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Candidacidal Activity of Recombinant Human Salivary Histatin-5 and Variants

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Human salivary histatins possess fungicidal and bactericidal activities. The current investigation evaluates the structure-function relationship of histatins with regard to their candidacidal activity by using recombinant histatin-5 and its variants produced in *Escherichia coli*. The purified recombinant histatins were examined for their candidacidal activity and secondary structure. The m21 (with Lys-13 replaced by Thr [Lys-13→Thr]) and m71 (Lys-13→Glu) variants are significantly less effective than recombinant histatin-5 in killing *Candida albicans*, suggesting that Lys-13 is critical for candidacidal activity. The m68 (Lys-13→Glu and Arg-22→Gly) variant is significantly less potent than the recombinant histatin-5 as well as m71, indicating that Arg-22 is crucial for the cidal activity. The candidacidal activities of m1 (Arg-12→Ile), m2 (Arg-12→Ile and Lys-17→Asp), m12 (Arg-12→Lys and His-21→Leu), and m70 (His-19→Pro and His-21→Arg) variants, however, are comparable to that of recombinant histatin-5, indicating that Arg-12, Lys-17, His-19, and His-21 are not functionally important. The conformational preferences of histatin-5 and variants were determined by circular dichroism. The results indicate that all proteins have a strong tendency to adopt α-helical conformation in trifluoroethanol. Previously, we have shown that the α-helical conformation is one of the important structural requirements for eliciting appreciable candidacidal activity. Collectively, the data suggest that in addition to the helical conformation, specific residues such as Lys-13 and Arg-22 in the sequence of histatin-5 are, indeed, important for candidacidal activity.

Human salivary histatins (Hsns) are a group of small, neutral to very basic histidine-rich polypeptides (23, 29, 41). They are important members of the human nonimmune defense system and possess a variety of functions. They exhibit remarkable growth inhibitory activity on *Candida albicans* (32, 33, 42). They also possess bactericidal activities against *Streptococcus mutans* (20), *Porphyromonas gingivalis* (7) and *Actinomyces* species (17). They inhibit hemagglutination of *P. gingivalis* by specifically binding to the bacterial cells through a single class of receptor (26, 27) and inhibit the coaggregation between *P. gingivalis* and *Streptococcus mitis* (24). Hsns are also involved in the formation of acquired enamel pellicle and participate in the mineral solution dynamics of the oral fluids, thereby providing an ideal environment for tooth integrity (13, 31, 35). Hsns also play an important role in chondrocyte proliferation (25).

There are at least 12 salivary Hsns which have been isolated from saliva, and their primary structures have been determined (41). Hsn-1, -3, and -5 are the three major forms (30), with Hsn-5 being the most effective in killing the blastospore and the germinated forms of *C. albicans*. However, Hsn-3 is reported to be the most potent in inhibiting the germination of *C. albicans* (42). A recent study of synthetic Hsn-5 and its fragments suggested that a sequence at the C terminus (residues 9 to 24), a peptide chain length of at least 14 residues, and an α-helical conformation are the major structural requirements for eliciting appreciable candidacidal activity (33, 34).

We have previously subcloned cDNA encoding Hsn-1 into an *Escherichia coli* expression vector, pGEX-3X, and shown that the fusion protein was produced in large quantity and in a soluble form in the *E. coli* cytoplasm (4). However, we have experienced difficulty with the cleavage of the fusion protein with factor X. Production of recombinant Hsn-1 and -3 in *E. coli* has also recently been described by Driscoll et al. (9) and Zuo et al. (43), respectively. To further elicit the structure-function relationship of Hsns, in this study, we produced recombinant Hsn-5 (reHsn-5) and its variants, since Hsn-5 was shown to have the highest blastosporicidal activity of the three most abundant Hsns secreted in saliva.

MATERIALS AND METHODS

Materials. A cDNA clone encoding Hsn-3 was isolated previously in our laboratory from a human submandibular gland cDNA library (5). Oligonucleotides were made by the Oligonucleotide Synthesis Facility (Department of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo) or Amitof Inc. (Allston, Mass.). *C. albicans*, a clinical isolate from the palate of a denture-induced-stomatitis patient, was generously provided by M. Edgerton (Department of Oral Biology, State University of New York at Buffalo) (33). Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Promega Co. (Madison, Wis.). Glutathione agarose beads, thrombin (from human plasma; 1,000 U/ml), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, Mo.). Aprotinin was from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.), Sabouraud dextrose agar was from Difco Laboratories (Detroit, Mich.), and Centriplus-10 was from Amicon, Inc. (Beverly, Mass.).

Generation of Hsn-5 cDNA and its random mutations. A Hsn-5 cDNA fragment was generated by PCR with the Hsn-3 cDNA clone in λgt11 *Sfi-Not* vector (5) as a template and the following primers: P1 (forward primer), 5′-GTGGATCCATGCAAAGAGACAT-3′; P2 (reverse primer), 5′-CCCTTAATCAAACCTCGAGTGA-3′. A unique *Bam*HI site (underlined) and a stop codon (underlined) were incorporated into the forward and reverse primers, respectively. The PCR was carried out in an automatic thermal cycler for 30 cycles. Each cycle consisted of the following three steps: a denaturation temperature of 94°C for 30 s, an annealing temperature of 52°C for 45 s, and an extension temperature of 72°C for 45 s, followed by a 10-min incubation at 72°C to elongate the products to their full length. About a 2-μl aliquot of λgt11–Hsn-3 phage stock and 1 μM each primer were used in 100 μl of reaction mixture containing buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1% Triton X-100), 100 μM deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, and 5 U of *Taq*I DNA polymerase. The PCR-amplified Hsn-5 cDNA was analyzed on a 4% low-melting-point agarose

