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Isolation and Partial Characterization of an Adhesin from *Candida albicans*

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Candida albicans produces extracellular polymeric material (EP) which contains a mannoprotein adhesin. EP isolated from culture supernatants of *C. albicans* GDH 2346 consisted of a mixture of glycoprotein components and inhibited yeast adhesion to buccal epithelial cells by up to 60%. Partial purification of the adhesin was achieved by a two-step procedure involving chromatography of EP on concanavalin A-Sepharose and DEAE-cellulose. The purified adhesin inhibited adhesion to buccal cells 30 times more efficiently (on a weight basis) than unfractionated EP. Pretreatment of EP with heat, dithiothreitol or proteolytic enzymes either partially or completely destroyed its ability to inhibit adhesion, whereas pretreatment with sodium periodate or α-mannosidase had little or no effect. These results suggest that the protein portion of the mannoprotein adhesin is more important than the carbohydrate moiety in mediating yeast attachment to buccal epithelial cells.

INTRODUCTION

The essential role of microbial adhesion in the pathogenesis of a number of infectious diseases is now well established. Most research in this area relates to bacterial adhesion, but recently there has been increasing interest in adhesion of the most important yeast pathogen, *Candida albicans*, to epithelial surfaces (reviewed by Douglas, 1985, 1987). Adhesion of some *C. albicans* strains *in vitro* can be promoted by growing the yeast in defined medium containing a high concentration of galactose as the carbon source (Douglas *et al.*, 1981; McCourtie & Douglas, 1981, 1984). These conditions stimulate the production of a fibrillar–floccular layer on the yeast surface, a change which is presumed to account for the enhanced adhesion (McCourtie & Douglas, 1981). A surface layer of similar morphology has been shown to mediate attachment of *C. albicans* to mucosal surfaces (Marrie & Costerton, 1981) and renal endothelium (Barnes *et al.*, 1983) *in vivo*.

Prolonged incubation of *C. albicans* in medium containing galactose causes release of surface fibrils, and extracellular polymeric material (EP) can be isolated from culture supernatants; EP appears, by adhesion inhibition tests, to contain the yeast adhesin (McCourtie & Douglas, 1985). The present paper describes a partial purification and characterization of this adhesin.

METHODS

Organism. C. albicans GDH 2346 (NCYC 1467) was used throughout the study. This strain was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis. It was maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every two months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

Growth conditions. The yeast was grown at 37 °C, with shaking, in yeast nitrogen base medium (Difco) containing 500 mM-galactose as described previously (McCourtie & Douglas, 1981). It grows exclusively in the budding yeast phase under these conditions. Cells were harvested after 24 h (stationary phase of growth) and washed twice in 0·15 M-phosphate-buffered saline, pH 7·2 (PBS).

Abbreviations: AII, adhesion inhibition index; Con A, concanavalin A; EP, extracellular polymeric material.

Adhesion assays. Yeast adhesion to exfoliated buccal epithelial cells was determined by light microscopy as described by Douglas *et al.* (1981), except that lower yeast cell concentrations (5×10^7 or 1×10^7 organisms ml^{-1}) were used. In some experiments, washed buccal cells were preincubated with crude EP or EP fractions as follows. Epithelial cell suspensions (1×10^5 cells ml^{-1} in PBS; 1 ml) were centrifuged in a Beckman microfuge and the pellet was resuspended in EP solution (1 ml). After incubation at 37 °C for 30 min with gentle shaking, the buccal cells were recovered by centrifugation and resuspended in PBS (1 ml) for use in adhesion assays.

Isolation of EP. EP was prepared by freeze-drying dialysed culture supernatants. Batches of medium (500 ml in 2 litre Erlenmeyer flasks) were inoculated with overnight yeast cultures (50 ml) and incubated at 37 °C for 5 d in an orbital shaker operating at 150 r.p.m. Yeasts were removed by centrifugation and the culture supernatant fluid was dialysed at 4 °C for one week against five changes (12 litres each) of distilled water. The retentate (crude EP) was freeze-dried and weighed.

