ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1997, p. 2224–2228 Vol. 41, No. 10
0066-4804/97/$04.00+0

Studies of the Mechanism of Human Salivary Histatin-5 Candidacidal Activity with Histatin-5 Variants and Azole-Sensitive and -Resistant *Candida* Species

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Received 7 April 1997/Returned for modification 10 June 1997/Accepted 16 July 1997

Histatins are a group of small, cationic, antifungal peptides present in human saliva. A previous molecular modeling analysis suggested structural similarity between the Phe^{14}-His^{15} and His^{18}-His^{19} dipeptide sequences in histatin-5 (Hsn-5; a 24-amino-acid polypeptide) and the sequence of miconazole (one of the azole-based antifungal therapeutic agents), implying that the mechanisms of killing of *Candida albicans* by these two molecules may be similar. To further elaborate on this observation, we have produced two variants of Hsn-5 in which Phe^{14}-His^{15} or His^{18}-His^{19} dipeptide sequences were replaced by Ala-Ala (F14A/H15A and H18A/H19A) to eliminate the phenyl and imidazole rings of the side chains and assessed their candidacidal activities against *C. albicans*. In addition, we tested azole-resistant *C. albicans* and *Candida glabrata* strains for their susceptibilities to Hsn-5. Analysis of the purified recombinant proteins for their candidacidal activities indicated that both variants were significantly less effective (the molar concentrations required to kill half of the maximum number of cells [ED$_{50}$s], ~67 and ~149 μM for F14A/H15A and H18A/H19A, respectively) than the unaltered Hsn-5 (ED$_{50}$, ~8 μM) at killing *C. albicans*, suggesting that the two dipeptide sequences are important for the candidacidal activity of Hsn-5. Assessment of the candidacidal activity of Hsn-5 with the well-characterized azole-resistant strains of *C. albicans* and *C. glabrata*, however, suggested that the mode of action of histatins against *Candida* is distinct from that of azole-based antifungal agents because Hsn-5 kills both azole-sensitive and azole-resistant strains equally well.

With the steady increase in the number of immunocompromised patients during the past decade, there has been a concomitant rise in the frequencies and types of severe fungal infections. The most common AIDS-associated fungal infections are oral and esophageal candidiases, which occur in more than 70% of patients (3, 30). Pseudomembranous candidiasis, the most common form of oral thrush, is seen in 10 to 15% of debilitated, elderly people (20, 22). Increased use of prosthetic devices has also resulted in a rise in fungal infections (3). Candida-associated denture stomatitis affects 25 to 65% of denture wearers (2, 37). The opportunistic pathogens in infected individuals have the potential to spread into the bloodstream, which can carry them into the brain, heart, kidney, eyes, and other tissues (20, 30), where they can cause invasive or even life-threatening infections.

The treatment of candidal infections usually involves antifungal agents which are highly toxic for mammalian cells and which are not suitable for long-term treatment. In addition, many drug-resistant strains are emerging in response to the widespread and prolonged treatments (30). Therefore, the search for more effective but less toxic antifungal therapeutic agents cannot be overemphasized. Histatins (Hsns) are a group of small, cationic, antifungal peptides present in human saliva (21, 23). They were also detected in human serum (16). In saliva, secretory immunoglobulin A, lactoferrin (11, 17), lysozyme (31), and Hsns (23) have all been demonstrated to exert anticandidal activities. Among these molecules, Hsns are most likely to be the proteins responsible for the in vitro salivary candidacidal activity (12). In fact, among the several protective proteins in saliva, only the Hsn concentration decreased (statistically significant decrease) in the saliva of AIDS patients who developed candidiasis (15). The decrease in the Hsn concentration in the saliva of AIDS patients (13) may partially explain why more than 70% of AIDS patients develop oral candidiasis over the course of the disease (30). Since Hsns are naturally occurring molecules, they may be nontoxic to mammalian cells (unpublished data). The lack of toxicity and the candidacidal potencies similar to those of the azole-based antifungal drugs (29) make Hsns promising natural therapeutic agents against fungal infections. Among the three major Hsns (Hsn-1, Hsn-3, and Hsn-5), Hsn-5 is the most potent peptide in killing the blastospore and germinated forms of *Candida albicans* (36). The level of killing of the blastospore form of *C. albicans* can reach 80 to 100% at the physiologic concentration (15 μM) of Hsn-5 (32).

