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Isolation and physico-chemical characterization of an antifungal and antibacterial peptide produced by *Bacillus licheniformis* A12

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Abstract. An antifungal substance named peptide A12-C has been purified to homogeneity from supernatants of sporulated cultures of *Bacillus licheniformis* A12. It consists of a 0.77-kDa hydrophilic peptide containing two residues of Glu and one of Arg, Ala, Pro, Tyr and Orn. No fatty acids, phosphorus or carbohydrates have been detected. Peptide A12-C is active on several fungi (*Microsporum canis* CECT 2797, *Mucor mucedo* CECT 2653, *M. plumbeus* (CCM F 443, *Sporothrix schenckii* CECT 2799 and *Trichophyton mentagrophytes* CECT 2793) and bacteria (*Bacillus megaterium, Corynebacterium glutamicum, Sarcina* and *Mycobacterium*), although the latter are less sensitive.

Introduction

Production of antibiotics by species of the genus *Bacillus* has long been recognized (Bodansky and Perlman 1964; Bérdy 1974; Katz and Demain 1977) and there is still an interest from various points of view in the search for new antibiotics. Many of them show interesting antifungal properties, of which the major family are lipopeptides comprising iturins (Delcambe and Devignat 1957), surfactins (Kluge et al. 1988), bacillomycins (Peypoux et al. 1981) and mycosubtilins (Besson and Michel 1990). Most of these antibiotics are cyclic peptides containing D-and L-amino acids closed by a β-amino acid carrying a long aliphatic chain, and they all appear to be active against fungi and yeasts. A second family comprises hydrophilic and antifungal metabolites, of which only a few have been reported in the literature, including the dipeptides bacilysin (Walker and Abraham 1970) and chlorotetain (Rapp et al. 1988a). Bacteria, yeasts and other budding fungi are sensitive to bacilysin, whereas hyphal fungi require considerably higher concentrations for inhibition. The related compound chlorotetain, which is produced by a plasmid-carrying strain of *B. subtilis* (Rapp et al. 1988a) exhibits different antifungal activity. Another antifungal substance belonging to this category, the hydrophilic phosphono-oligopeptide rhizocticin has also been characterized (Rapp et al. 1988b) and shown to be active against a variety of budding and filamentous fungi as well as the cultivated nematode *Caenorhabditis elegans,* but not against bacteria or protozoa (Kluger et al. 1990).

Several strains of *B. licheniformis* are known for the production of bacitracin, but only a few have been reported to produce other types of antibiotics, such as the phosphorus-containing triene proticin (Nesemann et al. 1972), bacilysin (Kluger et al. 1990) and the licheniformins (Callow and Work 1952).

In a study carried out in our laboratory on the production of amoebicidal substances by bacterial strains isolated from natural habitats, three isolates of *B. licheniformis* were selected. One of them, strain A-12, produces two types of inhibitory compounds: the first shows marked inhibitory activity against the human pathogenic amoeba *Naegleria fowleri* (currently under study) and the second one exhibits antifungal and antibacterial activity. The isolation, purification and characterization of the antifungal compound produced by *B. licheniformis* A-12 is presented in this manuscript.

Materials and methods

Microorganisms and culture conditions. The producer strain *B. licheniformis* A12 was isolated from a natural cave containing thermal vents (Cueva de los Murciélagos, Murcia, Spain) and identified in our laboratory according to Norris et al. (1981). *Mucor plumbeus* CCM F 443 was used as the indicator sensitive strain. Other microbial strains used to establish an inhibitory spectrum are listed later in Table 3. Fungal strains were grown at 28°C with shaking on CM broth (1% glucose, 0.5% yeast extract, 0.5% malt extract, pH 5.0) or CM agar. Bacterial strains were cultivated in brain-heart infusion broth (BHI, BBL, Cockeysville, Mass., USA) or Luria broth (LB, BBL).

