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Effect of pH and Human Saliva on Protease Production by *Candida albicans*

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The elaboration of extracellular proteolytic activity by *Candida albicans* during growth in laboratory broth or in human whole salivary supernatant was investigated. Growth of the organism in broth at pH 3 to 7 followed by assay of culture supernatants at pH 4 (optimum for activity) indicated protease was only present in cultures grown at a pH of <5. In contrast, growth in sterile human whole salivary supernatant over the pH range of 3 to 7 uniformly failed to result in production of protease. Growth of the organism at pH 4 in broth supplemented with saliva resulted in a saliva-dependent inhibition of protease production. Although the addition of up to 16% (vol/vol) saliva had essentially no effect on growth, 4% saliva caused a 50% reduction in proteolysis of substrate protein. Due to the low pH requirement for protease production and activity and the demonstration that saliva is a potent inhibitor of protease synthesis, we conclude *C. albicans* most likely does not produce extracellular protease in the human oral cavity.

Candida albicans is indigenous to the human oral cavity (9) and is a major cause of palatal inflammation in denture wearers (2). The organism is known to produce an extracellular acid protease (pH optimum of 3.5 to 4) and an intracellular neutral protease (pH optimum of 6.6) (3, 8). Prompted by an earlier report (8) that proteolytic *C. albicans* strains were more virulent in mice than nonproteolytic strains, a comparison of the proteolytic potential of isolates from denture wearers was published (1). It was found that production of extracellular protease activity was widespread among strains isolated from both healthy subjects and from those with palatal inflammation. It was suggested that proteolytic *C. albicans* was not uniquely associated with denture stomatitis. Failure to establish an association of in vitro extracellular protease production with the origin (healthy versus inflamed sites) of isolates does not constitute proof that candida proteolytic activity is not involved in the development of inflammation. Furthermore, it remains possible that the oral maintenance and proliferation of *C. albicans* might be enhanced by elaboration of proteolytic activity. Since saliva is the principal fluid that bathes those sites colonized by *C. albicans*, we have investigated the effect of saliva on expression of the proteolytic potential of the organism. In a previous communication (4), we reported that growth of *C. albicans* at neutral pH in saliva was not accompanied by degradation of any of several anionic salivary proteins. Incubation of crude extracellular protease with human parotid proteins, however, did result in degradation of several anionic proteins at pH 4 to 5. It remained possible, therefore, that growth in intraoral locations that might experience periods of low pH (such as on the tissue side of denture plates [6, 7]) might support elaboration of proteolytic activity. In this study, we report that saliva is a potent inhibitor of *C. albicans* acid protease synthesis over the pH range of 3 to 7 and thus conclude that it is unlikely that the organism is proteolytic in vivo.

MATERIALS AND METHODS

Organism, medium, and saliva. A proteolytic strain of *C. albicans* was used (4). The organism was cultured in the medium described earlier (4) supplemented with 10^{-2}% yeast extract. Unstimulated, human whole saliva was collected by expectoration. A pool of fresh saliva from at least five donors was always used. The saliva was centrifuged (10,000 × *g*, 20 min, 4°C), and the supernatant solution was sterilized by passage through a 0.45-μm membrane filter unit (Nalge/Sybron Corp., Rochester, N.Y.).

Cultivation of *C. albicans* in broth and saliva. In experiments where broth alone or saliva-supplemented broths were used, the inocula (1%) were obtained from overnight broth cultures. In cases where saliva served as the growth medium, *C. albicans* was removed from a blood plate and suspended in 10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.0, to an optical density of 1.0 (540 nm). Sterile, whole saliva supplemented with 0.1% glucose (wt/vol) was inoculated with the *C. albicans* suspension to give a 1% inoculum. Cultures, including uninoculated con-

trols where required, were incubated and kept stationary in air at 37°C. At appropriate intervals, growth was estimated by measuring the optical density of the culture at 540 nm.

Protease assay. Samples to be assayed consisted of either crude enzyme preparations (4) or culture supernatant solutions. Enzyme samples were routinely assayed by incubation with bovine serum albumin (BSA), at 2 mg/ml, in sodium acetate buffer (50 mM, pH 4.0) at 37°C. In some experiments, bovine hemoglobin served as the substrate. At appropriate times, samples were mixed with trichloroacetic acid to give a 5% (wt/vol) final concentration and chilled for at least 30 min. Supernatant solutions were collected by centrifugation. Acid-soluble products of proteolysis were estimated by either absorbance at 280 nm or by the method of Lowry et al. (5). In several experiments the degradation of substrate BSA during growth of the organism was estimated. In these cases, samples of the cultures were removed at the desired times, and the cells were removed by centrifugation. The supernatant solutions were supplemented with carrier BSA (to give 2.5 mg/ml) and immediately mixed with ice cold trichloroacetic acid to give 5%. After at least 30 min in ice, the precipitated material was removed by centrifugation, and the supernatant solutions were assayed for trichloroacetic acid-soluble products by the method of Lowry et al. (5).

