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Proteolytic Activity of *Candida albicans*: Action on Human Salivary Proteins

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The susceptibility of human salivary proteins to degradation by *Candida albicans* was studied. The organism was cultivated in either whole-salivary supernatant or parotid fluid, both of which were supplemented with glucose (0.1%). The culture pH's were at, or above, neutrality. After growth, the culture supernatant solutions were examined by polyacrylamide gel electrophoresis for alterations in their profiles of salivary proteins. No evidence of proteolysis of whole-saliva or parotid fluid proteins was found. Salivary proteins, however, are susceptible to degradation by preparations of *C. albicans* protease. *Candida* protease was incubated with parotid fluid adjusted to several pH values. After incubation the reaction mixtures were subjected to polyacrylamide gel electrophoresis. Extensive degradation of parotid proteins was found at pH 4, very slight proteolysis at pH 5, and no degradation at pH 6 or 7. No selectivity in proteolysis of the several parotid proteins was noted. These results indicate that *C. albicans* protease is strictly dependent upon low (ca. 4) pH for activity on salivary proteins. Furthermore, it is suggested that due to the pH requirements of the enzyme, it is unlikely to be of major significance to the pathogenesis of *Candida*-induced oral inflammatory lesions.

Candida albicans is a member of the normal human oral flora (5, 8, 17). *C. albicans* is found in the oral cavity of over 50% of the human population. The organism colonizes mucosal surfaces preferentially and may compete for establishment with other genera of microorganisms (10). *C. albicans* is also the primary pathogen associated with palatal inflammation in full-denture wearers (denture stomatitis) (1, 5). It has been suggested that proteases elaborated by *Candida* organisms contribute to their virulence. For example, mice were infected with *C. albicans* that produce proteases and with other strains that failed to produce proteases. It was reported that enhanced virulence was exhibited by protease-producing strains (15). On the other hand, no evidence exists of increased frequency of isolation of proteolytic strains of *C. albicans* from inflamed versus noninflamed mouths of human subjects (3). Furthermore, the pH optimum for *Candida* proteolysis of some standard protein substrates such as bovine serum albumin and hemoglobin is low and in the range of 3 to 4 (6, 15; G. R. Germaine and L. M. Tellefson, submitted for publication). This pH is not reached in either denture plaques or human saliva even after exposure to acidogenic substrates (12). In addition, recent experiments have shown that the acid protease may be irreversibly inactivated at pH values of 6 and above (Germaine and Tellefson, submitted for publication). Any assessment of the role of *C. albicans* protease in oral inflammation must consider the above. Further, it is unknown whether (i) protease is produced during growth of *C. albicans* in vivo, and (ii) whether proteolysis of salivary proteins is as pH dependent as is proteolysis of hemoglobin and bovine serum albumin. In an attempt to approach these questions, we have studied the growth of *C. albicans* in saliva with respect to utilization of salivary proteins and also have demonstrated the mode of susceptibility of salivary proteins to preparations of *Candida* protease.

MATERIALS AND METHODS

Organism. *C. albicans* was obtained from the culture collection of the Department of Microbiology, University of Minnesota.

Media. Routine passage of the organism was in sheep blood agar (per liter of water): Trypticase soy (30 g), yeast extract (5 g), menadione (0.5 mg), hemin (5 mg), agar (15 g), and sheep blood (50 ml). Growth of *C. albicans* for protease production was in a modification (substitution of yeast extract for *Protovita*) of the medium of Remold et al. (15) (per liter of water): yeast extract (100 mg), glucose (20 g), bovine serum

albumin (2 g), $MgSO_4$ (500 mg), KH_2PO_4 (1 g). The medium was adjusted to pH 4.0 and sterilized by filtration.

