Original article

Antimicrobial activity of a protein purified from the latex of *Hevea brasiliensis* on oral microorganisms

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Summary

This study aimed at screen for antimicrobial activity present in the non-rubber constituents of rubber latex of *Hevea brasiliensis* against various microbial strains. An antimicrobial protein, hevein was extracted from the bottom fraction after centrifugation and purified by acetone fractionation and anion exchange chromatography on a DEAE-Sepharose Fast Flow column. This procedure was more efficient and rapid than the previously described procedures. The antimicrobial activity was investigated and revealed that hevein, a small (4.7 kDa) cysteine-rich protein, had strong antimicrobial activity, especially against *Candida* spp. including *Candida albicans*, *Candida tropicalis* and *Candida krusei*. The MIC_{80} value for hevein was as low as 12 μg ml^{-1} with *C. tropicalis* ATCC 750. Higher MIC_{80} values were obtained for *C. albicans* ATCC 10231 (95 μg ml^{-1}) and *C. krusei* ATCC 6258 (190 μg ml^{-1}). To confirm the antifungal activity, hevein also inhibited the growth of those fungi in a disk diffusion assay and its inhibition was enhanced when a *Hevea* latex protease inhibitor was also included. Hevein at a concentration of 30 μg ml^{-1} also caused a Ca^{2+}-dependent aggregation of *C. tropicalis* yeast cells. These data indicate that hevein can inhibit the growth of certain potential oral fungal pathogens.

Key words: *Hevea brasiliensis*, hevein, antimicrobial activity, *Candida* spp., oral organisms, rubber latex.

Introduction

Hevea brasiliensis is a tropical rubber tree belonging to the family Euphorbiaceae. It is extensively cultivated in Southeast Asia for the production of natural rubber. Although numerous plants can synthesize rubber in latex, the *Hevea* rubber tree remains to be the most suitable and viable source of commercial natural rubber. A few hundred milliliters of latex can be obtained from each tree by simply incising the bark, the common practice of tapping the rubber trees. Besides rubber particles that constitute 30–40% of the whole latex volume, the latex contains numerous other non-rubber constituents including proteins that are presently overlooked and discarded as waste in the rubber industry. In our laboratory, we had already purified and characterised several biological-active molecules present in the non-rubber constituents from latex such as β-1,3-glucanase isozymes,[1] a polyphenol oxidase,[2] NAD(P)H quinone reductase[3] and a protease inhibitor.[4] These proteins are involved in the defense mechanisms of the rubber tree against fungal pathogens. In this study, hevein was found to inhibit various *Candida* spp. As the rubber trees are tapped or wounded almost everyday for latex collection, it has been suggested that the rubber tree must be well equipped with antimicrobial compounds to protect itself from any invaders. Defense-related proteins reported to be present in latex include chitinase, β-1,3-glucanase, hevamines, hevein, glucosidase, β-galactosidase, β-*N*-acetyl-glucosaminidase, polyphenol oxidase, and a protease inhibitor.[5,6]

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This has been supported by the finding that whole latex had antifungal activity against a broad spectrum of potential plant fungal pathogens. Such antifungal activity may be related to the presence of glycosidase activities such as α-mannosidase, *N*-acetyl-β-D-glucosaminidase, chitinase, chitinase/lysozyme and β-1,3-glucanase that hydrolyse the polymers in the fungal cell wall.[1,7–9] Another report showed that another substance with antifungal activity was hevein, a major protein component of the B-serum derived from the bottom fraction of centrifuged fresh latex. Hevein was active against several phytopathogenic fungi included *Botrytis cinerea*, *Fusarium culmorum*, *Fusarium oxysporum*, *Phycomyces blakesleeanus*, *Pyrenophora tritici-repentis*, *Pyricularia oryzae*, *Septoria nodorum* and *Trichoderma hamatum*.[10] Hence, the B-serum normally treated as waste could be a great biotechnological resource because of the large quantity produced and its potential for possible use as an antimicrobial agent, a value added product. In this study, we determined the antimicrobial activity of freshly prepared latex serum against various potentially pathogenic oral microorganisms and identified a compound that possesses antimicrobial activity.

