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Purification, biochemical characterization and antifungal activity of a new lipid transfer protein (LTP) from *Coffea canephora* seeds with α-amylase inhibitor properties

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ABSTRACT

Background: A growing number of cysteine-rich antimicrobial peptides (AMPs) have been isolated from plants and particularly from seeds. It has become increasingly clear that these peptides, which include lipid transfer proteins (LTPs), play an important role in the protection of plants against microbial infection.
Methods: Peptides from *Coffea canephora* seeds were extracted in Tris–HCl buffer (pH 8.0), and chromatographic purification of LTP was performed by DEAE and reverse-phase HPLC. The purified peptide was submitted to amino acid sequence, antimicrobial activity and mammalian α-amylase inhibitory analyses.
Results: The purified peptide of 9 kDa had homology to LTPs isolated from different plants. Bidimensional electrophoresis of the 9 kDa band showed the presence of two isoforms with pIs of 8.0 and 8.5. *Cc*-LTP$_1$ exhibited strong antifungal activity, against *Candida albicans*, and also promoted morphological changes including the formation of pseudohyphae on *Candida tropicalis*, as revealed by electron micrograph. Our results show that *Cc*-LTP$_1$ interfered in a dose-dependent manner with glucose-stimulated, H^+-ATPase-dependent acidification of yeast medium and that the peptide permeabilized yeast plasma membranes to the dye SYTOX green, as verified by fluorescence microscopy. Interestingly, we also showed for the first time that the well characterized LTP$_1$ family, represented here by *Cc*-LTP$_1$, was also able to inhibit mammalian α-amylase activity in vitro.
Conclusions and general significance: In this work we purified, characterized and evaluated the in vitro effect on yeast of a new peptide from coffee, named *Cc*-LPT1, which we also showed, for the first time, the ability to inhibit mammalian α-amylase activity.

1. Introduction

Lipid transfer proteins (LTPs) are antimicrobial peptides that cluster into two multigenic families according to their observed molecular masses: LTP$_1$ (9 kDa) and LTP$_2$ (7 kDa). The LTP$_1$ family is composed of small, basic peptides (isoeletric point (pI) 8–10) containing 90 to 94 amino acids, arranged in a three-dimensional structure formed by a bundle of four to five α-helixes and a hydrophobic cavity for loading a large variety of lipids or hydrophobic molecules but not sterols [1–3]. Different functions are reported for LTPs: transport of hydrophobic molecules such as phospholipids between membranes [4], cutin formation [5,6] and protection against pathogens [7–11].

The antimicrobial activity of the LTPs was discovered through an in vitro anti-phytopathogenic screen of plant protein extracts [12]. The LTPs inhibited both bacterial and fungal growth; however, the antimicrobial activity was stronger against fungi [2]. The most potent peptide belonging to the LTP class was obtained from onion seeds, *Ace*-AMP$_1$ [13]. This peptide was able to inhibit growth of all 12 fungi tested and of the Gram-positive bacteria, *Bacillus megaterium* and *Sarcina lutea*, at concentrations below 10 μg/ml. Like other LTPs, this peptide was not efficacious against the Gram-negative bacteria tested [13]. Despite its strong antimicrobial activity, *Ace*-AMP$_1$ was not toxic to mammalian cells (fibroblasts) and did not cause hemolysis of erythrocytes until concentrations of 200 μg/ml were reached; the same lack of cytotoxicity against mammalian cells was demonstrated for LTPs from other plant species [13,14].

Since the discovery of the LTPs as anti-phytopathogenic peptides, it has been speculated that this activity could result from the

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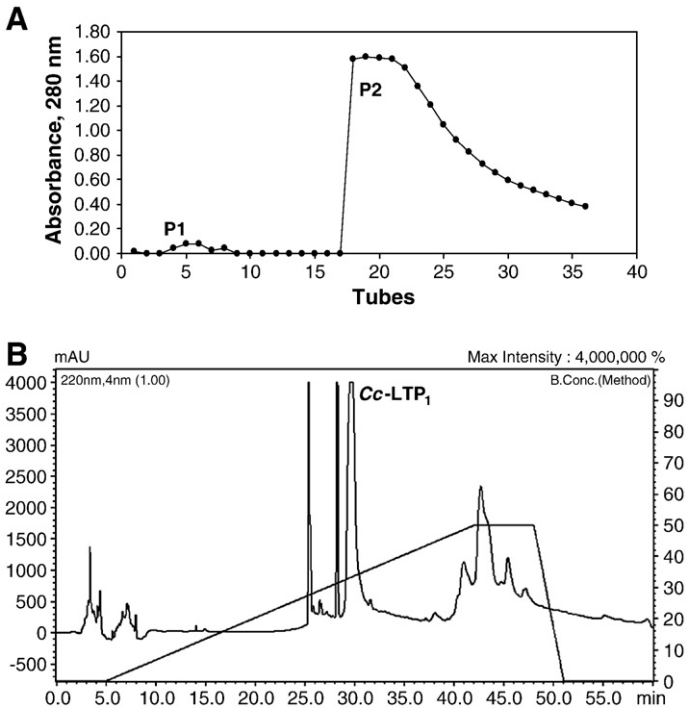

Fig. 1. Purification of *Cc*-LTP$_1$ from coffee seeds. (A) Partial purification of the antifungal peptide from *C. canephora* seeds by anion-exchange DEAE-Sepharose chromatography. The column was equilibrated and the sample was initially eluted with 50 mM Tris–HCl pH 8.0 followed by the same buffer containing 1 M NaCl at 60 ml/h. (B) RP-HPLC chromatography. The DEAE-Sepharose fraction (P1) was applied to a C18 reverse-phase column and run in a Shimadzu apparatus. Elution was carried out as described in Section 2.3. The oblique line represents the propanol gradient and the other line represents the protein elution profile at 220 nm.

interaction of the LTPs with biological membranes, possibly leading to loss of membrane integrity and membrane permeabilization [2,15]. LTPs have been purified from many plants like cowpea [8], sunflower [16], chili pepper [17], tomato [18], cumin [19] and others; however, their physiological functions in vivo and the mechanism of microbial inhibition are still unknown.