Saliva. Unstimulated, human whole saliva was collected by expectoration. A pool of saliva from at least five donors was always used. The saliva was centrifuged (10,000 × *g*, 20 min, 4°C followed by 100,000 × *g*, 120 min, 4°C), and the supernatant solution was passed through a 0.45-μm membrane filter unit (Nalge, Rochester, N.Y.). Parotid fluid was collected as described earlier (16). Up to 20 ml of stimulated (lemon candy) fluid was obtained per donor. Parotid fluid was passed through a filtration unit as above.

Cultivation of *C. albicans* in saliva. *C. albicans* was removed from a blood plate and suspended in 10 mM phosphate (sodium and potassium salts) buffer–0.9% NaCl (pH 7.0) (phosphate-buffered saline) to an optical density of 1.0 (540 nm). Sterile parotid fluid and sterile whole saliva (see above) supplemented with 0.1% glucose (wt/vol) was inoculated with the *C. albicans* suspension to give a 1% inoculum. Saliva cultures, including uninoculated controls, were incubated stationary either in air or in an anerobic (GasPak, BBL) atmosphere at 37°C. At appropriate intervals, growth was estimated by measuring culture optical density at 540 nm.

Assessment of salivary protein degradation by *C. albicans*. Saliva cultures of *C. albicans* were centrifuged (ca. 9,000 × *g* for 10 min), and the supernatant solution was dialyzed against two changes of distilled water (2,000 volumes per change) at 4°C. Dialyzed supernatants were lyophilized and stored at −20°C until used. Lyophilized salivary supernatant was reconstituted in phosphate-buffered saline to 1/10 the original volume. Reconstituted salivary supernatant, or dilutions thereof, was mixed with 0.2 volumes of 50% glycerol containing bromophenol blue. The samples so prepared were subjected to polyacrylamide gel electrophoresis (PAGE).

Preparation of crude protease. The effect of a wide range of yeast extract supplementation of the basic medium on protease production by *C. albicans* was initially investigated. Maximum growth and protease production was achieved within 48 h when 10^{-2}% (wt/vol) yeast extract supplementation was used. Accordingly, depending upon protease level as determined by assay, supernatant solutions from 24- to 48-h cultures of *C. albicans* were treated with ammonium sulfate (90% of saturation) at 0°C for 3.5 to 4 h. Precipitates were collected by centrifugation and dissolved in, and dialyzed against, 1 mM phosphate buffer, pH 7.2. All of the above procedures were performed at ice-bath temperatures. Enzyme solutions were lyophilized and stored at −20°C.

Protease assay. Enzyme preparations were routinely assayed by incubation with bovine serum albumin, at 2 mg/ml, in sodium acetate buffer (50 mM, pH 4.0) at 37°C. At appropriate times samples were mixed with trichloroacetic acid to give a 5% (wt/vol) final concentration and chilled in ice for at least 30 min. Supernatant solutions were collected by centrifugation. Acid-soluble products of proteolysis were estimated by absorbancy at 280 nm in the supernatants. In addition, Folin-reactive material was also estimated in the supernatants by the Lowry et al. (11) method. One unit of protease will release 1 μg of tyrosine equivalents per min at 37°C from bovine serum albumin.

Degradation of salivary proteins by *C. albicans* protease. Twenty milliliters of freshly collected parotid fluid was titrated with 1 N HCl. At the following pH values, 3-ml samples were removed: pH 7.02, 6.07, 5.03, and 4.01. Next, the parotid samples at desired pH's were incubated with *C. albicans* protease at a final concentration of 100 μg/ml (1 to 1.3 U/mg of protein) in the presence of 1 drop of toluene as preservative. Incubation continued for 24 h at 37°C under an anaerobic atmosphere (GasPak, BBL). The reaction mixtures were clarified by centrifugation (9,000 × *g* for 10 min), and the supernatant solutions' final pH was determined. Then the solutions were dialyzed against two changes (2,000 volumes each) of cold distilled water, lyophilized, and stored at −20°C until used. Lyophilized parotid fluid samples were reconstituted in 1/5 volume of phosphate-buffered saline containing 10% (wt/vol) glycerol and sufficient (ca. 0.001%, wt/vol) bromophenol blue. Samples so prepared were subjected to PAGE.

