecting the effect due to PgAFP degradation.

Given that PgAFP digestion was run under extreme conditions, for 12 h at favorable temperature and pH values for each enzyme, the observed residual activity reveals a remarkable ability to withstand proteases. Only the mixture of exopeptidases and endopeptidases from *S. griseus* contained in pronase E was able to efficiently inactivate 130 μg/mL PgAFP. With the latter result, the potential use of PgAFP might be argued, when proteolytic organisms were present. However, highly proteolytic molds from dry-ripened meats, such as *P. chrysogenum* Pg222 and *A. oryzae* CECT 2095 (ATCC 9362) (Trigueros et al., 1995; Benito et al., 2002), were among the highly sensitive molds. The observed protease resistance can be attributed to the compact structure of the small, basic, cysteine-rich antifungal proteins, as it has been proposed for AFP (Lacadena et al., 1995).

Among the main physicochemical factors affecting protein activity in foods are pH and heat treatments. To study the impact of these two factors on PgAFP activity, one sensitive strain from each genus was selected as reference. PgAFP was highly resistant to a wide range of temperature (Fig. 3). Intense heat treatments partially inactivated 300 μg/mL PgAFP, as can be inferred from the loss of inhibition on the less sensitive *A. niger* and the residual effect on the more sensitive *P. restrictum.* Similarly to its heat stability, PgAFP withstood a broad range of pH values (Fig. 4). These characteristics are similar to those of other antifungal proteins from molds. AFP from *A. giganteus* is stable to protease degradation, high temperature, and a broad pH range due to a specific folding pattern (Lacadena et al., 1995). Similarly, AcAFP from *A. clavatus* is also resistant to 100 °C and is effective in a 5–12 pH range (Skouri-Gargouri et al., 2010). AFP folds into a small and compact β-barrel, stabilized by four disulfide bridges formed by cysteine residues (Campos-Olivas et al., 1995). It has been assumed that AcAFP, Anafp, NAF, and PAF also have a similar tertiary structure, with three or four disulfide bridges formed by the cysteine residues present in these proteins (Marx, 2004; Skouri-Gargouri et al., 2009). PgAFP has characteristics common to these antifungal proteins, including six cysteine residues and a quite high similarity in the amino acid sequence, leading a tertiary structure based on a compact β-barrel (Rodríguez-Martín et al., 2010).

Treatment with the two PgAFP concentrations assayed was effective in reducing growth of *A. flavus* and *P. restrictum* in sausage samples (Table 2, Fig. 5). The counts of *A. flavus* in treated samples were always below 3.5 log CFU/g. This effect can serve to prevent development of hazardous levels at which aflatoxin B1 production has been described on dry-cured hams (Rodríguez et al., 2012). Given the observed fungistatic effect of PgAFP, it is not expected that sufficient inhibition would be maintained throughout the whole ripening time. Thus, the antifungal protein would be most efficient if applied at the time when the product is most susceptible to mold development. Direct application of PgAFP by spraying on the product in the key stages could be useful to delay mold growth to allow corrective actions to be taken before mycotoxins are produced. The low identity of PgAFP in relation to other antifungal proteins from molds, as well as the differences in site of action and possible targets (Marx, 2004) sustain the combined use of PgAFP with other antifungal proteins to take advantage of complementary effects.

Although the inhibitory activity of some antifungal proteins produced by molds has been reported (Lee et al., 1999; Kaiserer et al., 2003; Theis et al., 2003; Skouri-Gargouri and Gargouri, 2008), this is the first time that the inhibitory effect against toxigenic molds has been proved in foods. Given that *P. chrysogenum* is used to obtain generally recognized as safe (GRAS) compounds and has a long record of industrial use, the potential application of PgAFP in foods should be considered additionally with good manufacturing practice through HACCP procedures for reducing the health hazard due to mycotoxins in dry-fermented sausages. In addition, *P. chrysogenum* CECT 20922 is able to limit growth of aflatoxin and OTA-producing molds and OTA accumulation in dry-cured Iberian ham throughout the processing (Bernáldez et al., 2014; Rodríguez et al., 2015).

In conclusion, PgAFP showed an efficient inhibition of undesired molds, including some of the main toxigenic species for dry-ripened foods, when these fungi were grown in culture medium and in a dry-fermented sausage. Moreover, PgAFP withstands most proteases, intense heat and a wide range of pH values. This protein can be of interest to control unwanted fungi in dry-ripened foods.

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