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Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*

A.-L. Moyne[1], R. Shelby[2], T.E. Cleveland[3] and S. Tuzun[1]

[1]Department of Entomology and Plant Pathology, Auburn University, [2]USDA/ARS Fish Diseases and Parasites Research Laboratory, Auburn, AL, and [3]USDA/ARS, Southern Regional Research Center, New Orleans, LA,USA

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Aims: In a search for an antifungal peptide with a high activity against *Aspergillus flavus*, *Bacillus subtilis* AU195 was selected from a collection of isolates with antagonistic activity against *A. flavus*.

Methods and Results: To identify the antifungal peptides, a protein purification scheme was developed based on the detection of the antifungal activity in purified fractions against *A. flavus*. Two lipopeptides were purified with anion exchange and gel filtration chromatography. Their masses were determined to be 1045 and 1059 m/z with mass spectrometry, and their peptide moiety was identical to bacillomycin D.

Conclusions: AU195 synthesized a mixture of two antifungal bacillomycin D analogues with masses of 1045 and 1059, the 14 mass unit difference representing the difference between a C15 and a C16 lipid chain.

Significance and Impact of the Study: Both bacillomycin D analogues were active at the same concentration against *A. flavus*, but the different lipid chain length apparently affected the activity of the lipopeptide against other fungi.

INTRODUCTION

Aflatoxins are extremely toxic chemicals which can cause liver cancer in animals and humans when contaminated feed or food is consumed. Aflatoxin production occurs when *Aspergillus flavus* or *A. parasiticus* invades peanuts, cotton seed, corn and certain nuts under favourable conditions of temperature and humidity. *Aspergillus* spp. are also involved in respiratory infection in immunodeficient patients (Lotholary *et al*. 1993). Currently available control methods, such as use of optimal cultural practices, have reduced but have not eliminated pre-harvest aflatoxin contamination in susceptible crops. Identification of new antifungal peptides with activity against *A. flavus* could lead to the development of biotechnological strategies for controlling aflatoxin contamination and, for example, increase plant resistance to fungal invasion through genetic engineering. During the screening of bacteria for antagonistic activity against *A. flavus* *in vitro*, a *Bacillus subtilis* isolate, AU195, was identified as having the highest antifungal activity.

The potential of *Bacillus* spp. to synthesize a wide variety of metabolites with antibacterial and/or antifungal activity has been intensively exploited in medicine and industry, and is one determinant of their ability to control plant diseases when applied as a biological control agent (McKeen *et al*. 1986; Silo-suh *et al*. 1994; Leifert *et al*. 1995). Most of the antifungal peptides secreted by *B. subtilis* have a molecular weight of less than 2000 Da and are synthesized non-ribosomally via a multi-enzyme-catalysed synthesis (Zuber *et al*. 1993). The antibiotics synthesized non-ribosomally include the lipopeptides iturin (Maget-Dana and Peypoux 1994), surfactin (Kluge *et al*. 1988), fengycin (Vanittanakom *et al*. 1986), plipastatin (Tsuge *et al*. 1996), the di- and tripeptides such as bacilysin (Walker and Abraham 1970), and the phosphono-oligopeptide, rhizocticin (Kugler *et al*. 1990). Iturin is a group of cyclic lipopeptides with a peptide moiety and a β-amino fatty acid linked by amide bonds to the constituent amino acid residues. They share a common sequence (β-hydroxy fatty acid-Asx-Tyr-Asx) and show variation at the other four positions (Maget-Dana and

Correspondence to: Dr A.-L. Moyne, Department of Entomology and Plant Pathology, 209 Life Sciences Bldg, Auburn University, Auburn, Alabama 36849, USA (e-mail: amoyne@acesag.auburn.edu).