PAGE of salivary samples. The gel system is a modification of a published method (16). A triple-layer slab gel system was used. The lower-most gel (8.5 by 18 by 0.2 cm), middle gel (1.5 by 18 by 0.2 cm), and stacking gel (2 by 8 by 0.2 cm) contained 10, 6, and 3% (wt/vol) acrylamide, respectively. Buffer for the 10% gel consisted of 500 mM tris(hydroxymethyl)-aminomethane-sulfate, pH 9.0. The buffer used for the 6 and 3% gels was 375 mM tris(hydroxymethyl)-aminomethane-sulfate, pH 9.0. The electrode buffer was 65 mM tris(hydroxymethyl)aminomethane-borate, pH 9.0. Samples (25 μl) in phosphate-buffered saline containing glycerol and tracking dye were applied into sample slots formed in the 3% gel. Electrophoresis was performed at ambient temperature for 3 h at 30 mA (constant current). Next, the gel was fixed in 25% (wt/vol) trichloroacetic acid for 30 min, stained for 20 min in a solution of 0.1% Coomassie brilliant blue in 25% trichloroacetic acid (wt/vol), and destained with several changes of 10% acetic acid over 18 to 24 h.

RESULTS

Growth of *C. albicans* in saliva. Initial experiments indicated that *C. albicans* grew very poorly, if at all, in human saliva culture if no carbohydrate supplementation was provided. Therefore, we routinely used 0.1% glucose in this regard. Growth of the organism in filter-sterilized whole-saliva supernatants was always superior to that obtained in filter-sterilized parotid fluid. In addition, incubation of the stationary cultures in air yielded greater growth than when an anaerobic atmosphere was used. In the experiments reported here, incubation of the cultures proceeded for 48 h. Microscopic examination at this time indicated the presence of blastospores (yeast form) and hyphae. Optical densities (540 nm) of the cultures at 48 h ranged from 0.214 (whole saliva, aerobic) to 0.130 (pa-

rotid fluid, anaerobic). In some cases, turbidities did develop in sterile saliva. This may be due to pH and/or calcium solubility changes. Thus, absolute estimates of growth by optical density measurements may not always be reliable and must be considered in the light of uninoculated controls. The range of culture optical densities given above are such adjusted values. None of the pH values at 48 h was less than 6.85. Aerobic cultures had elevated pH values (8.84, parotid fluid; 7.37, whole saliva) due to loss of CO_2 from the salivas. Anaerobic incubation tended to stabilize pH rises, since the atmosphere is enriched in CO_2. In these experiments the time course of pH rise could not be followed. However, in other studies with whole saliva and parotid fluid, slight pH changes occur within several hours and appear to reach stable levels after ca. 24 h.

Salivary proteins in saliva cultures. The protein profiles of the culture supernatants from whole-saliva and parotid fluid systems are displayed in Fig. 1. Each sample was examined at three concentrations to assist in detection of altered protein patterns. Comparison of the PAGE profiles of inoculated to uninoculated cultures failed to yield any evidence of significant alteration of protein profiles by growth of *C. albicans*. This was true for both whole-saliva and parotid fluid cultures incubated either aerobically or anaerobically. Thus, growth of *C. albicans* in oral fluids was not accompanied by salivary protein digestion.