Materials and methods

Microorganisms

A total of 49 microbial strains, potential pathogens of the oral cavity and respiratory tract, were tested in this study. These were *Porphyromonas gingivalis* A, *P. gingivalis* W 50, *P. gingivalis* 381, *P. gingivalis* ATCC 33277, *Prevotella intermedia* 25611, *Tannerella forsythia* ATCC 43037, *Aggregatibacter actinomycetemcomitans* ATCC 33384, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus mutans* ATCC 25175, *Lactobacillus fermentum* ATCC 14931, *Candida albicans* ATCC 90028, *C. albicans* ATCC 10231 and 28 clinical isolates. *Candida glabrata* ATCC 90030, *C. glabrata* ATCC 66032, *Candida tropicalis* ATCC 750, *C. tropicalis* ATCC 13803, *C. tropicalis* ATCC 66029, *Candida krusei* ATCC 6258, *C. krusei* ATCC 34135, *Cryptococcus neoformans* ATCC 90112, *C. neoformans* ATCC 90113. Organisms were maintained on 5% blood agar, brain heart agar or Sabouraud dextrose agar as appropriate to their growth requirements.

Preparation of B-serum from fresh latex

Fresh latex was collected from rubber trees of the *H. brasiliensis* species (clone RRIM 600) grown at Songkhla Rubber Research Center, Songkhla, Thailand. The latex was filtered through cheese cloth and centrifuged at 5000 *g* for 15 min at 4 °C to separate the bottom fraction from the latex serum. The bottom fraction was subject to ultracentrifugation at 59 000 *g*, 4 °C for 45 min and four main layers were obtained. The upper white creamy layer consisting mostly of rubber particles was discarded, a thin layer of yellow Frey-Wyssling particles beneath the top layer, the less coloured serum fraction called C-serum and the yellowish bottom fraction containing lutoid particles were kept for antimicrobial activity screening. After rinsing the bottom fraction with isotonic buffer [50 mmol l^{-1} Tris–HCl, pH 7.4 containing 0.9% NaCl (w/v)] to remove any contaminating latex, the lutoid particles in the bottom fractions were burst by freezing and thawing several times until a majority had lysed. This was then centrifuged at 8000 × *g* for 15 min at 4 °C and the brownish aqueous fraction called B-serum was collected. The Frey-Wyssling layer, C-serum and B-serum were screened for antimicrobial activity. The B-serum from the bottom fraction was the only one to show antimicrobial activity.

Purification of an antimicrobial protein from B-serum

B-serum was fractionated by acetone precipitation with increasing concentrations from 0–40%, 40–60% and 60–80% acetone saturation and centrifuged. The pellets were collected and any acetone was removed by blow drying. Pellets of each fraction were dissolved in 20 mmol l^{-1} Tris–HCl, pH 7.4 and were screened for antimicrobial activity. The active fraction was submitted to anion exchange chromatography on a DEAE-Sepharose Fast Flow column equilibrated in 20 mmol l^{-1} Tris–HCl, pH 7.4 and washed with 20 mmol l^{-1} Tris–HCl, pH 7.4 until the A_{280} fell below 0.01. The bound protein was then eluted with a gradient of 0–0.5 mol l^{-1} NaCl in the same buffer. The peak fractions containing antimicrobial activity were pooled, dialysed against water and lyophilised. Protein concentrations were determined by the Bradford method using bovine serum albumin as standard.[11]

N-terminal amino acid sequencing

The 11 amino acids of the NH-terminal sequence were determined by Edman degradation using the Applied Biosystems-Procise HT Instrument (Mayo Proteomic Research, Minneapolis, MN, USA).

