Antifungal activity of Histatin-5 against non-*albicans Candida* species.

Abstract

Fungicidal effects of Histatin-5 against 26 oral isolates belonging to 5 non-albicans *Candida* species were examined. Fifty μM of Histatin-5 killed more than 95% of *C. tropicalis* and *C. guilliermondii* isolates, and more than 90% of *C. parapsilosis* and *C. krusei.* However, and *C. glabrata* were less sensitive to the peptide (mean 62.9%). Our results, taken together, demonstrated that Histatin-5 possessed the fungicidal activity against *Candida* species, other than *C. glabrata.*

The AIDS epidemic, improved life-sustaining therapy, and aggressive anticancer therapy have contributed to the rise in the number of severly immunocompromised patients (3). This has led to an increase in oral and systemic fungal infection, and the concominant increased usage of antifungal agents for prophylaxis is most likely the main cause of the development of antifungal drug resistance (20).

Recently, the importance of non-albicans *Candida* species in the candidal infection and candidaemia has been reported(5,10,21). Chakrabarti et al. (1996) has suggested that non-albicans *Candida* spp. with fluconazole resistance have become more prominent in recent years(1). In addition, unfortunately, most of currently available antifungal drugs, such as polyene and azole antimycotics, have undesirable toxic and other side effects (16). Therefore, the search for more effective but less toxic anticandidal theurapeutic agents cannot be overemphsized. Peptide antibiotics, which are believed to interact with the microbial membrane leading to the disruption of cellular integrity and cell death, may be a promising new class of antifungal agents (3).

Histatins are a group of small, cationic antifungal peptides present in human saliva (11,12) They were also detected in human serum (7). In saliva, secretary IgA, lactoferrin (8,9), lysozyme (19), and histatins (12) have all been demonstrated to exert anticandidal activities. Among these molecules, histatins are most likely to be the protein responsible for the in vitro salivary candicidal activity (4). In fact, among several protective proteins in saliva, only the histatin concentration decreased (statistically significant decrease) in the saliva of AIDS patients who developed candidiasis (6). The decrease in histatin concentration in the saliva of AIDS patients (4) may partially explain why more than 70% of AIDS patients develop oral candidiasis over the course of the disease (17). Since histatins are naturally occuring molecules they are considered to be nontoxic to mammalian cells (18). This property and the

fungicidal potencies make histatins promising natural therapueic or preventive agents against fungal infection. Among the three major histatins (Hsn-1, -3 and -5), Hsn-5 is the most potent peptide in killing *Candida albicans* isolates (22).

However, little is known about the antifungal activity of this peptide agaist non-*albicans Candida* species from oral cavity. Thus, in the present study, we analysed the fungicidal potential of Hsn-5 against 26 isolates of *Candida* spp. representing 5 species.

Four isolates of *C. guilliermondii* (GU1, GU2, GU3, GU4), 8 of *C. glabrata* (GL1, GL2, GL3, GL4, GL5, GDH 1306, GDH 1407, GDH 2269), 4 of *C. parapsilosis* (P1, P2, P3, P5), 4 of *C. krusei* (BOX1, CK2, CK14, CK 16) and 6 of *C. tropicalis* (T1, T2, T3, T5, GDH 1362, ATCC13803) were used in the present study. All the isolates, other than ATCC strain were oral isolates gifted from Prof. Samaranayake, and identified by sugar assimilation test using the API 20C system (API Products, Biomeroux, Lyon, France) and "germ tube" test (15) in Oral Biology Unit of Hong Kong University.

A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250 mM glucose and grown aerobically at 37 °C. After 18 h incubation, the yeast was harvested in the mid exponential growth phase, washed twice with PBS containing 1 mM phosphate (pH 6.8) and resuspended to a final concentration using a haemocytometer. All yeast cells suspended, remained in the blastospore phase.

Hsn-5 (DSHEKRHHGYKRKFHEKHHSHRGY) was purchased from Sigma Chem. Co. .(St Louis, MO, USA).