Analysis of EP. Protein was determined by the Lowry method and phosphorus by the method of Chen *et al.* (1956). Total carbohydrate was estimated according to the procedure of Dubois *et al.* (1956), using mannose as a standard.

Fractionation of EP. Crude EP (30–50 mg in 5 ml PBS) was applied to a column (1·5 × 10·7 cm) of concanavalin A-Sepharose 4B (Con A-Sepharose 4B, Sigma) and eluted with PBS until the absorbance at 280 nm of the eluate was negligible. The flow rate was 34 ml h^{-1} and the fraction volume was 3 ml. Bound material was eluted with 0·5 M-methyl α-D-mannoside. Fractions were analysed for protein, phosphorus and total carbohydrate. Appropriate fractions were pooled, dialysed against distilled water and concentrated using Aquacide II (Calbiochem).

All of the material eluted by methyl α-D-mannoside was subjected to further chromatography on DEAE-cellulose. The pooled fractions were first dialysed against distilled water, concentrated and then applied to a column (2·6 × 58 cm) of DE 52 (Whatman). The column was eluted with a linear gradient of NaCl (0–0·5 M) in 0·15 M-potassium phosphate buffer (pH 7·2) at a flow rate of 20 ml h^{-1}. Fractions (3·2 ml) were analysed, and appropriate fractions were pooled, dialysed and concentrated as described above. Before any concentrated fraction was used as a potential inhibitor in adhesion assays, its content of protein and carbohydrate was determined.

Heat, chemical and enzyme treatments of EP. In some experiments, crude EP used to pretreat epithelial cells was first processed as follows.

(*a*) *Heat treatment.* Solutions of EP (10 mg ml^{-1} in PBS) were heated at 50 °C, 80 °C and 100 °C in small, screw-capped bottles for 15 min.

(*b*) *Chemical treatments. Mild alkali treatment* involved dissolving EP (20 mg ml^{-1}) in 0·1 M-NaOH in a screw-capped bottle; the solution was incubated at 25 °C for 24 h and then neutralized with HCl to give a final EP concentration of 10 mg ml^{-1}. For *mild acid-treatment*, EP (20 mg ml^{-1}) was heated in 0·01 M-HCl at 100 °C for 30 min in a sealed ampoule and neutralized with NaOH to a final EP concentration of 10 mg ml^{-1}. *Dithiothreitol treatment* was accomplished by dissolving EP (10 mg ml^{-1}) in 12 mM-dithiothreitol and incubating the solution at 37 °C for 60 min. *Periodate treatment* involved adding sodium periodate (final concentration 20 mM or 50 mM) to a solution of EP (5 mg ml^{-1}) in 0·05 M-sodium acetate buffer (pH 4·5). The mixture was incubated in the dark at 4 °C for 30 min and then the reaction was terminated by adding excess ethylene glycol. EP was recovered by precipitation with acetone, dried in a desiccator and redissolved in PBS.

(*c*) *Enzyme treatments.* Trypsin, chymotrypsin, pronase (from *Streptomyces griseus*; pronase E) and papain (all from Sigma) were used at concentrations of 0·1 mg ml^{-1} in 0·01 M-potassium phosphate buffer (pH 7·2; pH 6·2 for papain). EP (10 mg ml^{-1}) was incubated in these enzyme solutions at 25 °C for 30 min and then equivalent concentrations of enzyme inhibitor were added: trypsin inhibitor was used for trypsin, chymotrypsin inhibitor for chymotrypsin, and α_2-macroglobulin for papain and pronase. Bromelain (Boehringer) and α-mannosidase (Sigma) (both 1 mg ml^{-1}) in 0·04 M-sodium acetate buffer (pH 4·5) were incubated with EP (10 mg ml^{-1}) at 37 °C for 60 min. For treatment with endoglycosidase H (Miles), EP (1 mg) was incubated at 37 °C for 2 h with 1 μg enzyme at pH 5·6. Endoglycosidase-treated EP was applied to a small column of Con A-Sepharose. Unbound components were eluted from the column with PBS while bound material was eluted with 0·5 M-methyl α-D-mannoside. Both fractions (bound and unbound) were dialysed against distilled water at 4 °C, freeze-dried and redissolved in PBS (1 ml).

