Gelatin fragments block adherence of *Candida albicans* to extracellular matrix proteins

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The adherence of *Candida albicans* to extracellular matrix proteins may be a critical step in the pathogenesis of candidiasis. Yeast cell adherence to type I and IV collagen, fibronectin and laminin was blocked by peptide fragments from denatured type I collagen (gelatin). Gelatin fragments were obtained by digestion of the reduced protein with trypsin or CNBr. The fragments did not have antifungal properties, presumably inhibiting adherence by blocking receptors (adhesins) on the surface of the fungus. A 10-mer (GQRGVVGLPG) fashioned from the α -1 chain of type I collagen reduced adherence by 68%. However, a gelatin peptide possessing 47 amino acids reduced fungal adherence to type I collagen by 100%. Peptides derived from the biocompatible protein gelatin, therefore, may have a potential role in reducing the adherence of the fungus to host proteins.

Keywords: *Candida*, extracellular matrix, adherence, peptides, gelatin

INTRODUCTION

The adherence of *Candida albicans* to host tissue is presumed to be a critical and necessary step in the pathogenesis of all forms of candidiasis, including the gravest manifestation of the disease, disseminated candidiasis (Calderone & Braun, 1991; Klotz, 1992). One of the fungal targets for adherence in candidiasis may be proteins of the extracellular matrix (ECM) (Klotz, 1992). Since adherence is a necessary step in the establishment of disease by *C. albicans*, it follows that preventing or interrupting adherence may ameliorate or abrogate disease. In this report we show that gelatin fragments inhibit or block the adherence of *C. albicans* yeast cells to ECM proteins.

Gelatin contains primarily the denatured form of type I collagen, a glycoprotein which, along with fibronectin, laminin, and type IV collagen, may constitute some of the adherence targets of bloodborne yeast cells in disseminated candidiasis (Klotz, 1992). These ECM glycoproteins may serve as targets for adherence in mucosal and skin infections with this fungus as well. For example, *C. albicans* binds avidly to the ECM proteins type I and IV collagen, laminin and fibronectin when these glycoproteins are immobilized on plastic surfaces (Klotz, 1990). Furthermore, the fungus binds soluble fibronectin and in

doing so prevents fungal adhesion to immobilized ECM proteins and basement membrane (Klotz & Smith, 1991). Previous work has shown that the adherence of *C. albicans* yeast cells to immobilized ECM proteins and to sub-endothelial ECM is also inhibited by peptides derived from fibronectin, such as RGD, or from collagen, such as GRGESP (Klotz & Smith, 1991).

Gelatin is a protein available in large quantities which is tolerated by humans even when given intravenously (Adelmann-Grill, 1981). Gelatin contains both RGD and RGE sequences and therefore may yield fragments containing these sequences which possess inhibitory activity against *C. albicans* adherence to ECM proteins. The present work was undertaken to identify biocompatible fragments of gelatin which are also effective inhibitors of adherence of *C. albicans* to ECM glycoproteins. Fragments of gelatin obtained by treatment of the protein with trypsin or CNBr were separated and tested individually in a systematic fashion to detect their effect on the adherence of yeast cells to ECM proteins.

METHODS

Trypsin digestion. Porcine gelatin (Sigma) (1 mg ml⁻¹) in Earle's balanced salt solution (EBSS) containing 10⁻⁷ M trypsin (Sigma) was incubated for varying times at 37 °C as stated. Digestion was stopped with twofold excess amounts of soybean trypsin inhibitor (Sigma). Tryptic gelatin fragments were desalted on a Biogel P-2 (Bio-Rad) column, lyophilized, dissolved in 0.1 M acetic acid and then separated on a Sephadex

Abbreviation: ECM, extracellular matrix.

G-25, G-50 or G-100 (Sigma) column (2.5 × 95 cm) equilibrated with 0.1 M acetic acid.