Antimicrobial assay of antimicrobial protein

The broth microdilution method

For determining the minimal inhibitory concentration (MIC) values, bacteria were grown to stationary phase in Brain Heart Infusion (BHI) broth under aerobic or anaerobic conditions, according to the species being tested. Each cell suspension was adjusted to approximately 10^6 CFU ml^{-1}. For yeast, MIC testing was performed using a modified broth microdilution method following that of the National Committee for Clinical Laboratory Standards.[12] Yeasts were grown on Sabouraud dextrose agar (SDA) and incubated at 35 °C for 24 h. The inoculum was prepared by picking five colonies from these culture plates and suspending them in RPMI medium pH 7.0, the cell density was adjusted to the density of a 0.5 McFarland standard unit at 530 nm, which resulted in a yeast stock suspension of 1×10^6 to 5×10^6 cells ml^{-1}. A working suspension was prepared by diluting this 1 : 1000 with appropriate medium to obtain 1×10^3–5×10^3 CFU ml^{-1}. The broth microdilution test was performed by using 96 well plastic flat bottom plates with covers. Then, 50 µl of sample was added and serially diluted by two fold dilutions to appropriate concentrations. A control was included for the determination of the growth of each species in the absence of antimicrobial protein. 50 µl of working inoculum was added to 100 µl final volumes. After inoculation, plate was incubated at 35 °C for 24 h. The optical density at 620 nm (OD_{620}) was measured with a microplate reader. The OD of the blank wells were subtracted from the OD of the inoculated wells.

For a growth inhibition plot, *Candida* spp. were mixed with RPMI 1640 medium and hevein at various concentrations and incubated for 24 h. The growth inhibition was represented as a percentage of the growth inhibition of the control cultures.

The disk diusion assay

This was performed as described previously[13] with several modifications. Briefly, SDA was autoclaved and cooled to 45 °C. A suspension of *C. albicans* ATCC 10231 and *C. tropicalis* ATCC 750 in RPMI 1640 medium was added to a concentration of 1×10^4 organisms ml^{-1} and 20 ml portions of the warm liquid agar suspension were added to a Petri dish. Sterile paper discs were placed on the solidified agar, and 40 µl of diluted sample preparations was added to each disc, and the plates were incubated overnight at 37 °C. Clear zones of fungal growth were visualised around the discs containing inhibitory concentrations of antimicrobial proteins.

The disk diffusion assay was similarly employed for investigating the effect of a *Hevea* proteinase inhibitor (HPI) on the antifungal activity of hevein. The HPI was partially purified, as an 80–95% acetone precipitated fraction from the C-serum isolated from ultracentrifuged fresh latex, according to the method described by Sritanyarat *et al.* [4]. Hevein at 40 µg was mixed with either 200 or 400 µg HPI before adding it to each paper disc. The resulting clear zones of fungal growth were compared to those of the controls containing either hevein (40 µg) or HPI (400 µg) alone.

Aggregation analysis

Candida tropicalis ATCC 750 was used in this experiment. The experiments were carried out as previously described.[14] Briefly, the yeast cells were suspended in calcium-binding buffer (CBB): 20 mmol l^{-1} Tris–HCl, pH 6.75, 0.5 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} $MgSO_4$, 0.1 mmol l^{-1} DTT, 154 mmol l^{-1} NaCl, (or CBB containing 10 mmol l^{-1} EDTA) and diluted to the required absorbance at 620 nm (A_{620}). Appropriate concentrations of hevein or buffer were added to the final volume of 800 µl for all samples. After protein supplementation, the A_{620} of each sample was measured every minute for 2 h. Aggregation is recorded by a decreasing A_{620} greater than that observed in the negative control (without protein addition) as the aggregated material collected at the bottom of the assay tube as sedimentation.