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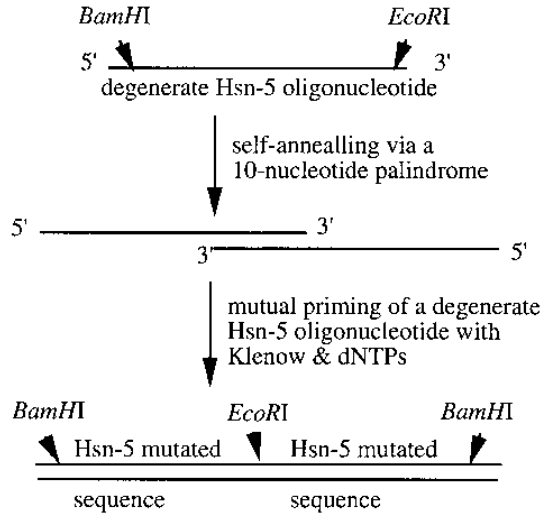

FIG. 1. Schematic representation of the conversion of single-stranded degenerate Hsn-5 oligonucleotide to double-stranded DNA containing different Hsn-5 mutations.

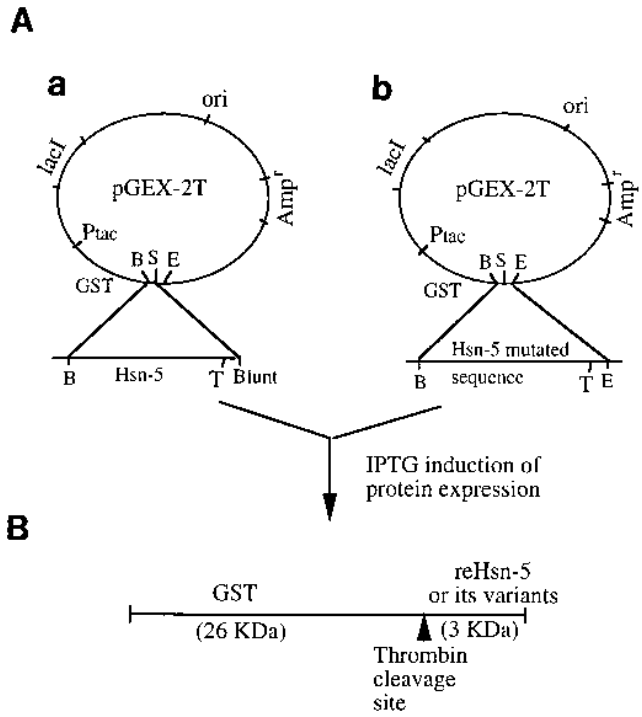

FIG. 2. Schematic representation of construction of pGHsn-5 and pGHsn-5-mN expression plasmids as well as the expressed fusion proteins. (A) Construction of pGHsn-5, a plasmid encoding reHsn-5 (Asp-1 to Gly-1 change) (a), and pGHsn-5-mN, plasmids encoding reHsn-5 variants (b). m, mutation; N, mutation number. (B) Schematic representation of the expressed fusion proteins. See the text for more details. Abbreviations: B, *Bam*HI; S, *Sma*I; E, *Eco*RI; T, termination codon. The figure is not drawn to scale.

gel, and the correct-size fragment was excised and purified by a freeze-squeeze method, followed by phenol-chloroform extraction and ethanol precipitation.

DNA fragments containing Hsn-5 random mutations were generated as follows. An 87-base degenerate oligonucleotide which contains a full-length Hsn-5 coding region flanked by *Bam*HI and *Eco*RI sites was custom synthesized. A 4% mutation rate at the 42-base region, which encodes the C-terminal 14 amino acids of Hsn-5, was introduced by incorporation of a mixture of 96% wild-type nucleotide and 4% of the other three nucleotides into each nucleotide position. The 87-mer was converted to double-stranded DNA by mutually primed synthesis (Fig. 1) as described by Ausubel et al. (1) with some modifications. Briefly, 200 pmol (about 5 μg) of 87-mer, dissolved in 7 μl of deionized water, was incubated at 70°C for 5 min, and then 1 μl of 10× DNA polymerase buffer was added. The sample was cooled to room temperature and incubated for 1 h. After incubation, 2 μl of a 2.5 mM dNTP mixture (2.5 mM each dNTP) and 5 U of Klenow fragment of DNA polymerase I were added to the reaction mixture, and the mixture was incubated for 1 h at room temperature. Finally, another 5 U of Klenow fragment was added and the sample was further incubated overnight. The reaction was stopped by adding 1 μl of 0.5 M EDTA. The sample volume was adjusted to 50 μl with Tris-EDTA buffer and extracted once with phenol-chloroform, precipitated with ethanol, and resuspended in 15 μl of Tris-EDTA buffer.

Construction of recombinant plasmids. The *E. coli* expression vector pGEX-2T was used for cloning. We chose to use the pGEX-2T vector because we have successfully used pGEX-2T for the production of large quantities of recombinant human salivary cystatin S as well as cystatin SN and its variants (4, 6). The pGEX-2T vector contains the *tac* promoter, which controls the expression of the recombinant protein as a fusion protein with the C terminus of a 26-kDa glutathione *S*-transferase (GST) upon induction by IPTG (38). The GST carrier protein can be cleaved from the cloned protein by thrombin treatment (Fig. 2B). For construction of the pGHsn-5, the Hsn-5 cDNA fragment was blunt-ended with T4 DNA polymerase, treated with *Bam*HI, and ligated to the *Bam*HI-*Sma*I-digested pGEX-2T vector (Fig. 2Aa). For the construction of the plasmids with random mutations in the Hsn-5 sequence, pGHsn-5-mN (N = number of the mutated version of pGHsn-5), the converted double-stranded DNAs which contained 2 oligonucleotide units of Hsn-5 random mutations (or the wild type) were cleaved with *Bam*HI and *Eco*RI restriction enzymes, gel purified as described above, and then cloned into *Bam*HI-*Eco*RI-digested pGEX-2T vector (Fig. 2Ab). The ligated DNAs were transformed into *E. coli* AR68 by electroporation. Transformants were randomly picked, and the expression of foreign proteins, induced by IPTG, was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (4). Transformants which expressed the foreign protein were selected, plasmids were extracted with the Wizard Minipreps plasmid DNA purification kit, and the DNA was sequenced by the method of Sanger et al. (37).