RESULTS

Yield and chemical composition of EP

EP was isolated by freeze-drying dialysed culture supernatants of *C. albicans* GDH 2346. Growth for 5 d in 1 litre medium produced 8·23 ± 0·62 g yeast (dry wt) (mean ± SEM) and 1·82 ± 0·04 g EP (dry wt). This represents an EP yield of 18%, similar to that obtained previously by precipitation of culture supernatants with acetone (McCourtie & Douglas, 1985). The chemical

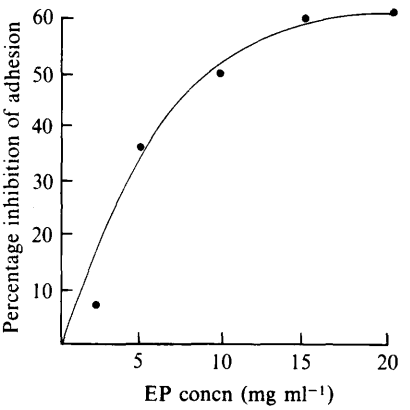

Fig. 1. Effect of crude EP on adhesion of *C. albicans* GDH 2346 to buccal epithelial cells. Buccal cells were pretreated with a solution of EP (2–20 mg ml^{-1} in PBS) at 37 °C for 30 min, and then used in adhesion assays with a yeast concentration of 5×10^7 organisms ml^{-1}. Results shown represent mean values from assays done in triplicate.

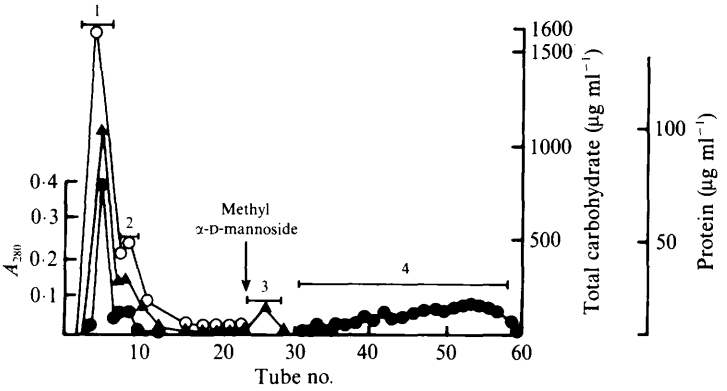

Fig. 2. Affinity chromatography of crude EP from *C. albicans* GDH 2346 on Con A-Sepharose. EP (30 mg in PBS) was applied to the column and eluted with PBS until the absorbance at 280 nm of the eluate was negligible. Bound material was eluted with 0·5 M-methyl α-D-mannoside as shown by the arrow. ●, A_{280}; ○, total carbohydrate; ▲, protein. Horizontal bars (numbered 1–4) indicate fractions which were pooled after analysis.

composition was also similar to that of acetone-precipitable material, comprising 70·0 ± 2·6% carbohydrate (mean ± SEM), 10·0 ± 0·7% protein and 0·49 ± 0·04% phosphorus.

Effect of EP on yeast adhesion in vitro

Preincubation of buccal epithelial cells with increasing concentrations (2–20 mg ml^{-1}) of EP inhibited yeast adhesion by up to 60% (Fig. 1). There was no further increase in inhibition at higher concentrations of EP. Adhesion to mouse vaginal epithelial cells *in vitro* was inhibited to a similar extent; pretreatment of these cells with EP (10 mg ml^{-1}) at 37 °C for 30 min resulted in a 51% decrease in adhesion.