Results

Purification and characterisation of protein that inhibits microbial growth

B-serum was fractionated by precipitation of protein with increasing concentrations of acetone, 0–40%, 40–60% and 60–80%. Each fraction was assayed for antimicrobial activity by the broth microdilution method. Only the 60–80% acetone fraction inhibited the growth of the microbes tested as shown by the MIC values (Table 1). *C. albicans* ATCC 90028 was the most sensitive microbe with an MIC 112 µg ml^{-1} protein but *C. neoformans*, the other fungus tested at this time, was not sensitive at 1.8 mg ml^{-1}. All Gram-negative bacteria tested except the *P. aeruginosa* species had MIC values of 896 µg ml^{-1} protein. However, the *Pseudomonas* and Gram-positive bacteria were not affected by 1.8 mg ml^{-1} protein from this fraction. When this 60–80% acetone fraction was tested against 28 clinical isolates of *C. albicans*, the average MIC of those isolates was 91.3 ± 40.45 µg ml^{-1} and two standard strains of

Table 1 Screening of a crude 60–80% acetone fraction from the *Hevea brasiliensis* against various potential microbial pathogens of the oral cavity and respiratory tract

Microbial	MIC (μg ml^{-1})
Gram-negative bacteria	
Porphyromonas gingivalis A	896
Porphyromonas gingivalis W50	896
Porphyromonas gingivalis 381	896
Porphyromonas gingivalis ATCC 33277	896
Prevotella intermedia 25611	896
Tannerella forsythia ATCC 43037	896
Aggregatibacter actinomycetemcomitans ATCC 33384	896
Pseudomonas aeruginosa ATCC 27853	>1800
Gram-positive bacteria	
Streptococcus mutans ATCC 25175	>1800
Lactobacillus fermentum ATCC 14931	>1800
Fungi	
Candida albicans ATCC 90028	112
Cryptococcus neoformans ATCC 90112	>1800
Cryptococcus neoformans ATCC 90113	>1800

Controls were cell suspensions added to broth without hevein.

C. albicans ATCC 10231 and ATCC 90028 had MIC of 56 and 112 μg ml^{-1} respectively. Thus, *C. albicans* ATCC 10231 was used for the rest of the study to identify the active substance during further purification of the 60–80% acetone fraction. This first step using DEAE-Sepharose Fast Flow chromatography separated a protein with antimicrobial activity. The active fractions were pooled and contained a homogenous protein of 0.3 mg of purified active protein per 1 l of fresh latex. The mass spectrum of the active fraction was determined by MALDI-TOF mass spectrometry and exhibited major peaks corresponding to an M_r value of 4717 Da (data not shown). The N-terminal sequence (11 residues) was analysed and showed 100% similarity to the hevein sequence (Fig. 1) that had been previously reported.[15] Previously, the antifungal activity of hevein had been examined by a hyphal extension-inhibition assay in which it had shown potent antifungal activity against several plant pathogenic fungi.[10] In this study, the antimicrobial activity of hevein was investigated with other ATCC strains of *Candida* spp. including *C. albicans* ATCC 10231 and ATCC 90028, *C. tropicalis* ATCC 750, ATCC 66029 and ATCC 13803, *C. krusei* ATCC 34135 and ATCC 6258 and *C. glabrata* ATCC 90030 and ATCC 66032. The concentration of protein required for 80% growth inhibition (MIC_{80}) was used as a measure of the inhibitory potency of hevein on these fungi. Hevein was able to inhibit the growth of *C. tropicalis* strains in suspension cultures at as little as 12 μg ml^{-1}. Although *C. tropicalis* strains were the most sensitive species of *Candida* tested, strains of *C. albicans*, and *C. krusei* were quite sensitive with strains of *C. glabrata* being the least sensitive with MIC values of >190 μg ml^{-1} (Fig. 2).