Coffee, one of the most widely traded commodities in international markets, is an agricultural crop of significant economic importance [20]. *Coffea arabica* L. (Arabica-type coffee) is typical of the highland growing regions and constitutes almost 75% of world production. The remaining 25% of coffee produced is *Coffea canephora* p. ex Fr. (Robusta-type coffee), which grows in lowland regions [21]. In this work, we report the characterization and purification of a new LTP from *Coffea canephora* seeds, *Cc*-LTP$_1$, which has in vitro fungicidal activity, especially against pathogenic yeast, and we also demonstrate for the first time α-amylase inhibitive properties for this peptide family.

2. Materials and methods

2.1. Plant materials

Seeds from *Coffea canephora* (p. ex Fr.) cultivar Robusta Tropical (EMCAPER – 8151) were collected at the pinhead stage from coffee trees on the experimental farm of INCAPER, Linhares, ES, Brazil.

2.2. Yeast

Saccharomyces cerevisiae (1038), *Candida albicans* (CE022) and *C. Tropicalis* (CE017) were obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The yeasts were maintained on agar Sabouraud (1% peptone, 2% glucose and 1.7% agar–agar).

2.3. Purification of Cc-LTP$_1$

The purification of peptides from coffee seeds was performed as described by [7] with some modifications. Peptides from seed flour

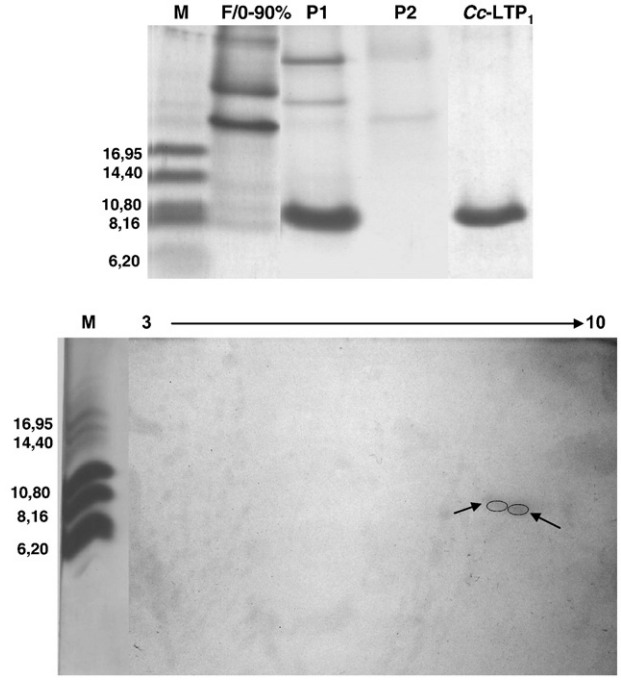

Fig. 2. (A) Tricine–SDS-PAGE of *C. canephora* proteins during the partial purification process. F/0–90, extract rich in peptide; P1, non-retained fraction; P2, retained fraction eluted with 1 M NaCl; M, molecular mass markers (kDa). (B) Two-dimensional electrophoresis of the *Cc*-LTP$_1$ peak from the HPLC chromatography; arrows show spots on the gel.

Cc-LTP$_1$	1	I	T	C	Q	Q	V	T	S	E	L	G	P	C	V	P	Y	L	T	G	Q	G	21	I	P
Gossypium barbadense	28	V	T	C	G	Q	V	T	S	S	L	A	P	C	I	G	Y	L	T	G	N	G	48	66	76
Setaria italica	30	I	T	C	G	Q	V	A	S	S	L	A	P	C	I	P	Y	A	T	G	-	-	48	68	73
Gossypium hirsutum	28	V	T	C	G	Q	V	T	S	S	L	A	P	C	I	G	Y	L	T	G	N	G	48	66	76
Capsicum annum	27	-	-	C	G	Q	V	T	S	A	M	A	P	C	V	P	Y	L	M	G	R	G	45	68	78

Fig. 3. Comparison of the N-terminal amino acid sequences of the peptide of approximately 9 kDa (arrow) purified from *Coffea canephora* seeds with other homologous peptides described in the literature. Sequences from the following proteins were presented: gi: 40792591 from *Gossypium barbadense*; gi: 16904376 from *Setaria italica*; gi: 133907522 from *Gossypium hirsutum*; and gi: 60459407 from *Capsicum annuum*. (I) represents the percentage of identical amino acids, (P) represents the percentage of positive amino acids, and gaps (–) were included for better alignment. The numbers flanking sequences are the amino acid positions in the sequence of the peptides.

(100 g) were extracted for 2 h (at 4 °C) with 500 mL of extraction buffer (0.1 M Tris–HCl pH 8.0, 1 mM phenylmethanesulfonyl fluoride (PMSF)). The precipitate formed between 0 and 90% relative ammonium sulfate saturation was re-dissolved in distilled water and heated at 80 °C for 15 min. The resulting suspension was clarified by centrifugation, and the supernatant was extensively dialyzed against distilled water. The dialyzed solution was recovered by freeze drying (F/0–90) and was further purified by ion exchange chromatography. A DEAE-Sepharose column (1.2×14.6 cm) equilibrated with 50 mM Tris–HCl (pH 8.0) was employed for separation of proteins from the F/0–90 fraction. We eluted at a flow rate of 60 ml/h, first with the equilibrium buffer to obtain fraction P1, and then with 1 M NaCl in equilibrium buffer to obtain bound proteins (P2). The P1 fraction was dialyzed against distilled water, recovered by freeze drying (Frezone 4.5, Labconco) and diluted in 0.1% (v/v) trifluoracetic acid (TFA, Fluka) and injected onto an HPLC (Prominence, Shimadzu) Vydac C18 reverse phase column (250×4.6 mm, Shimadzu) (attached to a C8 pre-column (20×4.6 mm, Pelliguard, Sigma-Aldrich). The solvent flow rate was 0.5 ml/min and the solvent progressed from 100% solvent A (0.1% TFA in water) for 10 min, 0 to 50% solvent B (100% 2-propanol (Merck) containing 0.1% TFA) for 50 min, 50% solvent B for five min and finally returned to 0% of solvent B for 10 min. Elution of proteins was monitored by on-line measurement of the absorbance at 220 and 280 nm.