Several mechanisms of antifungal action of Hsns against *C. albicans* have been proposed (see Discussion). One of these postulates that the mechanism of antifungal action of Hsn may resemble those of azole-based antifungal molecules, which act through inhibition of sterol 14α-demethylase, a cytochrome P-450 enzyme involved in the biosynthesis of ergosterol, a major sterol component of the fungal cell membrane. This hypothesis was substantiated by molecular modeling of the Hsn-5 C-terminal 16-amino-acid fragment and one of the azole antifungal drugs, miconazole, which suggested that the structure of Phe^{14}-His^{15} and His^{18}-His^{19} was similar to that of miconazole (26).

In order to determine if the mode of action of Hsns against *C. albicans* is similar to that of azole-based antifungal agents, we constructed two Hsn-5 variants in which Phe^{14}-His^{15} or His^{18}-His^{19} was replaced by Ala-Ala and examined if the replacement of these two dipeptide sequences affected the can-

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didacidal activity of Hsn-5. Furthermore, we have tested the candidacidal activity of Hsn-5 against well-characterized azole-sensitive and azole-resistant *C. albicans* and *Candida glabrata* strains.

MATERIALS AND METHODS

Materials. The pET30b(+) expression system was from Novagen Inc. (Madison, Wis.). The Transformer site-directed mutagenesis kit was from Clontech Inc. (Palo Alto, Calif.). Oligonucleotides were made by the Bio · synthesis Inc. (Lewisville, Tex.). Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Promega Corp. (Madison, Wis.). Phenylmethylsulfonyl fluoride was from Sigma Chemical Co. (St. Louis, Mo.), aprotinin was from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.), and Sabouraud dextrose agar was from Difco Laboratories (Detroit, Mich.).

Strains. The *C. albicans* strain used in the amino acid replacement analysis was a clinical isolate from the palate of a patient with denture-induced stomatitis (D15) that was generously provided by Mira Edgerton (Department of Oral Biology, State University of New York at Buffalo) (24). The azole-sensitive and azole-resistant *C. albicans* isolates (isolates 2-76 and 12-99, respectively) were kindly provided by Theodore C. White (Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, and Seattle Biomedical Research Institute, Seattle, Wash.). Both of these strains were originally isolated by Spencer Redding (School of General Dentistry, University of Texas Health Science Center at San Antonio) from a human immunodeficiency virus-infected patient. The resistant isolate contains the following changes: a point mutation in lanosterol 14α-demethylase (R467K) (34), overexpression of lanosterol 14α-demethylase, overexpression of the efflux pumps, CDR1 (an ABC transporter) and the MDR1 (a major facilitator) (35), alterations in the promoter region of lanosterol demethylase, and a gene conversion in the gene region surrounding lanosterol demethylase (personal communication). The azole-sensitive and -resistant *C. glabrata* strains (strains NCCLS 84 and 65C, respectively) were kindly provided by John E. Bennett (National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