For the production of inhibitory activity, flasks containing brain-heart infusion broth in 100 mM sodium phosphate buffer, pH 7.2, (BHI-B) were inoculated with an overnight culture of the

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producer strain (4% v/v) and incubated at 28°C with shaking at 130 rpm. At regular intervals, aliquots (5 ml) were removed and centrifuged (5000 rpm for 20 min), and the supernatants were filter-sterilized (0.45-μm filters, Millipore) and tested for antimicrobial activity.

Assay of antifungal and antibacterial activity. Liquid samples (100 μl) were assayed by the agar-well diffusion method (Tagg and McGiven 1971). Plates containing CM agar or buffered brain-heart infusion agar (BHA-B) were overlayed with 6 ml of semi-solid CM agar or BHA-B, respectively, previously inoculated with approximately 10^6 colony-forming units of exponential-phase cultures of the fungal or bacterial strain being tested, respectively. Following adequate incubation, the plates were examined for halos of inhibition around the wells. Titration of activity was carried out by serial dilution. One arbitrary unit (AU) was defined as the highest dilution to exhibit a halo of inhibition (10 mm diameter) on a lawn seeded with bacteria or fungi. The titre, in AU/ml, was calculated from the inverse of the dilution factor.

Purification of active component. Crude supernatants of 72-h cultures of the producer strain were applied to a 2 × 10 cm glass column filled with Q-Sepharose (Pharmacia) equilibrated in 20 mM sodium phosphate buffer, pH 7.0. The column was washed with 2 vol. of the above buffer and 2 vol. of buffer plus 250 mM sodium chloride. Antifungal activity was eluted with 1 M sodium chloride in sodium phosphate buffer. Aliquots (10 ml) were applied to a Supelcosil LC-8 column (1 × 30 cm, Supelco) equilibrated with water, and the retained material was eluted with a 0–100% linear gradient of methanol, at a flow rate of 2.5 ml/min. Fractions were lyophilized, redissolved in 1 ml water and tested for inhibitory activity. The fraction of this column containing antifungal activity was loaded onto a 4.6 × 25 mm Vydac 218TP54 column (The Separation Group, Calif., USA) equilibrated with 10 mM trifluoroacetic acid (TFA), and the column was eluted with two combined linear gradients (0–40% over 3 min, then to 80% over 12 min) of isopropyl alcohol:acetonitrile (2:1) in 4 mM TFA, at a flow rate of 1 ml/min. The eluted fractions were lyophilized and tested for antifungal and antibacterial activity as above. Protein of the different fractions was determined according to Bradford (1976) using crystallized bovine serum albumin (Sigma) as the standard.

Molecular mass estimation. The relative molecular mass (M_r) of the purified peptide was determined by gel filtration on a Sephadex G-15 column (1 × 60 cm). Blue dextran (M_r 2 × 10^6), bacitracin (M_r 1400), polymyxin B (M_r 1280), actinomycin (M_r 1200) and bromcresol green D (M_r 750) were selected as markers. The column was eluted with water at a flow rate of 15 ml/h and the absorbance at 280 nm of the effluent was monitored.

Physico-chemical analyses. Aliquots of the purified substance were heated at pH 7.0 for 30 min at 50°C, 75°C and 100°C and then tested for activity. Alternatively, samples were adjusted to pH 2.5 or 9.5 and incubated for 30 min at room temperature, and then tested for remaining activity after readjusting the pH to neutrality and compensating the volumes.

Sensitivity to the following enzymes (from Sigma) was tested as follows: trypsin, pronase, proteinase K, lipase, β-glucosidase, β-glucuronidase and lysozyme (1 mg/ml each) were dissolved in 50 mM TRIS-HCl, pH 7.2. Carboxypeptidase A (100 μg/ml) was assayed in 200 mM ammonium bicarbonate, pH 6.0. Alkaline phosphatase (1 mg/ml) was assayed in 50 mM TRIS-HCl, pH 8.5, plus 20 mM magnesium sulphate. Incubation with enzymes was carried out at 37°C for 2 h, except for pronase (28°C for 2 h) and lysozyme (28°C for 18 h). After incubation, the activity of the samples was tested and compared with that of a control.