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Peypoux 1994). Iturin A, C, D and E (Besson *et al*. 1978a; Besson and Michel 1987), bacillomycin D (Peypoux *et al*. 1984), F (Mhammedi *et al*. 1982) and L (Peypoux *et al*. 1984), bacillopeptin (Kajimura *et al*. 1995) and mycosubtilin (Peypoux *et al*. 1986) belong to the iturin group. Surfactin is also a cyclic lipopeptide, containing seven residues of D- and L-amino acids and one residue of a β- hydroxy fatty acid (Kluge *et al*. 1988), with an amino acid sequence completely different from the iturin group. Fengycin (Vanittanakom *et al*. 1986) and plipastatin (Tsuge *et al*. 1996) are lipopeptides with 10 amino acids and a lipid attached to the N-terminal end of the molecule. They differ from iturin and surfactin by the presence of unusual amino acids such as ornithine and allo-threonine.

The purpose of this study was to isolate, purify and characterize the antifungal substance(s) produced by *B. subtilis* AU195 from the culture filtrate, and to assess the antifungal spectrum of the purified antifungal compound(s).

MATERIALS AND METHODS

Micro-organisms

Bacillus subtilis AU195 was selected from a bacterial collection of Auburn University for its ability to inhibit *A. flavus in vitro*. The strain AU 195 was identified as close to *B. subtilis* upon determination of fatty acid methyl ester profiles by gas chromatography (Sasser 1990). An *A. flavus* strain was isolated and identified from an Alabama peanut kernel. *Alternaria solani*, *Fusarium oxysporum*, *Botryosphaeria ribis*, *Helminthosporium maydis*, *Phomopsis gossypii*, *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* were provided by the Plant Diagnostic Lab (Auburn University).

Culture conditions

A single bacterial colony was inoculated into 1 ml Luria-Bertani (LB) medium and cultured overnight at 27 °C with constant shaking at 140 rev min^{-1} (Orbit shaker, Lab-line, Melrose Park, IL, USA). For the production of the antifungal compounds, 2 l flasks each containing 1 l LB medium were inoculated with this overnight culture and incubated at 27 °C with constant shaking at 80 rev min^{-1}. Every day, samples (5 ml) were removed and centrifuged (10 000 *g* for 10 min). The supernatant fluids were filter-sterilized (0·22 μm Acrodisc HT Tuffryn membrane, Gelman Sciences, Ann Arbor, MI, USA) and tested for antifungal activity.

Antifungal assay

Microtitre plate assay. Culture supernatant fluids were tested for their antifungal activity against *A. flavus* in a microtitre plate (Falcon 3918, Becton-Dickinson, Franklin Lakes, NJ, USA). Each well contained 100 μl Potato Dextrose broth (PDB, Difco) and 100 μl culture filtrate, and each was inoculated with 500 spores of *A. flavus*. In the control well, culture filtrate was replaced by LB medium. Fungal growth was monitored by reading optical density (O.D.) at 560 nm (Dynatech microplate reader, Chantilly, VA, USA).

Disc plate diffusion assay. Mycelial plugs from the edges of actively-growing fungal cultures were placed in the centre of the Petri plate, containing LB medium supplemented with 15 g l^{-1} agar for *A. flavus* and PDA for *A. solani*, *F. oxysporum*, *B. ribis*, *H. maydis*, *P. gossypii*, *C. gloeosporioides* and *S. rolfsii*. After incubation at 27 °C to allow vegetative growth, samples were applied to sterile filter paper discs laid on the agar surface at a distance of 5 mm from the edge of the colony. The antifungal activity of each fraction at the different purification steps was evaluated against *A. flavus*. The purified peptides analogues were tested against *A. solani*, *F. oxysporum*, *B. ribis*, *H. maydis*, *P. gossypii*, *C. gloeosporioides* and *S. rolfsii*. The results were observed after 24 h and the antifungal activity was evaluated by the presence of an inhibition zone.

Iturin A (I-1774) was purchased from Sigma and was composed of C14 and C15 β-amino acids.

Protein determination

Protein concentration was measured by the method of Bradford (Bradford 1976) using Bio-Rad reagents and bovine serum albumin as a standard.