Production and purification of recombinant proteins. Overnight cultures (20 ml) of each of the pGHsn-5 and pGHsn-5-mN transformants were added to 1 liter of L broth containing 100 μg of ampicillin per ml and grown at 37°C to an optical density at 600 nm of ~0.8. IPTG (0.1 mM) was then added to induce the expression of the fusion proteins, and the cultures were allowed to grow for an additional 3 h. The bacteria were then harvested by centrifugation.

For purification of GST fusion proteins, the method described by Ausubel et al., with some modifications, was followed (2). The bacterial pellets were resuspended in 10 ml of ice-cold 1× phosphate-buffered saline (PBS; pH 8.0). Cell lysis was achieved by using lysozyme (1.2 mg/ml) and sonication (30 s, with pulse on for 1 s and pulse off for 1 s) with a microtip at a power setting of 3, in the presence of the proteinase inhibitors PMSF (1 mM) and aprotinin (5 μg/ml). After centrifugation, the supernatant containing soluble GST–Hsn-5 or GST–Hsn-5 variants was added to a 50% slurry of glutathione agarose beads (70 mg of beads) and the fusion protein was allowed to bind for 45 min at 4°C while gently rotating. The beads were washed three times with ice-cold PBS and equilibrated with thrombin-cutting buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl). The reHsn-5 and the variants were released from GST (~26 kDa) by thrombin treatment (20 U, 1 U/μl) (Fig. 3B) in the presence of aprotinin (5 μg/ml) for 90 min at 4°C. The reHsn-5 and its variants were eluted from the beads with wash buffer (same as the thrombin cutting buffer mentioned above).

The partially purified reHsn-5 and variants were further purified as follows. The samples were boiled for 5 min and then cooled on ice for 10 min and ultrafiltered with Centriplus-10, as described in the manufacturer's recommendations. The samples were then desalted on a Sephadex G-10 column equilibrated with 50 mM acetic acid. Final purification of reHsn-5 and variants was achieved by reverse-phase high-performance liquid chromatography (RP-HPLC) under conditions described previously (33). Briefly, the samples were dissolved in 10% acetonitrile in water and subjected to RP-HPLC purification with a Rainin Dynamax-60A reverse-phase C-18 column (10 by 250 mm), coupled to a guard column (10 by 50 mm), with acetonitrile and water (both containing 0.1% trifluoroacetic acid) as the solvent system.

Amino acid analysis. Each sample was hydrolyzed with 6 N HCl for 24 h at 110°C in evacuated sealed tubes. Hydrolysates were analyzed with a Beckman model 6300 amino acid analyzer, with norleucine as an internal standard. Peaks were quantitated with a Gilson model 620 Data Master integrated to an Apple IIe computer.

N-terminal sequencing. One nanomole of reHsn-5 was dissolved in 20 μl of deionized water, and N-terminal amino acid sequencing was performed with an Applied Biosystems (model 471) protein sequencer interfaced with a Hewlett-Packard HP3394 A integrator. Fifteen cycles were analyzed.

Gel electrophoresis. Hsns were analyzed on an SDS–15% polyacrylamide gel by the method of Laemmli (18) and an SDS–20% polyacrylamide gel with 10% glycerol included in the separating gel (14). Hsns were visualized by Coomassie blue staining.

CD analysis. Circular dichroism (CD) spectra were obtained with a Jasco J-600A Spectropolarimeter interfaced to an IBM ps/2 microcomputer. The polypeptide (ca. 30 to 40 μM) was dissolved in trifluoroethanol (TFE), and spectra were recorded between 180 and 250 nm at 27°C by use of a 0.1-cm-path-length cell. CD band intensities are expressed as molar ellipticities $[\theta]_M$ in degrees square centimeter per decimole.

Candidacidal blastosporicidal assay. A modified assay method of Raj et al. (33) was performed so that decreased amounts of proteins, cells, and Na phosphate buffer could be used. Briefly, one colony of *C. albicans* was inoculated into 10 ml of yeast synthetic growth medium (40) and incubated for 48 h (optical density at 600 nm, ~0.5 to 1.0) at room temperature in a shaker rotating at 200 rpm. Cells were then harvested, washed, and resuspended to a concentration of 10^5 cells per ml in 0.01 M Na phosphate buffer (pH 7.4). For the testing of the inhibition of blastospore viability as a function of protein concentration, 20 μl of yeast suspension was added to an equal volume of twofold serial dilutions protein (final concentrations, 3.125 to 100 μM in 0.01 M Na phosphate buffer [pH 7.4]) in sterile glass test tubes. The samples were incubated at 37°C for 1.5 h and mixed every 15 min. For the testing of the inhibition of blastospore viability as a function of incubation time, yeast suspension was mixed with an equal volume of each variant protein (final concentrations, 15 and 30 μM) and incubated at 37°C for 45 min, 1.5 h, or 3 h. At the end of each incubation, samples were diluted 20 times, and 60 μl (150 cells) of the diluted cells was plated on Sabouraud dextrose agar plates in triplicate and grown overnight at 37°C. Blastosporicidal activity of the test protein was calculated as percent loss of cell viability (1 minus the ratio of colonies per test plate to the number of colonies on control [no-protein] plates).

Statistical analyses. The ED_{50} (molar concentration of peptide required to kill half of the maximum number of cells) was determined by the procedure PROBIT (SPSS software package release 4.1 for VAX/VMS). Differences in candidacidal activity between synthetic Hsn-5, reHsn-5, and its variants at each concentration were assessed by analysis of variance by use of the StatView SE + Graphics software package on a Macintosh computer.