Construction of the pET–Hsn-5 expression plasmid. For the construction of pET–Hsn-5, Hsn-5 cDNA was recloned from the plasmid pGHsn-5 that we constructed previously (32) into an *Escherichia coli* pET-30b(+) expression system (18), as follows: the Hsn-5 coding region was PCR amplified from the pGHsn-5 plasmid with primer P1 (5′-GCGCCATGGATTCACATGCAAAG AGACATC-3′), which annealed to the 5′ end of the coding region, and primer pGEX#4 (5′-TTTCACCGTCATCACCGAAA-3′), which annealed to the pGEX-2T sequence about 50 bp downstream of the Hsn-5 stop codon. An *Nco*I restriction site (CCATGG; underlined in the sequence of primer P1) and a Met codon (ATG; double underlined in the sequence of primer P1) were introduced at the 5′ end of Hsn-5 for cloning and cyanogen bromide (CNBr) cleavage purposes, respectively. PCR was carried out in an automatic thermal cycler for 30 cycles. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 45 s. This was followed by a 10-min incubation at 72°C to elongate the products to their full lengths. About 50 ng of plasmid DNA and 1 μM (each) primer were used in the reaction mixture. The resulting PCR fragment also contained an *Eco*RI site (derived from the pGHsn-5 plasmid) at the 3′ end of the Hsn-5-coding region. The amplified product was analyzed on a 4% low-melting-temperature agarose gel, and the fragment of the correct size was excised and purified by a freeze-squeeze method followed by phenol-chloroform extraction and ethanol precipitation. The purified Hsn-5 PCR fragment was cleaved with *Nco*I and *Eco*RI and was ligated to the *Nco*I-*Eco*RI-digested pET30b(+) vector. This resulted in an in-frame gene fusion between the 3′ end of the His · Tag carrier DNA and the 5′ end of the Hsn-5 cDNA. The ligated DNA was transformed into an *E. coli* BL21(DE-3) proteinase-deficient strain by electroporation. Transformants which expressed the foreign protein upon induction by IPTG, as indicated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), were selected. The plasmid DNAs from the selected transformants were extracted with the Wizard Minipreps plasmid DNA purification kit (Promega), and their nucleotide sequences were determined by using a vector-specific primer (which anneals to the T7 promoter site) and the dideoxynucleotide termination method of Sanger et al. (28).

Site-directed mutagenesis. Two Hsn-5 mutations were created on the basis of the unique site elimination method (4) with the Transformer site-directed mutagenesis kit. For these constructs, the following three primers were made: a selection primer, which eliminates the *Alw*NI site in the pET30b(+) vector, and two mutagenic primers, which introduce codons for Ala-Ala in place of Phe^{14}-His^{15} or His^{18}-His^{19}. The primers were phosphorylated with T4 polynucleotide kinase. The mutagenesis was performed according to the manufacturer's protocol. Briefly, the double-stranded pET30b(+) plasmid was denatured by boiling and was annealed with the selection and mutagenic primers. The second strand was synthesized with T4 DNA polymerase, and gaps were sealed with T4 DNA ligase. The newly synthesized DNA was digested with *Alw*NI and was transformed into the *E. coli* BMH 71-18 *mutS* strain. The plasmid without the *Alw*NI site was not cleaved by the enzyme and was transformed into the cells. The transformed cells were grown overnight at 37°C in an orbital shaker, and then the plasmid DNA was isolated. The plasmid DNA was again digested with *Alw*NI, to further eliminate the unaltered plasmid, and was transformed into an *E. coli* BL-21(DE3), a proteinase-deficient strain. Several transformants were picked and screened for the desired mutations in the Hsn-5 sequence by DNA sequencing by the method of Sanger et al. (28).

Production of recombinant proteins. The pET–Hsn-5 expression vector expresses the recombinant protein under the control of the T7 promoter and *lac* operator. Upon induction with IPTG, Hsn-5 was expressed as a fusion protein with a carrier protein containing six histidine residues (His · Tag) at the N-terminal end of Hsn-5. The expression of His · Tag carrier–Hsn-5 facilitates the purification of the recombinant protein by Ni^{2+} affinity chromatography. An overnight cell culture (20 ml) of BL21(DE-3) containing a pET-Hsn-5, pET-F14A/H15A, or pET-H18A/H19A plasmid was added into a 1 liter of L broth containing 30 μg of kanamycin per ml and was grown at 37°C to an optical density at 600 nm of ~0.8. IPTG (1 mM) was then added to induce the expression of the fusion proteins, and cultures were allowed to grow for an additional 2.5 h. Bacteria were then harvested by centrifugation.

