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Selection of antifungal protein-producing molds from dry-cured meat products

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ABSTRACT

To control unwanted molds in dry-cured meats it is necessary to allow the fungal development essential for the desired characteristics of the final product. Molds producing antifungal proteins could be useful to prevent hazards due to the growth of mycotoxigenic molds. The objective has been to select *Penicillium* spp. that produce antifungal proteins against toxigenic molds. To obtain strains adapted to these products, molds were isolated from dry-cured ham. A first screening with 281 isolates by the radial inhibition assay revealed that 166 were active against some of the toxigenic *P. echinulatum*, *P. commune*, and *Aspergillus niger* used as reference molds. The activity of different extracts from cultured medium was evaluated by a microspectroscopic assay. Molds producing active chloroform extracts were eliminated from further consideration. A total of 16 *Penicillium* isolates were screened for antifungal activity from both cell-free media and the aqueous residues obtained after chloroform extraction. The cell-free media of 10 isolates that produced a strong inhibition of the three reference molds were fractionated by FPLC on a cationic column. For protein purification, the fractions of the three molds that showed high inhibitory activity were further chromatographed on a gel filtration column, and the subfractions containing the highest absorbance peaks were assayed against the most sensitive reference molds. One subfraction each from strains AS51D and RP42C from *Penicillium chrysogenum* confirmed the inhibitory activity against the reference molds. SDS-PAGE revealed a single band from each subfraction, with estimated molecular masses of 37 kDa for AS51D and 9 kDa for RP42C. Although further characterisation is required, both these proteins and the producing strains can be of interest to control unwanted molds on foods.

1. Introduction

Toxigenic molds pose a hazard to different foods. Mold growth can be efficiently controlled on various foods with different treatments such as chemical preservatives or modified atmosphere packaging. However, such treatments are not adequate for mold-ripened foods, where fungal development is essential for the desired characteristics of the final product. When the raw material can be heated to eliminate the wild mold population, as it is common for most ripened cheeses, fungal starter cultures may minimize the risk due to toxigenic molds. On the contrary, heating is not adequate for ripened raw meat products, such as dry-cured ham and sausages. Therefore, a different means to control unwanted molds on dry-cured meat products is required.

The antimicrobial proteins described in recent years could offer an option to control undesirable molds. The use of small, basic proteins secreted from filamentous fungi has been suggested for biopreservation purposes in food (Geisen, 2000). They show a quite interesting inhibition spectrum on fungi, with neither activity against prokaryotes nor detrimental effects on mammalian cells (Marx, 2004). In addition, either the purified protein or the producing strain could be used for meat products.

Only a very limited number of antifungal protein producers have been described among molds including *Aspergillus giganteus* (Nakaya et al., 1990; Lacadena et al., 1995), *Penicillium chrysogenum* (Marx et al., 1995), *Aspergillus niger* (Lee et al., 1999), *Penicillium nalgiovense* (Geisen, 2000), and *Aspergillus clavatus* (Skouri-Gargouri and Gargouri, 2008). They show inhibitory activity against a high proportion of the filamentous ascomycetes tested (Marx, 2004), including *Aspergillus* spp. and *Fusarium* spp., whereas *Penicillium* spp. seem to be less sensitive. In spite of the potential benefits, there is no information on the occurrence of antifungal protein producers in mold-ripened foods. It would be of great interest to have strains adapted to the particular environment of these products, showing activity against the unwanted molds.

Dry-cured meat products are suitable ecosystems for the development of a wide variety of molds; thus, they seem appropriate to find mold strains showing antifungal capability. It was decided to search for antifungal *Penicillium* species in dry-cured meats because penicillia are commonly found in dry-cured meat products all through the

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ripening process (Núñez et al., 1996), and some of their species are used as starter cultures (Sunesen and Stahnke, 2003). In addition, there is very little concern about hazards due to mycotoxins by most *Penicillium* spp.

Numerous methods have been described to evaluate the inhibitory activity of antimicrobial substances or producing microorganisms by formation of inhibition zones, including agar plug test (Larsen and Knochel, 1997), spot on lawn assay (Larsen and Knochel, 1997), agar plate inhibition assay (Geisen, 2000) or agar-well diffusion (Von der Weid et al., 2003). Microtiter methods have been also designed (Amsterdam, 1996; Broekaert et al., 1990; du Toit and Rautenbach, 2000; Steinberg and Lehrer, 1997). However, no standard procedure has been established to select active isolates, and the precise conditions for the assays must be specifically considered.

The aim of this work has been to select *Penicillium* strains that produce antifungal protein active against toxigenic molds for an eventual use in dry-cured meat products.

2. Materials and methods

2.1. Collection of fungal isolates

Samples were taken at nine factories, experienced in traditional processing of dry-cured ham and located at different towns in the Southwest of Spain belonging to the Protected Designations of Origin "Guijuelo", "Jamón de Huelva", and "Dehesa de Extremadura". Five hams were randomly sampled at each of the different ripening stages from every selected factory. A total of 135 hams were sampled.