Purification of the active compound

Step 1. Ammonium sulphate fractionation. After 7 days, the culture was centrifuged (Sorvall RC5C centrifuge with a GSA rotor, 10 000 *g*, 30 min at 4 °C) to remove the bacteria. The culture supernatant fluid was subjected to sequential ammonium sulphate precipitation to achieve 20, 40, 60 and 80% saturation at 0 °C with constant and gentle stirring. The precipitated proteins were pelleted by centrifugation at 10 000 *g* for 20 min. The protein pellet was dissolved in 0·02 mol l^{-1} sodium phosphate buffer, pH 7·0, and dialysed using a 3500 MW cut-off membrane (Spectra/por 3, Spectrum, Houston, TX, USA), overnight, at 4 °C, against the same buffer. The crude preparation was then stored at –80 °C for further analysis.

Step 2. Anion exchange chromatography. The crude preparation was loaded onto a 5 ml anion exchange column (HiTrap Q Pharmacia, Piscataway, NJ, USA). The column was connected to an FPLC system (ConSep LC 100,

PerSeptive Biosystems, Fragmingham, MA, USA) and a linear gradient of 0–1·6 mol l^{-1} NaCl, including 20 mmol l^{-1} phosphate buffer, pH 7, was applied. Twenty fractions of 5 ml were collected and 50 μl per fraction were tested in the disc diffusion assay. All the fractions with antifungal activity were pooled, dialysed against water and freeze-dried.

Step 3. Gel filtration chromatography. Gel filtration chromatography was performed with a Superdex peptide PE 7·5/300 column (Pharmacia) for analytical analyses and a Superdex peptide HiLoad 16/60 column (Pharmacia) for preparative separation connected to an HPLC system (Waters, Milford, MA, USA). The absorbance was monitored at 210 and 280 nm with a photodiode array (PDA, Waters). Proteins (100 μg) from step 2 were analysed on a Superdex peptide PE 7.5/300 column which was eluted with 20 mmol l^{-1} phosphate buffer, pH 7·5, isocratically at a flow rate of 0·5 ml min^{-1}. All the peaks were collected as separate fractions, freeze-dried and tested for antifungal activity in a disc plate diffusion assay. To purify a sufficient amount of the antifungal peptides, 33 mg of the freeze-dried material with antifungal activity was loaded on a preparative Superdex peptide HiLoad 16/60 column (Pharmacia) and eluted with 20 mmol l^{-1} phosphate buffer, pH 7·5, isocratically at a flow rate of 1 ml min^{-1}.

Detection of antifungal activity in SDS-PAGE gel electrophoresis

SDS PAGE was performed in a Mini-Protean II electrophoresis system (Bio-Rad) by the discontinuous method of Laemmli (Laemmli 1970). Protein samples were prepared as described by Laemmli (Laemmli 1970) and boiled for 5 min. The crude 20% ammonium sulphate fraction (50 μl) was separated on 16% SDS-PAGE gel and the electrophoresis was stopped when the blue dye reached 1 cm from the end of the gel. A suspension of *A. flavus* spores (5000 spores ml^{-1}) in melted and cooled PDA medium (50 °C) was plated in a Petri dish and incubated for 4 h at 27 °C. After electrophoresis, a 16% SDS-PAGE gel was overlaid for 1 min on the PDA plate containing *A. flavus* spores and removed for further silver staining. The PDA plate was incubated at 37 °C for 24 h until the inhibition zone was visible.

Isoelectric Focusing (IEF) electrophoresis

Acrylamide (7%) tube gels containing 2% ampholite (Bio-Rad), pH range 2–8, and 10% glycerol were cast in a Hoefer GT1 unit (Amersham Pharmacia Biotech). Electrophoresis was carried on overnight at 400 V, 4 °C, with 0·02 mol l^{-1} sodium hydroxide for the cathode solution and 10% phosphoric acid for the anode solution. A 50 μl (50 μg) aliquot of the 20% ammonium sulphate-precipitated proteins, mixed with 10% (v/v) glycerol, was loaded per tube gel. Afterwards, electrophoresis gels were cut into 1 cm pieces. The pH of each gel fraction (total of 15) was determined and the fractions were placed on LB agar media pre-inoculated with *A. flavus*.

Mass spectrometry and sequencing

Mass spectrometry and peptide sequencing were performed by the Protein Chemistry Core Facility at Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030–3498, USA.