For the initial purification of recombinant fusion proteins, the affinity chromatography method described in the pET system manual (18) was followed, with some modifications. Briefly, the bacteria were washed once with 50 mM Tris buffer (pH 8.0) and were then resuspended in a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris buffer [pH 7.9]) containing 1 mM phenylmethylsulfonyl fluoride and lysozyme. The solution was sonicated with a microtip and then centrifuged. The fusion recombinant proteins were then purified from the supernatant with a His · Bind metal chelation resin. The fusion proteins were eluted with an elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris buffer [pH 7.9]) (see Fig. 1A). The eluent was dialyzed against water and then lyophilized.

The His · Tag carrier–Hsn-5 fusion and the two variant fusion proteins were purified further. They were first treated with CNBr to remove the carrier protein (~5 kDa). The samples were then passed through a DE-52 anion exchanger by using 10 mM Tris buffer (pH 8.0) to remove the neutral and acidic peptides generated by CNBr cleavage of the carrier protein. Final purification of Hsn-5 and the two variants was achieved by reversed-phase high-pressure liquid chromatography (RP-HPLC) as described previously (32).

Cationic PAGE. Cationic PAGE was performed essentially by method of Baum et al. (1) and MacKay et al. (14), with some modifications. Briefly, the sample loading buffer contained 20% glycerol instead of sucrose. After electrophoresis, the gel was stained with 0.5% Coomassie blue R-250 in 50% methanol–10% acetic acid, followed by fixation in 50% methanol–10% acetic acid and destaining in 10% methanol–10% acetic acid.

Amino acid analysis and CD analysis. Amino acid analysis and circular dichroism (CD) analysis of purified Hsn-5 and the variants were both performed as described previously (32).

Anticandidal assay. The blastosporicidal activities of recombinant Hsn-5 (reHsn-5) and its variants were determined by incubation of twofold serially diluted recombinant proteins with an equal volume of *C. albicans* DIS or *C. glabrata* (10^5 organisms/ml) at 37°C for 1.5 h as described previously (32). For comparison of the candidacidal activity of Hsn-5 against the azole-sensitive and -resistant strains, unaltered reHsn-5 was used and the assay was performed as described previously (32). The molar concentration of peptide required to kill half of the maximum number of cells (ED_{50}) was determined by the procedure PROBIT (SPSS software package, release 4.1, for VAX/VMS). Differences in candidacidal activity between reHsn-5 and its variant at each concentration were assessed by analysis of variance by use of the StatView SE + Graphics software package on a Macintosh computer.

RESULTS

Cloning, expression, and purification of Hsn-5 and its variants in pET30b(+). Previously, we have produced several Hsn-5 variants in *E. coli* using the expression vector pGEX-2T and showed that the fusion proteins were expressed in large quantities and in a soluble form in the *E. coli* cytoplasm (32). However, the final yields of the purified proteins were only in the range of 100 to 200 μg from 1 liter of bacterial culture. Consequently, other expression systems were assessed for the production of Hsn-5. pET30b(+) turned out to be a much more efficient Hsn-5 producer than pGEX-2T. Using this vector, we were able to obtain ~1 mg of the purified recombinant protein from 1 liter of bacterial culture. Additionally, we were able to produce Hsn-5 that is unaltered at the N-terminal amino acid residue (see Materials and Methods).

