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A Novel Antimicrobial Peptide from *Crotalaria pallida* Seeds with Activity Against Human and Phytopathogens

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Abstract An actual severe problem in agriculture consists of an expressive increase of economical losses caused by fungi and resistant bacteria toward antibiotics. In order to find a solution to this problem, several studies have been concentrating on the screening of novel plant defense peptides with antimicrobial activities. These peptides are commonly characterized by having low molecular masses and cationic charges. The present work reports the purification and characterization of a novel plant peptide with molecular mass of 5340 Da, named *Cp*-AMP, from seeds of *C. pallida*, a typical plant from Caatinga biome. Purification was achieved using a size exclusion S-200 column followed by reversed-phase chromatography on Vydac C18-TP column. In vitro assays indicated that *Cp*-AMP was able to inhibit the development of filamentous fungi *Fusarium oxysporum* as well as the gram-negative bacterium *Proteus* sp. The identification of *Cp*-AMP could contribute, in the near future, to the development of biotechnological products, such as transgenic plants with enhanced resistance to pathogenic fungi and/or of antibiotics production derived from plant sources in order to control bacterial infections.

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Introduction

An actual worldwide problem consists of a severe agricultural crops production decrease caused by phypathogenic fungi. Among them, there are many saprophytic fungi which, under certain conditions, are able to colonize seeds, such as *Fusarium oxysporum*, which has the ability to colonize agricultural plants including cotton, beans, soybeans, sugarcane, potatoes and other important crops [1]. This fungus is able to cause typical symptoms such as vascular wilt, yellows, corm rot, root rot and damping-off [1]. In order to control *Fusarium*, some procedures are the application of used soil disinfectants, the use of planting material with fungicidal chemicals, crop rotation with *F. oxysporum* non-hosts or by using resistant cultivars [2]. Nevertheless, these techniques are not completely effective and the application of antifungal peptides can be utilized as an additional strategy to control phytopathogens. Additionally, some pathogens can also cause human infections. This could be considered another serious problem, which also faced World population, especially with the increasing of bacteria resistant to antibiotics. Urinary and gastrointestinal infections have commonly being related to bacteria from the genus Proteus [3]. Furthermore, the enhancement of antibiotic resistance by Gram-negative bacteria has also facilitated hospital infections, making illness treatment each day more difficult.

Hence, the screening and isolation of defense plant proteins have been performed in order to develop alternative strategies to control bacteria and fungi that cause economical, healthy and social problems. Among different sources, plant seeds are able to produce a wide variety of proteins and peptides which inhibit microorganism development, such as defensins [4, 5], lectins [6, 7], 2S albumins [8], lipid-transfer proteins [9, 10] and several others. Since the literature concerning plant producing proteinaceous antibiotics is extremely vast, few studies report the identification of antimicrobial peptides from wild plants, especially from South America. Indeed, this communication reports the isolation and biochemical characterization of an antimicrobial peptide from *C. pallida* seeds, a plant growing in tropical areas. Phytopatogenical fungi and human pathogenic gram-negative bacteria were also challenged against the same peptide, in order to find novel resistance sources to agricultural crops, as well as alternative tools toward bacterial infections.

Materials and Methods

Extraction and Isolation of *Cp*-AMP

C. pallida (Fabaceae) mature seeds were obtained from the IBAMA (Brazilian Environmental Institute) seed bank in Natal/RN, Brazil. Finely ground Crotalaria seed was extracted (1:10, w/v) with 0.05 M Tris–HCl buffer pH 7.5. After centrifugation at 12,000×*g* for 30 min at 4°C, the crude extract was precipitated with ammonium sulphate in a range of 30–60%. The sample was submitted to dialysis (cut off of 3,000 Da), and the fraction was applied in size exclusion S200 chromatography, yielding several peaks [11]. After new dialysis and lyophilization, 1.0 mg of this specific fraction was diluted with 0.1% trifluoroacetic acid (TFA) and applied onto a reverse-phase Vydac C-18TP column coupled to a HPLC system and equilibrated with 0.1% TFA, from where the retained proteins were eluted with a linear acetonitrile gradient (0–100%) at a flow rate of 1.0 ml min^{-1}. Major peak was re-applied onto the same column and peptides were eluted with a non-linear acetonitrile gradient with a retarded step (20–50%) of 40 min, in order to improve peptide purity quality.

Molecular Mass Analyses

The peptide fractions eluted from the reversed-phase chromatography were analysed by 15% SDS-PAGE according to Laemmli [12] with minor modifications, using bromophenol blue as tracking dye.