RESULTS

Production of the antifungal substances

Bacillus subtilis strain AU 195 inhibited the growth of *A. flavus* on a PDA plate as indicated by the inhibition zone. The active substance was secreted in the culture filtrate when AU 195 was cultured in LB medium, and the production was monitored during 7 days with a microtitre plate assay. *Aspergillus flavus* growth, measured by O.D. at 560 nm, was inhibited by the filtrate collected 2, 3, 4, 5, 6 and 7 days after starting the bacterial culture (Fig. 1). The antifungal substance production increased every day to reach a maximum at 6 days. Culture filtrate collected at 6 and 7 days completely inhibited fungal spore germination and hyphal growth (Fig. 1).

Partial purification of the antifungal substance

All protein fractions and supernatant fluids precipitated sequentially with 20, 40, 60 and 80% ammonium sulphate were tested before and after dialysis with a disc plate diffusion assay. Ammonium sulphate salt did not inhibit *A. flavus* at the concentration used and did not modify the result of the assay. The antifungal activity against *A. flavus* was present mainly in the fraction precipitated with 20% ammonium sulphate. Fractions precipitated with 40 and 60% ammonium sulphate exhibited a weak inhibition. Supernatant fluid and 80% ammonium sulphate precipitate did not exhibit any antifungal activity. The antifungal substance present in the 20% fraction inhibited both hyphal growth and spore germination. Even after one month, spores had not germinated in the presence of the 20% ammonium sulphate fraction.

Characterization of the antifungal substances

To determine the pI of the antifungal protein, proteins in the 20% ammonium sulphate fraction were separated by

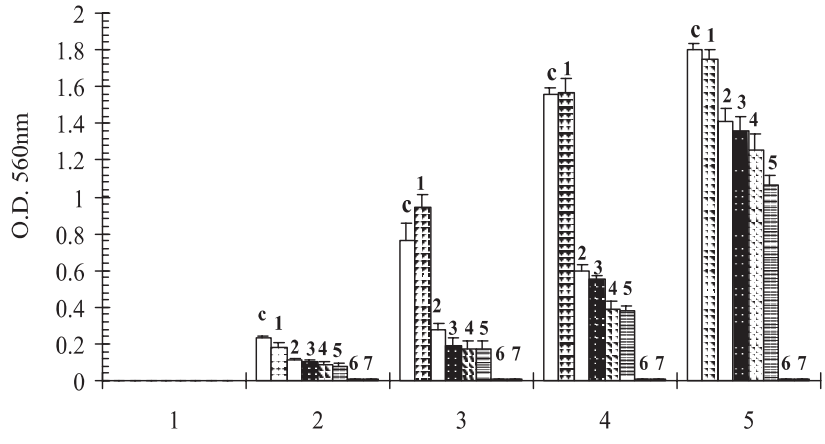

Fig. 1 Effect of culture filtrates from the bacterium AU195 on *Aspergillus flavus* growth. Culture supernatant fluids were collected daily and tested for antifungal activity against *A. flavus* in a microtitre plate. The control (□) represents the fungal growth in medium uninoculated with the bacterium (500 spores well^{-1} in 100 μl LB and 100 μl PDB). Other histograms represent the growth of the fungus in the presence of bacterial culture filtrates obtained at 1 (▪), 2 (□), 3 (■), 4 (▨), 5(▤), 6 (▣) and 7 (▩) days of incubation. Fungal growth was monitored for 5 days by recording the optical density at 560 nm. Vertical bars represent the means of 3 replicates

IEF. Upon IEF and bioassay of gel sections, the active proteins were demonstrated to have an isoelectric point of 4·5, indicating that the antifungal substances have an acidic isoelectric point.

When the 20% ammonium sulphate protein fraction was separated on a 16% acrylamide gel, the antifungal activity remained in a single band co-migrating with the bromophenol blue front (Fig. 2), indicating a low molecular weight for the antifungal protein (less than 6 kDa). The antifungal substance was able to diffuse very rapidly into the gel; 1 min overlaid was enough to obtain an inhibition zone. Boiling the samples prior to electrophoresis did not destroy the antifungal activity and it was therefore concluded that the antifungal substance was heat stable.