Susceptibility of salivary proteins to *C. albicans* protease. Incubation of *C. albicans* protease (100 mU/ml, final concentration) with parotid fluid was performed at several pH values (Table 1). The reaction mixtures were incubated in an anaerobic atmosphere enriched in CO_2 to minimize pH changes. We purposely avoided the use of exogeneous buffers. The final pH's of the

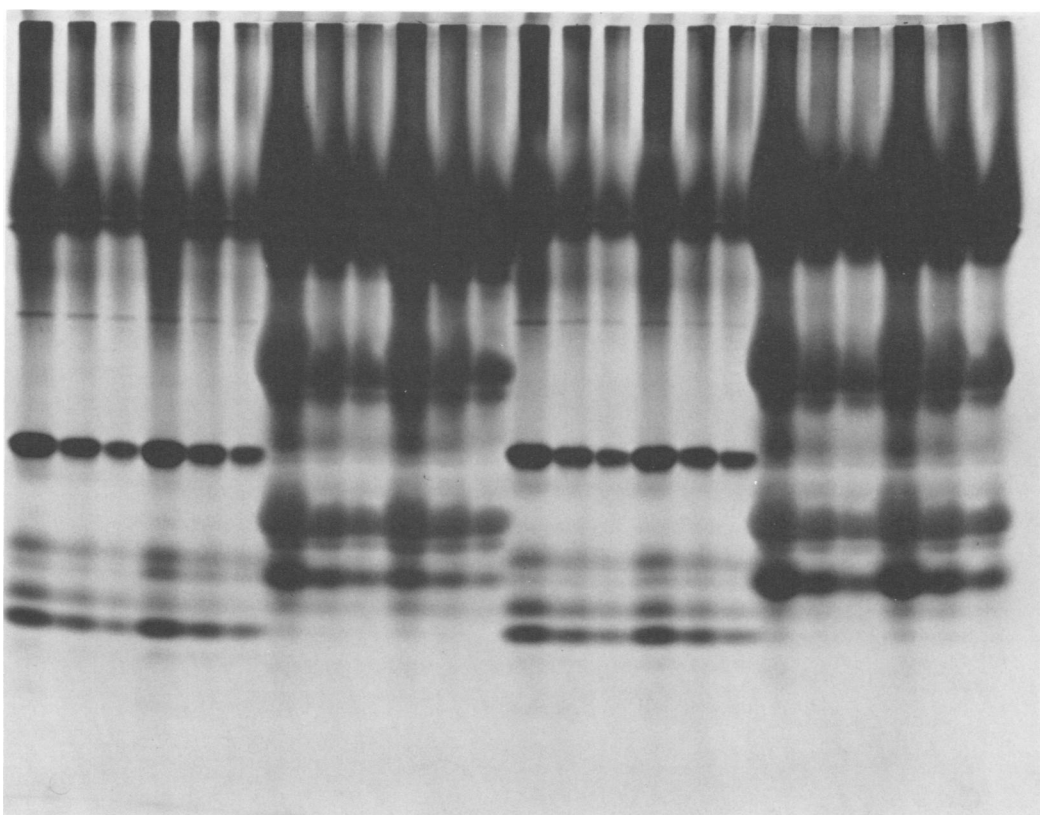

FIG. 1. *PAGE of saliva supernatants after growth of C. albicans. Samples are whole saliva (lanes 1 to 6, 13 to 18) and parotid fluid (lanes 7 to 12, 19 to 24) from C. albicans cultures and uninoculated controls incubated either aerobically (lanes 1 to 12) or anaerobically (lanes 13 to 24). The first three lanes of each of the four groups are samples from C. albicans-inoculated cultures. The last three lanes of each group are samples from the corresponding uninoculated controls. The three lanes with either inoculated or control samples from each of the four groups represent salivary supernatants that were reconstituted to (read left to right) eight-, four-, and two-fold higher concentrations than the original saliva.*

reaction mixtures after 24 h of incubation are given in Table 1. Note that, in general, increases of 0.5 to 1 pH unit were found. Based on initial pH, a sufficiently wide range of values was examined. The pH range spanned the region of protease pH optimum as assayed on bovine serum albumin (Germaine and Tellefson, submitted for publication) and, in addition, spanned those pH values found in vivo under a variety of conditions (12).