Molds were collected from hams by scraping 25 cm^2 of the ham surface with a sterile scalpel. After homogenization and decimal dilution in sterile 0.1% peptone water, 0.1 ml of the resulting suspension was plated onto creatine sucrose neutral agar (CSN; Pitt, 1993), and dichloran 18% glycerol agar (DG18; Hocking and Pitt, 1980), and incubated at 25 °C for 7 days.

Colonies showing different macroscopic characteristics were picked from grown agar plates and subcultured on CSN or malt extract agar (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar; MEA) for isolation. Conidia were harvested by washing the surface of agar plate with 10% glycerol in sterile water and stored at −80 °C. At least three different isolates showing differential characteristics on CSN or MEA were selected from every ripening stage and factory sampled. *P. nalgiovense* BFE66, kindly supplied by Dr. Rolf Geisen (Federal Research Centre for Nutrition, Kalrsruhe, Germany) was also included for reference.

2.2. Radial inhibition assay

The agar plate inhibition assay described by Geisen (2000) was run against three reference toxigenic molds: *P. echinulatum* Pe321, *P. commune* Pc332, and *A. niger* An261 isolated from dry-cured ham (fungal collection of Food Hygiene of the University of Extremadura). Growth of reference molds and conidia harvest were carried out as described above for selected isolates, using 1% Tween 20 in sterile water instead of the glycerol solution to harvest conidia. All isolates were grown on MEA pH 4.5 at 25 °C for 4 days. A 25 mm diameter plug was cut out with a sterile corer from the actively growing part of the colony and transferred to an empty 9 cm diameter Petri dish. Plugs from three different isolates were placed per plate. Molten MEA (*ca.* 20 ml), cooled to 50 °C, was mixed with spores of one reference mold and poured onto the agar plates containing plugs from three isolates. This molten medium was added right to the height of agar plugs, ensuring that the mycelium on the excised agar was not covered with medium. This process was repeated for each reference mold. Plates were incubated at 25 °C for 48 h, and inspected for growth of the reference molds. The intensity of the inhibition was estimated by the area of the inhibition zone around the colony.

2.3. Classification of active isolates

Active isolates on plate inhibition assay were grouped by their morphological characteristics and media reaction on MEA (Raper and Thom, 1949), Czapeck yeast extract autolysate agar CYA (Pitt, 1973), and glycerol nitrate agar G25N (Pitt, 1973) according to classical taxonomical concepts (Pitt and Hocking, 1997). Isolates classified as *Penicillium* subgenus *Penicillium* were further grouped by the morphological aspect and substrate reaction after growing on CSN at 25 °C for 7 days (Pitt, 1993).

2.4. Preparation of fungal extracts

Selected molds were inoculated in 150 ml of both malt extract broth (2% malt extract, 2% glucose, 0.1% peptone; MEB) and potato dextrose broth (PDB, Scharlab, Barcelona, Spain), pH 4.5, and incubated at 25 °C for 15 days. Three different fractions were obtained from each culture: whole cell-free medium (CFM), chloroformic extract (ChE) and aqueous residue (AR).

To obtain CFM, 50 ml of the culture broth was filtered through a 0.22 µm-pore-size nylon membrane (MSI, Westboro, USA). Filtered medium was vacuum-dried in a Speedvac (Savant Instruments, Farmingdale, USA) to a final volume of 5 ml. To obtain ChE, 100 ml of medium including the mycelium was transferred to a plastic bag and macerated with 50 ml chloroform in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 4 min. After 1 h, the slurry was centrifuged at 3000 ×*g* for 10 min. The chloroformic phase was filtered through anhydrous sodium sulphate with Whatman no. 1 filter paper (Whatman Int., Maidstone, UK) and evaporated in a rotary evaporator model VV2000 (Heidolph, Kelheim, Germany) at 40 °C. Then, ChE was resuspended in 1 ml chloroform and filtered through a 0.45 µm-pore-size nylon membrane (MSI). Aliquots of 500 µl were evaporated to dryness under a gentle stream of nitrogen and stored at 4 °C in the dark until required. Just before use in the inhibition assay, dried ChE was resuspended in 500 µl of dimethyl sulfoxide. The AR was obtained by filtering 50 ml of aqueous phase left after chloroform extraction through a 0.22 µm-pore-size nylon membrane, and vacuum-drying in a Speedvac set at low temperature (43 °C) until a final volume of 5 ml. Extracts from both non inoculated culture media were obtained to be used as negative control in the antifungal assay.

2.5. Microspectrophotometric inhibition assay

Extracts from the assayed molds were tested in microtiter plates against the reference molds according to a microspectrophotometric method adapted from Broekaert et al. (1990). Aliquots of 100 µl from CFM, ChE, or AR extracts were loaded in each well of a 96-well microplate in triplicates. As a negative control, extracts obtained from sterile culture medium were run in triplicate wells as required. Then, every well in the same plate was filled with 100 µl of double-strength of either MEB or PDB containing 10^6 fungal spores/ml of a single indicator mold. Growth was recorded at 24, 48, 72, and 96 h of incubation at 25 °C. The absorbance at 595 nm served as a measure for fungal growth. Any given extract was considered to be active when the average absorbance in the wells differed significantly from that in control wells. Data were statistically analyzed using SPSS software package (version 15.0. SPSS Inc., Chicago, USA) by a variance analysis (one way ANOVA) and the means were separated by the Tukey honest significant difference test.