RESULTS

Construction and expression of Hsn-5 cDNA and its mutations. A Hsn-5 cDNA fragment was generated by PCR as described in Materials and Methods. To clone the Hsn-5 fragment into the *Bam*HI site of the pGEX-2T plasmid (Fig. 2Aa), the 5′ end of the Hsn-5 cDNA was slightly modified. This resulted in an in-frame fusion between the 3′ end of the GST and the 5′ end of Hsn-5 with an Asp-to-Gly change at position 1. For generation of Hsn-5 mutations, we chose the method of random mutagenesis by degenerate oligonucleotide (see Materials and Methods and Fig. 1). Since Raj et al. (33) have shown that the C terminus of Hsn-5 with a minimum chain length of 14 residues is required for eliciting appreciable candidacidal activity, we randomly mutated only the C-terminal 14 amino acids of Hsn-5. A 4% mutation rate introduced at the C-terminal 42-base region of Hsn-5 gives, on average, 1 to 3 nucleotide substitution(s) per oligonucleotide. After the cloning of the mutagenic DNAs, each of the resulting recombinant plasmids should contain a different Hsn-5 mutation (or the wild type). Approximately 100 different recombinants were analyzed (by expression screening on the SDS-PAGE gel and DNA sequencing), and 7 were selected for further analysis (Table 1). We found that not all 14 amino acid residues in the C-terminal region were changed. This was presumably due to the high (>96%) ratio of the wild type to the three non-wild-type oligonucleotides at some nucleotide positions during oligonucleotide synthesis. The seven selected Hsn-5 variants had only one or two amino acids replaced by other amino acids. The m1 variant (Arg-12 replaced by Ile) was chosen to examine whether the positive charge or the hydrophobic effect of the side chain of Arg-12 is important for candidacidal activity. The m2 variant (Arg-12 and Lys-17 replaced by Ile and Asn, respectively) was selected to examine the importance of the positive charge at position 17 for candidacidal activity. The m12 (Arg-12 and His-21 replaced by Ile and Leu, respectively) and m70 (His-19 and His-21 changed to Pro and Arg, respectively) variants were chosen to examine the influence of the imidazole rings at positions 19 and 21 on candidacidal activity.

TABLE 1. Amino acid sequences and candidacidal activities of synthetic Hsn-5 (syHsn-5) and reHsn-5 and its variants

Hsn	Sequence[a]	ED_{50}[b]
syHsn-5	DSHAKRHHGYKRKFHEKHHSHRGY	6.03 (5.37–6.78)[c]
reHsn-5	G----------	4.79 (4.11–5.56)
m1	----------I----------	4.86 (3.83–6.03)
m2	----------I----N-------	5.45 (4.23–7.01)
m12	----------I--------L---	3.62 (2.85–4.44)
m21	----------T----------	14.70 (10.85–20.14)
m68	----------E--------G--	49.59 (34.97–77.74)
m70	----------P-R---	4.57 (2.79–6.92)
m71	----------E----------	19.37 (14.7–25.74)

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

The m21 variant (Lys-13 replaced by Thr) and m71 variant (Lys-13 changed to Glu) were selected to examine the influence of Lys at position 13 on fungicidal activity. The m68 variant (Lys-13 to Glu and Arg-22 to Gly) was chosen to examine whether Lys-13 and Arg-22 are crucial for the candidacidal activity.

Production and purification of reHsns. Large-scale (1-liter) cultures were grown and processed as described in Materials and Methods. Large amounts of GST–Hsn-5 fusion proteins (~30 mg/liter; the Hsn-5 portion represents 1/10 of the fusion) were produced after induction of the foreign proteins by IPTG (Fig. 3A). The fusion proteins were produced in a soluble form. This allowed for their initial purification by affinity chromatography on glutathione agarose beads (Fig. 3B). During the purification steps, we encountered several problems which negatively influenced the final yield of the reHsns. First, we found that only a small portion of the fusion protein bound to the beads and repeated binding did not increase the binding efficiency. This finding suggested that only a portion of the fusion protein was properly folded for the binding. Second, Hsn-5 and the majority of its variants were sensitive to proteolytic degradation, even when the proteinase-deficient *E. coli* strain and proteinase inhibitors (PMSF and aprotinin) were used. Since a previous study by Lal et al. (19) showed that boiling does not affect the Hsn activity, we boiled the samples immediately after thrombin cleavage. Third, in the process of purification, reHsn-5 was spontaneously polymerizing in H_2O. Finally, the reHsn-5 could not be desalted by dialysis because it appeared to adhere to the dialysis tubing. Sephadex G-10 chromatography was found to be the best way to desalt the samples before the final purification step, RP-HPLC. The reHsn-5 and all the variants were purified by several rounds of RP-HPLC. The HPLC peak integration at 230 nm indicated ~99% purity of the proteins (see examples of the purified protein profiles in Fig. 3C). The final yields of the purified reHsn-5 and some of the variants were rather low (~100 to 200 μg/liter). The amino acid composition of each recombinant peptide was confirmed by amino acid analysis. The N-terminal sequence of the purified reHsn-5 was verified by amino acid sequence analysis, and as expected, the first amino acid was found to be a Gly instead of an Asp. An Asp-to-Gly change at position 1 in all variants was confirmed by DNA sequencing and amino acid analysis.

