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Original

Fungicidal activity of a phospholipase A_2-derived synthetic peptide variant against *Candida albicans*

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SUMMARY

pEM-2, a 13-mer synthetic peptide variant derived from myotoxin II, a phospholipase A_2 homologue present in Bothrops asper *snake venom, has shown potent bactericidal activity in previous studies due to the combination of cationic and hydrophobic amino acids, including three tryptophan-substituted residues in its sequence. This study reports that pEM-2 also exerts potent fungicidal activity against a variety of clinically relevant* Candida *species, killing 100% of yeasts at concentrations near 10 mg/l (5 μM), as indicated by plate counting assays. Thus, this peptide displays a broad-spectrum antimicrobial activity, in the absence of hemolytic activity. The fungicidal action of pEM-2 against* Candida *can be partially inhibited by increasing concentrations of extracellular divalent cations (Ca^{+2} or Mg^{+2}), in agreement with its proposed membrane-permeabilizing mechanism of action.*

Key words: Fungicidal - Microbicidal - Synthetic peptide - *Candida* - Phospholipase A_2

Actividad fungicida sobre Candida albicans *de una variante de péptido sintético derivada de una fosfolipasa A_2*

RESUMEN

pEM-2, una variante de péptido sintético derivada de la miotoxina II, un homólogo de la fosfolipasa A_2 presente en el veneno de la serpiente Bothrops asper, *ha mostrado en estudios previos una potente actividad bactericida debida a la combinación de aminoácidos catiónicos e hidrófobos en su secuencia, incluyendo tres residuos sustituidos por triptófano. Este estudio describe que el pEM-2 también ejerce una potente actividad fungicida contra una variedad de especies de* Candida *clínicamente relevantes, matando el 100% de las levaduras a concentraciones cercanas a 10 mg/l (5 μM), mediante ensayos de recuento en placas. De tal modo, este péptido muestra una acción antimicrobiana de amplio espectro, en ausencia de actividad hemolítica. La acción fungicida del pEM-2 sobre* Candida *es parcialmente inhibida por concentraciones crecientes de cationes divalentes extracelulares (Ca^{+2} o Mg^{+2}), en concordancia con su mecanismo de acción propuesto, permeabilizante de membranas.*

Palabras clave: Fungicida - Microbicida - Péptido sintético - Candida - Fosfolipasa A_2

INTRODUCTION

Infectious diseases are among the leading causes of death worldwide, especially in the less developed nations. The rise in the number of immunocompromised patients due to the AIDS epidemic, the use of anticancer drugs, as well as immunosuppressive regimens in transplantation, has led to an increase in fungal infections. Among them, candidiasis is the most frequently observed clinical expression and *Candida albicans* is the predominant species recovered (1). Over the last decade, many *Candida* species other than *C. albicans* have also emerged as pathogens of clinical relevance (2) and drug resistance among these isolates is of great concern (3). In addition, many of the currently available antifungal therapies have undesirable toxic effects (4), and there is a great need for the development of new classes of antimycotic agents and strategies.

Over the last two decades, an increasing number of peptides with antimicrobial activity have been isolated from various natural sources (5), emerging as potential new therapeutic agents. The majority of these peptides can be categorized into four distinct structural classes: (a) extended peptides with a high content of one (or two) amino acids (e.g., histatins and indolicidin); (b) those that contain intramolecular disulfide bonds, often stabilizing a predominantly β-sheet structure (e.g., defensins and protegrins); (c) those with amphiphilic regions assuming an α-helical structure (e.g., magainins and cecropins); and (d) the loop-structured peptides like bactenecin (6). These antimicrobial peptides are often cationic and amphipatic, and most of them kill bacteria by permeabilizing the target cell membrane. Their net positive charge presumably facilitates interactions with negatively charged moieties present in membranes and/or bacterial cell walls, whereas their amphiphilic character enables membrane penetration and permeabilization (7). A number of natural antifungal agents of proteinaceous nature have been found in animal secretions such as saliva, including lactoferrin, lysozyme and histatins. Synthetic peptides derived from those proteins have shown high fungicidal potency against *C. albicans* (8) and other *Candida* spp., with the exception of *C. glabrata* (9).