2.6. Analysis of secondary metabolites

The dried ChE was dissolved in 200 µl of acetonitrile just before analysis. Secondary metabolites in extracts were analyzed by micellar electrokinetic capillary electrophoresis (MECC) and liquid chromatography coupled to mass spectrometer detector (HPLC-MS).

The MECC analysis was carried out according to Martín et al. (2004) in a Beckman P/ACE 5500 model with a photodiode array detector (Beckman Instruments, Fullerton, USA). Previous to analysis, 100 µl of ChE dissolved in acetonitrile was mixed with an equal volume of water. A fused silica capillary column of 75 µm diameter by 57 cm total length (50 cm until window detector) was used for separation with 25 mM sodium tetraborate and 50 mM SDS (pH 9) as running buffer, at 15 kV, maximum current at 200 µA, and 23 °C. The absorbance was recorded at 200 and 280 nm. For each peak, a spectrum of absorbance between 190 and 600 nm was obtained in the photodiode array detector.

HPLC-MS analysis of the ChE dissolved in acetonitrile was performed according to Núñez et al. (2000) in a Hewlett Packard series 1100 apparatus (Hewlett Packard, Palo Alto, USA). A Supelcosil LC-18 column (SUPELCO, Bellafonte, USA) was used with mobile phases (A) 100% water and (B) 0.05% trifluoroacetic acid in acetonitrile in a gradient from 10% to 99% B. The secondary metabolites were identified in a Finnigan LCQ Mass Spectrometer (Finnigan, San Jose, USA) with atmospheric pressure chemical ionization source (APCI), according to their retention time and molecular mass.

Mycotoxins produced by molds commonly found in dry-cured meat products, including viridicatum toxin, roquefortin C, citreoviridin, xanthomegmine, verruculogen, secalonic acid D, fumitremorgin B, paxilin, deoxynivalenol, penitrem A, citrinin, penicillic acid, ochratoxin A, cyclopiazonic acid, mycophenolic acid, zearalenone, aflatoxins B1 and G1, sterigmatocystin, griseofulvin, and patulin (Sigma Chemical, St. Louis, USA) were used as standards. Verrucosidin standard was kindly supplied by Dr. L. Leistner (Institute for Microbiology, Toxicology and Histology, Kulmbach, Germany).

2.7. Purification of antifungal proteins

The isolation of antifungal proteins was carried out by fast protein liquid chromatography (FPLC) using an ÄKTA FPLC equipped with a UV detector at 214 nm and a fraction collector FRAC-950 (Amersham Pharmacia Biotech, Uppsala, Sweden). Filtered CFM, obtained as described for preparation of fungal extracts, was applied to a cationic exchange column HiTrap SP HP (Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 20 mM sodium acetate, pH 4.5 (buffer A). Unadsorbed material was eluted with buffer A, while adsorbed proteins were eluted with 20 mM sodium acetate buffer (pH 4.5) containing 1 M NaCl (buffer B) under the following gradient: initial 0% B, linear change to 25% B in 15 column volumes (CV), linear change to 100% B in 2 CV, keeping this percentage for 15 additional CV. Flow rate was set at 5 ml/min. Twenty eight fractions of 5 ml each were collected per sample. The antifungal activity of every collected fraction was assessed by the microspectrophotometric inhibition assay.

Those fractions showing antifungal activity were gel filtered on a HiLoad 26/60 Superdex 75 column for FPLC (Amersham Biosciences) in 50 mM sodium phosphate buffer (pH 7) containing 0.15 M NaCl. Fractions showing the highest protein concentration from every peak eluted were assayed for antifungal activity by the microspectrophotometric method.

The presence of proteins in the active fractions was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Separation gels consisted of a 4% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel. Proteins were denatured by boiling the sample for 5 min in 0.0625 M Tris–HCl buffer (pH 6.8) with 20% glycerol, 2% SDS, and 5% 2-mercaptoethanol. Sigma Marker Low Range from 6500 to 66,000 Da (Sigma Chemical) was used as molecular weight standards. Electrophoresis was carried out at 100 V for 90 min at room temperature. Proteins were visualized by Coomassie Brilliant Blue R-250 staining. The molecular mass of the antifungal proteins was determined by comparison of their electrophoretic mobility with those of the proteins in the molecular mass marker using 1D Image Analysis Software by Kodak Eastman (Rochester, USA).

3. Results

3.1. Selection of active strains

A total of 281 isolates were obtained from dry-cured hams at the different stages of ripening at the nine processing plants sampled. A first screening with the radial inhibition assay revealed that 166 isolates were active against at least one reference mold. The ratio of active isolates at the different processing stages showed no apparent trend related to ripening time. Eighty eight active isolates representative from every sampling and processing plant were classified by their macroscopic and microscopic characteristics in four culture media (Pitt, 1993; Pitt and Hocking, 1997) into 18 different groups (Table 1).