Candidacidal blastosporicidal activity. The efficacy of synthetic Hsn-5 and reHsns to induce loss of viability of the blastospore form of *C. albicans* was measured at various concentrations and at different time points. The results are

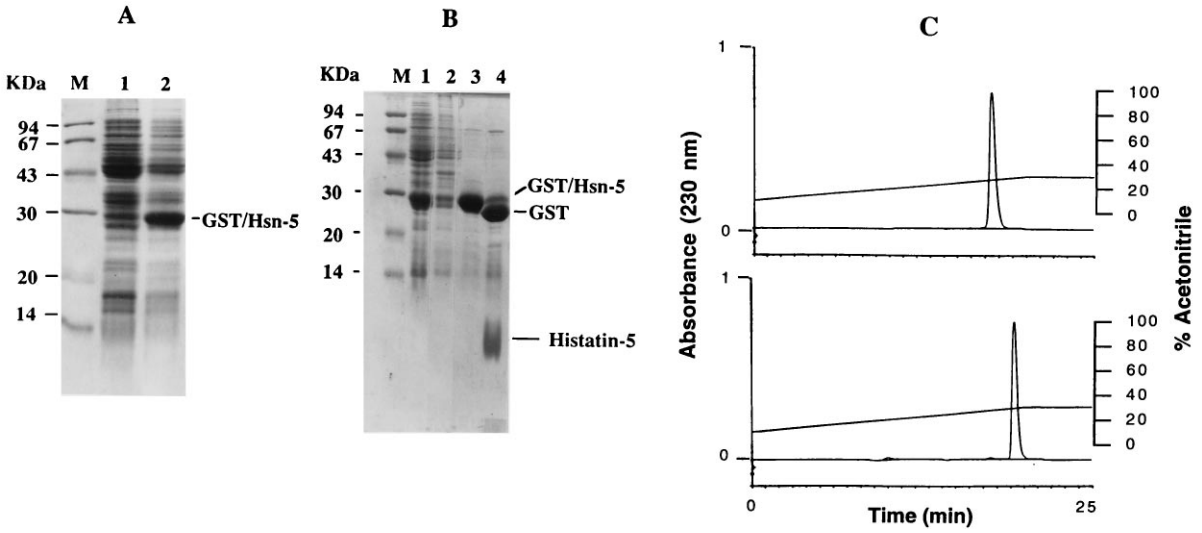

FIG. 3. Production and purification of reHsns. (A) Production of Hsn-5 in *E. coli* AR68 cells monitored by SDS–15% PAGE. Lanes: M, molecular weight markers; 1 and 2, proteins present in total cell lysates of uninduced and IPTG-induced cultures, respectively, containing pGEX-2T/Hsn-5 plasmid. (B) Expression of Hsn-5 in *E. coli* AR68 cells and its initial purification monitored by SDS–15% PAGE. Lanes: 1 and 2, proteins present in the supernatant and pellet, respectively, of IPTG-induced cultures (note that the GST/Hsn-5 fusion protein [30 kDa] is present in the cell lysate supernatant); 3, material bound to the glutathione beads before thrombin treatment; 4, a sample of material after thrombin treatment. (C) RP-HPLC profiles of purified reHsn-5 (top) and m2 (bottom) variant.

summarized in Fig. 4 and 5 and in Table 1. The data were obtained by a modified assay system and with the DIS strain of *C. albicans*, as described in Materials and Methods.

The concentration-dependent loss of viability of cells induced by reHsn-5 was first compared with that of synthetic Hsn-5 (Fig. 4A). The candidacidal activity of synthetic Hsn-5 has previously been reported to be consistent with that of the native Hsn-5 (33). If the candidacidal activity of reHsn-5 was comparable to that of synthetic Hsn-5, the reHsn-5 would be a proper control for the examination of Hsn-5 variants. The results showed that even though the first amino acid of natural Hsn-5, Asp, was replaced by Gly in the reHsn-5, the percent loss of viability induced by reHsn-5 was comparable to that of synthetic Hsn-5 ($P > 0.05$ for each concentration of protein tested). The 95% confidence limits of their ED_{50} values overlap each other (Table 1), suggesting that there is no statistically significant difference between reHsn-5 and synthetic Hsn-5. This indicates that Asp, at position 1, may not be necessary for the candidacidal activity of Hsn-5, which is consistent with the previous finding (33). The activity data of reHsn-5 and synthetic Hsn-5 are also comparable to those reported for native Hsn-5 (42).

The concentration-dependent losses of viability induced by seven Hsn-5 variants were then compared with that of reHsn-5. The results obtained with variants m1, m2, m12, and m70 are shown in Fig. 4B and Table 1. The ED_{50} values of these variants showed no statistically significant difference from that of reHsn-5 (the 95% confidence limits overlap each other), indicating that they possess comparable candidacidal activities. On the other hand, the results obtained with variants m21, m68, and m71, shown in Fig. 4C and Table 1, indicated that these variants have ED_{50} values significantly higher than that of reHsn-5, suggesting that they exhibit a diminished candidacidal potency.

In the time course study, the candidacidal potency of each variant was tested at three different time points (0.75, 1.5, and

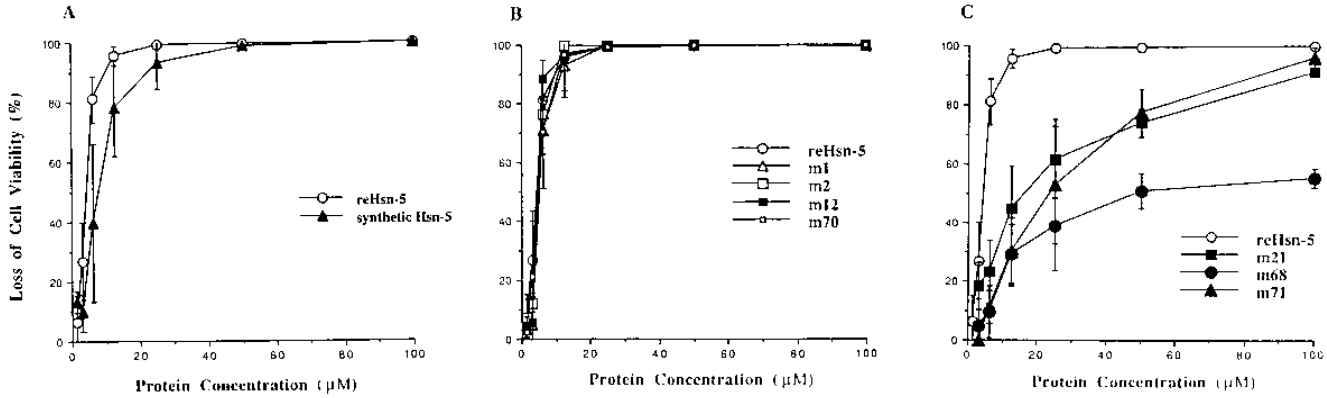

FIG. 4. Dependence of percent loss of viability of *C. albicans* on concentration induced by synthetic Hsn-5 and reHsn-5 and its variants. (A) Loss of cell viability induced by synthetic and reHsn-5; (B and C) loss of cell viability induced by m1, m2, m12, and m70 variants (B) and by m68, m21, and m71 variants (C). Bars indicate the mean percentages of candidacidal activities of each recombinant protein for each concentration tested, and error bars indicate the standard deviations of the means. Values are based on at least three separate experiments, each run in triplicate.