RESULTS

Effects of pH on protease activity. The proteolysis of hemoglobin as a function of pH is shown in Fig. 1. Optimal pH for activity was 3.8 to 4.0. Note that the pH activity profile is particularly sharp. The rather sharp pH activity profile suggested that irreversible inactivation of the enzyme may occur at pH values over 4. To test this possibility, protease was preincubated alone at a variety of pH's for 2 h. Substrate was then added in sufficient acetate buffer to drop all reaction mixtures to pH 4 for protease assay. The results (Fig. 2) indicated that preincubation of protease at pH values of 6 or higher led to irreversible loss of activity. A sharp transition from active to inactive protease occurred between pH 5 and 6. Inclusion of 1 mM phenylmethylsulfonyl fluoride completely prevented loss of protease activity during preincubation at pH 6 and 7.

Effect of saliva and pH of growth on protease activity. *Candida albicans* was grown in either broth or whole salivary supernatant at a variety of pH's over the range 3 to 7. Culture supernatant solutions were examined for the presence of proteolytic activity after adjustment of all samples to pH 4. The results indicated protease activity was only present in samples from the broth cultures (Fig. 3). Furthermore, a pH-dependent decrease in the protease activity of the broth samples was noted. Protease content decreased as the pH of growth approached neutrality. None of the saliva cultures exhibited protease activity. Thus, growth of the organism in saliva, even at pH's that promoted protease elaboration in broth, failed to yield enzyme activity.

In the preceding type of experiment, cultivation of the organism in saliva yielded only ca. 40% of the growth yield obtained in the broth cultures. Therefore, in an effort to minimize problems of nonequivalent growth in broth and

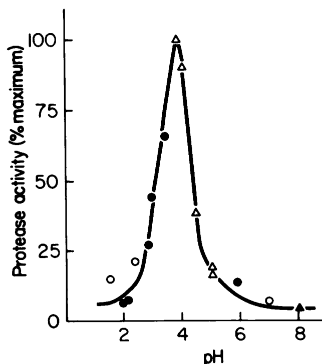

FIG. 1. *Effect of pH on the activity of C. albicans extracellular protease. Bovine hemoglobin was dissolved to 5 mg/ml in the appropriate buffer system (50 mM) and equilibrated to 37°C. At zero time, crude enzyme was added to 2 U/ml, and incubation was continued for 60 min. Remaining acid-insoluble substrate was precipitated with trichloroacetic acid, and the acid-soluble products were estimated by absorbance at 280 nm. Symbols for the buffer systems are as follows:* (○) *phosphate;* (●) *citrate;* (△) *acetate;* (▲) *tris(hydroxymethyl)aminomethane.*

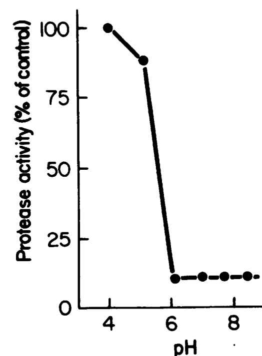

FIG. 2. *Effect of pH of preincubation on subsequent protease activity at pH 4. Crude enzyme (0.6 U/ml) in either 1 mM acetate buffer (pH 4 to 6) or tris(hydroxymethyl)aminomethane buffer (pH 7 to 8.5) was incubated for 2 h at 37°C. An equal volume of a BSA solution (4 mg/ml in 100 mM acetate buffer, pH 4.0) was then added, and the reaction mixture was incubated for 18 h at 37°C. Acid-soluble products were estimated by the method of Lowry et al. (5).*