Enzyme Assays

Peptide contents were measured according to the procedure described by Bradford [13], using bovine serum albumin (Sigma) as protein standard. The trypsin inhibitory assay was performed using casein as substrate. Ten micrograms of trypsin were incubated for 20 min at 40°C with 100 μg ml^{-1} of *Cp*-AMP (*C. pallida* antimicrobial peptide) and 120 μl of 0.05 M Tris–HCl pH 7.9. Reaction was stopped by adding TCA 75%, following a new incubation. Proteolytic activity was measured by absorbance at 280 nm. All assays were performed in triplicate.

Bioassays Against Pathogenic Agents

Bioassays against filamentous fungi *F. oxysporum*, *R. solani*, *A. fumigatus* and *T. harzianum* were carried out according to Pelegrini et al. [14] with minor modifications. Bioassays were also performed against *E. coli* ATCC 8739, *K. pneumoniae* ATCC 13883, *S. typhimurium* ATCC 14028 *Proteus* sp., *Rathaybacter* sp, *X. campestris*, *S. aureus*, *R. solanaceum* and *Erwinia* sp. according to Pelegrini et al. [15] with minor modifications. Fungi and bacteria pathogenic to humans were obtained from clinical isolates at the Hospital of the Universidade Catolica de Brasilia, Brasilia—Brazil. Phytopathogens were gently obtained from EMBRAPA Collection.

Mass Spectrometry Analysis

Molecular mass peptides were analysed by MALDI-TOF/TOF on an ABI 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). For analyses, samples were mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid (1:3) and spotted on a sample plate. The MS spectra were carried out in the reflector mode with external calibration, using the 4700 Standard kit (Applied Biosystems) and Peptide Calibration Standard—Starter Kit 4 (Bruker Daltonics).

Results

Purification of Antimicrobial Peptide from *C. pallida*

In order to identify novel antifungal peptides in *C. pallida*, seed crude extract was challenged against *F. oxysporum*, showing the ability to reduce fungal development. After dialyses, this fraction was submitted to a reversed-phase chromatography HPLC (Fig. 1a), showing a major peak at approximately 40% acetonitrile. This peak was further re-chromatographed in the same column (Fig. 1b) with a

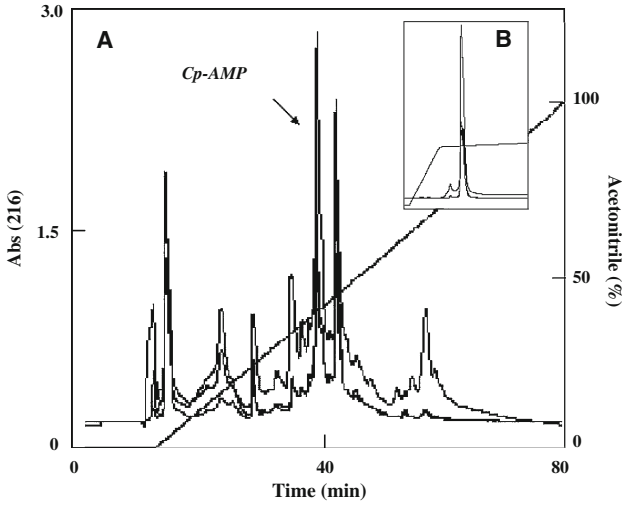

Fig. 1 A Reversed-phase chromatogram profile (Vydac C18-TP column) of size exclusion S-200 last fraction [8]. The diagonal line indicates the linear acetonitrile gradient (0–100%). B Re-chromatography of *Cp*-AMP (*black arrow*), using a retarded step gradient of acetonitrile (20–40%)

retarded step in acetonitrile gradient (20–50%), improving purification quality.

Molecular Mass Analysis of *Cp*-AMP

SDS-PAGE analyses of HPLC major peaks showed a single band that corresponds to peptide with approximately 5 kDa (Fig. 2a). Moreover, a major peak with 5340.72 Da with minor contaminants was observed by mass spectrometry analyses. No dimer or multimers of *Cp*-AMP were observed (Fig. 2b).