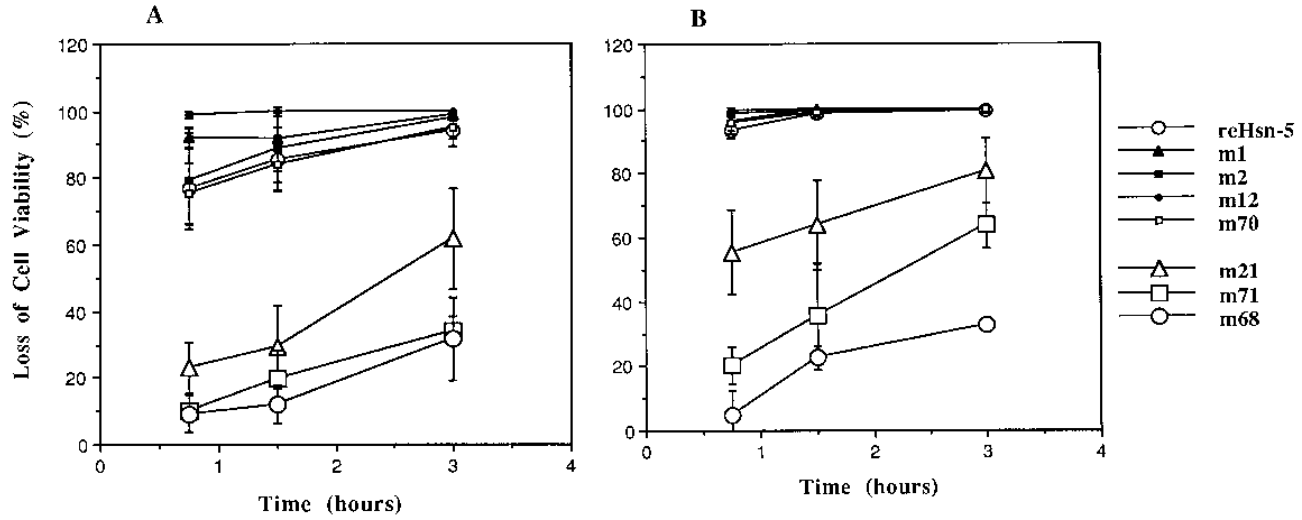

FIG. 5. Percent loss of *C. albicans* viability at different time points (0.75, 1.5, and 3 h) induced by 15 μM (A) and 30 μM (B) reHsn-5 and its variants. Bars indicate the mean percentages of candidacidal activities of each recombinant protein for each concentration tested, and error bars indicate the standard deviations of the means. Values are based on at least three separate experiments, each run in triplicate.

3 h) with both 15 μM (within the range of physiological concentration) and 30 μM peptide concentrations. At the 15 μM peptide concentration (Fig. 5A), the time-dependent loss of viability of cells induced by reHsn-5 and variants m1, m2, m12, and m70 showed nearly 70 to 99, 80 to 100, and 100% killing within 45 min, 1.5 h, and 3 h, respectively (top five curves). On the other hand, the loss of viability induced by m21, m68, and m71 showed only 5 to 25% killing within 45 min, about 5 to 30% killing at 1.5 h, and 30 to 60% killing at 3 h (bottom three curves). At the 30 μM peptide concentration (Fig. 5B), for reHsn-5 and variants m1, m2, m12, and m70, nearly 90 to 100% killing was achieved within 45 min of incubation, and the killing reached 100% within 1.5 h. However, for m21, m68, and m71, only 5 to 55% loss in cell viability was observed within 45 min after incubation. Even after 3 h of incubation, only about 30 to 80% killing was achieved. These results suggest that the candidacidal activity of m1, m2, m12, and m70 is comparable to that of reHsn-5, whereas m21, m68, and m71 have much lower cidal activities than reHsn-5 at both 15 and 30 μM peptide concentrations ($P < 0.05$ by analysis of variance with the Scheffe F-test multiple-comparison procedure). These results are consistent with the concentration-dependent loss of viability results (Fig. 4).

CD studies. The CD spectrum of the reHsn-5 was first compared with that of synthetic Hsn-5 to determine whether reHsn-5 would be a proper control for examination of the CD of Hsn-5 variants. The spectrum of the reHsn-5 in TFE was in good agreement with that of synthetic Hsn-5 (Fig. 6A and Table 2). Both peptides exhibited two negative bands between ~221 and ~209 nm and a strong positive band at ~194 nm, characteristic of helical structures (15). The two negative bands observed for these peptides correspond to the long-wavelength component of exciton split of the π-π^* and the n-π^* transitions, respectively (12). The use of absolute ellipticity values to estimate the helix content in peptides of low molecular mass has been reported to often yield underestimation of the helix content (16, 22, 28, 39). The ratio of the two negative bands (R), $\theta_{n\text{-}\pi^*}/\theta_{\pi\text{-}\pi^*}$, has generally been taken as an index of α-helical structures, with values of ~1 being ascribed to α-helical polypeptides (12). Hence, we used the R values to compare the tendency of the peptides to adopt helical structures. The R