Amino sugars were determined by a modified Morgan-Elson reaction (Ghuysen et al. 1966) after hydrolysis in 3 M HCl for 4 h at 95°C. The contact of phosphorus (Ames 1966) and neutral sugars (Dubois et al. 1956) was determined. Lipid content was determined with the Total Lipids test from Boehringer (Mannheim, Germany). The optical absorbance spectrum of the purified compound was digitized in a UV/VIS Beckman DU-70 spectrophotometer. Aliquots (approx. 10^{-3} optical absorbance units at 280 nm) were analysed for their amino acid composition according to Giménez-Gallego and Thomas (1987) and the protein content correlated with the recorded absorbance. N-Terminal amino acid sequence was carried out by modified manual Edman degradation (Chang 1981).

For fatty acid analysis, the purified peptide was hydrolysed in 6 M HCl for 8 h at 150°C or 12 h at 120°C in vacuum-sealed ampoules under N_2. The hydrolysate was extracted with chloroform, lyophilized and methylated by diazomethane and the products were analysed by gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard HP5890 gas chromatograph equipped with a 0.2 mm × 25 m methyl-silicone capillary column on-line with a Hewlett-Packard HP5988A mass spectrograph. The GC was operated under an injector temperature of 270°C, with He as the carrier gas. The MS was operated under electronic ionization mode at 70 eV, at a source temperature of 200°C.

Results

Production and purification of the antifungal substance

The strain *B. licheniformis* A-12 was selected for its capacity to produce antibiotic activity against a variety of microorganisms such as bacteria, fungi and human pathogenic amoebae. Antifungal activity was best produced in buffered brain-heart infusion at 28°C under agitation. The highest titre of activity (14 AU/ml against *M. plumbeus* CCM F 443) was reached after a prolonged period of incubation (72 h) under these conditions.

The antifungal activity from 100 ml cell-free supernatant was retained by Q-Sepharose at 0.25 M NaCl, and eluted at a salt concentration of 1 M. This bulk purification step allowed recovery of 85.7% of the initial activity, and a 5.2-fold increase in purification (Table 1). The active material from this step was fractionated on a semi-preparative RP-HPLC column into several peaks, of which components 1 and 2 showed amoebicidal activity (unpublished data) and component 3 showed a strong inhibition against *M. plumbeus* CCM F 443, with a specific activity of 1.5 AU/μg of protein. This fraction, which represents 42.8% of the initial activity, was repurified on a Vydac C-18 column (Fig. 1). Antifungal activity eluted in a single fraction, with a specific activity of 1.71 AU/μg of protein. This fraction will be referred to as peptide A12-C. The purified peptide eluted as a single peak on a Sephadex G-15 gel filtration column. Its elution volume corresponded to an M_r of 800.

Physical characterization and chemical composition

Peptide A12-C was resistant to heat (100°C for 30 min at pH 7.0) and to incubation at room temperature under acidic conditions (pH 2.5), but lost 75% of the activity after incubation at pH 9.5 for 30 min at room

Table 1. Purification of peptide antibiotic A12-C from 100 ml of a 72-h-old culture supernatant of *Bacillus licheniformis* A12

Fraction	Total protein (μg)	Total activity (AU)	Specific activity (AU/μg prot)	Recovery (%)[a]	Purification (-fold)
Crude supernatant	20000	1400	0.07	100.0	1.0
Q-Sepharose	3280	1200	0.36	85.7	5.2
Supelcosil LC-18	400	600	1.50	42.8	21.4
Vydac C18	280	480	1.71	34.2	24.4

Activity is expressed in arbitrary units (AU) against *Mucor plumbeus* CCM F 443
[a] Percentage of initial activity