Biological Activity

Initially, isolated peptide *Cp*-AMP was capable of inhibited 80% of *Proteus* sp growth (Fig. 3). With this preliminary data, MICs of *Cp*-AMP1 were calculated against *Proteus* sp., *E. coli* ATCC 8739, *K. pneumoniae* ATCC 13883 and *S. typhimurium* ATCC 14028. In fact *Cp*-AMP showed a clear bactericidal activity, where MIC values obtained for this peptide were 32 μg ml^{-1} for *E. coli*, 30 μg ml^{-1} for *Proteus* sp, 12 μg ml^{-1} for *R. solanaceum* and 68 μg ml^{-1} for *Erwinia* sp (Table 1). No activity was obtained towards *S. typhimurium*, *K. pneumoniae*, *S. aureus*, *Rathaybacter* sp. and *X. campestris* (Table 1). Purified *Cp*-AMP was also tested against human and plant pathogenic fungi: *F. oxysporum*, *A. fumigatus* and *R. solani* (Fig. 4). Evaluation was also conducted against *T. harzianum*, which, although it is not a pathogenic organism, it is a widely used model filamentous fungus for biochemical studies. Only *F. oxysporum* (70%) and *R. solani* (45%) were affected by

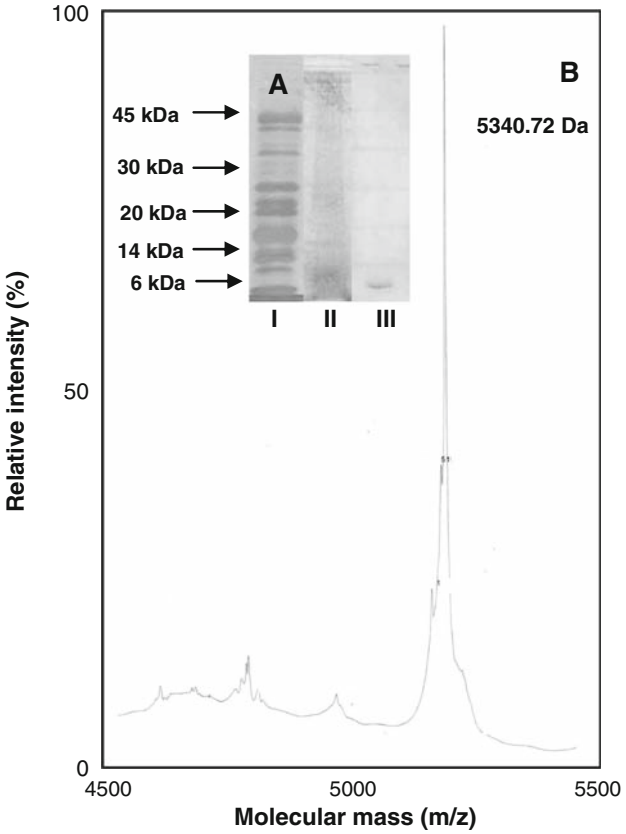

Fig. 2 A SDS-PAGE analyses of crude extract (*lane I*), S200 fraction (*lane II*) and *Cp*-AMP (*lane III* and MALDI-ToF spectrum B)

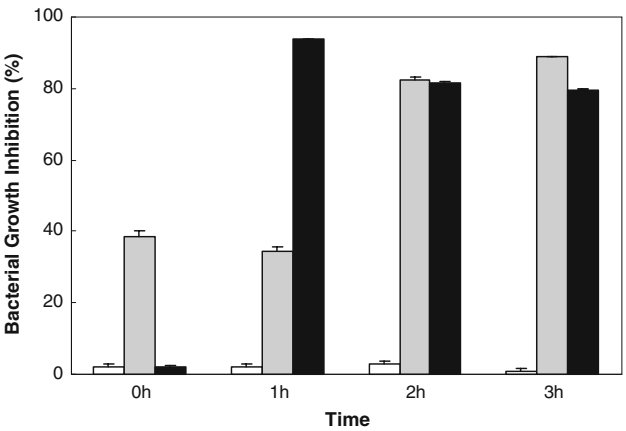

Fig. 3 Bactericidal activity of *Cp*-AMP against *Proteus* sp, during the 0–3 h of incubation. Positive control (*grey columns*) corresponds to 50 μg ml^{-1} chloramphenicol and negative control (*white columns*) to distilled water. *Black columns* correspond to bacterial inhibition in the presence of *Cp*-AMP. Vertical bars correspond to standard deviation

purified peptide at 50 μg ml^{-1} standard concentration. Finally, no inhibitory activity of *Cp*-AMP was observed against any proteinase assayed (data not shown).