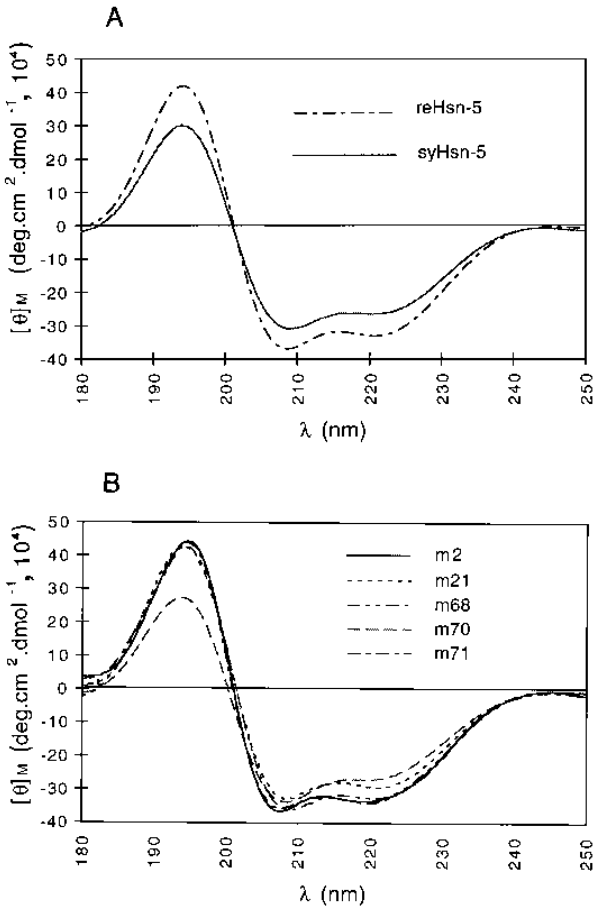

FIG. 6. CD analysis of Hsn-5 and variants in TFE. (A) CD spectra of reHsn-5 and synthetic Hsn-5; (B) CD spectra of Hsn-5 variants m2, m21, m68, m70, and m71.

TABLE 2. CD parameters for Hsns and variants

Hsn	Parameter in TFE	
	λ (nm)	$[\theta]_M$ $(10^4)^a$
reHsn-5	194	+41.82
	209	−36.84 (0.898)
	221	−33.09
Synthetic Hsn5	195	+32.96
	208	−30.91 (0.890)
	219	−27.51
m2	195	+44.23
	208	−36.55 (0.918)
	219	−33.57
m21	196	+42.96
	208	−32.92 (0.896)
	221	−34.10
m68	196	+42.56
	209	−36.10 (0.907)
	220	−32.74
m70	196	+27.24
	209	−33.65 (0.807)
	221	−27.14
m71	195	+43.56
	208	−35.71 (0.956)
	220	−34.10

[a] $[\theta]_M$ expressed as degrees square centimeter per decimole. Values in parentheses are R values ($\theta_{n\text{-}\pi^*}/\theta_{\pi\text{-}\pi^*}$).

values of both the synthetic Hsn-5 and reHsn-5 were ~0.9, indicating populations of largely α-helical conformations in hydrophobic environments.

Among the variants which had comparable candidacidal activities (m1, m2, m12, and m70), only m2 and m70 were selected for CD analysis. The variants m1, m2, and m12 were expected to have secondary structures comparable to that of reHsn-5, since the substituted amino acids Ile-12, Asn-17, and Leu-21 had a comparable α-helical propensity to that of Arg-12, Lys-17, and His-21, respectively. The m70 variant was expected to have less α-helical content because of the presence of Pro at position 19 instead of a His residue. The variants which had diminished candidacidal activity (m21, m71, and m68) were all examined to correlate the secondary structure with their candidacidal activity. The Hsn-5 variants m2, m21, m68, m70, and m71 showed CD spectra similar to that of reHsn-5 and synthetic Hsn-5 in TFE (Fig. 6B and Table 2). Like the synthetic Hsn-5 and reHsn-5, all variants except m70 had R values of ~0.9. The m70 variant had less α-helical content ($R = 0.807$), presumably because of the replacement of His-19 with a Pro residue. The tertiary amide bond of the Pro residue in the place of secondary amide could destabilize and stretch the α-helical backbone conformation, which might have lowered the helical content.

DISCUSSION

As part of our ongoing investigation of the structure-function relationship of Hsns with respect to their candidacidal activity, we have expressed and purified the reHsn-5 and its random variants. As indicated in Results, we overcame the degradation problems of Hsn-5 in *E. coli* and were able to purify intact Hsn-5. Although the N-terminal amino acid, Asp, is replaced by Gly, both the helical propensity and the candidacidal activity of the reHsn-5 remain unaffected. The candidacidal activity and helical propensity of synthetic Hsn-5 have previously been reported to be consistent with those of native Hsn-5 (33). Therefore, in the present study, reHsn-5 is used as a control for analysis of the candidacidal activity of the Hsn-5 variants.