From these 88 active isolates, the inhibitory potential of ChE was further evaluated in a second screening by the microspectroscopic assay. This test revealed that 59% of the isolates originated ChE active against *P. commune* Pc332, while only 11% against *A. niger* An261 (Table 1). The ChE from all isolates characterised as *P. aurantiogriseum*, *P. commune*, *P. solitum*, or *P. echinulatum* inhibited at least one reference mold. On the contrary, just ChE from 16 isolates showed no inhibition on any of the three reference molds used.

The 16 isolates characterised as *Penicillium* spp. with no active ChE were further screened with two additional tests: 1) antifungal activity from CFM and AR obtained from both MEB and PDB, and 2) mycotoxin analysis. All 16 selected isolates gave active CFM from both culture media against at least one of the reference molds (Table 2). Pe321 and Pc332 seem to be more sensitive than An261. Although most AR proved to be active, the inhibition was in some instances weaker than that of CFM. The isolates producing strongly active ($p<0.001$) CFM on the three reference molds were: *P. chrysogenum* AS51D, AMB11, RB41C, and RP42C, *P. camemberti* AMP61, *P. verucosum* AB11C, *P. viridicatum* DBI23, and *Penicillium* sp. DBII41.

Analysis of hazardous secondary metabolites revealed the production of secalonic acid by isolates AS51D, RB41C, and AMB11

Table 1
Inhibitory activity of tested isolates against *P. echinulatum* Pe321, *P. commune* Pc332, and *A. niger* An261 by radial inhibition assay on malt extract agar (RI) and by the microspectroscopic inhibition test from chloroform extracts obtained from malt extract broth and potato dextrose broth (ChE).

Group	Initial characterisation	No. isolates	Reference mold					
			Pe321		Pc332		An261	
			RI[a]	ChE[a]	RI	ChE	RI	ChE
I	*P. chrysogenum*	9	3	–	7	–	1	–
II	*P. olsonii*	4	2	1	2	2	1	1
III	*P. camemberti*	1	–	–	1	–	–	–
IV	*P. verrucosum*	6	1	–	6	5	–	–
V	*P. viridicatum*	7	7	1	6	3	2	2
VI	*P. polonicum* or *P. aurantiogriseum*	9	6	5	8	7	2	3
VII	*P. commune*	4	4	4	4	1	–	–
VIII	*P. griseofulvum*	8	7	2	7	6	–	–
IX	*P. solitum*	21	20	10	19	16	1	1
X	*P. echinulatum*	4	2	2	3	4	1	–
XI	*P. canescens*	3	1	–	3	2	–	–
XII	*P. crustosum*	1	–	1	1	1	–	1
XIII	*P. brevicompactum*	2	1	1	2	1	–	–
XIV	*P. griseoroseum*	1	1	1	–	1	–	1
XV	*P. citrinum*	1	–	–	1	–	–	–
XVI	*P. jensenii* (*nalgiovense*)	1	1	–	–	–	–	–
XVII	*Penicillium* spp.	3	1	2	2	–	1	–
XVIII	*Eupenicillium* spp.	3	3	2	3	3	–	1
	Total	88	60	32	75	52	9	10

[a] Number of isolates that showed inhibition against the reference mold.

Table 2
Activity of extracts from selected isolates against *P. echinulatum* Pe321, *P. commune* Pc332, and *A. niger* An261 after 96 h of incubation on malt extract broth (MEB) and potato dextrose broth (PDB).

Identification	Isolate	Extract[a]	Pe321		Pc332		An261	
			MEB	PDB	MEB	PDB	MEB	PDB
P. chrysogenum	AS51D	CFM	+++[b]	+++	+++	+++	+++	++
		AR	+++	++	+++	−	+++	−
P. chrysogenum	RB41C	CFM	+++	+++	+++	+++	++	++
		AR	+++	+++	+++	+++	+++	+++
P. chrysogenum	AMB11	CFM	+++	+++	+++	+	+++	−
		AR	+++	+++	+++	++	+++	−
P. chrysogenum	RP42C	CFM	+++	+++	+++	+++	+++	−
		AR	+++	+++	+++	+++	+	−
P. camemberti	AMP61	CFM	+++	+++	+++	+++	−	+++
		AR	+++	+	+++	+++	+++	−
P. verrucosum	AB11C	CFM	++	+++	−	+++	−	+++
		AR	−	++	−	−	−	++
P. viridicatum	DBI23	CFM	+++	+++	+++	+++	+++	+++
		AR	+++	+++	+++	+++	+++	+++
P. polonicum	CSII52	CFM	−	++	+++	+++	+	+++
		AR	−	−	+++	−	−	−
P. polonicum	CSII42	CFM	−	+++	+++	+++	−	++
		AR	−	−	−	−	−	−
P. solitum	CSII33	CFM	+++	+++	+++	+++	++	−
		AR	++	+	−	+++	−	−
P. solitum	CSII16	CFM	+++	+++	+++	+++	++	−
		AR	+	−	−	+++	−	−
P. solitum	CBI25	CFM	+++	+++	+++	+++	−	−
		AR	+	−	−	+++	−	−
Pencillium sp.	DBII41	CFM	+++	+++	+++	+++	++	+++
		AR	−	+++	+++	+++	−	−
Pencillium sp.	CSI54	CFM	+++	+++	+++	+++	++	−
		AR	−	++	−	+++	−	−
Pencillium sp.	CBII57	CFM	+++	+++	+++	+++	−	−
		AR	−	−	−	+++	−	−
Pencillium sp.	CBI42	CFM	−	+++	+++	+++	−	−
		AR	−	−	−	++	−	−
P. nalgiovense	BFE66	CFM	+	+++	−	+	−	−
		AR	+++	−	++	−	−	−

[a] CFM: cell-free medium; AR: aqueous residue.
[b] Statistically significant differences compared to control: +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$.

characterised as *P. chrysogenum*, cyclopyazonic acid by *P. camemberti*, ochratoxin A by *P. verrucosum*, and verrucosidin by the two isolates characterised as *P. polonicum*.