Fractionation of EP

Affinity chromatography of EP on Con A-Sepharose (Fig. 2) resulted in elution of two components (fractions 1 and 2) before the addition of methyl α-D-mannoside. Both components contained carbohydrate and protein. Material eluted after mannoside addition (fractions 3 and 4), and monitored by its absorbance at 280 nm or protein content, appeared to separate poorly. A more satisfactory separation was achieved by chromatography of this material on DEAE-cellulose (Fig. 3) which produced two distinct components (fractions 5 and 6), each containing both carbohydrate and protein.

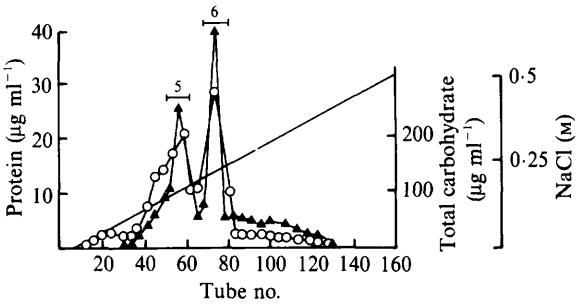

Fig. 3. DEAE-cellulose chromatography of Con A-bound EP. Material eluted from Con A-Sepharose with methyl α-D-mannoside (Fig. 2) was dialysed, concentrated and applied to a column of DE 52. This column was eluted with a linear gradient (0–0·5 M) of NaCl in 0·15 M-potassium phosphate buffer (pH 7·2). ▲, Protein; ○, total carbohydrate. Horizontal bars (numbered 5 and 6) indicate fractions which were pooled after analysis.

Table 1. *Effect of EP fractions isolated by chromatography on Con A-Sepharose and DEAE-cellulose on adhesion of C. albicans GDH 2346 to buccal epithelial cells*

Buccal epithelial cells were incubated at 37 °C for 30 min either in PBS or in an EP fraction (1 ml) obtained by column chromatography. After this pretreatment, buccal cells were recovered by centrifugation, resuspended in PBS and used in adhesion assays. Assays were done three times in triplicate with a yeast concentration of 5×10^7 organisms ml^{-1}.

EP fraction*	Mean no. (± SEM) of adherent yeasts per 100 epithelial cells	Percentage inhibition of adhesion†
1	1738 ± 25	9
2	1572 ± 72	17
3	1728 ± 54	9
4	977 ± 67	49
None (PBS control)	1902 ± 36	0
5	975 ± 60	44
6	1658 ± 65	5
None (PBS control)	1739 ± 45	0

* Numbers refer to column fractions indicated by bars in Figs 2 and 3. Fractions 1–4 were isolated by chromatography of crude EP on Con A-Sepharose; fractions 5 and 6 were obtained by further separation of Con A-bound material on DEAE-cellulose.

† Inhibition obtained with EP-treated epithelial cells when compared with adhesion to PBS-treated epithelial cells.

The ability of each fraction to inhibit yeast adhesion *in vitro* was tested in standard adhesion assays. Preincubation of buccal cells with Con A-bound material (fraction 4) resulted in maximum inhibition; after further chromatography on DEAE-cellulose, inhibitory activity was localized in fraction 5 (Table 1). To determine the extent to which purification of yeast adhesin had been achieved, an adhesion inhibition index (AII) was calculated for each fraction (Table 2). AII is a measure of the relative efficiency with which each fraction inhibits adhesion as compared with crude, unfractionated EP. At a concentration of 10 mg ml^{-1}, crude EP inhibits adhesion by 50% (Fig. 1). In calculating AII, the weights of protein and carbohydrate in each fraction required to inhibit adhesion by 50% were determined. The respective weights of protein and carbohydrate in 10 mg crude EP (i.e. 1 mg and 7 mg) were then divided by these values. The results (Table 2) indicate that this two-step separation protocol, involving chromatography on Con A-Sepharose and DEAE-cellulose, allows a 30-fold purification of yeast adhesin (fraction 5).