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CLUSTAL W (1.82) multiple sequence alignment

hevein      EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKD 43

unknown     EQCGRQAGGKL---------------------------------- 11
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Figure 1 N-terminal sequence analysis of hevein purified in this study showing that its first 11 residues had 100% identity to hevein previously isolated.[15]

The disk diffusion assay showed inhibition of growth of *C. tropicalis* ATCC 750 (Fig. 3a1) and *C. albicans* ATCC 10231 (Fig. 3a2) by hevein, added to the disc at a concentration of as little as 125 μg ml^{-1} (5 μg per disc). At each concentration, the inhibition zone was bigger with *C. tropicalis* ATCC 750.

An enhancing effect of HPI on the antifungal activity of hevein was also revealed from the clear zone diameter size obtained upon combination with hevein (Fig. 3b). The HPI (at 400 μg) when tested alone showed no antifungal activity as no clear zone could be seen from disc 2. However, when it (at either 200 or 400 μg) was combined with 40 μg of hevein, the clear zone diameter obtained (discs 4 or 5) was significantly bigger than that of the hevein control, disc 3.

The interaction with hevein and *C. tropicalis* ATCC 750 yeast cells was investigated. We performed aggregation experiments using purified hevein. Hevein was added to the diluted yeast cells and the aggregation was tested by measuring changes in absorbance at 620 nm as a function of time. This provided a method for evaluating the interaction of hevein under a variety of conditions. The addition of hevein (30 μg ml^{-1}) resulted in a decrease in absorbance owing to the aggregation of yeast cells to form sediment in the presence of 0.5 mmol l^{-1} $CaCl_2$. This effect was inhibited by the addition of 10 mmol l^{-1} EDTA. Binding of hevein to yeast cells is therefore calcium dependent because the addition of EDTA completely inhibited the aggregation (Fig. 4).

Discussion

Hevein was previously shown to have antifungal activity against various plant pathogenic fungi.[10] In our study, this is the first testing of its activity against various commensals and potential pathogens of the oral cavity and respiratory tract. The inhibitory effect of a pure sample obtained by a new procedure, that was far more simple than the previously described methods,[10]

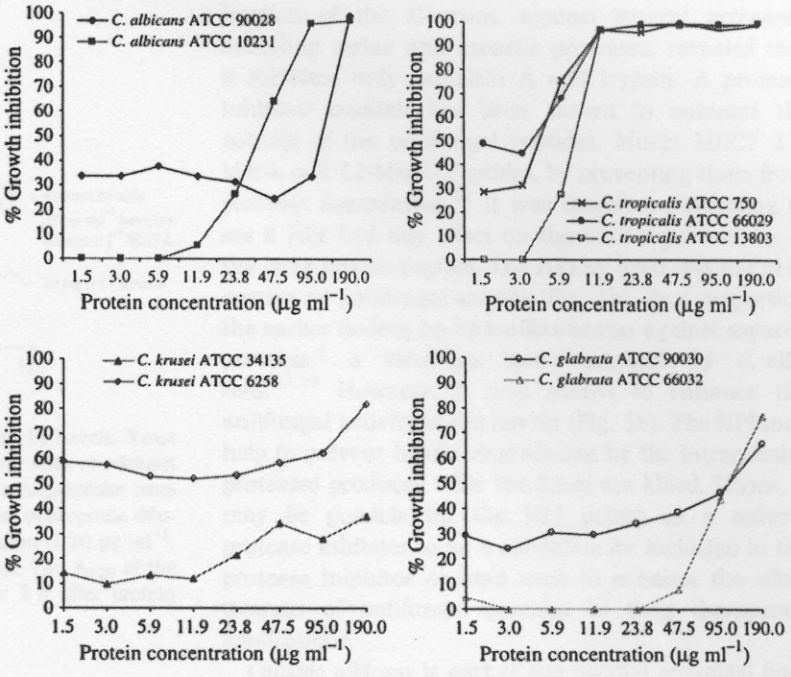

Figure 2 Antimicrobial activity of purified hevein. The percentage of growth inhibition was measured at varying concentrations of hevein with the following test organisms: *Candida albicans* ATCC 90028 (♦), ATCC 10231 (■), *C. tropicalis* ATCC 750 (*), ATCC 66029 (●), ATCC 13803 (□), *C. krusei* ATCC 34135 (▲), ATCC 6258 (◇), *C. glabrata* ATCC 90030 (○), ATCC 66032 (△). The growth inhibition is represented as a percentage of the growth of the control cultures.