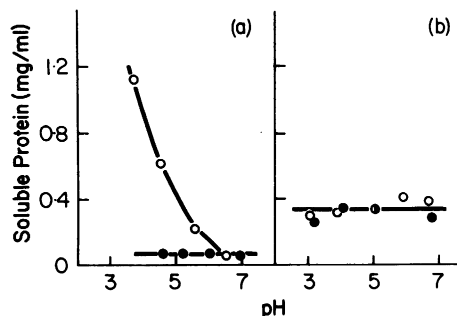

FIG. 3. *Elaboration of proteolytic activity during growth of C. albicans in broth and saliva. Cultures of C. albicans were grown in broth (48 h) and sterile, whole salivary supernatant (72 h), which were adjusted to the indicated pH values. Samples of each culture supernatant solution (○) and uninoculated controls (●) were adjusted to pH 4 and mixed with an equal volume of a BSA solution (4 mg/ml in 100 mM acetate buffer, pH 4). Incubation proceeded for 18 h at 37°C. Acid-soluble products were estimated as described in the legend to Fig. 2. (a) Broth cultures; (b) saliva cultures.*

in saliva, additional experiments were performed in broth supplemented with 0 to 16% (vol/vol) sterile, whole salivary supernatant. It was observed that the growth effects of saliva could be separated from effects on the proteolytic activity of the organism (Fig. 4). The results clearly indicated that the presence of very low (1 to 8%) saliva concentrations had a dramatic effect on the proteolysis of BSA during growth. Saliva-supplemented cultures showed less than a 10% difference in growth from the control (no saliva) culture. Saliva present at very low concentrations thus exhibited a marked inhibitory effect on the degradation of extracellular protein but not on growth.

DISCUSSION

The experiments reported here clearly indicate that human whole salivary supernatant is inhibitory to the expression of the proteolytic potential of *C. albicans*. Relief of the inhibitory effects of saliva required substantial dilution. Indeed, supplementation of broth with only 4% saliva resulted in about 50% of the inhibitory effect of undiluted saliva. This suggests that undiluted saliva contains considerable reserve inhibitory capacity. We previously reported (4) that crude protease caused extensive degradation of several salivary proteins at pH 4. It follows that the primary effect of saliva at pH 4 is to inhibit synthesis of protease by the growing microorganism.

The low optimal pH for activity of the extracellular *C. albicans* protease reported here is

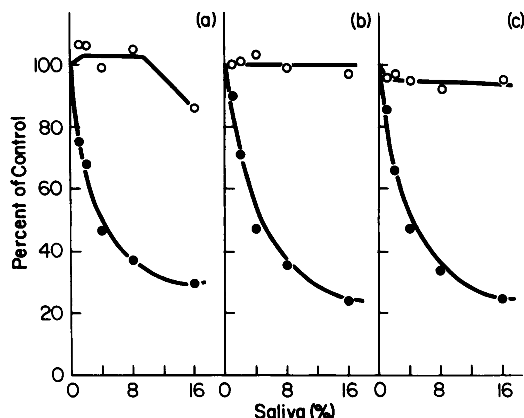

FIG. 4. *Effect of saliva on the proteolysis of BSA during growth of C. albicans in broth. The organism was grown in broth supplemented with 0, 1, 2, 4, 8, and 16% (vol/vol) sterile, whole salivary supernatant. All cultures were initially adjusted to pH 4. After incubation for 24 (a), 48 (b), and 72 (c) h, samples were removed, and culture supernatants were obtained and treated with trichloroacetic acid (5% final concentration). Acid-soluble protein (●) was estimated as described in the legend to Fig. 3. Growth of the organism (○) was estimated by determining the absorbance of cultures at 540 nm. Absorbance of the control culture was 0.640 (a), 0.960 (b), and 1.05 (c) at 24, 48, and 72 h, respectively. Acid-soluble protein present in the control culture at 24, 48, and 72 h was 286, 237, and 212 μg/ml (BSA equivalents), respectively.*

consistent with earlier studies (3, 4, 8). Our examination of small pH intervals revealed the particularly narrow (sharp) response of the protease to pH. The studies reported here suggest that the narrow pH requirement of the protease is due to inactivation of the enzyme at a pH of >5. Loss of protease activity by preincubation at pH 6 to 7 was prevented in the presence of phenylmethylsulfonyl fluoride. Thus, loss of protease activity at pH 6 to 7 may be due to proteolysis by an undetected phenylmethylsulfonyl fluoride-sensitive protease (perhaps the neutral protease reported earlier [3]).

Finally, under optimal pH conditions for activity, stability, and production of protease, saliva completely inhibited expression of proteolytic activity by *C. albicans*. Thus, it seems especially unlikely that *C. albicans* would produce (and therefore rely on) extracellular protease in the human oral cavity.

ACKNOWLEDGMENTS

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