CNBr treatment. Porcine gelatin was first reduced by solubilizing 100 mg gelatin in 15 ml 0.1 M ammonium bicarbonate to which 7 ml 2-mercaptoethanol was added. The gelatin was placed in a waterbath overnight at 60 °C. The gelatin was desalted by passage over a Biogel P-2 (Bio-Rad) column in 0.1 M acetic acid and lyophilized. Equal amounts of gelatin (100 mg) and CNBr (100 mg) in 0.1 M HCl (20 ml) were then incubated at 40 °C for 4 h. This was desalted on a Biogel P-2 column into 0.1 M acetic acid and lyophilized. The lyophilized CNBr-treated gelatin fragments were dissolved in 6 ml 0.1 M acetic acid and applied to a Sephadex G-100 column.

Separation of CNBr-treated gelatin fragments. Four peaks of CNBr-treated gelatin fragments were identified from the G-100 column. The fractions from the four peaks were pooled, lyophilized, solubilized in EBSS at 1 mg ml⁻¹ concentration and individually tested for adherence inhibitory activity. Peaks from the G-100 column were then separated further by reversed-phase HPLC (Gilson, Model 260) using a 0.45 × 25 cm C₁₈ large-pore column (Vydac) with a water/acetonitrile gradient. Mobile phase A consisted of 0.1 % trifluoroacetic acid in distilled water whereas mobile phase B consisted of acetonitrile/water/trifluoroacetic acid (95:4.9:0.1, by vol.). Flow was constant at 1 ml min⁻¹ and the linear gradient at injection was 98 % phase A, 2 % phase B, at 30 min 78 % A, 22 % B and at 40 min 38 % A, 62 % B. The solvent was evaporated from the fractions with dry N₂ and the fractions stored at 4 °C.

***C. albicans*.** Four clinical isolates of *C. albicans* were maintained on Sabouraud dextrose agar (Difco) slants and subcultured monthly. For purposes of an adherence assay a loopful of micro-organisms from the slant was inoculated into 50 ml Sabouraud dextrose broth (Difco) and incubated at room temperature with shaking for 20 h. This yields early-stationary phase yeast cells. Yeast cells were then washed in EBSS by centrifugation and resuspended at desired concentrations by use of a haemocytometer (Klotz & Smith, 1991).

Adherence test. The adherence assay was conducted as previously described by Klotz & Smith (1991). Target proteins were coated onto the bottom of the plastic wells of 24-well tissue culture trays. Bovine corneal subendothelial ECM in 24-well tissue culture trays was purchased from Accurate Chemical and Scientific Corporation. Each well was inoculated with 150 c.f.u. of *C. albicans* in 200 µl EBSS with or without protein fragments and left stationary at 37 °C for 30 min. The number of c.f.u. added was determined by also spreading samples onto Mycosel agar (BBL Laboratories) plates and counting the c.f.u. the following day. The wells were then washed three times with 1 ml EBSS, covered with molten Mycosel agar and incubated overnight at 37 °C. The number c.f.u. in each well was determined the following day and the percentage adherence was calculated by dividing this number by the number c.f.u. added to each well and multiplying by 100.

Protein sequencing. Peaks obtained from HPLC were submitted for protein sequencing by Edman degradation (Biotechnology Services, University of Kansas Medical Center, Kansas City, KS, USA).

RESULTS

Trypsin digest

Digestion of gelatin was first carried out using trypsin solubilized in EBSS, a buffer which allows for maximal adherence of yeast cells to immobilized ECM substrates.

Table 1. The inhibitory activity of non-fractionated trypsin-digested gelatin fragments on the adherence of *C. albicans* to immobilized gelatin

Percentage adherence equals number of c.f.u. of yeast cells adherent to gelatin as a percentage of the total c.f.u. added. Proteins were added at 1 mg ml⁻¹ concentration. Four wells were used for each experimental variable and the experiments repeated a minimum of three times. Digestion of the protein was at 37 °C.

Addition to gelatin	Percentage adherence ± SEM
Buffer only	87 ± 4
Trypsin + trypsin inhibitor (added simultaneously, no digestion)	72 ± 7
Trypsin for 60 min, then trypsin inhibitor	73 ± 8
Trypsin for 24 h, then trypsin inhibitor	25 ± 5
Trypsin for 48 h, then trypsin inhibitor	22 ± 6

The digestion was terminated at various time points including zero time of incubation and 24 and 48 h at 37 °C. The results of measuring adherence to immobilized gelatin when the crude digest of gelatin was added simultaneously with yeast cells are shown in Table 1. As can be seen, by 24 h digestion the crude trypsin digest was effective in inhibiting the adherence of yeast cells to immobilized gelatin. In order to test the generality of this inhibitory effect by gelatin fragments, digests were added simultaneously with yeast cells to a variety of immobilized ECM proteins. The results of such an experiment are shown in Table 2. Again it is clear that the gelatin fragments inhibit the ability of the yeast cells to adhere to various ECM glycoproteins.