The Hsn-5 variants m1, m2, m12, and m70 possess candidacidal activity comparable to that of reHsn-5. As shown in Table 1, m1, m2, and m12 sequences have Ile in the place of Arg at position 12. In addition to this change, Lys at position 17 is replaced by Asn in m2, whereas in m12, His at position 21 is changed to Leu. The m70 has Pro at position 19 and Arg at position 21 instead of His in the original sequence (Table 1). These changes do not significantly affect the candidacidal activity of these molecules, suggesting that cationic Arg-12 and Lys-17 and imidazole rings of His-19 and His-21 may not be essential for candidacidal activity of Hsn-5. Instead of Arg-12, the hydrophobic Ile appears to be compatible, indicating that a hydrophobic side chain interaction with the yeast cell membrane may be important at this position and not the positive charge. Asn at position 17 appears to be comparable to Lys when the cidal potencies of reHsn-5 and m2 are compared, indicating that the interaction of the peptide with the *C. albicans* membrane requires a polar side chain with a hydrogen bonding potential. This also suggests that the positive charge of Lys at position 17 is not critical for candidacidal activity. The Lys residue is expected to be susceptible to trypsin-like proteinase degradation in saliva. The m2 variant, in which Lys-17 is replaced by Asn, could be more resistant to proteolytic degradation and at the same time is equipotent to the parent molecule. In fact, we have purified a larger amount of m2 peptide (~1.5 mg/liter) than that of reHsn-5 by the same purification procedure, presumably because of its resistance to proteolysis. The replacement of His-19 and His-21 in m70 with Pro and positively charged Arg, respectively, elicits candidacidal activity similar to that of the parent molecule, suggesting that the imidazole moieties in these positions may not be essential for fungicidal activity. At position 19, the hydrophobic Pro residue in the place of histidine did not affect the cidal potency, suggesting that partial hydrophobicity of His at this position may be important for cidal activity. The linear relation established between hydrophobicity and the fractional accessibility of amino acid side chain to the solvent within the structure, particularly for moderately polar amino acids such as histidine and tyrosine, provides support for this argument (36). The Arg residue at position 21 would provide both hydrophobic and polar effects as a result of its alkyl and guanidine side chain groups, respectively. Therefore, both the partial hydrophobicity and/or polarity of His-21 may be necessary for candidacidal activity. The candidacidal potency of the m70 variant at various pHs has not been determined in the present study. The imidazole groups which could serve as a buffer may be important for fungicidal activity at altered pH levels.

The CD spectra of reHsn-5 and the variants m1, m2, m12, and m70 shown in Fig. 6B are reminiscent of an α-helical conformation. With the exception of m70, the α-helical propensity is high, with an R value of ~0.9 (Table 2). The R value of the m70 variant, in which His-19 and His-21 are replaced by Pro and Arg, respectively, is slightly lower ($R = 0.8$) than those of other molecules. The replacement of His-21 by Arg may not have a significant effect on the backbone conformation, since the helical propensities of the two residues are quite comparable. The presence of Pro in the sequence instead of His at position 19 results in the formation of a tertiary amide in the

place of a secondary amide, with the loss of an intramolecular hydrogen bond, which might cause a slight distortion in the helical backbone. This accounts for the slightly lower α-helical content of this variant. However, the candidacidal activity of m70 is not significantly lowered, suggesting that the small variation in the α-helical backbone may not affect the interaction of the side chains with the *C. albicans* cell membrane. Alternatively, it is also probable that the negative cidal effect due to the decreased α-helical content might be balanced by the increased positive charge caused by Arg-21.

The Hsn-5 variants m21, m68, and m71 are less effective in killing *C. albicans* than reHsn-5 is. Variants m21 and m71 have Thr and Glu in place of Lys-13, respectively. The sequence of m68 has Glu and Gly instead of Lys-13 and Arg-22, respectively. The cidal potencies of these variants are much lower than that of reHsn-5 (Table 1). The replacement of Lys-13 by Thr or Glu results in a threefold loss in activity (according to ED_{50} values), suggesting the importance of a positive charge at this position for candidacidal activity. The substitution of the negatively charged Glu in the place of Lys-13 and Gly in the place of Arg-22 markedly lowers the cidal potency (nearly 10-fold as shown by the activity of m68; see Table 1). The data suggest that Lys-13 and Arg-22 are crucial for candidacidal activity. The CD spectra of these three variants show that they prefer largely an α-helical conformation in a nonaqueous solution. Collectively, the results indicate that in addition to the α-helical backbone conformation, specific functional elements such as the side chains of Lys-13 and Arg-22 are essential to elicit high candidacidal activity. It has also been previously reported that Arg-22 in a Hsn fragment is important for inhibiting hemagglutination of *P. gingivalis* (27).

All three variants with diminished candidacidal activity were analyzed on a cationic gel before and 1.5 h after the incubation with *C. albicans*. No measurable degradation of peptides was observed (data not shown). These results suggest that the diminished candidacidal activity observed for these peptides may not be related to the susceptibility of the peptides for proteolytic degradation.

The growth inhibitory effects of Hsns on *C. albicans* have been reported to be concomitant with the loss of potassium from yeast cells, suggesting that these peptides may alter membrane permeability (32), presumably as a result of the formation of ion channels in cell membranes. However, the spontaneous insertion of the peptide into the hydrophobic barrier of membranes appears unlikely as the helical structure lacks sufficient amphipathic character (34). Cationic antimicrobial peptides such as magainins from frog skin exhibit a broad spectrum of antimicrobial activity, presumably through the formation of cation-selective channels in cell membranes. Interestingly, magainins have also been reported to prefer an α-helical conformation in TFE-water and in micelles (3, 21) and random coil conformation in water. Magainin 2 has been suggested to form cation-selective channels by superficially associating with the lipid bilayer. The peptide serves only to stabilize the channel lining which is actually formed by lipid head groups (8). Hsn-5 may have the same potential to form this type of ion channel without being inserted into the lipid bilayer. Although an ion channel may be formed by the interaction of Hsn-5 with the lipid bilayer, other mechanisms may also be involved in the candidacidal activity of Hsns. Putative receptors for histatins have been reported to exist on *C. albicans* membranes (10, 11). Further experimental evidence is necessary to determine the precise mechanism of interaction of Hsns with *C. albicans*.

In summary, the present study of Hsn-5 and its variants (under our assay conditions) suggests that Lys-13 and Arg-22 are important functional elements, while Arg-12, Lys-17, His-19, and His-21 may not be essential for eliciting high candidacidal activity. The present and future investigations may delineate variants (such as m2) that have high cidal potency and resistance to proteolytic degradations. Such variants might serve to overcome microbial adaptive resistance to conventional antibiotics. Ultimately, we strive to produce molecules with enhanced antifungal activity and resistance to proteolytic degradation which may serve as novel therapeutic agents.

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