THE PROTEOLYTIC POTENTIAL OF *CANDIDA ALBICANS* IN HUMAN SALIVA SUPPLEMENTED WITH GLUCOSE

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SUMMARY. The production of proteases by *Candida albicans* in batch cultures of human saliva supplemented with glucose was investigated with two clinical strains of *Candida* and both individual and pooled samples of whole saliva from volunteers. Salivary proteolysis during a 48-h period was estimated by biochemical and isoelectric focusing techniques. Candidal growth in saliva was associated with acid production and salivary proteolysis and there was a highly significant positive correlation between these two activities. Neither candidal growth nor proteolysis was observed in glucose-free control samples and with one strain of *Candida* cultured in the saliva of one individual. Isotachophoretic analysis of culture liquor showed a significant increase in acetate and pyruvate ions. The oral cavity provides niches that have a low pH and are periodically supplemented with dietary carbohydrates. The acidic proteases of *C. albicans* may play a role in the pathogenesis of oral candidoses.

INTRODUCTION

Candida albicans, an opportunist pathogen found in the oral cavity of more than half of the human population is the major cause of the palatal inflammation observed in chronic atrophic candidosis (syn: denture stomatitis; denture sore mouth) (Budtz-Jørgensen, 1974*b*). The acidic proteases produced by *C. albicans* may contribute to the pathogenesis of this disease which is particularly prevalent in elderly denture wearers (Budtz-Jørgensen, 1974*a*; 1981; Olsen and Birkeland, 1975, 1976; Zraggen and Graf, 1975). Remold, Fasold and Staib (1968) demonstrated that protease-producing strains of *C. albicans* caused more extensive infection in mice than protease-deficient strains, although Budtz-Jørgensen (1974*a*) could find no direct relationship between protease production and infectivity.

Germaine, Tellefson and Johnson (1978) performed experiments *in vitro* to demonstrate the expression of the proteolytic potential of *C. albicans* in human whole

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saliva and observed that purified yeast proteases degraded salivary proteins at low *pH* values (*c.* 3.0–4.0), confirming similar previous studies by other workers (Staib, 1965; Staib *et al.*, 1972; Budtz-Jørgensen, 1974a). However, on further investigation, Germaine and Tellefson (1981) observed that *C. albicans* cultured in human whole saliva lost its proteolytic potential and they concluded that saliva was a potent inhibitor of candidal protease synthesis and, therefore, that protease production did not contribute to the virulence of the organism in oral candidoses.

We have performed experiments, similar to those of Germaine and Tellefson (1981), with two oral strains of *C. albicans* and samples of whole saliva from a small group of volunteers. Additionally, the saliva samples were supplemented with glucose to evaluate the effect of a dietary carbohydrate on growth and protease production by *C. albicans*.

MATERIALS AND METHODS

Organisms. Two strains of *C. albicans* designated GDH 1957 and GDH 1261, isolated from patients with chronic atrophic candidosis who attended Glasgow Dental Hospital and School were used throughout the study. Both strains were identified by the results of sugar assimilation and fermentation tests (Lodder, 1970) and a germ-tube test (Mackenzie, 1962). Stock cultures were maintained on Sabouraud's dextrose agar slopes at 4°C, and subcultured monthly. Before the experiments, the yeasts were cultured for 18 h at 37°C in 1% (w/v) Mycological Peptone Medium (Oxoid) supplemented with 50 mM glucose.

Saliva. Unstimulated whole saliva was collected by expectoration from healthy volunteers and held at 4°C until required. Saliva samples obtained from three different donors were pooled for growth and proteolysis studies and samples from another donor, designated LPS saliva, were investigated separately. Because the concentration of salivary proteins in whole saliva varies and depends upon a number of factors (Mason and Chisholm, 1975), saliva collection was standardised as far as possible and the ratio of individual samples within the saliva pool was always the same.

The samples were centrifuged twice, first at 10 000 *g* for 20 min at 4°C and then at 100 000 *g* for 120 min at 4°C, and the supernates carefully collected and used immediately in the experiments. An appropriate volume of 1 M glucose sterilised by filtration was added to the supernate to obtain a final glucose concentration of 200 mM and, penicillin 100 µg/ml and streptomycin 100 µg/ml were added to kill any remaining viable, salivary bacteria.