In this study, the Phe^{14}-His^{15} and His^{18}-His^{19} dipeptide sequences were selected for mutagenesis because, as described in the introduction, their structures mimic that of a currently available antifungal therapeutic agent, miconazole. The dipeptide sequence His^{7}-His^{8} was not selected for mutagenesis be-

TABLE 1. Amino acid sequences and candidacidal activities of reHsn-5 and its variants

Peptide	Sequence[a]	ED_{50} (μM)[b]
reHsn-5	DSHAKRHHGYKRKFHEKHHSHRGY	8 (6.9–10.8)
F14A/H15A	----------AA---------	67 (42–142)
H18A/H19A	----------AA-----	149 (88–400)

[a] The amino acid sequences begin at position 1 and end at position 24.
[b] The results are based on three separate experiments, each of which was run in triplicate. Values in parentheses represent the 95% confidence limits of the ED_{50}.

cause it falls in the N-terminal region of the Hsn-5 sequence, which is presumably irrelevant to the candidacidal activity of Hsn-5 (24). The peptide sequences of reHsn-5, F14A/H15A, and H18A/H19A are presented in Table 1. The junction between the His · Tag carrier and the foreign sequences, as well as the sequence of the full-length Hsn-5 cDNA and its variants, were confirmed by DNA sequencing.

The constructs were expressed in the host *E. coli* BL-21 (DE3), a proteinase-deficient strain, and the three recombinant proteins were purified as described in Materials and Methods. Each protein was finally purified by two to three rounds of RP-HPLC to ~99% purity, as indicated by the peak integration at 230 nm (data not shown). The purity of each protein was also shown by SDS–15% PAGE and cationic PAGE analysis (Fig. 1B and 1C, respectively). The amino acid composition of each molecule was analyzed and was in good agreement with the predicted value. The concentration of each recombinant peptide was also determined by amino acid analysis. The molecular weight of the purified reHsn-5 and one of the variants (F14A/H15A, used as an example) was determined by mass spectral analysis (Bio · synthesis Inc.). The values that were obtained were in good agreement with the calculated values.

Candidacidal activities of reHsn-5, F14A/H15A, and H18A/H19A. The candidacidal activity of reHsn-5 produced in the pET–Hsn-5 expression plasmid was similar to that of the native Hsn-5 reported previously (24). The candidacidal activities of the two variants were compared with that of reHsn-5 at different protein concentrations. The results are summarized in Fig.

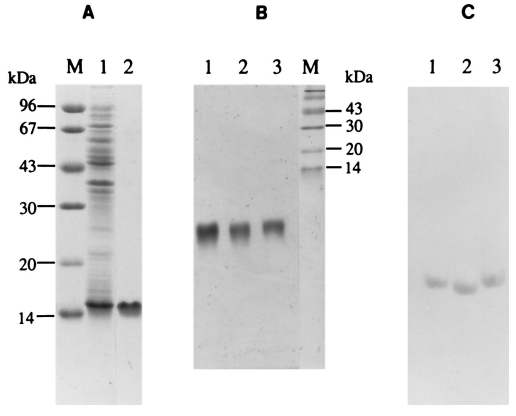

FIG. 1. Analysis of reHsn-5, F14A/H15A, and H18A/H19A. (A) SDS–15% PAGE. Lane M, molecular size marker; lane 1, proteins present in the supernatant of the *E. coli* BL-21(DE3) lysate containing IPTG-induced His · Tag carrier–Hsn-5 fusion protein; lane 2, the eluent from the Ni^{2+} affinity chromatography containing His · Tag carrier–Hsn-5. (B and C) SDS–15% PAGE and cationic PAGE (15%) analysis, respectively, of purified proteins after CNBr cleavage and RP-HPLC. Lanes 1, reHsn-5; lanes 2, F14A/H15A; lanes 3, H18A/H19A; lane M, molecular weight marker.