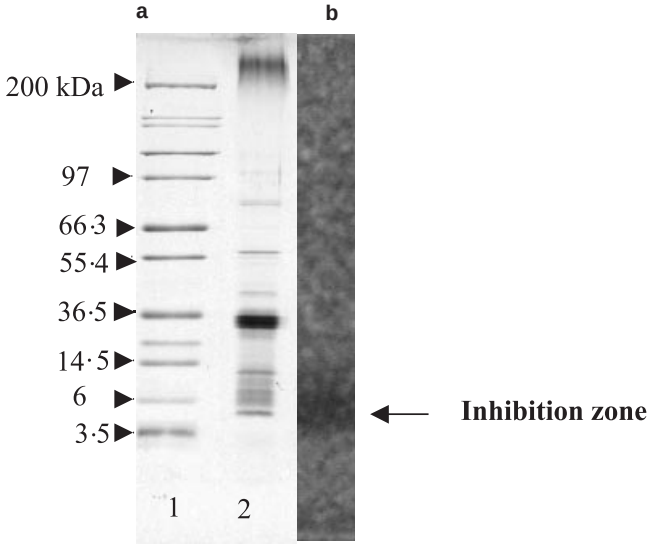

Fig. 2 'In-gel' antifungal assay. (a) 16% SDS polyacrylamide gel silver-stained. Lane 1: Mark12^{TM} Novex molecular weight marker; lane 2: 10 μl of the 20% ammonium sulphate fraction was separated on the gel. (b) Detection of antifungal activity. A suspension of *Aspergillus flavus* spores (5000 spores ml^{-1}) in PDB medium was plated in a Petri dish and incubated for 4 h at 27°C. After electrophoresis, the 16% SDS PAGE gel was overlaid on the PDA plate containing *A. flavus* spores for 1 min and removed for further silver-staining. The antifungal inhibition zone was observed 24 h after incubating the PDA plate containing *A. flavus* at 37°C

Purification of the antifungal peptides by anion exchange and gel filtration

Because of its acidic pI, the antifungal peptides were further purified with an anion exchange chromatography column. All the antifungal activity was contained in fractions eluting with 0·5–1 mol l^{-1} sodium chloride. Antifungal fractions

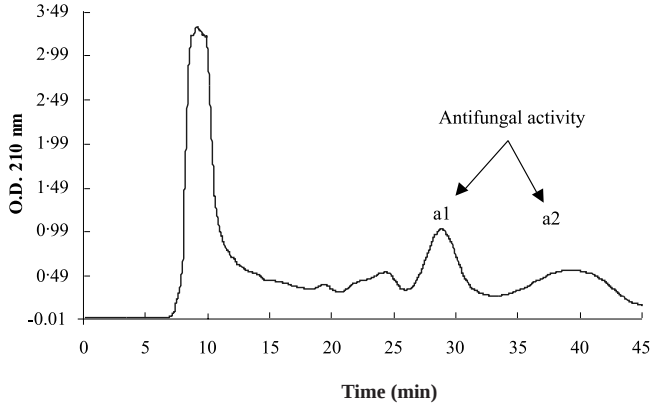

Fig. 3 Gel filtration chromatogram of 100 μg of protein from the partially-purified fraction after anion exchange chromatography separation. Fractions were analysed on a Superdex peptide PE 7·5/300 column connected to an HPLC system. The column was eluted with 20 mmol l^{-1} phosphate buffer, pH 7·5, isocratically at a flow rate of 0·5 ml min^{-1}. All peaks were collected as separate fractions and tested for antifungal activity using a disc plate diffusion assay