Protein content was determined as described by Bradford [22].

2.4. Tricine gel electrophoresis

Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine–SDS-PAGE) was performed according to the method of Schägger and von Jagow [23]. Gels were fixed and proteins were visualized with 0.1% Coomassie brilliant blue R250 (Sigma) in methanol: water:acetic acid (Merck) (1:8:1). The bidimensional analysis in acrylamide gel was performed according to O'Farrell [24] with modifications [25].

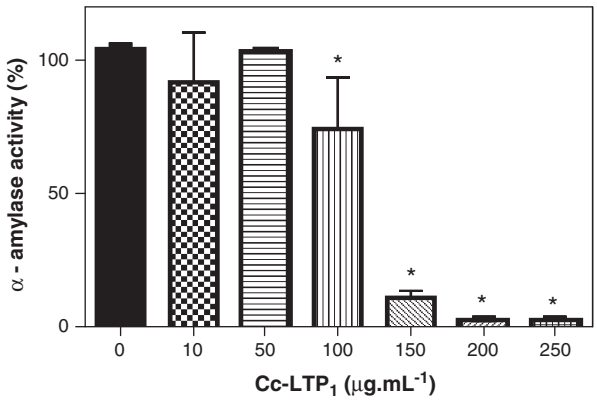

Fig. 4. Human salivary α-amylase activity in the absence and presence of different concentrations of *Cc*-LTP$_1$. Concentrations are in μg/ml. The assays were performed in water and at 37 °C. The values are means (±SD) of triplicates. Asterisks indicate significant differences (P<0.05) between experimental treatment and control.

2.5. Amino acid sequence analysis

For the amino acid sequence analysis, peptides from the P1 fraction were separated by tricine–SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF, Millipore) membrane and stained with Ponceau-S (0.1%). The 9 kDa band was excised from the membrane and briefly washed in different substances as follows: in 100 μl water, then in 400 μl methanol with vortexing and then in 100 μl chloroform with vortexing. The last wash was removed, and finally the membrane was air dried. N-terminal amino acid sequences of the peptides blotted onto PVDF were determined by Edman degradation carried out in a Shimadzu PSQ-23A

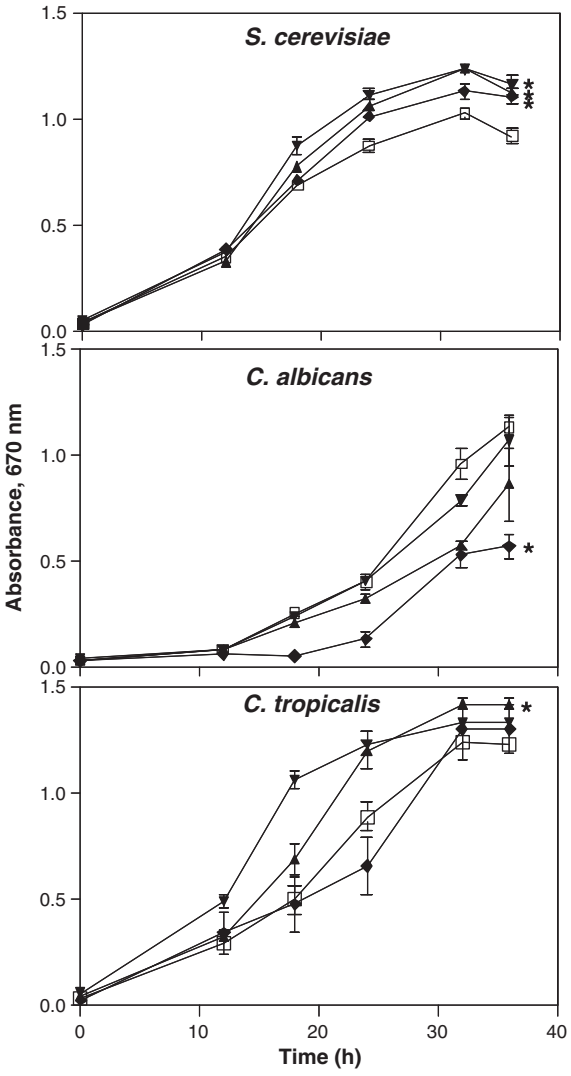

Fig. 5. Inhibition growth assay of *S. cerevisiae*, *C. albicans* and *C. tropicalis* in the presence of *Cc*-LTP$_1$. The growth was observed until 36 h. (-□-) Control; (-▲-) 100 μg/ml; (-▼-) 200 μg/ml and (-◆-) 400 μg/ml. The values are means (±SD) of triplicates. Asterisks indicate significant differences (P<0.05) between experimental treatment and control at the 36 h of experiment.