Table 1 *Cp*-AMP effects against pathogenic bacteria

Species	MICs	Host
E. coli ATCC 8739	32 μg ml^{-1}	Human
K. pneumoniae ATCC 13883	NA	Human
S. typhimurium ATCC 14028	NA	Human
Proteus sp.	30 μg ml^{-1}	Human
S. aureus	NA	Human
X. campestris	NA	Plants
R. solanaceum	12 μg ml^{-1}	Plants
Rathaybacter sp.	NA	Plants
Erwinia sp.	68 μg ml^{-1}	Plants

MICs (minimum inhibitory concentration) correspond to peptide concentration with the ability to reduce 50% of bacterial growth. NA corresponds to not activity

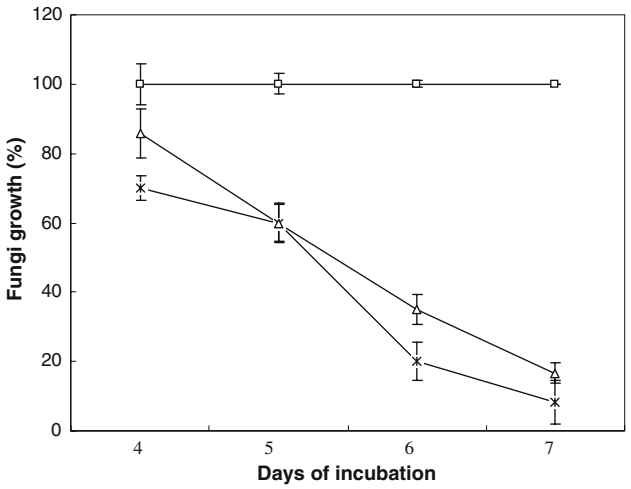

Fig. 4 Antifungal activity of *Cp*-AMP against *F. oxysporum* at 4, 5, 6 and 7th days after inoculation. Positive control (*asterisks*) corresponds to 50 μg ml^{-1} fungizone and negative control (*white squares*) to distilled water. *White triangles* correspond to fungus inhibition in the presence of *Cp*-AMP. Vertical bars correspond to standard deviation

Discussion

The *Cp*-AMP reported here shows similar biochemical properties than those from defensin [5] and 2S albumin [8] protein families. Hence, the acetonitrile concentrations elution [4, 8] and the molecular masses from *Cp*-AMP showed similarities with both groups of proteins, as well as the antimicrobial activity features. Nevertheless, once that masses and elution profiles are similar in so different peptide classes, peptide sequencing must be carried out in order to shed some light over *Cp*-AMP classification.

Furthermore, similar specificity with *Cp*-AMP was also observed with antifungal peptides from different sources, which include animal secretions such as snake venoms [16] and shrimp hemolymph [17] as well as plant seeds [18, 19]. *Cp*-AMP was also capable to inhibit growth of a gram-negative bacteria *Proteus* sp. and *E. coli*, one of the main causes of gastro-intestinal infections. Earlier studies have shown that, although there are several antimicrobial peptides described, most of them inhibit only phytopathogens or only human pathogens [20–24]. Therefore, analysing the antimicrobial peptides from plant sources described earlier in literature, we can conclude that *Cp*-AMP is the first peptide from *C. pallida* to present activity against gram-negative human pathogenic bacterium as well to a phytopathogenic fungi. Finally, purified peptide was evaluated against bovine trypsin, since a proteinase inhibitor was previously purified from *C. pallida* seeds utilizing similar procedures. However, no inhibitory activity was observed, suggesting that the peptide isolated in this report is different from the inhibitor isolated by Gomes et al. [11]. In summary, data here reported show the isolation of a novel plant peptide with the ability to control fungi and bacteria infections. These compounds demonstrate a clear potential utility in agribusiness, medicine, food processing and several other areas where pathogen inhibiting are needed, by using biotechnological techniques.