3.2. Selection of active proteins

The 10 strains showing stronger inhibition were selected (Table 3). Every fraction obtained from CFM on a cationic column was tested against the reference molds by the microspectroscopic assay. Control fractions from uninoculated media delayed growth of *P. echinulatum* Pe321 and *P. commune* Pc332 when NaCl concentrations were over 0.15 and 1 M respectively, whereas An261 was unaffected. A total of 164 active fractions were obtained from the 10 tested strains. For simplicity, results given in Table 3 are grouped, according to the molarity required for their elution, into ranges of low (0–0.1 M NaCl), medium (0.1–0.25 M NaCl), and high (0.25–1 M NaCl) ionic strength. Every tested strain showed fractions able to inhibit ($p<0.01$) at least one reference mold.

Most active fractions confirmed the activity shown by whole CFM, but some discrepancies were found for various isolates (indicated with shaded background in Table 3). In some instances, no fraction obtained from MEB or PDB confirmed the activity of whole CFM. On the other hand, some fractions showed activity against *A. niger* An261 not observed with whole CFM. For most isolates, the fractions inhibiting ($p<0.05$) the three reference molds eluted with medium or high NaCl concentrations.

For the next step of protein purification, the fractions chosen were those active against two reference molds and from both culture media, reaching strong effects ($p<0.001$) at least for one of them. Every fraction selected was chromatographed on a gel filtration column (Fig. 1). Fraction A11 from *P. chrysogenum* AS51D produced two neat peaks eluting at 47% (peak A11.1) and 54% column volume (peak A11.2) that inhibited growth of *P. commune* Pc332 (Table 4). Fraction B5 from *P. chrysogenum* RP42C produced just one peak (B5.1) eluting at 71% column volume that confirmed the strong inhibition ($p<0.001$) against Pe321, Pc332, and An261. Fraction A11 from *P. viridicatum* DBI23 contained two neat peaks (A11.3 and A11.4), but no significant inhibition ($p>0.05$) was recovered. Fractions A6 and B4 contained just one small peak each (A6.1 and B4.1) that showed only a limited inhibition ($p<0.05$) for the first 48 h, but not at 72 h incubation time.

The molecular mass of the proteins in subfractions showing an outstanding inhibitory activity ($p<0.01$) was estimated by SDS-PAGE (Fig. 2). Only one band was observed in subfraction A11.1 from *P. chrysogenum* AS51D of 37.7 kDa, and another in subfraction B5.1 from *P. chrysogenum* RP42C of 9.1 kDa.

4. Discussion

From the 281 isolates obtained from dry-cured ham, 166 showed inhibitory activity by the radial inhibition assay against some of the most common mycotoxin-producers on dry-cured meat products (Núñez et al., 1996, 2007; Sosa et al., 2002). Even though the ability of molds to produce antimicrobial compounds is well known, the high ratio of active isolates could be related to the ecological advantage on a substrate where a heavy fungal growth occurs (Córdoba et al., 2002). To select a wide range of active isolates for a closer study, a representative mold from every step and processing plant showing differential macroscopic or microscopic characteristics was chosen. Most of the 88 resulting isolates were able to inhibit *P. echinulatum* Pe321 and *P. commune* Pc332, but not *A. niger* An261 (Table 1). A much lower ratio of active isolates was obtained from ChE by the microspectroscopic assay. In addition, some isolates producing active ChE against a particular reference mold were not active by the RI assay, and vice versa. Given that chloroform extraction involves concentration from 50 ml culture medium to 500 µl extract, the chloroform-soluble compounds cannot be regarded as the main compounds responsible for the inhibition observed by the RI assay. On the other hand, various chloroform-soluble metabolites showing antifungal activity are known to be produced by molds, including non proteinaceous antimicrobials, like griseofulvin, or haemolytic lipopeptides, like echinocandins (Iwamoto et al., 1994a,b). Thus, molds producing active ChE were eliminated from further consideration.

Strong differences were found in the sensitivity of the reference molds to ChE. Pe321 showed the lowest rate of active ChE to positive RI (*ca.* 53%), whereas *P. commune* 332 was inhibited by chloroform extracts from most (*ca.* 70%) tested isolates. This could be due to a higher sensitivity of Pc332 to chloroform-soluble compounds, thus making it less selective to detect antifungal protein producers. An261 seems more resistant to potential inhibitors than the *Penicillium* strains, which can be of value when looking for broad-spectrum activities.