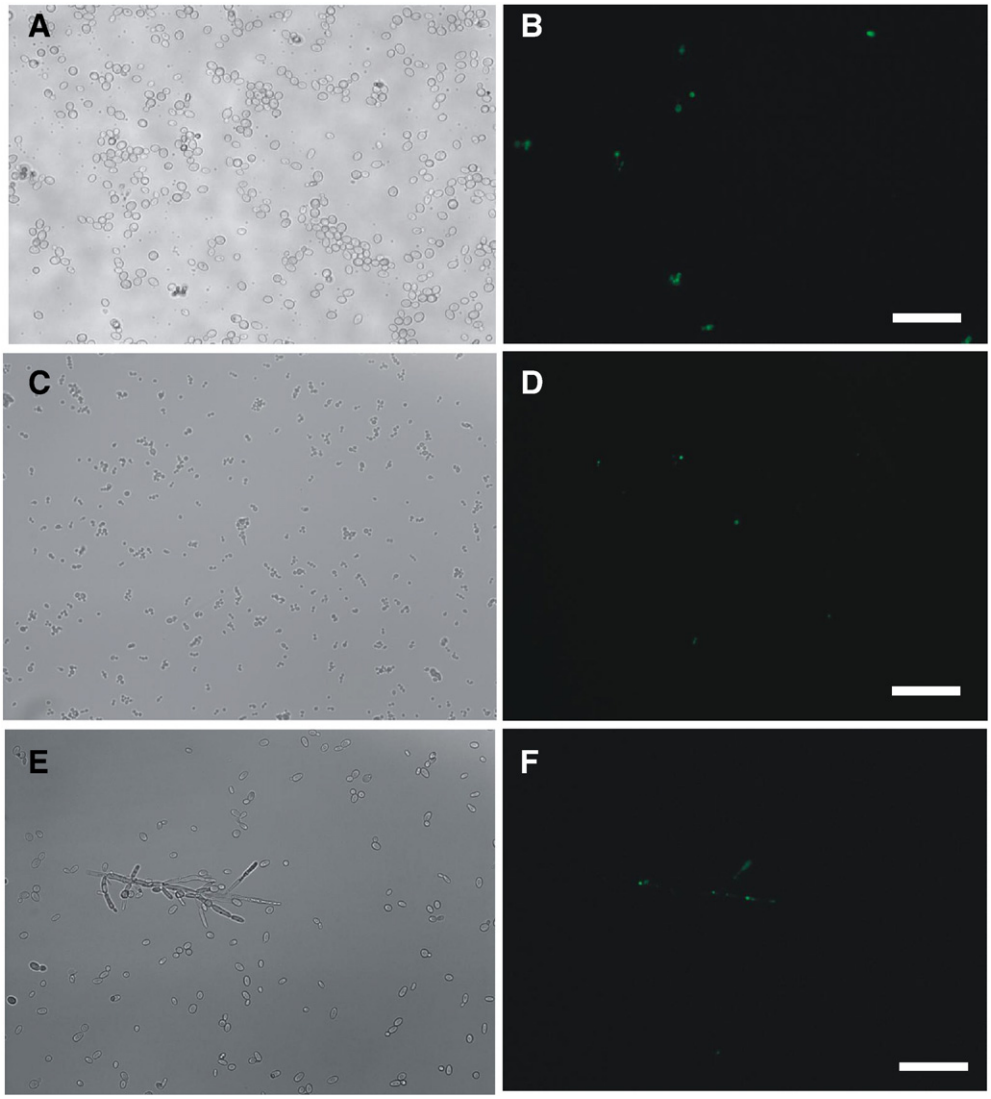

Fig. 6. Membrane permeabilization assay of *S. cerevisiae* (A and B), *C. albicans* (C and D) and *C. tropicalis* (E and F) cells treated for 36 h with 400 μg/ml of *Cc*-LTP$_1$ and incubated with SYTOX green. A, C and E bright field; B, D and F fluorescence. Scale bars = 20 μm.

protein sequencer (Shimadzu). PTH-amino acids were detected at 269 nm after separation on a reverse-phase C18 column (250 × 4.6 mm) under isocratic conditions, according to the manufacturer's instructions. Searches for sequence homology were performed with the BLASTp program [26].

2.6. Human salivary α-amylase inhibition assay

Human α-amylase was generously furnished by Dr. Viviane Veiga do Nascimento. The enzymatic activity assay for human salivary α-amylase (EC 3.2.1.1) was performed according to the method of Bernfeld [27]. Initially, the reaction mixture for determination of enzymatic activity was assayed in water at 37 °C for 15 min using 1% starch (Sigma, Co.) as the substrate. After the addition of 3,5-dinitrosalicilic acid (DNS), the reaction was stopped by heating to 100 °C, and absorbance at 540 nm was monitored (UVVIS-1203, Shimadzu). One unit of α-amylase activity was defined as the quantity of the enzyme that increased the absorbance at 540 nm by 0.1 absorbance units over 30 min.

Human salivary α-amylase inhibitory assays were carried out as described above after a 15 min pretreatment at 37 °C with 5 U of human α-amylase and different concentrations of *Cc*-LTP$_1$ (1 to 25 μg) in a final volume of 100 μl. After this incubation, 1% starch substrate solution was added; the reaction mixture was incubated under the same conditions for additional 15 min; and the reaction was developed as described above. All inhibition assays were performed in triplicate. Data were normalized to enzyme activity in the control reaction (100% activity).

2.7. Effect of *Cc*-LTP$_1$ on yeast growth and cell viability

For the preparation of yeast cell cultures, an inoculum of each yeast was transferred to Petri dishes containing agar Sabouraud and allowed to grow at 28 °C for 3 days. After this period, cells were transferred to sterile Sabouraud broth (1 mL). Yeast cells were quantified in a Neubauer chamber for further calculation of appropriate dilutions. To monitor the effect of *Cc*-LTP$_1$ on the growth of yeasts, cells (10^4 in 1 ml Sabouraud broth) were incubated in the presence of the peptide (0, 100, 200 and 400 μg/ml) at 28 °C in 200 μl microplates. The spectrophotometer was blanked with culture medium alone then optical readings at 670 nm were taken at the zero time point and then every 6 h for the following 36 h [28]. The optical densities were plotted as a function of peptide concentration.

For the cell viability assay, cultures were grown for 36 h, and then the cells in the control sample were quantified in a Neubauer chamber. The control and test samples (treated with 100 μg/ml *Cc*-LTP$_1$) were diluted

as necessary in Sabouraud broth and were plated with a Drigalsky loop on Petri dishes containing agar Sabouraud. The cells were evenly distributed on the plate and were incubated at 30 °C for 24 h so that the colonies could develop. After this period, the colonies were counted [29]. Data were analyzed with respect to the control reaction (100% viability).