Culture technique. An 18-h culture in mycological peptone was washed in phosphate buffered saline (PBS, 0.1 M, *pH* 7.2) and centrifuged at 1500 *g* for 10 min; 50 µl of the deposit was added to 100 ml of saliva supernate supplemented with glucose to obtain an initial inoculum of $(1.0\text{--}2.0) \times 10^6$ blastospores/ml. Glucose-supplemented saliva inoculated with heat-killed (70°C for 120 min) blastospores, and glucose-free saliva with a viable inoculum were the controls.

Sterility of the control and the test saliva samples was ensured by routinely inoculating portions on to Sabouraud's dextrose agar and blood agar plates at the beginning and at the end of the 48-h incubation period. The plates were incubated aerobically at 37°C for 48 h and observed for yeast or bacterial growth.

Batch cultures of *C. albicans* were grown in 250-ml conical flasks containing 100 ml of salivary suspension. The flasks, plugged with loose cotton wool, were held at 37°C and the contents agitated continuously by magnetic stirring. Samples (2 ml) were taken at 8-h intervals, except during the overnight incubation period, for 48 h. Each sample was divided into two portions. One was used for *pH* measurement and to assess the number of yeasts, the other for protein and acid analyses.

The *pH* of each sample was measured by an EIL 2320 *pH* meter (Electronic Instruments Ltd, Surrey) fitted with a Beckmann narrow diameter glass electrode (Beckmann R II C Ltd, Fife). For maximum accuracy the *pH* electrode and meter were calibrated before use at two *pH* values, one above and the other below the measurements to be made, with standard buffers.

The number of yeasts/ml of saliva was measured by microscopic counting in an improved Neubauer Haemocytometer Chamber (Hawksley Ltd, London). This method of estimating yeast growth was preferred to a turbidimetric method because opacities develop in saliva due to pH or calcium solubility changes (Germaine *et al.*, 1978).

At the end of each experiment, the test and control samples of saliva were subjected to isoelectric focusing to assess the proteolysis of salivary isozymes. Each experiment, with either pooled or individual samples of saliva, was repeated at least three times.

Salivary protein estimations. The total salivary protein of each portion was determined by the spectrophotometric method of Bradford (1976). The protein standard was a saliva sample in which the protein concentration had been estimated previously by the method of Lowry *et al.* (1951), as modified by Wilcox, Cohen and Tan (1957). Each portion was centrifuged at 10 000 *g* for 10 min to remove yeast cells and the supernate was used for protein assay.

Isoelectric focusing of saliva was performed by the method of Eckersall, Mairs and Beeley (1981). The saliva samples were centrifuged at 10 000 *g* for 30 min and the supernates dialysed extensively against 1 mM ethyl-diamine-tetra-acetate and further concentrated during a 16-h period by absorption with polyethylene glycol placed over the dialysis bags. Isoelectric focusing was performed on a LKB 2117 Multiphor apparatus (LKB-Produkter AB, Bromma, Sweden) according to the manufacturer's directions. A dried polyacrylamide gel block was placed in the saliva concentrate for 18 h at 4°C and the swollen block then placed on the isoelectric focusing gel. The absorbed proteins migrated into the pH gradient that was adjusted to a range of pH 4-8. The gels were then subjected to electro-focusing at a maximum of 10 W for 90 min and the salivary proteins then fixed with trichloroacetic acid, stained with Coomassie blue and, finally, washed in acetic acid.

Analysis of acids. Because the growth of *C. albicans* in saliva was associated with a marked decrease in pH, the acidic metabolic products of *Candida* that were present in the culture medium were analysed. The short-chain carboxylic acids were identified and quantified by isotachopheresis (Samaranayake *et al.*, 1982). An LKB 2127 Tachophor (LKB-Produkter) equipped with a two-turn (61 mm) capillary tube and maintained at 23°C was used. The leading electrolyte was 10 mM aqueous HCl, with Triton X-100 0.1% adjusted to pH 4.0 by the addition of β -alanine. The terminating electrolyte was 5 mM caprylic acid and the current flowing past the thermal and UV detectors was 50 μ A. The culture liquor was used directly for isotachopheresis.