Bothrops asper is a crotalid snake species distributed in Central America and Southern Mexico (10). Its venom contains a variety of phospholipases A_2, including several catalytically inactive phospholipase A_2 homologues of the Lys49 family such as myotoxin II (11). In previous studies, a non-hemolytic 13-mer peptide (KKYRYYLKPLCKK), derived from the C-terminal region of myotoxin II, showed bactericidal activity against both Gram-positive and Gram-negative strains (12). Ten synthetic peptide variants were constructed and one of them, named pEM-2 (KK*W*R*W*LK*A*L*A*KK), with a triple Tyr→Trp and single CysAla and Pro→Ala substitutions, showed high bactericidal potency, endotoxin-neutralizing activity and, importantly, a reduced toxicity upon muscle cells (13, 14). In this study, we investigated the possible fungicidal effect of peptide pEM-2 against a variety of *Candida* species, with the aim of evaluating its potential as a novel antimycotic drug lead.

MATERIALS AND METHODS

Peptide synthesis

pEM-2 was synthesized by F-moc chemistry (15), purified by preparative reverse-phase HPLC to a purity of at least 97% and analyzed by mass spectrometry to confirm its sequence. Dry peptide was stored at –20 °C, and dissolved in 10 mM sodium phosphate buffer, pH 7.4, to a final concentration of 50 mM immediately before the experiments.

Candidacidal activity

To determine the minimal concentration of peptide with fungicidal activity, a pilot experiment was performed using a common laboratory strain of *C. albicans* (SC5314) and a series of peptide dilutions (in 10 mM sodium phosphate pH 7.4). Antifungal activity was examined according to the method of Edgerton et al. (16) with some modifications. A loopful of the yeast was inoculated in yeast nitrogen base medium (YNB pH 6.8) (Difco Laboratories), containing 4.5% of glucose, grown at 30 °C and harvested in the mid-exponential phase by centrifugation at 2,100×g for 10 min at room temperature. Cells were washed once with phosphate-buffered saline (PBS) and twice in 10 mM sodium phosphate buffer (pH 7.4) to a final concentration of 1.8×10^5 yeast/ml. Twenty µl of cell suspension were mixed with 20 mcl of pEM-2, at different concentrations (ranging from 80 to 0.15 µg/ml in 2-fold dilutions), and incubated for 90 min at 37 °C with shaking. Cells incubated with 20 µl of phosphate buffer alone were used as a control. Finally, the suspensions were diluted by adding 360 µl of YNB, and 40 µl were spread onto each of three Sabouraud dextrose agar plates (pH 7.4) and incubated for 48 h at 37 °C. Colonies were counted and the cell survival percentage was calculated as previously described (9).

Effect of divalent cations

Candidacidal experiments were performed as described above, except that varying concentrations of Ca^{2+}

and Mg^{2+} (0, 0.5, 5 and 25 mM) were added to the solutions during the 90-min incubation of *C. albicans* SC5314 and pEM-2 at 5 μM concentration.

RESULTS AND DISCUSSION

As shown in Fig. 1, pEM-2 caused a complete fungicidal effect at concentrations of 5 μM (10 mg/l) and higher. In order to determine if this activity was also exerted upon other *Candida* species, and since sensitivity to peptides varies among them (9), we extended this assay to several non-*C. albicans* species and other yeasts such as *S. cerevisiae*, including the most frequently clinically isolated strains (2). These included *C. parapsilosis*, *C. guilliermondii*, *C. lusitanie*, *C. viswanathii*, *C. krusei*, *C. albicans* SC5314, *C. glabrata*, *C. valida*, *C. tropicalis* and *C. kefyr*; *S. cerevisiae* and one *C. albicans* clinical isolate. We followed the same protocol for cell preparation and tested the peptide at 5 μM concentration to evaluate its fungicidal activity. Interestingly, all of the strains tested were completely killed at this peptide concentration, indicating that all of them were at least as sensitive as *C. albicans* SC5314, and suggesting that pEM-2 is a potential antifungal agent with a broad spectrum of activity.