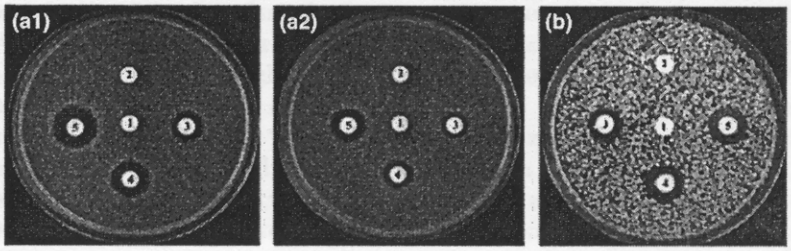

Figure 3 (a) Growth inhibition (clear zone) of hevein against *Candida tropicalis* ATCC 750 (a1) and *C. albicans* ATCC 10231 (a2) and (b) The enhancing eect on antifungal activity of hevein against *C. albicans* ATCC 10231 by *Hevea* protease inhibitor (HPI). Discs in both A1 and A2 contained 40 μl hevein solution added at dierent concentration, where disc 1 is the negative control containing 40 μl of 20 mmol l^{-1} Tris–HCl buer, disc 2–5 contained 40 μl of hevein solution at 25, 125, 250, 750 μg ml^{-1} or 1, 5, 10 and 30 μg per disc respectively. For the discs in C, disc 1, negative control containing 40 μl of 20 mmol l^{-1} Tris–HCl buer; disc 2, 400 μg of HPI; disc 3, 40 μg of hevein; disc 4–5, 40 μg of hevein combined with 200 and 400 μg of HPI respectively.

showed a strong inhibition against *Candida* spp. (MIC range 12–190 μg ml^{-1}) but less for *C. krusei* ATCC 34135 and *C. glabrata* ATCC 90030 with MIC (>190 μg ml^{-1}). It was much less active against oral Gram-negative species (MIC 896 μg ml^{-1}) and inactive against Gram-positive species at concentrations of 1.8 mg ml^{-1}. Hevein is one of the major proteins in lutoids, which are small vacuole-derived organelles.[16] Lutoids contain proteins encoded by defense- or stress-related genes.[17] This indicates that defense is one of the functions of laticifers. Hevein is accumulated more than 10-fold in the latex when compared with the leaf tissues.[18] The antifungal property of hevein has been investigated against potential human pathogenic fungi including *C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata*. The structural components of the cell wall of these fungi are mainly chitin, (1, 3)β-D-glucan, (1,6)β-glucans, lipids, and peptides embedded in a protein matrix.[19] Hevein is a small chitin-binding protein that targets the chitin of the pathogenic fungal cell wall. An estimate of the fungal wall pore size predicts that proteins larger than 15–20 kDa will not pass through the fungal wall.[20] Thus, hevein with a molecular size of 4.7 kDa could penetrate the fungal cell wall matrix. As