2.8. Plasma membrane permeabilization

Plasma membrane permeabilization was measured by SYTOX green uptake as described previously by Thevissen et al. [30] with some modifications. One-hundred microlitres aliquots of the suspensions of *S. cerevisiae*, *C. albicans* and *C. tropicalis* that were grown in the presence of *Cc*-LTP$_1$ (400 μg/ml), as described in item 2.7, were incubated with 0.2 μM SYTOX green in 96-well microplates for 30 min at 25 °C with periodic agitation, followed by observation using a DIC microscope (Axiophoto Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450 to 490 nm; emission wavelength, 500 nm). Negative (no *Cc*-LTP$_1$ added) controls were also run to evaluate the baseline membrane permeability.

2.9. Inhibition of glucose-stimulated acidification of the medium by yeast

Yeast cultures in Sabouraud broth (200 mL) were grown in a rotating incubator (Innova) for 16 h at 30 °C with aeration. The culture (ABS 670 nm = 0.24) was pelleted by centrifugation at 3000 × *g* (5 min, 4 °C), followed by two washes with water. The activity of the peptide *Cc*-LTP$_1$ was determined by incubation of yeast cells (1×10^4 ml) with 0.8 ml of 5 mM Tris–HCl, pH 6.0. *Cc*-LTP$_1$ was added to the final concentrations of 100 μg/ml and 200 μg/ml. After a 60 min incubation, a 0.5 M glucose solution was added to a final concentration of 0.1 M. Measurements of pH were taken each minute for the next 30 min. Controls, without the addition of *Cc*-LTP$_1$, were run to evaluate the influence of the peptide on H^+ extrusion by yeast cells. The extent of H^+ extrusion was calculated as the difference (ΔpH) between the initial (T = 0) and final (T = 30 min) pH, and the resultant values were input into the equation $pH = -\log[H^+]$. Again, data were analyzed with respect to the negative control (100% acidification).

2.10. Scanning electron microscopy

For scanning electron microscopy, yeast cells grown for 36 h in Sabouraud broth in the presence or absence of *Cc*-LTP$_1$ (400 μg/ml) were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde and 4.0% formaldehyde in 0.05 M cacodylate buffer, pH 7.0. Subsequently, the cells were rinsed three times in 0.1 M cacodylate buffer, pH 7.0; post-fixed for 30 min at room temperature with 2.0% osmium tetroxide diluted in 0.1 M cacodylate buffer, pH 7.0; and rinsed again with this same buffer. After this procedure, the yeast cells were dehydrated in alcohol, critical point dried in CO_2, covered with 20 nm gold and observed in a DSEM 962 Zeiss scanning electron microscope. The yeast cells grown without the addition of peptide were also imaged.

2.11. Statistical analysis

Data were obtained from experiment performed in triplicate. To test the effects of treatment wound on the cell viability the data were analyzed using Student's t test. Data for yeast growth, α-amylase inhibition and glucose-stimulated acidification assay were evaluated by using the one-way ANOVA. Mean differences at $P<0.05$ were considered to be significant. All statistical analyses were performed with software GraphPad Prism (version 5.0 for Windows).

3. Results

3.1. Purification and characterization of *Cc*-LTP$_1$

Initially, proteins that were precipitated with ammonium sulfate (F/0–90) were separated into two different fractions, P1 and P2, by means of anion exchange chromatography in DEAE-Sepharose (Fig. 1A). The basic fraction P1, which exhibited antifungal activity, was further fractionated by reverse-phase chromatography (RP) on a C18 column (Fig. 1B).

Because of its antifungal activity, the P1 fraction was chosen for further characterization. An analysis of the protein profile of the P1 fraction obtained from anion-exchange chromatography showed the

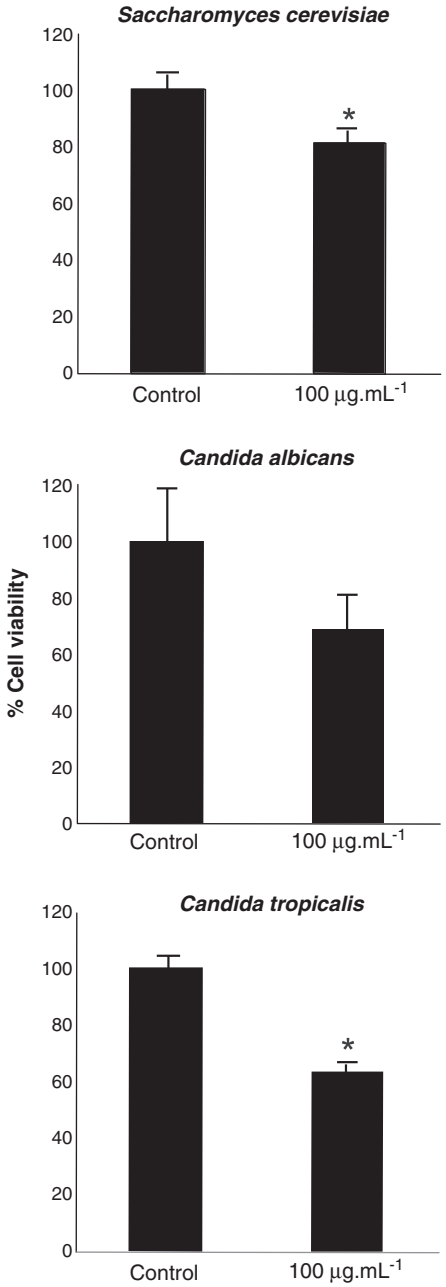

Fig. 7. Cell viability test after the inhibition growth assay with 100 μg/ml of *Cc*-LTP$_1$; data are represented by the number of colonies formed after 12 h on Sabouraud agar. (A) *S. cerevisiae*, (B) *C. albicans*, (C) *C. tropicalis*. (Control) without addition of *Cc*-LTP$_1$. The values are means (±SD) of triplicates. Asterisks indicate significant differences ($P<0.05$) between experimental treatment and control.

presence of a major peptide in tricine–SDS-PAGE under reducing conditions. The P1 fraction was mainly composed of one peptide with a molecular mass of ~9 kDa; other proteins were bigger than 16 kDa (Fig. 2A). The main peak obtained after RP chromatography was also analyzed through tricine–SDS-PAGE. This peak was composed of one unique peptide with a molecular mass around 9 kDa (Fig. 2A). Bidimensional electrophoresis showed that the single 9-kDa band from tricine–SDS-PAGE was actually composed of two peptide isoforms with pIs between 8.0 and 8.5 (Fig. 2B).