Treatment of EP with heat, chemicals and enzymes

Crude EP preparations were subjected to a variety of treatments with heat, chemicals and enzymes in an attempt to determine the minimum structure necessary for inhibition of yeast

Table 2. *Purification of yeast adhesin by chromatography on Con A-Sepharose and DEAE-cellulose: AII of EP fractions*

EP fraction*	Composition of EP fraction: Carbohydrate (μg ml^{-1})	Protein (μg ml^{-1})	Carbohydrate: protein ratio	AII†: Based on carbohydrate content	AII†: Based on protein content
1	728	70	10·4	2	3
2	528	40	13·2	5	9
3	0	10	–	0	18
4	240	70	3·4	28	14
5	190	28	6·7	32	31
6	342	44	7·7	2	3

* Numbers refer to column fractions indicated by bars in Figs 2 and 3. Fractions 1–4 were isolated by chromatography of crude EP on Con A-Sepharose; fractions 5 and 6 were obtained by further separation of Con A-bound material on DEAE-cellulose. All fractions were dialysed and concentrated before use in adhesion assays.

† AII is a measure of the relative efficiency with which each fraction inhibited adhesion as compared with crude EP, and was calculated as described in the text.

Table 3. *Effect of heat, chemical and enzyme treatments on the ability of crude EP to inhibit adhesion of C. albicans GDH 2346 to buccal epithelial cells*

Buccal epithelial cells were incubated at 37 °C for 30 min in a solution (10 mg ml^{-1}) of EP pretreated as indicated. Control buccal cell preparations were incubated under the same conditions in PBS. Adhesion assays, with parallel controls, were done three times in triplicate, normally with a yeast cell concentration of 1 × 10^7 organisms ml^{-1}.

Pretreatment of EP	Relative adhesion (%)†	Pretreatment of EP	Relative adhesion (%)†
None	50	Bromelain	84
50 °C, 15 min	64	Trypsin	73
80 °C, 15 min	85	Chymotrypsin	90
100 °C, 15 min	99	Pronase	76
Periodate (20 mM)	51	Papain	42
Periodate (50 mM)	55	Papain + mild alkali	27
Dithiothreitol	97	Endoglycosidase H*	
Mild acid	92	Con A-bound fraction	83
Mild alkali	45	Con A-eluted fraction	54
α-Mannosidase	59	Control (no EP)	100

* Endoglycosidase H-treated EP was separated into 2 fractions on Con A-Sepharose as described in Methods.

† Adhesion is expressed as a percentage of that to control (PBS-treated) buccal cells for which, typically, a value of 897 ± 38 adherent yeasts per 100 epithelial cells (mean ± SEM) was obtained.

adhesion *in vitro*. These studies indicated that the protein portion of the mannoprotein adhesin is more important than the carbohydrate moiety in mediating attachment to buccal epithelial cells. Pretreatment of EP with heat, dithiothreitol or 0·01 M-HCl destroyed its ability to inhibit adhesion (Table 3). Similarly, all of the proteolytic enzymes tested, except papain, significantly decreased ($P < 0·001$, by Student's *t*-test) the inhibitory activity of EP. On the other hand, treatment with periodate or α-mannosidase had no significant effect. Preincubation of EP with papain or dilute alkali appeared to enhance its ability to inhibit adhesion, while a combined treatment of papain followed by alkali produced substantially greater inhibition ($P < 0·001$; Table 3). In all of these experiments, control assays were included which demonstrated that none of the pretreatments *per se* (i.e. in the absence of EP) significantly affected yeast adhesion. Additional experiments also indicated that periodate and α-mannosidase treatments effectively degraded the carbohydrate component of EP.

Further evidence for the importance of the protein portion of EP in mediating adhesion came from experiments with endoglycosidase H, an endo-β-*N*-acetylglucosaminidase capable of

hydrolysing the di-*N*-acetylchitobiose units which link polymannose chains to asparagine residues in yeast mannoproteins (Tarentino *et al.*, 1974). Endoglycosidase H-treated EP was chromatographed on Con A-Sepharose and both Con A-bound and Con A-eluted fractions were tested for their ability to inhibit yeast adhesion. The eluted, protein-rich fraction inhibited adhesion more effectively ($P < 0{\cdot}001$) than the Con A-bound, carbohydrate-rich material (Table 3).