Because the growth of *C. albicans* in saliva resulted in a marked reduction in pH (see below), two more controls were included: one to examine the effect of acid anions such as acetate and pyruvate and the other to monitor the effect of low pH on the protein concentration in the salivary supernate. The first control was a mixture of acid anions simulating the anionic composition of a 48-h candidal culture (Samaranayake, 1981); this was added with a killed inoculum to a pooled whole saliva sample to lower the pH to 3.5. The second control saliva was inoculated with killed *Candida* and acidified to pH 3.5 by the addition of a few drops of 1 N HCl. These controls were treated and investigated in the same way as the test samples.

RESULTS

Growth of C. albicans in saliva

Both strains of *C. albicans* gave sigmoid growth curves in pooled saliva samples with a mean specific growth rate (μ) of 0.029/h (table). In LPS saliva, *C. albicans* strain GDH 1957 had a faster growth rate overall; this was particularly evident on the first day ($\mu = 0.102$ /h) but there was negligible growth on the second day ($\mu = 0.008$ /h). In contrast the growth of strain GDH 1261 in LPS saliva was relatively slow on the first day ($\mu = 0.020$ /h) and completely inhibited on the second day.

No discernible growth of yeasts occurred in glucose-free control saliva samples. Subculture of the test and control suspensions, on blood agar, failed to reveal any bacterial growth, confirming the efficacy of the antibiotic supplements.

FIG. 1.—Salivary proteolysis (a, c) and pH variations (b, d) associated with *C. albicans* cultured in glucose-supplemented saliva. *C. albicans* strain GDH 1957 (test $\blacktriangle$, control $\triangle$) and *C. albicans* strain GDH 1261 (test $\bullet$, control $\circ$) were cultured in pooled saliva from three individuals (a,b) or in saliva from individual LPS (c,d). Each point represents the mean of three separate determinations.

FIG. 1.—(Continued)

TABLE

Relationship between growth rate, acid production and proteolytic potential of two strains of C. albicans cultured in 200 mM glucose-supplemented saliva

Source of saliva	<i>C. albicans</i> strain	Incubation period (h)	Specific growth rate (μ)*	Rate of decrease in pH (units/h)	Rate of protein degradation (μ g/ml/h)
Pooled	GDH 1957	{ 0-24	0.030	0.15	6.04
		{ 24-48	0.029	0.02	5.00
	GDH 1261	{ 0-24	0.027	0.16	10.20
		{ 24-48	0.030	0.03	1.04
Subject LPS	GDH 1957	{ 0-24	0.102	0.06	1.66
		{ 24-48	0.008	0.09	3.33
	GDH 1261	{ 0-24	0.020	0.01	NC
		{ 24-48	NG	NC	NC

NG = no growth; NC = no change.

* Specific growth rate (μ) = $\frac{0.693}{\text{mean generation time}}$; values are the mean of three determinations.

Rate of pH change

The pH curves of both *C. albicans* strains cultured in pooled and individual saliva samples are shown in fig. 1 (b, d). Initially, the pH of all saliva samples rose slightly (not shown), apparently because of the loss of dissolved CO₂ from the saliva. The pH of all the saliva samples that promoted the growth of *C. albicans* declined from *c.* 7.5 to 3.5 within 48 h. The mean fall in pH of the LPS saliva with strain GDH 1261 was only 0.2 units after 48 h, probably due to suppression of growth.

Proteolytic activity

Despite the standardised method for the collection of saliva employed throughout the study there were inevitable daily variations in the total salivary proteins between samples collected from different individuals. These variations ranged from 0.16–0.33 mg/ml. There was a highly significant positive correlation between the rate of protein degradation and the rate of increase in salivary acidity ($p < 0.01$, $r = 0.79$; table, fig. 1). Proteolytic activity was not discernible in the LPS saliva incubated with strain GDH 1261. Similarly, no significant proteolysis or reduction in pH was observed in any of the control cultures. Isoelectric focusing of the culture supernates confirmed the protein analyses (fig. 2). The principal difference between the control and the test profiles, which demonstrated proteolysis, appeared to be quantitative rather than qualitative, supporting similar observations of Germaine *et al.* (1978).

Acid production

Isotachophoresis of the control and the test culture supernates revealed varied concentrations of formate, pyruvate, lactate, acetate, succinate and propionate in the culture liquor. The acidic anions that appeared to cause the often dramatic reduction in pH were acetate and pyruvate. The concentrations of these anions increased at a

FIG. 2.—Isoelectric focusing profiles of salivary supernates obtained from cultures of *C. albicans* strain GDH 1957 (lanes 1 and 2) in pooled saliva and *C. albicans* strain GDH 1261 (lanes 3 and 4) in LPS saliva. Note the virtually complete salivary proteolysis in lane 2. (cf. fig. 1).

constant rate during the log phase of yeast growth and, generally, approached a maximum 28–33 h after inoculation.