The 9 kDa purified peptide underwent N-terminal amino acid sequencing, and the resulting sequence was homologous to LTPs isolated from different plants (Fig. 3), especially from the seeds of *Gossypium barbadense* (gi: 40792591), *Setaria italica* (gi: 16904376), *Gossypium hirsutum* (gi: 133907522) and *Capsicum annuum* (gi: 60459407) (Fig. 3). We named the purified peptide *Cc*-LTP$_1$.

3.2. Human α-amylase inhibitor properties of *Cc*-LTP$_1$

In addition to these previously described activities, in this work, we characterize for the first time a purified LTP with human salivary α-amylase inhibitory activity in vitro. Fig. 4 shows that *Cc*-LTP$_1$ completely inhibited the activity of human salivary α-amylase at a concentration of 200 μg/ml. Peptide concentrations of 100 and 150 μg/ml partially inhibited α-amylase activity, while concentrations of 10 and 50 μg/ml were ineffective (Fig. 4).

3.3. Antimicrobial activity against yeast

After the purification process, we tested *Cc*-LTP$_1$ against the yeasts *S. cerevisiae*, *C. albicans* and *C. tropicalis*. Fig. 5 shows the patterns of yeasts growth in the presence of 100, 200 and 400 μg/ml *Cc*-LTP$_1$. An inhibitory effect on the growth of only *C. albicans* was observed; no inhibitory effect was detected for *S. cerevisiae* and *C. tropicalis* (Fig. 5). However, dimorphic transitions with the development of pseudohyphae were observed in *C. tropicalis* cultures (Figs. 6 and 8F). This morphological change could explain our failure to detect growth inhibition in this yeast species: pseudohyphae formation might disrupt absorption measurements.

The ability of *Cc*-LTP$_1$ to permeabilize the plasma membrane of yeast was also examined using the dye SYTOX green, which cannot cross intact plasma membranes. If the permeability of the plasma membrane increases, the dye enters the cytoplasm where it binds to nucleic acids and fluoresces. When observed with a fluorescence microscope, all yeast cells incubated with *Cc*-LTP$_1$ showed SYTOX green fluorescence (Fig. 6), indicative of membrane permeabilization.

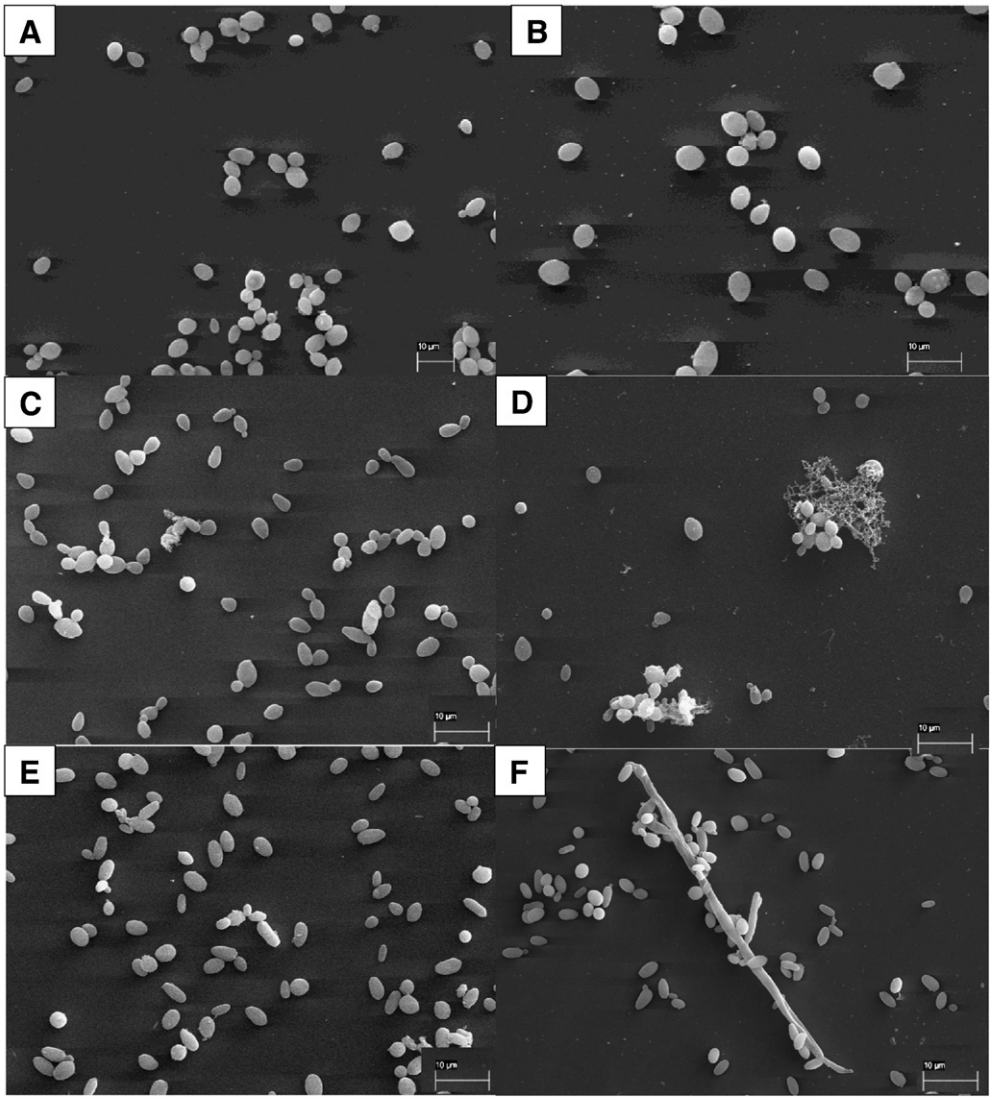

Fig. 8. Scanning electron microscopy of *S. cerevisiae*, *C. albicans* and *C. tropicalis* cells after the inhibition growth assay with *Cc*-LTP$_1$. (A, C and E) control of respective cells (absence of *Cc*-LTP$_1$); (B, D and F) cells in the presence of 400 μg/ml of *Cc*-LTP$_1$. Scale bars = 10 μm.