The crude digest obtained in the manner explained above was then passed over a G-25 column and fractions pooled, lyophilized, dissolved in EBSS at 1 mg ml⁻¹ concentrations and the adherence of *C. albicans* yeast cells to type I collagen determined. The inhibitory activity of the crude gelatin digest was contained in the earliest eluting fractions from the G-25 column (data not shown).

CNBr digests of gelatin

We also analysed CNBr digests to determine if the same inhibition of yeast cell adherence held true for gelatin fragments produced by this method. The results of Sephadex G-100 chromatography of reduced, CNBr-digested porcine gelatin is shown in Fig. 1. The fractions were pooled from each of the four peaks, lyophilized and tested at 1 mg ml⁻¹ concentrations in the yeast cell adherence assay. This was done in order to identify any inhibitory activity of the pooled samples to immobilized gelatin. The results are shown in Table 3. It is clear that peak 4 possessed significant inhibitory activity in the adherence assay. Therefore peak 4 from the Sephadex G-100 chromatography was separated further by reversed-phase HPLC using a C₁₈ column and an acetonitrile/water gradient. Over 20 HPLC sample peaks were then in-

Table 2. Inhibition of adherence of *C. albicans* yeast cells to ECM proteins in the presence of unfractionated trypsin digests of gelatin added at 1 mg ml⁻¹ concentration

Four wells were used for each experimental variable and experiments were repeated a minimum of three times.

Yeasts added to wells in:	Percentage of yeasts adhering to proteins $\pm$ SEM				
	Type IV collagen	Type I collagen	Fibronectin	Laminin	Gelatin
Buffer	90 $\pm$ 4	79 $\pm$ 1	90 $\pm$ 10	94 $\pm$ 1	88 $\pm$ 5
Buffer plus trypsin digest	4 $\pm$ 1	7 $\pm$ 2	1 $\pm$ 1	6 $\pm$ 2	4 $\pm$ 2

Fig. 1. Peaks and their respective fractions obtained from chromatography of CNBr-digested gelatin on Sephadex G-100.**Table 3.** Inhibition of *C. albicans* adherence to immobilized gelatin in the presence of CNBr digests as separated on a Sephadex G-100 column

Four wells were used for each experimental variable and experiments were repeated a minimum of three times.

Competitive inhibitor	Percentage adherence $\pm$ SEM
Buffer only	90 $\pm$ 2
Undigested gelatin (1 mg ml ⁻¹)	82 $\pm$ 5
Peak 1 (1 mg ml ⁻¹)	79 $\pm$ 3
Peak 2 (1 mg ml ⁻¹)	72 $\pm$ 3
Peak 3 (1 mg ml ⁻¹)	74 $\pm$ 4
Peak 4 (1 mg ml ⁻¹)	22 $\pm$ 2

Table 4. Inhibition of adherence of *C. albicans* to immobilized gelatin in the presence of peptides isolated by HPLC from peak 4 in Fig. 1

Total protein in each dried peak was used in the assay, and was quantified from the area under each peak which, in turn, was related to the areas of peaks collected on a separate chromatogram of this material and measured by the Pierce BCA protein assay using gelatin as standard. The experiments were repeated twice.

Added protein or peak	Protein (μ g)	Percentage adherence
EBSS (control)	—	100
Gelatin (1 mg ml ⁻¹)	200	100
Peak 12	5.5	5
13	6.6	6
14	8.5	7
15	9.6	0
16	5.5	3
17	4.4	4

shown before, does not have inhibitory activity against yeast cell adherence.