Antifungal activity of Hsn-5 was examined according to the method of Edgerton et al. (1998) with some modifications (2). Fungicidal assays were performed on exponential *Candida* cells in the presence or absence of 50 μM of Hsn-5. Briefly, *Candida* cells were washed twice with 1 mM sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4; pH6.8), and resuspended at 1.8 x 10^5 cells/ml. Twenty microliter of cell suspensions were mixed with 20 μl of 1 mM phosphate buffer containing the indicated protein and incubated for 90 min at 37 °C with shaking. Control tubes were incubated with 20 μl of 1mM phosphate buffer alone.

The reaction was stopped by addition of 360 μl of yeast nitrogen base (YNB); 40 μl of the suspension (containing 360 cells) were spread onto Sabouraud dextrose agar plates and incubated for 48 h at 37 °C.

The assays were carried out on two independent occasions with quadruplicated samples on each occasion. Loss of viability was calculated as [1 - (colonies from suspension with peptides/colonies from suspension with no protein)] $\underline{x}$ 100 (2).

x; a multiplication sign, not [ex].

All of the numerical data obtained here were analysed by an one-way analysis of variance (ANOVA) and subjected to Tukey's multiple range test.

Hsns are present in saliva of healthy adults at concentrations of 50 - 425 μg/ml (2). Hsn-5 is the most potent candicidal member of the family that kills pathogenic *Candida* species from 90 to 100% at physiological concentrations (15 - 30 μM)(13).Indeed, Edgerton et al. (1998) has examined the antifungal activities of histatins, including Hsn-5, and shown that concentration of Hsn-5 (31.25 μM) was required to kill more than 95% of *C. albicans* isolate derived from palate of denture stomatitis patients (2). Thai and Bobek (1997), and Helmerhorst et al. (1999), employed the range of concentration of Hsn-5 from 0 to 100 μM (3,18). In the preliminary study, loss of viability of isolates of *Candida* caused by Hsn-5 gradually increased at the concentration of 25-50 μM, and plateaued at 50-100 μM of peptide (data not shown). Thus, we employed 50 μM Hsn-5, to assess the fungicidal effects. At the concentration, *C. tropicalis* (ranged 91.4± 4.7 % to 100± 0 %; mean 97.7%) and *C. guilliermondii* (92.2± 1.7% to 99.6± 0.8%; mean 97.1%) was most sensitive to Hsn-5, followed by *C. parapsilosis* (74.1±7.4% to100±0%; mean 93.0 %) and *C. krusei* (82.0±4.1 % to 98.2±1.1%; mean 90.8%), and *C. glabrata* (44.4±10.6% to 87.5±2.37%; mean 62.9%) was significantly resistant to Hsn-5 (ANOVA; $p<0.01$; Fig. 1). The latter finding is consistent with the results of several studies (3,4,18). While several possible mechanisms of Hsn-induced killing of *C. albicans* has been proposed, the detail of the mechanisms is still unknown. Thus further studies on the mechanisms of the peptide should solve the problem of the peptide which is less effective against *C. glabrata.*

In conclusion, our results, taken together, demonstrated the therapeuic potentials of Hsn-5 against the infection by *Candida* species, though the antifungal effects against isolates of *C. glabrata* were relatively low. Further trials to improve the fungicidal potential of basic peptides should be required.