The inhibitory effect of *Cc*-LTP$_1$ on yeasts was finally confirmed by the viability assay of the yeasts on solid medium. Cells were seeded, incubated for 36 h in the presence of *Cc*-LTP$_1$, and cultured on fresh Sabouraud agar without the peptide (Fig. 7). For the yeast *S. cerevisiae*, we observed that this peptide reduced the viability of this yeast by 15%. A greater reduction in viability (35%) was observed for *C. albicans* and *C. tropicalis*.

Further tests to evaluate the inhibition of yeast growth were analyzed through scanning electron microscopy to verify possible alterations in yeast morphology. Normal growth development was observed for *S. cerevisiae* cells (Fig. 8B). Cultures of *C. albicans* and *C. tropicalis* treated with the *Cc*-LTP$_1$ fraction exhibited notable alterations such as cellular agglomeration and a reduction of cell number (Fig. 8D and F). An agglutinated material, which we suppose to be cytoplasmatic material that was liberated from the cell after treatment with *Cc*-LTP$_1$, was also observed (Fig. 8D). Development of pseudohyphae was observed during the growth of the cultured *C. tropicalis* (Fig. 8F). Normal growth and development, without pseudohyphae formation, was observed for all control cells (Fig. 8A, C and E).

3.3.1. Effects of Cc-LTP$_1$ on the glucose-stimulated acidification of medium by yeast

In this study, we investigated whether the LTP could interfere with yeast H^+-ATPase activity upon the supposed interaction of *Cc*-LTP$_1$ with the fungal plasma membrane. We monitored these effects by measuring the glucose-stimulated acidification of the culture medium of *S. cerevisiae*, *C. albicans*, *C. tropicalis* in the presence of 100 and 200 μg/ml *Cc*-LTP$_1$ (Fig. 9). We found that 100 μg/ml of the peptide enhanced the glucose-stimulated acidification of the fungal medium by 89% for *S. cerevisiae*. In the case of *C. albicans*, a dose-dependent effect was observed; at 200 μg/ml of the peptide, a 222% increase in medium acidification was measured. *C. tropicalis* behaved similarly to *C. albicans*: a dose-dependent effect was observed, with a 390% increase in extrusion of H^+ by the cells at 200 μg/ml of peptide.

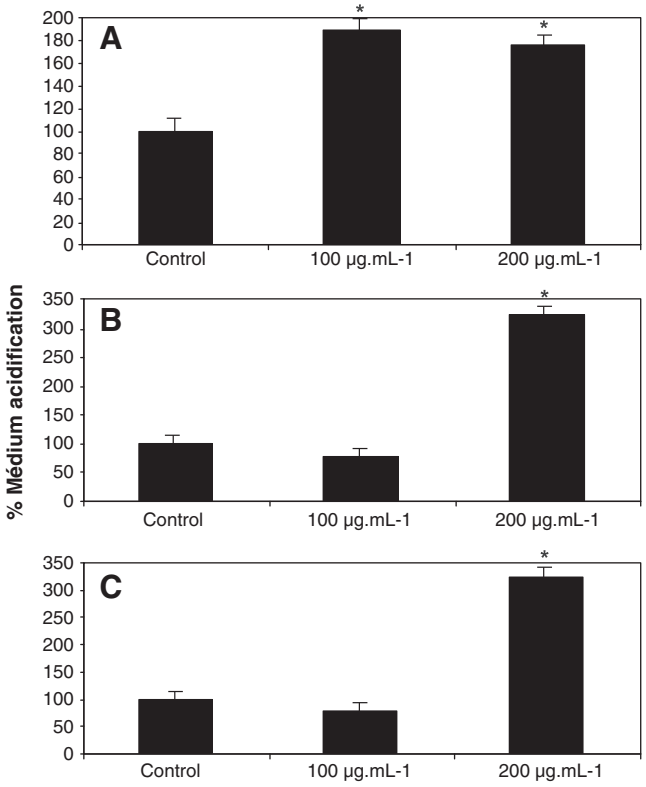

Fig. 9. Effects of 100 and 200 μg/ml of *Cc*-LTP$_1$ on the glucose-dependent acidification of the medium by (A) *S. cerevisiae*, (B) *C. albicans* and (C) *C. tropicalis* cells. Glucose (100 mM final concentration) was added after 60 min of cell incubation with *Cc*-LTP$_1$. The values are means (±SD) of triplicates. Asterisks indicate significant differences ($P<0.05$) between experimental treatment and control.

4. Discussion

In recent years, an increasing number of cysteine-rich antimicrobial peptides have been isolated from plants and particularly from seeds [31–33]. However, few antimicrobial peptides have been purified from *C. canephora* seeds. One of the few examples is a cyclotide characterized by Svangard et al. [34]. Therefore, we investigated the extent and mechanisms of in vitro antifungal activity of peptides isolated from *C. canephora* seeds. The 9 kDa purified was homologous to LTPs isolated from different plants. From the estimated molecular mass and the conserved residues – especially the cysteines – among the sequenced amino acids (C_3, Q_5, V_6, S_8, P_{12}, C_{13}, Y_{16}, G_{19}), we concluded that the isolated peptide belongs to the Family one LTPs, which have molecular masses of approximately 9 kDa and are basic (pI 8–10). These LTPs have 90–95 amino acid residues, of which eight are cysteines conserved in similar positions along the primary structure, and have antimicrobial activity [19,31].