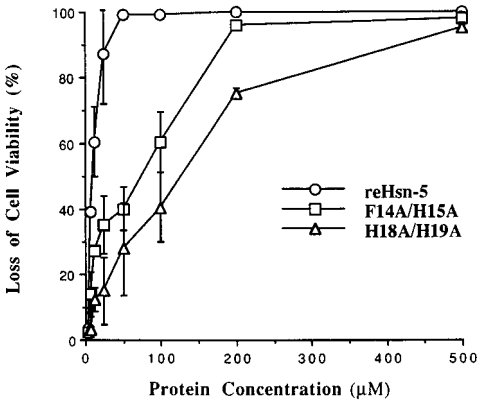

FIG. 2. Percent loss of *C. albicans* viability induced by reHsn-5, F14A/H15A, and H18A/H19A. Results are expressed as means ± standard deviations. Values are based on at least three separate experiments, each of which was run in triplicate.

2 and Table 1. The ED_{50}s of F14A/H15A and H18A/H19A are ~67 and ~149 μM, respectively, compared to an ED_{50} of 8 μM for the unaltered reHsn-5. The 95% confidence limits of the ED_{50}s of both variants do not overlap with that of reHsn-5, suggesting that the candidacidal activities of both variants are significantly lower than that of reHsn-5 ($P < 0.05$ by analysis of variance with the Scheffe F-test multiple-comparison procedure at each protein concentration). When the two variants are compared to each other, the 95% confidence limits of the ED_{50}s of the two variants overlapped each other, suggesting that there is no statistically significant difference in the candidacidal activities of these two variants ($P > 0.05$ by analysis of variance with the Scheffe F-test multiple-comparison procedure at each protein concentration). Collectively, these results indicate that the candidacidal activities of F14A/H15A and H18A/H19A are significantly lower than that of reHsn-5, suggesting that the dipeptide sequences Phe^{14}-His^{15} and His^{18}-His^{19} are important for candidacidal activity. However, on the basis of our experiments with Hsn-5 and azole-resistant *C. albicans* (and *C. glabrata*; see below), these results cannot be interpreted to imply that the mechanism of action of Hsn-5 against *C. albicans* is similar to that of azole-based antifungal agents, which act through inhibition of sterol 14α-demethylase. Instead, the importance of these two dipeptide sequences on Hsn-5 with respect to its candidacidal activity may be due to the interaction of these two regions with other *C. albicans* molecules (but not sterol 14α-demethylase), such as the putative Hsn receptor on the surface of *C. albicans* or unknown molecules in the candidal cells. The significant loss of candidacidal activity could be due to the removal of charged residues (histidine is charged or neutral, depending on the microenvironment around it) and/or the removal of the entire side chains.

CD studies. The CD spectrum of reHsn-5 was comparable to that of the native Hsn-5 reported previously (24). Both peptides contain a largely α-helical conformation under nonaqueous conditions. The secondary structures of F14A/H15A and H18A/H19A estimated by CD analysis were then compared with that of reHsn-5. In trifluoroethanol, all peptides exhibited two negative bands between ~220 and ~209 nm and a strong positive band at ~194 nm, characteristic of helical structures (Fig. 3). The ratio of the two negative bands, $\theta_{n-\pi^*}/\theta_{\pi-\pi^*}$ (*R* value), has generally been taken for the short peptides as an index of α-helical structures (7, 9). Hence, the *R* values of the reHsn-5 and its variants were calculated. The values for all of

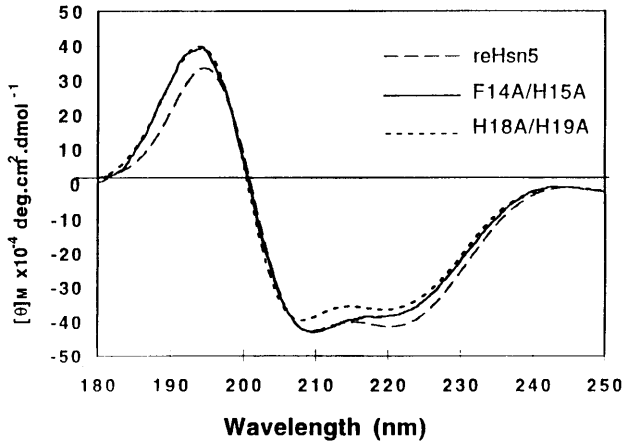

FIG. 3. CD spectra of reHsn-5, F14A/H15A, and H18A/H19A in trifluoroethanol.