Considering the potential clinical applications of this peptide, we evaluated its fungicidal activity in the presence of the two most abundant divalent cations, Ca^{2+} and Mg^{2+}. As shown in Fig. 2, the presence of Ca^{2+} at 0.5 μM concentration reduces by 50% the fungicidal activity of pEM-2,

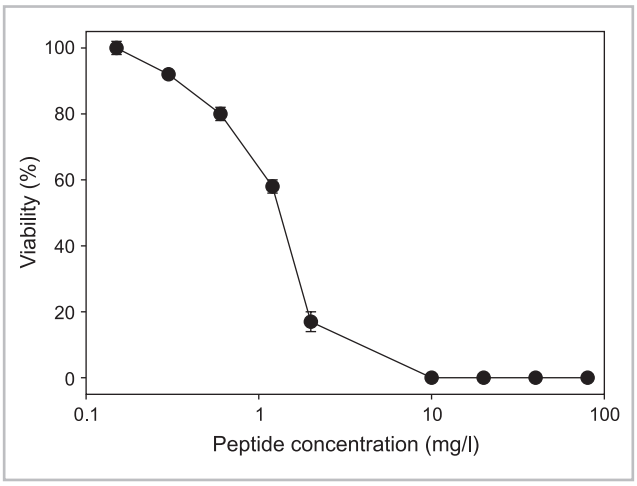

Figure 1. Fungicidal activity of peptide pEM-2 against *Candida albicans* SC5314. Yeasts were incubated with varying amounts of peptide (90 min at 37 ºC) and then plated on Sabouraud dextrose agar for viable counting, in triplicates. Each point represents mean ± SD for each peptide concentration. The experiment was performed twice, with identical results.

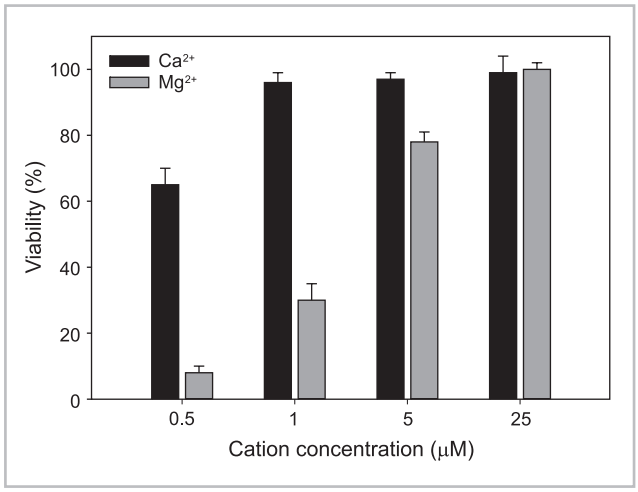

Figure 2. Inhibition of the pEM-2-induced killing of *Candida albicans* SC5314 by the presence of extracellular divalent cations. Yeast viability after incubation with pEM-2 (5 μM) in the absence of Ca^{+2} and Mg^{+2} was 0%. Each bar represents mean ± SD of three independent experiments.

whereas Mg^{2+} causes a comparable inhibition at concentrations between 1 and 5 μM. The reduction in candidacidal action of pEM-2 by divalent cations was dose-dependent (Fig. 2). An inhibitory effect of Ca^{2+} and Mg^{2+} on the activity of cationic peptides has also been documented for histatins isolated from saliva (17) and indolicidin, a tryptophan-rich peptide (6). This inhibition likely depends on the known stabilizing effect of Ca^{2+} or Mg^{2+} cations on membranes, mediated through electrostatic interactions with anionic sites of lipid components (18, 19).

In summary, the present results demonstrate that the peptide pEM-2, in addition to its previously reported bactericidal activity, is also able to exert potent fungicidal effects, which could be of interest to fight infections by *Candida* spp. Its low molecular weight (1742.9), and hence non-immunogenicity, as well as its relative ease of synthesis (13 amino acids), make pEM-2 an interesting drug lead for further evaluation of possible antifungal effects *in vivo*. Its fungicidal activity is likely to involve the permeabilization of fungal cell membrane, as suggested by the protective effects observed here with divalent cations, and in agreement with the permeabilizing mechanism previously shown for its bactericidal actions (12).

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