Many functions have been attributed to plant LTPs, including transport of hydrophobic molecules such as phospholipids between membranes [4], wax and cutin assembly [5,6,35], protection against fungal and bacterial pathogens [7,9–11,31,36], mobilization of seed storage lipids [37,38] and cell wall extension [39]. In addition to these previously described activities, in this work, we characterize for the first time a purified LTP with human salivary α-amylase inhibitory activity in vitro (Fig. 4). Previously, some authors have reported this biological activity for other AMPs such as the plant defensin family, which inhibits insect α-amylase [40–42]. An unusual bifunctional protein capable of inhibiting both α-amylase and trypsin was also purified to homogeneity from seeds of ragi (Indian finger millet). The complete amino acid sequence of this bifunctional amylase/trypsin inhibitor was determined, and the molecule consists of a single polypeptide chain of 122 amino acids that exhibits sequence homology with trypsin inhibitors from barley and maize and with an α-amylase inhibitor from wheat [43]. Later, this inhibitor was shown to have low homology with plant LTPs [44]. The ability of plant defensins to inhibit α-amylase activity suggests a possible role for these proteins in plant defense against herbivores. We also suggest that multifunctional LTPs could participate in plant defense against herbivores.

The antimicrobial activity of the LTPs was discovered by screening protein extracts from plants to identify proteins that could inhibit the growth of phytopathogens in vitro [12]. Since the discovery of the antimicrobial activity of plant LTPs in the beginning of the 1990s [12], several plant pathogens, including the fungi *F. solani*, *F. oxysporum*, *Pythium aphanidermathum*, *Sclerotium rolfsii*, *Pyricularia oryzae*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [12,16,17,45,46], and the bacteria *Pseudomonas syringae*, *Clavibacter michiganensis* subsp. *sepedonicus* and *Rhalstonia* (*Pseudomonas*) *sonanacearum* [12], have been shown to be sensitive to their effect. In regard to human pathogens, plant LTPs were shown to inhibit the pathogenic yeast *C. albicans* [17,47] and the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* [46,47].

Thus, we demonstrated that the membrane of yeast treated with *Cc*-LTP$_1$ was structurally compromised (Fig. 5). A LTP isolated from *Helianthus annuus* seeds has been previously reported to completely abrogate the germination of *F. solani* spores at 40 μg/ml [16]. Regente et al. [15] demonstrated, for first time, that the *H. annuus* LTP was able to permeabilize the membranes of *F. solani* spores, also demonstrated by the SYTOX green permeabilization assay. Other peptides have been studied and analyses have been performed to test the ability these peptides have to act on the plasma membrane. For example, Thevissen et al. [30] demonstrated an ion flux across the fungal plasma membrane

when the fungi *Neurospora crassa* and *Fusarium culmorum* were treated with the plant defensins *Rs*-AFP$_2$ and *Dm*-AMP$_1$.

Further tests to evaluate the inhibition of yeast growth were analyzed through scanning electron microscopy to verify possible alterations in yeast morphology. Interestingly, this cellular dimorphism might occur as a result of alterations in the pH of the growth medium due to an effect of the LTP on the plasma membrane of these yeasts. Osborn et al. [48] reported that plant defensins isolated from different seeds cause morphological alterations that are often very distinct in some, but not all, tested fungi. Diz et al. [17] and Ribeiro et al. [49] also showed that peptides present in the F1 fraction of chili pepper seeds promote a yeast–pseudohyphae transition state in *C. albicans* yeast following peptide fraction-induced permeabilization of the plasma membrane.

The plasma membrane H^+-ATPase plays an essential role in fungal cell physiology. Antagonist interference in the function of fungal H^+-ATPase commonly leads to cell death. We monitored these effects by measuring the glucose-stimulated acidification of the culture medium of *S. cerevisiae*, *C. albicans*, *C. tropicalis* in the presence of *Cc*-LTP$_1$ (Fig. 9). We found that *Cc*-LTP$_1$ enhanced the glucose-stimulated acidification of the fungal medium by all yeasts. In contrast to these results, other peptides have an inhibitory effect on medium acidification. The 2S albumins from passion fruit seeds might inhibit glucose-dependent acidification by *S. cerevisiae* yeast and also by phytopathogenic fungi [50,51]. Diz et al. [17] and Ribeiro et al. [49] demonstrated that the peptides present in the F1 fraction of chili pepper seeds inhibit glucose-dependent acidification of *S. cerevisiae* yeast medium by 100% at a concentration of 160 μg/ml. Our studies suggest that LTPs can bind not only lipids but also membrane proteins of eukaryotic cells, similarly to elicitins [4,52,53]. The phylogenetic relationship of members of the LTP family from diverse plant genomes is described in Carvalho et al. [54]. The phylogenetic tree shows that the plant LTPs share a common evolutionary origin based on conserved sequences and structural motifs. However, LTPs from *Spermatophytae Picea abies* were grouped with the LTP from *C. annum*. These two were the most divergent peptides among the plant LTPs and seem to be related to another class of lipid carrier protein, the elicitins, whose family forms an independent cluster. Thus, it is possible that *Cc*-LTP$_1$ is more closely related to elicitins because this LTP from coffee presented high degree of homology to the specie *C. annuum* (Fig. 3). As noted above, it is possible that *Cc*-LTP$_1$ could, through interaction with the proteinaceous components of the plasma membrane, cause changes in the pattern of ion permeability (H^+, K^+ and Cl^-), as demonstrates for the elicitins [52]. In fact, the biological activities assigned to *Cc*-LTP$_1$, as well as to the other members of the plant LTPs, may be due to the fact that LTPs are encoded by multigenic families whose members display low sequence identity and putatively accomplish distinct functions.

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