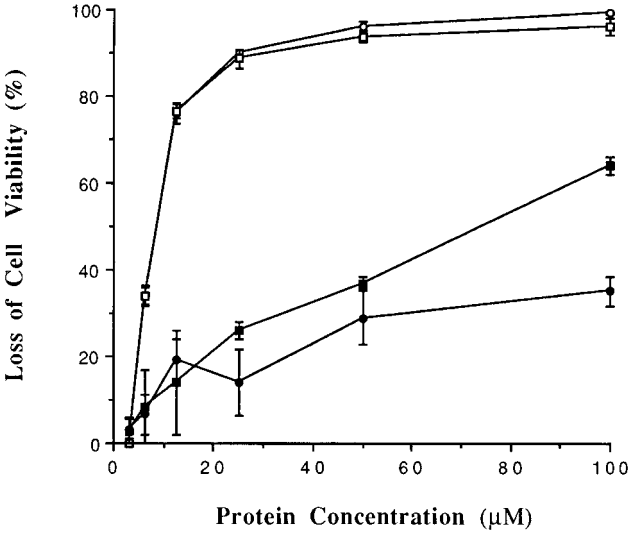

FIG. 4. Susceptibilities of azole-sensitive and azole-resistant *C. albicans* and *C. glabrata* to Hsn-5. Open and closed circles, azole-sensitive *C. albicans* and *C. glabrata*, respectively; open and closed squares, azole-resistant *C. albicans* and *C. glabrata*, respectively. The results were obtained from a single experiment that was run in triplicate. The values are means ± standard errors.

these peptides are close to 0.9 (Table 2), indicating populations of largely α-helical conformations in a hydrophobic environment. The α-helical conformation of Hsn-5 that has been suggested to be important for candidacidal activity (24, 25) may influence the insertion and/or the entrance of the Hsn-5 into *C. albicans* to exert its candidacidal activity. Since the α-helical contents of the two variants were similar to that of reHsn-5, this suggests that the decrease in the candidacidal activities of the variants is not due to the decrease in the α-helical content.

Candidacidal activity of reHsn-5 against azole-sensitive and azole-resistant *C. albicans* and *C. glabrata*. The antifungal activities of azole-based drugs are primarily derived from their binding to a sterol 14α-demethylase (a member of the cytochrome P-450 superfamily), which is involved in the synthesis of ergosterol (8, 33). Several azole-resistant *C. albicans* strains have been characterized as having increased levels of sterol 14α-demethylase (10, 35). As indicated in Materials and Methods, the azole-resistant *C. albicans* strain tested in this study contains an increased level of sterol 14α-demethylase as well as other defects which might also contribute to the azole resistance of this strain. The candidacidal activity assay indicated that this azole-resistant strain is as susceptible to killing by reHsn-5 as the azole-sensitive strain (Fig. 4), suggesting that the sterol 14α-demethylase may not be the target of the Hsn molecule. In addition to *C. albicans* strains, we have also examined azole-sensitive and azole-resistant *C. glabrata* strains for their susceptibilities to Hsn-5. Again, reHsn-5 killed both strains (Fig. 4) and at a 100 μM concentration of reHsn-5, the azole-resistant strain was even more susceptible to reHsn-5 than the azole-sensitive strain (statistically significant difference). However, Hsn-5 was less effective at killing *C. glabrata* than it was at killing *C. albicans*; this finding is consistent with those in a previous study by Rayhan et al. (27).