were pooled and 100 μg of proteins were analysed on a Superdex peptide PE 7·5/300 (Pharmacia) (Fig. 3). All the peaks were collected as separate fractions and only two peaks, eluting at 27 and 38 min, had antifungal activity (Fig. 3.). The elution times of both peptides corresponded to a molecular weight lower than the glycine (MW 75), which was used as a standard, due to interactions between the column media and the peptides. Thus, it was not possible to determine the molecular weight of the antifungal peptides by gel filtration. Complete purification of both peptides was achieved by preparative gel filtration using a Superdex peptide HiLoad 16/60 column (Pharmacia) (Figs 4, 6 a, b). Both antifungal fractions were de-salted on a C18 reverse phase HPLC column (Delta Pack C18 Waters) and the two peptides were eluted with a linear gradient of acetonitrile 5–60% containing 0·1% trifluoro-acetic acid (TFA). Single band homogeneity was demonstrated for the peptides by SDS gel electrophoresis (Fig. 5). The molecular mass was determined by mass spectroscopy (Fig. 6c, d). The molecular weight of the antifungal peptides was estimated to be 3·5 kDa with a 16% SDS gel and, respectively, 1045 for a1 and 1059 for a2 with mass spectrometry.

Amino acid sequence of a1 and a2

Initial Edman sequencing was unsuccessful. Tandem mass spectrometry of the molecular ion resulted in a highly complex spectrum which suggested a cyclic structure for both peptides. Peptide a1 was digested to give a linear molecule and the sequence after digestion at the Glu-C amino acid was NH2-STNYNPE-OH. A modification of

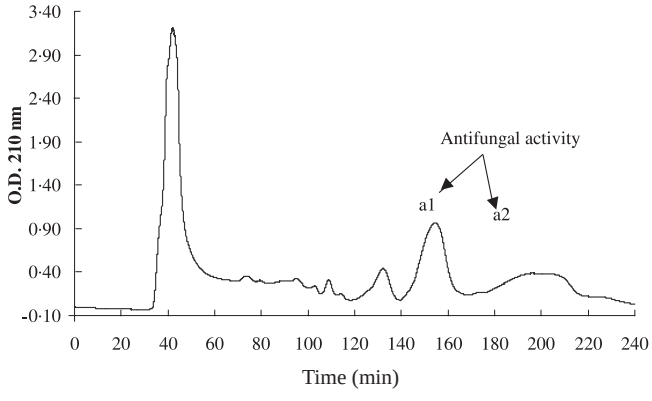

Fig. 4 Preparative chromatogram of the antifungal fraction after anion exchange chromatography separation. Fractions were separated on a Superdex peptide Hiload 16/60 column connected to an HPLC system. The column was eluted with 20 mmol l^{-1} phosphate buffer, pH 7·5, isocratically at a flow rate of 1 ml min^{-1}. All peaks were collected as separate fractions and tested for antifungal activity using a disc plate diffusion assay

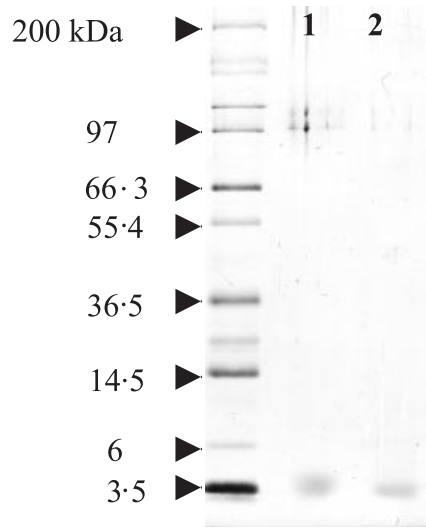

Fig. 5 Separation of the purified peptides on a 16% silver-stained SDS gel. Lane 1: 6 μg of a1; lane 2: 4 μg of a2

the molecule at the Thr residue suggested the attachment of hydroxy fatty acid. Peptide a1 has the same amino acid sequence and mass as reported for the bacillomycin D (Peypoux *et al.* 1984).

Tandem mass spectrometry of the cyclic form of a2 indicated a sequence similar to a1 with an additional CH2. Peptide a2 has an amino acid sequence identical to bacillomycin D, but its mass is higher (m/z 1059) than the one published by Peypoux *et al.* (1984) (m/z 1031 and 1045).