TABLE 1. *Reaction mixture pH values*

Condition	pH	
	Initial	Final
Protease + parotid fluid	4.01	4.52
	5.03	5.96
	6.07	7.39
	7.02	7.85
Protease alone	7.00	6.43
Parotid fluid alone	4.01	4.70
	7.02	7.86

PAGE analysis of the reaction mixtures clearly indicated that a pH-dependent proteolytic digestion of several parotid proteins occurred (Fig. 2). Comparison of the reaction mixtures (lanes 1 to 4) with the parotid controls (lanes 7 to 8) revealed that proteolysis was readily discernible at pH 4, barely detectable at pH 5, and inapparent at pH 6 and 7. Lanes 5 and 6 are enzyme controls that indicate the contribution of the protease preparation to the protein profile of the reaction mixtures.

DISCUSSION

Cultivation of *C. albicans* in human salivary secretions was investigated as an alternative approach to ultimately assess the potential role of

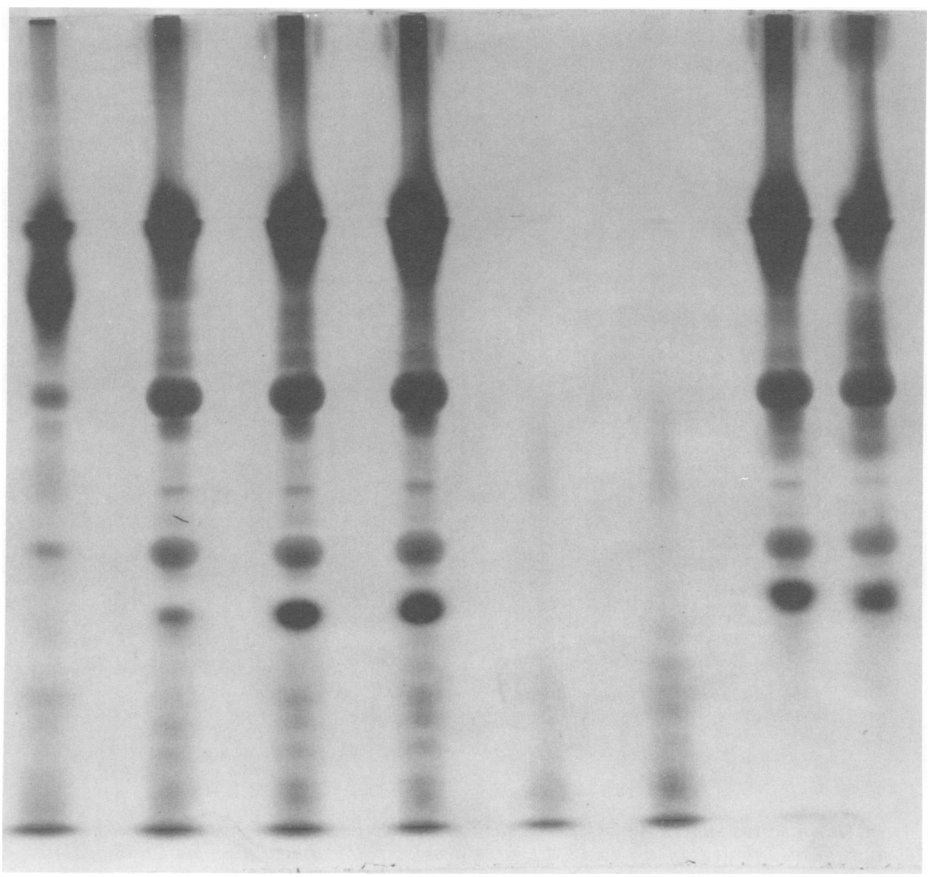

FIG. 2. *PAGE analysis of pH-dependent degradation of parotid fluid proteins by C. albicans protease. Samples were derived from the following reaction mixtures: (i) protease plus parotid fluid adjusted to pH's of 4 (lane 1), 5 (lane 2), 6 (lane 3), and 7 (lane 4); (ii) protease (pH 7) only, incubated 24 (lane 5) or zero (lane 6) h; (iii) parotid fluid only, incubated 24 h, pH 7 (lane 7), or pH 4 (lane 8).*