ACKOWLEDGEMENT

This study is supported by Grant-in-Aid 11470415

References

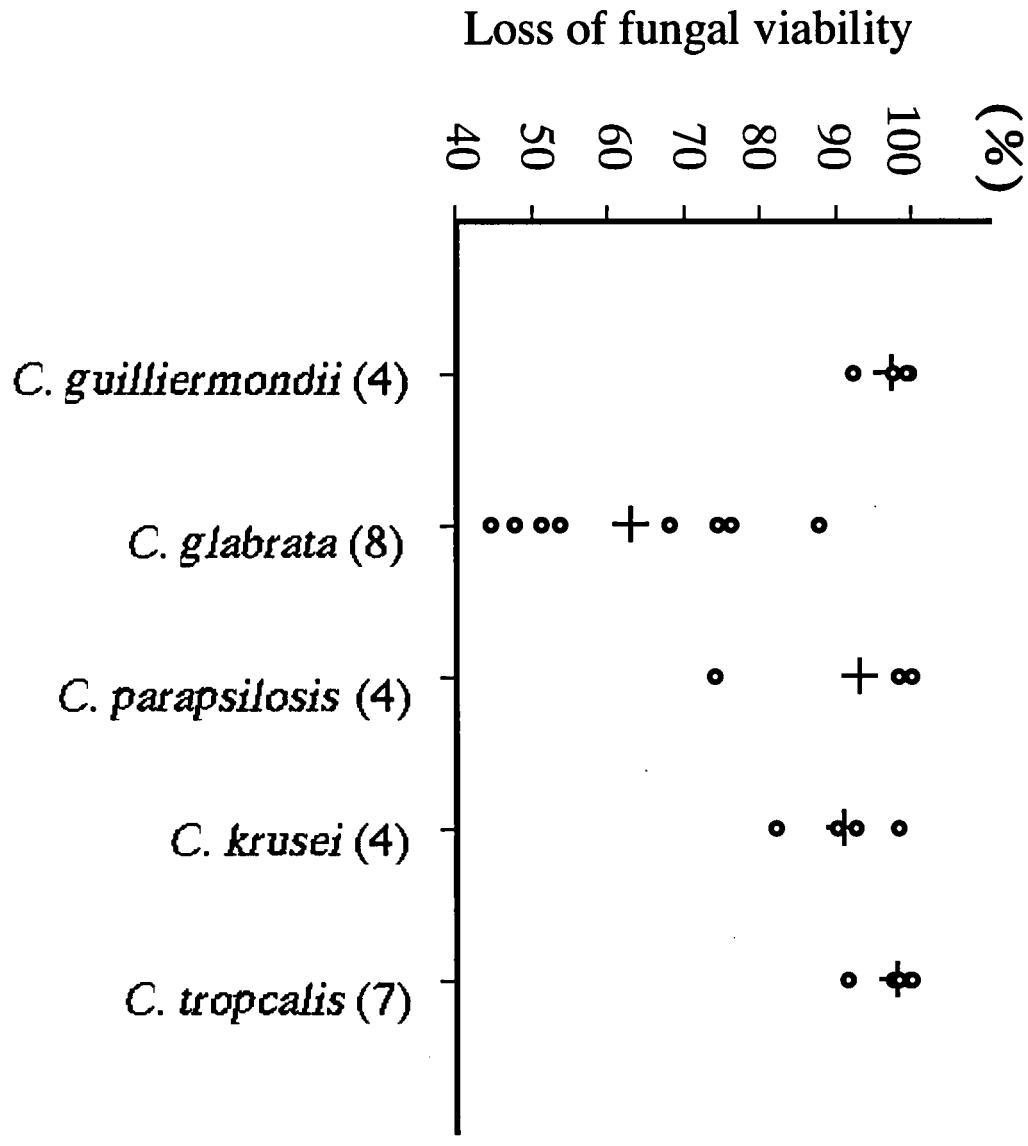
1. Chakrabarti, A., A. Ghosh, R. Batra, A. Kaushal, P. Roy, and H. Singh.1996. Antifungal susceptibility pattern of non-albicans *Candida* species and distribution of species isolated from Candidaemia cases over a 5 year period. Indian. J. Med. Res. **104:**171-176.
2. Edgerton, M., S.E. Koshlukova, T.E. Lo, B.G. Chrzan, R.M. Straubinger, and P.A. Raj. 1998. Candidacidal activity of salivary histatins. Identification of a histatin 5-binding protein on *Candida albicans*. J. Biol. Chem. **273:** 20438-20447.
3. Helmerhorst, E.J., I.M. Reijnders, W. van't Hof, I. Simoons-Smit, E.C.Veerman, and A.V. Amerongen. 1999. Amphotericin B- and fluconazole-resistant *Candida* spp., *Aspergillus fumigatus*, and other newly emerging pathogenic fungi are susceptible to basic antifungal peptides. Antimicrob. Agents Chemother. **43:** 702-704.
4. Lal, K., R.P. Santarpia, L. Xu, F. Manssuri, and J.J. Pollock.1992. One-step purification of histidine-rich polypeptides form human parotid saliva and determination of anti-candidal activity. Oral Microbiol. Immunol. **7:** 44-50.
5. Levy, I., L.G. Rubin, S. Vasishtha, V. Tucci, and S.K. Sood. 1998. Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. Clin. Infect. Dis. **26:**1086-1088.
6. Mandel, I.D., C.E. Barr, and L. Turgeon. 1992. Longitudinal study of parotid saliva in HIV-1 infection. J. Oral Pathol. Med. **21:**209-213.
7. Murakami, Y., H. Nagata, S. Shizukuishi, K. Nakashima, T. Okawa, M. Takigawa, and A. Tsunemitsu. 1994. Histatin as a synergistic stimulator with epidermal growth factor of rabbit chondrocyte proliferation. Biochem. Biophys. Res. Commun .**198:**274-280.
8. Nikawa, H., L.P. Samaranayake, J. Tenovuo, K.M. Pang, and T. Hamada. 1993. The fungicidal effect of human lactoferrin on *Candida albicans* and *Candida krusei*. Arch. Oral. Biol. **38:**1057-1063.
9. Nikawa, H., L.P. Samaranayake, J. Tenovuo, and T. Hamada. 1994. The effect of antifungal agents on the in vitro susceptibility of *Candida albicans* to apo-lactoferrin. Arch. Oral. Biol. **39:** 921-923.
10. O'Connell, B., D.C. Coleman, D. Bennett, D. Sullivan, S.R. McCann, and C.T. Keane. 1995. An epidemiological study of *Candida* species infection in cancer patients using genetic fingerprinting and morphotyping. J. Hosp. Infect. **31:**211-217.
11. Oppenheim, F.G., T. Xu, F.M. McMillian, S.M. Levitz, R.D. Diamond, G.D. Offner, and R.F. Troxler. 1988. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on