Antifungal activity of the purified bacillomycin D

The amount of bacillomycin D was estimated by the weight of the dry, purified and de-salted fraction, as the Bradford assay was not accurate to evaluate the concentration of small peptides. The antifungal spectra of bacillomycin D analogues a1 and a2 are shown in Table 1. Most of the fungal strains tested are plant pathogens and were found to be highly sensitive to both antibiotics. Although *A. flavus* hyphae are sensitive to the same concentration of a1 and a2, *A. solani*, *C. gloeosporioides* and *S. rolfsii* are differentially sensitive to a1 and a2. The lipid moiety of bacillomycin D plays a role in the differential antifungal properties observed when the fungi were screened. The antifungal activity of bacillomycin D was compared with iturin A against *A. flavus*; no inhibition zone was visible, even with 50 μg of iturin A.

DISCUSSION

In a search for an antifungal peptide with a high activity against *A. flavus*, *B. subtilis* AU195 was selected from a

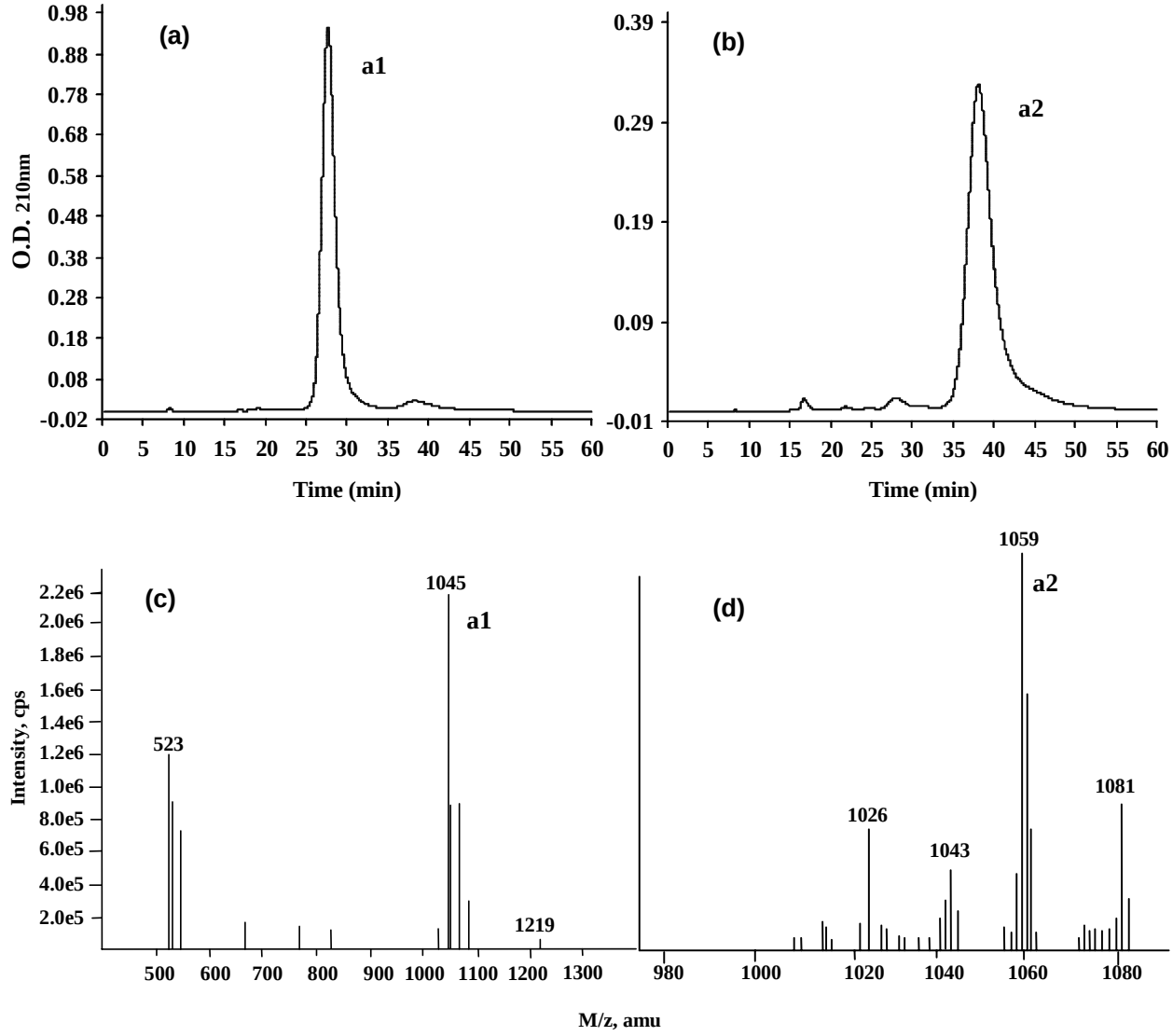

Fig. 6 Elution profile of the antifungal peptides a1 (a) and a2 (b) separated on a Superdex peptide 7·5/300 column. Mass spectrum of a1 (c) and a2 (d)

collection of isolates with antagonistic activity against *A. flavus*. To purify the antifungal peptides, a protein purification scheme was developed based on the detection of the antifungal activity in purified fractions against *A. flavus*. Two lipopeptides, purified from *B. subtilis* AU195, were shown to be antifungal and have an amino acid sequence identical to bacillomycin D described by Peypoux *et al.* (1984). Peypoux *et al.* (1984) reported that the FAB mass spectrum of bacillomycin D displayed two major peaks with a mass unit of 1031 and 1045. C14 and C15 β-amino acids were the major components of bacillomycin D, with a low percentage of C16 lipid components. AU195 synthesized a mixture of two antifungal bacillomycin D analogues with masses of 1045 and 1059, the 14 mass unit difference representing the difference between a C15 and a C16 lipid chain. Bacillomycin D exhibits a strong antifungal activity against *A. flavus* and a broad range of plant pathogenic fungi. Iturin A, which belongs to the same iturin group as bacillomycin D, has been reported to inhibit aflatoxin production by *A. flavus* and *A. parasiticus* (Ono and Kimura 1991). Although iturin A has already been patented for control of aflatoxin, it was later described not to be able to inhibit *A. flavus* growth and aflatoxin production (Klich *et al.* 1994). In the antifungal assay reported here, iturin A was not effective in controlling *A. flavus* growth. Antibiotics of the iturin group were found to act upon the sterol present