Active isolates by the RI assay with non-active ChE were selected for further characterisation. The microspectroscopic tests with CFM and AR (Table 2) not only corroborated the activity observed for most selected isolates by the RI test (Table 1), but also revealed a wider spectrum of inhibition against reference molds. Given that both CFM and AR extracts involved a 10 times concentration process, the higher rate of inhibition when compared to RI assay can be explained by the higher concentration of active compounds. For most isolates, the inhibitory activity of AR was lower than that of CFM. Just AR from three of the selected isolates characterised as *P. chrysogenum* and the only *P. viridicatum* completed the inhibition pattern obtained with CFM. Given that ChE from all selected isolates showed no activity, chloroform extraction may interfere by irreversible denaturing of

Table 3
Maximum activity of fractions from selected isolates grown on malt extract broth (MEB) or potato dextrose broth (PDB) against *P. echinulatum* Pe321, *P. commune* Pc332, and *A. niger* An261.

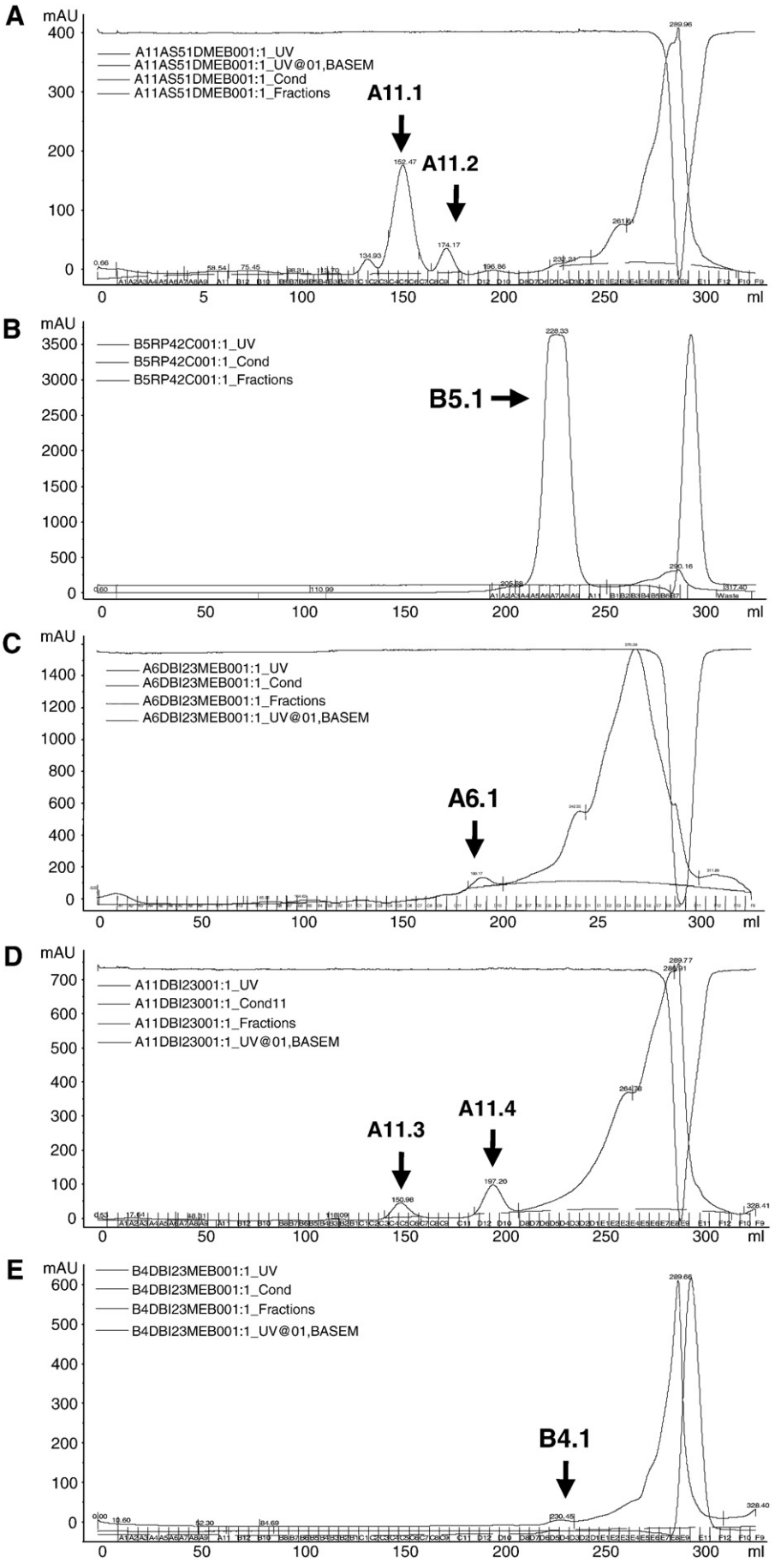
			Reference molds						
			Pe321		Pc332		An261		Selected fractions
	Isolate	Range of ionic strengh for elution	MEB	PDB	MEB	PDB	MEB	PDB	
P. chrysogenum	AS51D	Low				+++[a]			
		Medium			+++	+++	++	+	A11
		High				+++	+++	+++	
P. chrysogenum	RB41C	Low							
		Medium						+++	
		High				+			
P. chrysogenum	AMB11	Low				+		+	
		Medium			+++			++	
		High				++	++		
P. chrysogenum	RP42C	Low			+		+	+	
		Medium				+	+	+	
		High	+++	++	+	+++	+++	++	B5
P. camemberti	AMP61	Low							
		Medium				+++		+++	
		High		+		+++		+++	
P. verrucosum	AB11C	Low					++	++	
		Medium					+	+	
		High	++			+		++	
P. viridicatum	DBI23	Low			+	+	+++		A6
		Medium	+		+++	+		+	A11
		High		+		+++	++	++	B4
P. solitum	CSII33	Low		+			+	+	
		Medium			+		+++	+++	
		High	+				+	+	
Penicillium sp.	DBII41	Low							
		Medium							
		High	+			+	+++		
Penicillium sp.	CSI54	Low		+			++	+	
		Medium			+		+++	+++	
		High	++			+			