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References

1. Smith IM, Dunez J, Phillips DH, Lelliot RA, Archer SA (1988) European handbook of plant diseases. Blackwell Science Publications, Oxford, p 583
2. Jones JP, Jones JB, Miller W (1982) Fusarium wilt on tomato. Florida Department of Agriculture and Consumer Services Division of Plant Industry, Tallahassee, FL, Plant Path Cir. No. 237, 2 p
3. Sabbuba NA, Mahenthiralingam E, Stickler DJ (2003) Molecular epidemiology of *Proteus mirabilis* infections of the catheterized urinary tract. J Clin Microbiol 41:4961–4965
4. Franco OL, Murad AM, Leite JR, Mendes PAM, Prates MV, Bloch C Jr (2006) Identification of a cowpea γ-thionin with bactericidal activity. FEBS J 273:3489–3497
5. Pelegrini PB, Franco OL (2005) Plant γ-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins. Int J Biochem Cell Biol 37:2239–2253
6. Vasconcelos IM, Oliveira JT (2004) Antinutritional properties of plant lectins. Toxicon 44:385–403
7. Wang HX, Ng TB (2007) An antifungal peptide from red lentil seeds. Peptides 28:547–552
8. Pelegrini PB, Noronha EF, Muniz MA, Vasconcelos IM, Chiarello MD, Oliveira JT, Franco OL (2006) An antifungal peptide from passion fruit (*Passiflora edulis*) seeds with similarities to 2S albumin proteins. Biochim Biophys Acta 1764:1141–1146
9. Cammue BPA, Thevissen K, Hendricks M, Eggermont K, Goderis IJ, Proost P, Van Damme J, Osborn RW, Guerbette F, Kader JC, Broekaert WF (1995) A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. Plant Physiol 109:445–455

10. Carvalho AO, Souza-Filho GA, Ferreira BS, Branco AT, Araújo IS, Fernandes KVS, Retamal CA, Gomes VM (2006) Cloning and characterisation of a cowpea seed lipid transfer protein cDNA: expression analyses during seed development and under fungal and cold stresses in seedlings tissues. Plant Physiol Biochem 44:732–742
11. Gomes CEM, Barbosa AEAD, Macedo LLP, Pitanga JCM, Moura FT, Oliveira AS, Moura RM, Queiroz AFS, Macedo FP, Andrade LBS, Vidal MS, Sales MP (2005) Effect of a trypsin inhibitor from *Crotalaria pallida* seeds on *Callosobruchus maculatus* (cowpea weevil) and *Ceratitis capitata* (fruit fly). Plant Physiol Biochem 43:1095–1102
12. Laemmli UK (1970) Cleavage of structural proteins using assembly of the head of the bacteriophage T4. Nature 227:680–685
13. Bradford MM (1976) A rapid and sensitive method from quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal Biochem 72:248–254
14. Pelegrini PB, Murad AM, Silva LP, dos Santos RCP, Costa FT, Tagliari PD, Bloch C Jr, Noronha EF, Miller RNG, Franco OL (2008) Identification of a novel storage glycine-rich peptide from guava (*Psidium guajava*) seeds with activity against gram-negative bacteria. Peptides 29:1271–1279
15. Pelegrini PB, Lay FT, Murad AM, Anderson MA, Franco OL (2008) Novel insights on the mechanism of action of alpha-amylase inhibitors from the plant defensin family. Proteins 73:719–729
16. Gomes VM, Carvalho AO, Cunha MD, Keller MN, Bloch C Jr, Deodolindo P, Alves EW (2005) Purification and characterization of a novel peptide with antifungal activity from *Bothrops jararaca* venom. Toxicon 45:817–827
17. Chiou TT, Wu JL, Chen TT, Lu JK (2005) Molecular cloning and characterization of cDNA of penaeidin-like antimicrobial peptide from tiger shrimp (*Penaeus monodon*). Mar Biotechnol NY 7:119–127
18. Wang HX, Ng TB (2005) An antifungal peptide from the coconut. Peptides 26:2392–2396
19. Wang HX, Ng TB (2006) An antifungal peptide from baby lima bean. Appl Microbiol Biotechnol 73:576–581
20. Costa FT, Neto SM, Noronha EF, Bloch C Jr, Franco OL (2007) Susceptibility of human pathogenic bacteria to novel antimicrobial peptides from sesame kernels. Curr Microbiol 55:162–166
21. Grgurina I, Bensaci M, Pocsfalvi G, Mannina L, Cruciani O, Fiore A, Fogliano V, Sorensen KN, Takemoto JY (2005) Novel cyclic lipodepsipeptide from Pseudomonas syringae pv. lachrymans strain 508 and syringopeptin antimicrobial activities. Antimicrob Agents Chemother 49:5037–5045
22. Montesinos E (2007) Antimicrobial peptides and plant disease control. FEMS Microbiol Lett 270:1–11
23. Xiao-Yan S, Qing-Tao S, Shu-Tao X, Xiu-Lan C, Cai-Yun S, Yu-Zhong Z (2006) Broad-spectrum antimicrobial activity and high stability of trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. FEMS Microbiol Lett 260:119–125
24. de Lucca AJ, Cleveland TE, Wedge DE (2005) Plant-derived antifungal proteins and peptides. Can J Microbiol 51:1001–1014