Table 1 Antifungal spectrum of the purified bacillomycin D analogues

Fungi	Bacillomycin D analogues	
	a1 (μg)*	a2 (μg)*
Alternaria solani	6	10
Aspergillus flavus	3	3
Botryosphaeria ribis	3	3
Colletotrichum gloeosporioides	6	3
Fusarium oxysporum	6	6
Helminthosporium maydis	3	3
Phomopsis gossypii	1	3
Sclerotium rolfsii	10	1

*Minimum amount of bacillomycin D analogues a1 or a2 necessary to inhibit the listed fungi.
Hyphal growth inhibition was evaluated by the presence of an inhibition zone in the disc plate diffusion assay 24 h after inoculating the fungi with the bacillomycin D analogues.

in the cytoplasmic membrane of the organism (Quentin *et al*. 1982). *Aspergillus flavus* conidia membranes contain ergosterol and cholesterol (DeLucca *et al*. 1997) which could be the target of bacillomycin D. Both the peptide cycle and the lipid chain are important for the activity of the lipopeptides. The present results indicated that the length of the β-amino acid may play a role in specificity, leading to differential inhibitory effects against various fungal species.

Further research is being conducted to investigate the mechanism of action of bacillomycin D and its analogues against *A. flavus*. Biosynthesis of bacillomycin D is independent of the ribosomal process (Besson and Michel 1992) and the enzymes responsible for bacillomycin D production are complex peptide synthetases. Peptide synthetases are organized as modules which determine the order of the amino acid sequence in the lipopeptides. By recombining the different modules, it would be possible to modify bacillomycin D and create new antibiotics, as has already been shown for the surfactin (Stachelhaus *et al*. 1995) and the polyketide synthases (Gokhale *et al*. 1999). Thus, cloning the genes encoding the peptide synthetases will allow a better understanding of the relation between the activity and the structure of bacillomycin D, and perhaps create more potent antifungal peptides analogues.

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