TABLE 2. CD parameters for Hsn-5 and variants

Peptide	CD spectrum in trifluoroethanol		R^a
	λ (nm)	$[\theta]_M$ (deg · cm^2 · dmol^{-1} [10^4])	
reHsn-5	195	+38.77	0.971
	209	−49.22	
	221	−47.80	
F14A/H15A	194	+39.81	0.923
	208	−39.60	
	219	−36.54	
H18A/H19A	193	+37.94	0.881
	210	−43.33	
	220	−38.17	

[a] $R = \theta_{n-\pi^*}/\theta_{\pi-\pi^*}$

DISCUSSION

While the mechanism of Hsn-induced killing of *C. albicans* remains unknown, several possible mechanisms have been proposed. First, Raj et al. (24, 25) have suggested that the C-terminal 14 amino acids of Hsn-5 (residues 9 to 24) and an α-helical conformation are the major structural requirements for eliciting appreciable candidacidal activity. They proposed that Hsn may undergo a structural transition from a random coil in aqueous solution to an ordered α-helical conformation when the molecules are in close proximity to the plasma membrane. Second, Hsn may exert its candidacidal activity against *C. albicans* through the binding to the putative receptor on the *Candida* cell membrane (5, 6). Third, as mentioned in the introduction, the mechanism of antifungal action of Hsn may resemble that of azole-based antifungal molecules. This last hypothesis was substantiated not only by the molecular modeling that suggested that the structures of the Phe^{14}-His^{15} and His^{18}-His^{19} elements of Hsn-5 are similar to those of miconazole (26) but also by the following observations. Hsns are high in histidine content (seven histidine residues in 24 amino acids of Hsn-5); the histidines with their imidazole-containing moieties may interact with *C. albicans*, mimicking the action of the azole ring in azole-based antifungal molecules. The candidacidal potency of Hsn-5 is comparable to those of the imidazole antifungal agents miconazole and ketoconazole (23, 29). The loss of viability of *Candida* cells treated with Hsns is correlated with the loss of potassium from the cells; this phenomenon is similar to that in *Candida* cells treated with imidazole antifungal agents (23).

Although the mode of action of Hsn against *C. albicans* has

long been proposed to be similar to that of azole-based antifungal agents, the data required to support or reject this hypothesis were lacking. Our finding, together with an independent report by O'Connell et al. (19), who found that Hsn-3 kills both fluconazole-sensitive and -resistant *C. albicans* strains, suggests that the mechanism of the Hsn interaction with *C. albicans* is distinct from that of azole-based antifungal agents. In addition, our study indicated that both the azole-resistant and azole-sensitive *C. glabrata* strains are killed by Hsn-5 equally well. In the introduction we mentioned that the potential lack of toxicity and the candidacidal potencies of Hsns that are similar to those of the azole-based antifungal agents (29) make Hsns promising natural therapeutic agents against fungal infections. The fact that Hsn-5 kills azole-resistant *Candida* strains makes Hsn an even more potentially useful antifungal therapeutic agent, especially for the treatment of fungal infections in AIDS patients, many of whom harbor azole-resistant fungal species. The azole-based antifungal agents are generally toxic. The observation that Hsns may not be toxic to mammalian cells is now strengthened by the apparent distinct mechanisms of action exhibited by Hsns and azole-based antifungal agents on the killing of *Candida* spp. Additionally, Hsn receptors that have been suggested to exist on the *C. albicans* surface (5, 6) may be unique to *C. albicans*, which could also account for the lack of toxicity of Hsns to mammalian cells. From the data presented in this report, however, we cannot conclude whether Hsns are surface-acting proteins or target molecules inside the cells, or both. Thus, further investigation of the mechanism of interaction of Hsns with fungal cells is necessary.

ACKNOWLEDGMENTS

We thank T. C. White and S. Redding for providing the azole-sensitive and -resistant *C. albicans* strains and J. E. Bennett for the azole-sensitive and -resistant *C. glabrata* strains. We also thank M. J. Levine for use of facilities and for helpful advice and P. A. Raj for critical reading of the manuscript.

This work was supported by USPHS research grant DE09820.

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