In summary, growth of *C. albicans* in glucose-supplemented human, whole saliva was associated with a significant increase in acidity and degradation of proteins, whereas proteolysis was never observed in the absence of growth or acid production.

DISCUSSION

The present results demonstrate clearly that proteolytic strains of *C. albicans* cultured in whole saliva degrade salivary proteins. However, these observations differ from those of Germaine and Tellefson (1981) who were unable to induce salivary proteolysis by a proteolytic strain of *C. albicans*. One reason may be the very low (c. 5 mM) glucose supplements which Germaine and Tellefson used and another could be their use of a single strain of *C. albicans* throughout the studies. As clearly indicated by the present results, proteolysis by different strains of *C. albicans* varies with the source and the quality of the saliva samples. For instance, the proteolytic potential of

C. albicans strain GDH 1261 was completely inhibited by the LPS saliva, but not by the pooled saliva. This discrepancy could be explained in terms of pH, as acidic proteases of *C. albicans* are inactive in alkaline pH conditions (Germaine *et al.*, 1978). The latter conditions were prevalent when strain GDH 1261 was cultured in the LPS saliva (fig. 1), apparently as a consequence of growth suppression (table). Similar variations in growth patterns and proteolytic potential have been subsequently observed by us with several strains of *C. albicans* and *C. glabrata* cultured in pooled and individual saliva samples (unpublished data).

It is questionable whether the in-vitro conditions employed in this study prevail in the mouth and whether proteolytic enzymes have a role in the pathogenesis of candidosis. It is known that concentrations of glucose and sucrose of up to 500 mM can be found in saliva for variable periods, depending upon the frequency and duration of intake of a variety of carbohydrates (Darlington, 1978). This may be particularly relevant in relation to chronic atrophic candidosis because denture wearers frequently consume soft, carbohydrate diets. Additionally, regular and prolonged intake of carbohydrate foods lowers the pH under the dentures (Zraggen and Graf, 1975; Olsen and Birkeland, 1976), producing pH values in denture plaque of *c.* 4–5 (Olsen and Birkeland, 1975), conducive to the activity of candidal proteases. Several workers, therefore, support the theory that intra-oral proteolytic activity of *C. albicans* could contribute to the pathogenesis of chronic atrophic candidosis (Budtz-Jørgensen, 1974*a*; Olsen and Birkeland, 1975, 1976; Zraggen and Graf, 1975).

Furthermore, a high carbohydrate intake is associated with initiation and aggravation of chronic atrophic candidosis (Neil, 1965; Gentles and La Touche, 1969; Ritchie *et al.*, 1969). Not only would such an environment stimulate candidal proteases but high dietary carbohydrate could enhance the proliferation (Knight and Fletcher, 1971) and adhesion of *C. albicans* to palatal epithelial cells (Douglas, Houston and McCourtie, 1981; Samaranayake and MacFarlane, 1981, 1982) and acrylic denture surfaces (Samaranayake, McCourtie and MacFarlane, 1980; Samaranayake and MacFarlane, 1980; McCourtie and Douglas, 1981).

The acidogenic and aciduric nature of *C. albicans* cultured in glucose-supplemented media has been reported by several investigators (Thjøtta and Tørheim, 1955; Ramachandran and Walker, 1957; Nord, 1974; Sheperd and Sullivan, 1976). The present studies, while confirming such observations, also reveal that the major short-chain carboxylic acids mediating this shift in salivary acidity are acetate and pyruvate. We have demonstrated previously similar qualitative changes in *Candida* strains cultured in glucose-supplemented defined media (Samaranayake *et al.*, 1981, 1983*a*). Furthermore, on analysis of denture plaque with predominant yeast populations, obtained from patients with chronic atrophic candidosis, acetate was found to be the major acidic anion present (Samaranayake *et al.*, 1983*b*).

Conditions that are conducive to the production, activity and stability of *C. albicans* proteases exist in the human oral cavity. However, further studies are necessary to determine the mechanisms by which this opportunistic pathogen regulates and expresses its proteolytic potential.

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