Shaded background indicates discrepancies with results from cell-free medium.
[a]Positive results indicate that at least one fraction retarded growth of the reference mold, reaching statistically significant differences compared to control: +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$.

water-soluble active compounds (Khmelnitsky and Rich, 1999). Nevertheless, some results cannot be explained just by denaturation due to chloroform extraction. For example, isolates AS51D and CSII52 kept activity against Pc332 when cultured in MEB, but not when cultured in PDB, and vice versa for isolates CSII33, CSII16, CBI25, CSI54, CBII57, and CBI42. This could be due to the production of different inhibitory compounds. Media composition can exert a decisive role on the antifungal peptide production, as it has been reported for *P. chrysogenum* and *A. giganteus* (Marx et al., 1995; Meyer et al., 2002).

Some isolates showed the inhibition pattern wanted, with CFM inhibiting growth of the three reference molds. Even though losing activity after chloroform extraction would not rule out production of antifungal proteins, only isolates showing strongly active AR ($p<0.01$) against at least two reference molds were selected.

As a further characterisation for an eventual use as a protective culture, the selected molds were tested for hazardous secondary metabolites. The only mycotoxin of concern detected was ochratoxin A from *P. verrucosum*. The other toxic metabolites found (*i.e.* secalonic acid, cyclopiazonic acid, and verrucosidin) have not been linked to foodborne mycotoxicosis, not even under a low-level, long-term exposure (Richard, 2007). Additionally, the ability to produce hazardous secondary metabolites in culture media can be strongly limited in particular foods (Núñez et al., 2007). Even though the toxin-producing molds must be excluded as potential protective cultures,


A
mAU
A11AS51DMEB001:1_UV
A11AS51DMEB001:1_UV@01,BASEM
A11AS51DMEB001:1_Cond
A11AS51DMEB001:1_Fractions
A11.1
A11.2
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B
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B5RP42C001:1_UV
B5RP42C001:1_Cond
B5RP42C001:1_Fractions
B5.1
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mAU
A6DBI23MEB001:1_UV
A6DBI23MEB001:1_Cond
A6DBI23MEB001:1_Fractions
A6DBI23MEB001:1_UV@01,BASEM
A6.1
ml
D
mAU
A11DBI23001:1_UV
A11DBI23001:1_Cond11
A11DBI23001:1_Fractions
A11DBI23001:1_UV@01,BASEM
A11.3
A11.4
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E
mAU
B4DBI23MEB001:1_UV
B4DBI23MEB001:1_Cond
B4DBI23MEB001:1_Fractions
B4DBI23MEB001:1_UV@01,BASEM
B4.1
ml

Table 4
Antifungal activity of subfractions from selected peaks against *P. commune* Pc332, *P. echinulatum* Pe321, or *A. niger* An261 and molecular mass of purified proteins.

	Isolate	Selected fractions	Selected peaks	Sensitive mold	Inhibitory activity[a]	Molecular mass
P. chrysogenum	AS51D	A11	A11.1	Pc332	++[b]	37,667
			A11.2	Pc332	+	n.d.[c]
P. chrysogenum	RP42C	B5	B5.1	Pe321	+++	9067
				Pc332	+++	
				An261	+++	
P. viridicatum	DBI23	A6	A6.1	Pc332	+/−	n.d.
		A11	A11.3	Pc332	−	n.d.
			A11.4	Pc332	−	n.d.
		B4	B4.1	Pc332	+/−	n.d.

[a] Maximum level of activity reached at 48 and 72 h of incubation.
[b] Statistically significant differences compared to control: +, $p<0.05$; ++, $p<0.01$; +++, $p<0.001$; −, $p>0.05$; +/−, $p<0.05$ at 48 h, $p>0.05$ at 72 h.
[c] n.d., not detected.