protease(s) in the microbe-host relationship. Previous studies have relied upon protease screening of oral isolates (3), virulence testing of proteolytic and non-proteolytic isolates (15), and indirectly, pH determinations of the *C. albicans* oral habitat (12). The principal bathing fluid in the oral cavity is saliva. Thus, we elected to explore the use of saliva as a growth fluid for *C. albicans*. Our initial results reported here clearly indicate that the organism will grow in saliva to reasonable turbidities. Furthermore, exogenous buffers appear not to be essential, especially if an atmosphere enriched in CO_2 is used. Growth in either human whole-saliva supernatant or human parotid fluid exhibited evidence of differentiation as judged by the presence of yeasts and hyphae. Although the hyphal phase of *C. albicans* has been much discussed as the more invasive form (2, 4, 6), biochemical evidence of significant differences between yeast and hyphal forms is lacking (6). Indeed, numerous environmental and chemical parameters have been suggested to directly affect the yeast-hyphae transition (9), including low O_2 tension and elevated pH. Hyphal growth is commonly encountered in *C. albicans*-induced oral inflammatory lesions in humans (2, 8) and experimental denture stomatitis in monkeys (4, 13). Thus, although the basis is unknown, in vitro saliva cultivation of *C. albicans* does yield growth modes reminiscent of the in vivo situation.

Growth of *C. albicans* in saliva-based culture was not accompanied by utilization of salivary protein as judged by PAGE. All the cultures were at, or above, neutral pH throughout the growth time. The principal proteolytic activity of *C. albicans* is only active under acidic conditions and is inactive at pH values over 5 (6, 15; Germaine and Tellefson, submitted for publication). However, Chattaway et al. (6) reported the presence of an unstable, neutral protease (pH optimum of 6.6) found intracellularly. Thus, it seemed possible that digestion of saliva proteins during growth of *C. albicans* at, or above, neutral pH might occur. This clearly did not happen. Thus, any proteolytic potential of *C. albicans* remained unexpressed under these conditions of cultivation.

Salivary proteins are susceptible to *C. albicans* protease activity. However, as with other protein substrates mentioned earlier, a requirement for activity against salivary (parotid) proteins is a low pH. Proteolysis was most marked at pH 4 and not detected at pH 6. In fact, only minimal activity was noted at pH 5. From the PAGE profiles, it appeared that no selectivity of degradation of parotid proteins occurred. All proteins displayed by the gel technique we used were susceptible to degradation. A major shift in mobility of a band of protein(s) of very high molecular weight seen at pH 4 was confirmed in another gel system, in which the running gel was 5% acrylamide (data not shown). In the 5% gel the material formed a somewhat diffuse band with no evidence of multiple components. Considering both gel systems, all proteins appeared susceptible to *C. albicans* protease at low pH. No proteins appeared to be degraded at pH's above 5. Thus, even under conditions where active protease preparations are mixed with salivary proteins, no evidence of the presence of a protease active at or near neutral pH's was obtained. Lack of selectivity of *Candida* proteolysis of salivary proteins is in contrast to rather extreme selectivity by some proteases of oral bacteria. For example, a protease of *Streptococcus sanguis* has only one known substrate, immunoglobulin A1 (14). In addition, culture broths of *S. sanguis* may contain several proteases but exhibit a very selective action pattern on human salivary proteins (7).

The data presented here demonstrated that *C. albicans* proteolytic activity is not expressed during growth in saliva culture near neutral pH. Furthermore, rather low pH environs are required for activity of *C. albicans* protease. In vivo attainment of sufficiently long periods of appropriately low pH's seems unlikely. These observations taken together with data already mentioned above do not support a role of proteolysis in the pathogenesis of common inflammatory oral lesions induced by *C. albicans*.

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