Candida albicans. J. Biol. Chem. **263:** 7472-7477.

12. Pollock, J.J., L. Denepitiya, B.J. MacKay, and V.J. Iacono. 1984. Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptides on *Candida albicans*. Infect. Immun. **44:** 702-707.
13. Raj, P.A., M. Edgerton, and M.J. Levine. 1990. Salivary histatin 5: dependence of sequence, chain length, and helical conformation for candidacidal activity. J. Biol. Chem. **265:** 3898-3905.
14. Rayhan, R., L. Xu, R.P. Santarpia, C.A. Tylenda, and J.J. Pollock. 1992. Antifungal activities of salivary histidine-rich polypeptides against *Candida albicans* and other oral yeast isolates. Oral. Microbiol. Immunol. **7:** 51-52
15. Silverman, Jr. S., C.A. Migliorati, J.B. Epstein, and L.P. Samaranayake. 1990. Laboratory diagnosis of oral candidosis, p. 213 - 237. *In* Samaranayake, L.P. and T.W. MacFarlane (ed.), Oral Candidosis, Butterworth & Co. Ltd., London.
16. Situ, H., and L.A. Bobek. 2000. In vitro assessment of antifungal therapeutic potential of salivary histatin-5, two variants of histatin-5, and salivary mucin (MUC7) domain 1. Antimicrob. Agents. Chemother. **44:** 1485-1493.
17. Sternberg, S. 1994. The emerging fungal threat. Science **266:** 1632-1634.
18. Tsai, H., and L.A. Bobek. 1997. Human salivary histatin-5 exerts potent fungicidal activity against *Cryptococcus neoformans*. Biochim. Biophys. Acta. **1336:**367-369.
19. Tobgi, R.S., L.P. Samaranayake, and T.W. MacFarlane. 1987. The in vitro susceptibility of *Candida* species to lysozyme. Oral. Microbiol. Immunol. **3:** 35-39.
20. White, T.C., K.A. Marr, and R.A. Bowden. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. **11:**382-402.
21. Wingard, J.R. 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. Clin. Infect. Dis. **20:** 115-125.
22. Xu, T., S.M. Levitz, R.D. Diamond, and F.G. Oppenheim. 1991. Anticandidal activity of major human salivary histatins. Infect. Immun. **59:**2549-2554.

Legend to Figure

Fig. 1 Histatin-5 (50μM) induced loss of viability of isolates of *Candida* species.


Loss of fungal viability
40
50
60
70
80
90
100
(%)
C. guilliermondii (4)
C. glabrata (8)
C. parapsilosis (4)
C. krusei (4)
C. tropcalis (7)