Identification of peptide fragments

Attempts were made to sequence the proteins in the peaks obtained from reversed-phase HPLC which contained adherence inhibition properties. This was unsuccessful with one exception, mainly because several peptides were found in each peak. The one identified gelatin peptide with 100% inhibitory properties was a 47-mer known as CB4, residues 40–86 of the α -1 chain of type I collagen. CB4 contains three RGX sequences but no RGE or RGD sequences. Because we could not purify the peptides from the CNBr digest to absolute purity we turned to synthesizing several known peptide sequences with possible inhibitory activity. We used several known peptide sequences from the α -1 chain of type I collagen in adherence assays (Table 5). Only the 10-mer (residues

dividually lyophilized and resuspended in 200 μ l buffer and an adherence assay performed. Table 4 shows results from six active peaks. The parent molecule gelatin, as

Table 5. Inhibition of adherence of *C. albicans* to immobilized gelatin by two peptides from the α -1 chain of type I collagen, a 9-mer which is the first nine residues of the N-terminus of processed type I collagen, and a ~23-mer peptide, PepTite-2000, containing the RGD site of fibronectin

Peptides were added to EBSS at 1 mg ml⁻¹ concentrations.

Peptide	Percentage adherence of control
PQGIAGQRGVVGLPG-NH ₂ (residues 773–787)	100
GQRGVVGLPG-NH ₂ (residues 778–787)	32
QLSYGYDEK	100
XXXGRGDSPASSKXXX (PepTite-2000)	0

778–787) demonstrated significant inhibition of adherence whereas several other peptides did not.

DISCUSSION

The purpose of this study was to identify biocompatible, non-antifungal peptides which block the adherence of *C. albicans* yeast cells to ECM glycoproteins. ECM and its constituent glycoproteins comprise some of the major targets of adherence of this fungus to host tissue (Klotz, 1992) and adherence is one of several acknowledged important steps in the process of establishing disease with the fungus (Calderone & Braun, 1991). Based on our previous *in vitro* experiments it was reasonable to conclude that the ability of a protein to block adherence may have potential in ameliorating or abrogating disease with this micro-organism (Klotz & Smith, 1991). In fact, one *in vivo* investigation by us supports the concept that blocking adherence ameliorates disease with *C. albicans* (Klotz *et al.*, 1992).

Gelatin cleavage products are good candidates for effective blocking agents for the following reasons. Gelatin itself has little or no inhibitory activity against yeast cell adherence although when immobilized it is a surface to which *C. albicans* will adhere (Klotz, 1990). In work with fibronectin we learned that whole fibronectin and the amino acid sequences RGD and RGE were effective in inhibiting adherence of *C. albicans* yeast cells to immobilized ECM (Klotz & Smith, 1991) and cryptogenic fragments of fibronectin derived by enzymic digestion bound more avidly to yeast cells than did whole plasma fibronectin (Penn & Klotz, 1994).

Gelatin contains both RGD and RGE sequences and therefore its constituent peptides have the theoretical potential to inhibit adherence at least as well as the fibronectin peptides. Furthermore, gelatin is biocompatible (Adelmann-Grill, 1981) and has no detectable antifungal properties. From the work reported here it is obvious that peptides derived from gelatin do inhibit the

adherence of *C. albicans* to ECM and ECM proteins. This was apparent from results obtained in adherence experiments using the crude digests of gelatin obtained from digestion with trypsin and CNBr. Furthermore, when CNBr digests were further separated by column chromatography with Sephadex G-100 into peptides of differing molecular mass it was apparent that the later-eluting fractions presumably containing the lower molecular mass peptides possessed the ability to block adherence of the fungus to ECM and its proteins. Upon separation of the fraction responsible for the inhibition of adherence using HPLC it became apparent that several of the lower molecular mass peptides had significant adherence inhibitory properties. The inhibition of adherence by the peptides was extremely effective: very small amounts of the peptides separated by HPLC caused complete inhibition of adherence of *C. albicans* yeast cells to ECM glycoproteins.

Attempts to sequence the peptides responsible for the inhibitory activity were unsuccessful, with one exception, a 47-mer. Therefore several known short peptide sequences derived from the α -1 chain of type I collagen were synthesized and tried in the adherence assay. Only one, a 10-mer, showed significant inhibitory activity, but its activity was not comparable to that seen with the 47-mer. Future work will be directed towards improved isolation of these inhibitory proteins in greater quantity as well as defining their exact amino acid sequence.

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