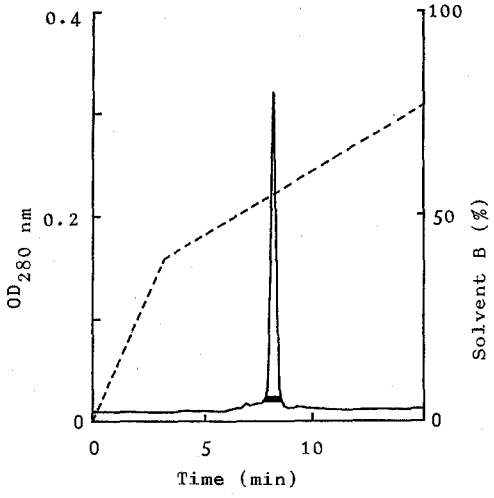

Fig. 1. Purification of the antifungal peptide A12-C by reversed-phase-HPLC. The active fraction from a Supelcosil LC-8 chromatography of a Q-Sepharose concentrate of 100 ml culture supernatant of the producer strain was fractionated on a 4.6 × 25 mm Vydac C18 TP54 column under the conditions described in Materials and methods. The *bar* indicates antifungal activity against *Mucor plumbeus* CCM F 443. The solvent gradient (----) and the recorded absorbance of the effluent at 280 nm (OD_{280}; ——) are also represented

Table 2. Amino acid composition of the purified peptide A12-C

Amino acid	Observed	Integer
Glu	1.00	2
Arg	0.43	1
Ala	0.42	1
Pro	0.53	1
Tyr	0.53	1
Orn	0.55	1
Lan	ND[a]	
D-Abu	ND	
DhAla	ND	

Lan, lanthionine; Orn, ornithine; D-Abu, D-aminobutyric acid; DhAla, dehydroalanine
[a] ND means that the amino acid was not detected in the assay

temperature. The crude supernatants as well as the purified substance retained 100% of the activity after storage for 1 month at 4°C or 6 months at −20°C.

Peptide A12-C was resistant to the proteolytic enzymes trypsin, pronase, and proteinase K. It was also resistant to carboxypeptidase A, suggesting the lack of a free carboxy-terminus. It also retained 100% of the activity after treatment with alkaline phosphatase, lipase, lysozyme, β-glucosidase and β-glucuronidase. None of the above-mentioned enzymes had any visible effect on the test strain at the final concentrations used in our experiments.

Aliquots of purified peptide A12-C each containing 100 μg protein were used in various determinations to establish its chemical composition. No phosphate, neutral sugars, amino sugars or lipids were detected. GC-MS of methylated chloroform extracts of samples hydrolysed at 120 or 150°C did not show any fatty acids.

The amino acid composition of peptide A12-C is shown in Table 2. Six different amino acids were detected, in the following molar ratios: glutamic acid (or glutamine) (2), arginine (1), alanine (1), proline (1), tyrosine (1) and ornithine (1). No lantionine, dehydroalanine or D-aminobutyric acid were detected. According to the amino acid composition, a minimum M_r of 770 can be estimated for peptide A12-C. The extinction coefficients E_{210} and E_{280} [in $(mg/ml)^{-1} \times cm^{-1}$] calculated from the absorption spectrum were 13.55 and 2.19, respectively. No amino-acid derivatives were found by Edman degradation, suggesting that peptide A12-C is either N-terminal-blocked, or cyclic.

Antimicrobial spectrum of inhibition

The antifungal and antibacterial spectrum of peptide A12-C is shown in Table 3. Most of the fungal strains tested were highly sensitive to the purified antibiotic, including *M. plumbeus* CCM F 443, *M. mucedo* CECT 2653, and *Trichophyton mentagrophytes* CECT 2793, whereas *Microsporum canis* CECT 2797 and *Sporothrix schenckii* CECT 2799 were less sensitive. Both cell growth and hyphal proliferation were inhibited, as shown by the persistence of the halos of inhibition after prolonged incubation of the test plates.