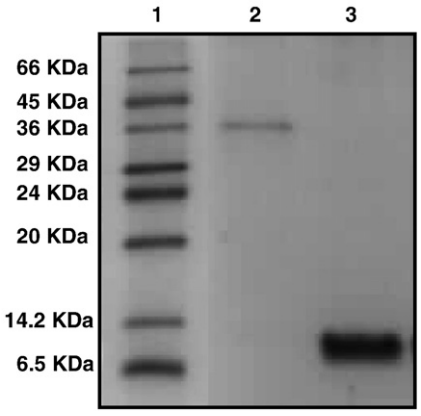

Fig. 2. SDS-PAGE of the purified antifungal proteins. Lane 1: molecular size marker (66 kDa, Bovine serum albumin; 45 kDa, Ovalbumin; 36 kDa, Glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, Carbonic anhydrase; 24 kDa, Trypsinogen; 20 kDa, Trypsin inhibitor; 14.2 kDa, α-lactalbumin; 6.5 kDa, Aprotinin). Lane 2: 20 µl of subfraction A11.1 from *P. chrysogenum* AS51D. Lane 3: 2 µl of subfraction B5.1 from *P. chrysogenum* RP42C.

the use of purified proteins would eliminate any potential hazards due to mycotoxins. Thus, even the ochratoxin A producer was taken for further evaluation.

To purify the inhibiting compounds, fractioned CFM from selected isolates was tested against the reference molds. All 10 strains produced active fractions eluting at high ionic strength, 9 with intermediate NaCl levels, and 6 also with low ionic strength (Table 3). As a consequence, most of these isolates seem to produce different active compounds. Various inhibitory proteins are synthesized as precursors with a signal sequence that is cleaved to release the mature protein (Wnendt et al., 1994). This could make that different active peptides appear as intermediate compounds along protein processing by the producer mold.

For various isolates, separate fractions did not precisely complete the inhibition pattern showed by CFM from either MEB or PDB, as it is indicated with shaded background cells on Table 3. In some instances, no fraction obtained from MEB and PDB confirmed the activity of whole CFM. This can be explained by the poor growth of Pe321 and Pc332 with fractions eluting at medium or high NaCl content. The delayed growth of reference strains could mask the inhibitory effect of the fractions, preventing the detection of differences between means at levels of statistical significance. Similarly, the efficient inhibition might require the joint action of active compounds eluting at different ionic strengths. This could be the case for *P. chrysogenum* AS51D or AMB11, where no fraction was active against Pe321, but different ones inhibited Pc332 and An261. Moreover, the production of active anionic compounds, not adsorbed to the cation exchange column, cannot be excluded. On the other hand, individual fractions from some strains were active against *A. niger* An261, whereas CFM was not (indicated as shaded background with positive results on Table 3). For these, the concentration effect by ion-exchange chromatography could be required to reach an effective inhibitory level.

The subfractions containing the highest absorbance peaks after gel filtration were tested against the more sensitive molds. Given that NaCl elutes separately, the results obtained (Table 4) revealed that most active compounds do not require high salt concentrations to inhibit the sensitive molds. Only the activity of *P. viridicatum* DBI23 was not recovered from the separate subfractions after gel filtration. Since two neatly separated peaks eluted from fraction A11, the combined action of two different compounds could explain the inhibition previously observed.

SDS-PAGE confirmed the isolation of two proteins from the most active subfractions: one of 37.7 kDa from *P. chrysogenum* AS51D and another of 9.1 kDa from *P. chrysogenum* RP42C. Given that no further bands were detected by SDS-PAGE, the low concentration of potentially active compounds seems to be responsible for the poor activity registered from other subfractions.

Data obtained from the two purified proteins revealed clear differences in molecular mass (9.1 vs. 37.7 kDa), activity against *P. echinulatum* Pe321 (Table 3), and ionic strength required to elute in the cationic exchange column (0.2 M vs. 1 M). This suggests that two different antifungal proteins have been isolated. The one in fraction B5.1 from *P. chrysogenum* RP42C shows common features with the new group of basic antifungal proteins from 5.8 to 6.6 kDa produced by ascomycetes (Marx, 2004), except for a slightly higher molecular weight. Even though mass determination by SDS-PAGE is far from precise, the estimated mass of 9.1 kDa points at considerable differences in electrophoretic mobility with the other basic antifungal proteins (Marx, 2004). The protein isolated in fraction A11.1 from *P. chrysogenum* AS51D does not show similar characteristics to previously described antifungal proteins from molds. 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 these proteins in foods should be considered. These apparently new proteins considerably widen the spectrum of antifungal proteins from molds with potential food use. In addition, the producer molds show interesting characteristics for a direct inoculation as protective cultures, thus making it possible to develop a new strategy to control unwanted fungi on mold-ripened foods. However, the use of the selected molds requires a previous evaluation of the impact in both ecology of starter cultures and sensorial characteristics of dry-cured meats.

In conclusion, only a low ratio of the *Penicillium* isolates active against unwanted molds showed the desired inhibition pattern of non-active ChE from active CFM. Those showing the deepest impact on the widest spectrum of sensitive molds were characterised as *P. chrysogenum* and *P. viridicatum*. Two strains of *P. chrysogenum* producing different antifungal proteins with potential commercial application have been isolated. Even though further characterisation is required, the two proteins and the producing strains can be of interest to control unwanted molds on foods.

Fig. 1. FPLC-gel filtration chromatography from selected cation exchange fractions: A) A11 from *P. chrysogenum* AS51D; B) B5 from *P. chrysogenum* RP42C; C) A6 from *P. viridicatum* DBI23; D) A11 from *P. viridicatum* DBI23; and E) B4 from *P. viridicatum* DBI23.

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