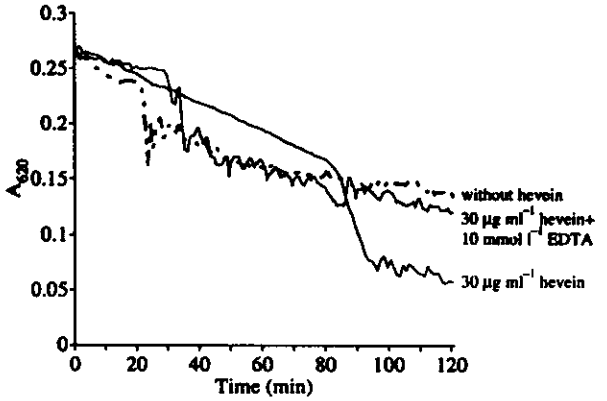

Figure 4 *Candida tropicalis* ATCC 750 aggregated by hevein. Yeast cells were suspended in calcium-containing buer with or without 10 mmol l^{-1} EDTA at room temperature. The suspensions were monitored at absorbance 620 nm (A_{620}) to the appropriate dilution and hevein was added to a final concentration of 30 μg ml^{-1}. Buer was added to the negative control sample. The A_{620} of the suspensions was monitored every minute for 2 h after protein supplement.

can be seen from the aggregation experiment, within 2 h of mixing, hevein caused a Ca^{2+}-dependent aggregation of *C. tropicalis*. Aggregation of the fungal cell was inhibited by EDTA. We assume that aggregation was because of the penetration of hevein through the protein matrix of the cell wall to bind to the chitin embedded in the protein matrix in the presence of Ca^{2+}. This binding then led to a modification of the wall to induce aggregation and finally death of the fungus within 24 h.

Our quantitative analyses of the growth-inhibitory effect of hevein gave some inconsistent results as shown by the broad distribution of MIC obtained against *C. albicans*, ranging from 12 to 95 μg ml^{-1} for strain ATCC 10231 and from 23 to 190 μg ml^{-1} for strain ATCC 90028 using different isolation and testing procedures (data not shown). For the disk diffusion assay, a higher concentration of hevein was required for growth inhibition (Fig. 3). The differences observed could be because of the different times of sample preparation. Different cultivation conditions, different protocols, or the different strains used had effects on the MIC values; for example, the test by the disc diffusion assay yielded higher MIC than those obtained by the broth microdilution method with the same material. This may also reflect different arrangements of the wall components. MIC values are therefore dependent on the choice of protocol.

A protease inhibitor (HPI) has recently been purified and characterised from *Hevea* latex in our laboratory.[4] The assay of HPI, isolated from an 80% to 95% acetone fraction of the C-serum, against several proteases, including serine and aspartic proteases, revealed that it inhibited only subtilisin A and trypsin. A protease inhibitor cocktail has been shown to enhance the activity of the antifungal peptides, Mucin MUC7 12-Mer-L and 12-Mer-D Peptides, by preventing them from protease degradation.[21] It was therefore interesting to see if HPI had any effect on the antifungal activity of the latex hevein peptide. The HPI by itself was found to possess no antifungal activity (Fig. 3b), thus supporting the earlier finding on its ineffectiveness against aspartic protease,[4] a virulence factor secreted by *C. albicans*.[22,23] However, it was shown to enhance the antifungal activity of the hevein (Fig. 3b). The HPI may help to prevent hevein degradation by the intracellular proteases produced after the fungi are killed. Hence, it may be possible for the HPI acting as a natural protease inhibitor to be a candidate for inclusion in the protease inhibitor cocktail used to enhance the effectiveness of antifungal peptides in their therapeutic applications.

Candida albicans is part of the normal microbial flora of the human host and can be found in the digestive, oral cavity and vaginal tracts[24] and is a major human opportunistic pathogen, causing both mucosal and systemic infections called candidiasis, especially in immunocompromised patients.[25,26] However, an increased prevalence of candidiasis has been attributed to the widespread use of antibiotics and immunosuppressive agents.[27] At present, synthetic antifungal drugs are available for the treatment of *Candida* infections some having very serious side effects to immunocompromised and AIDS patients who suffer from immune dysfunction. In the future, it may be necessary to seek out and test naturally occurring antifungal substances to treat these patients. However, as hevein is a known allergen (Hev b 6.02), its therapeutic application may be limited. Further work will be required to investigate this possibility. Ability to modify the hevein to eliminate its allergenicity without loosing its antifungal activity would obviously create more opportunities for the use of this biologically active 'waste protein'.

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