The antibacterial activity of peptide A12-C was much less pronounced, and restricted to some Gram-positive bacteria. Sensitive strains required a much higher concentration of antibiotic for inhibition than fungi, with the sole exception of *Corynebacterium glutamicum*. Only one strain of genus *Bacillus* was inhibited by this antibiotic, and the producer strain was resistant.

Table 3. Spectrum of inhibition of the purified peptide antibiotic A12-C

Organisms	AU/mg protein
Microsporum canis CECT 2797	470
Mucor mucedo CECT 2653	2430
M. plumbeus CCM F 443	1710
Sporothrix schenckii CECT 2799	850
Trychophyton mentagrophytes CECT 2793	3120
Bacillus megaterium	57
Corynebacterium glutamicum CECT 78	500
C. glutamicum CECT 80	464
Sarcina sp.	142
Mycobacterium phlei	43
Other bacterial and fungal species[a]	<10

Preparations of a known protein concentration were serially diluted and tested for activity as described in Materials and methods

[a] Other bacteria tested that were resistant to the antibiotic: *Alcaligenes faecalis* CCM 1052, *Bacillus cereus* CECT 131, *B. circulans* CCM 2084, *B. laterosporus* CCM 1612, *B. licheniformis* A12 (the producer strain), *B. licheniformis* D13, *B. licheniformis* M4, *B. subtilis, Enterobacter cloacae* CECT 194, *Enterococcus faecalis* S-47, *E. faecium S-29, E. faecium* CECT 410, *E. durans* CECT 411, *Escherichia coli* Delbruck, *Klebsiella pneumoniae* M5a 1, *Micrococcus luteus, Planococcus citreus* CCM 316, *Proteus* sp., *Pseudomonas reptilivora* N5, *Salmonella typhimurium* SV3, and *Staphylococcus aureus* ATCC 8. The following fungal species were also resistant: *Candida albicans* CECT 1394, *Cryptococcus neoformans* CECT 1075, *Penicillium notatum* A, and *Aspergillus niger* ATCC 9142

Discussion

In a study carried out in our laboratory on the production of inhibitory substances with antiamoebic and antifungal activity, three strains of *B. licheniformis* were isolated and produced a variety of antibiotic substances. In spite of the fact that the search for antibiotic production has focused mainly on species of *B. subtilis, B. brevis* and *B. polymyxa,* species of *B. licheniformis* isolated from natural habitats still seem a good source of new antibiotics, some of them with potential therapeutic applications.

The antifungal substance isolated in this study is an acidic hydrophilic peptide containing only six different amino acids. The M_r estimated from its amino acid composition is 770, which is in accordance with the value obtained by gel filtration (M_r 800). Peptide A12-C contains no fatty acids, phosphorus or carbohydrates, which makes it different from the lipopeptide antibiotics described from *B. subtilis*. It also differs in its amino acid composition from other peptide antibiotics previously described from *B. licheniformis*, such as the licheniformins (Callow and Work 1952), bacitracin (Weinberg 1967), and the phosphorus-containing triene proticin produced by *B. licheniformis* var. *mesentericus* (Nesemann et al. 1972). The only similarity with bacitracin is the presence of one ornithine residue.

The preferential action of peptide A12-C against eukaryotic microorganisms such as fungi and its low spectrum of activity against bacteria is outstanding. Also, its capacity to inhibit (at low concentrations) fungal strains causing human dermatomycoses (*M. canis* and *T. mentagrophytes*) and systemic mycoses (*S. schenckii*) opens new perspectives in antifungal therapy. This adds to the fact that the two more efficient antifungals used against the above pathogens (griseofulvin and amphotericin B) still have unpleasant side effects. Also, because of increasing frequency of topical and systemic infections caused by fungi the search for antifungal drugs is one of the more important challenges of chemotherapy today.

Future studies will determine the usefulness of the peptide antibiotic described here as a therapeutic agent against pathogenic fungi. In addition, the incidence of production of the antifungal activity in natural habitats and its possible role in controlling fungal populations deserves further investigation.

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