DISCUSSION

Adhesion of micro-organisms to mucosal surfaces is thought to involve specific, lectin-like interactions between microbial adhesins and complementary host-cell receptors. Attempts to characterize these adhesive surface structures often entail adding putative adhesin or receptor (or their analogues) to *in vitro* adhesion assays; either component will block adhesion by acting as a competitive inhibitor (Ofek & Beachey, 1980). This approach was used in a previous study of *C. albicans* adhesion (McCourtie & Douglas, 1985), where pretreatment of buccal epithelial cells with EP from galactose-grown yeasts was shown to inhibit subsequent yeast adhesion. The specificity of the interaction was demonstrated by the finding that EP from one *C. albicans* strain (GDH 2023) did not inhibit adhesion of a second strain (GDH 2346). In that earlier study, EP was isolated from culture supernatants by precipitation with acetone. In the present investigation, however, EP was obtained by freeze-drying to minimize the possibility of denaturation or aggregation of individual components. The two methods produced EP in similar yields and with a similar overall chemical composition.

Adhesion inhibition tests were used to monitor purification of an EP component which could block yeast adhesion to buccal epithelial cells. Unfractionated EP inhibited adhesion by up to 60%. During affinity chromatography on Con A-Sepharose, most of the inhibitory activity was localized in material that bound to the lectin. This finding is consistent with earlier analyses (McCourtie & Douglas, 1985), and with experiments involving the antibiotic tunicamycin (Douglas & McCourtie, 1983), which suggested that the yeast adhesin is mannoprotein in nature. Further chromatography of Con A-bound material on DEAE-cellulose produced two fractions, only one of which contained substantial inhibitory activity. On a weight basis (protein or carbohydrate content), this purified material inhibited yeast adhesion to buccal cells 30 times more efficiently than crude EP. It is not yet clear whether more than one type of adhesin is present in this fraction. Preliminary attempts at further separation by gel electrophoresis were unsuccessful. However, experiments described in the accompanying paper (Critchley & Douglas, 1987) indicate that *C. albicans* GDH 2346 does possess more than one adhesin for buccal cells.

C. albicans can adhere to a variety of epithelial cell types in addition to buccal cells. These include vaginal (King *et al.*, 1980; Sobel *et al.*, 1981) and uro-epithelial cells (Botta, 1981; Centeno *et al.*, 1983), as well as epidermal corneocytes (Botta, 1981; Ray *et al.*, 1984; Collins-Lech *et al.*, 1984). Crude EP inhibited adhesion to mouse vaginal cells and the extent of inhibition observed was similar to that obtained with buccal cells. This indicates that EP contains an adhesin for vaginal cells, although whether this yeast surface component is identical with the adhesin(s) responsible for attachment to buccal cells remains to be determined.

There is now considerable evidence that *C. albicans* adhesins for both buccal and vaginal epithelial cells are mannoprotein in nature (Sandin *et al.*, 1982; Douglas & McCourtie, 1983; Lee & King, 1983*a*, *b*; McCourtie & Douglas, 1985), but it is not yet certain whether the carbohydrate or protein portion of the mannoprotein molecule is more important in the adhesion process. Bacterial adhesion to animal cells can be mediated by carbohydrate–lectin interactions (Ofek & Perry, 1985) in three ways: (i) a bacterial surface protein (lectin) binds to glycosides on the animal cell membrane; (ii) extracellular lectin forms bridges between carbohydrates on the surface of both bacterial and animal cells; and (iii) lectin, present as an integral component of the animal cell membrane, binds to carbohydrate on the bacterial surface. With *C. albicans*, mannoprotein adhesins could permit all three types of interaction. In the present study, we tried to define more precisely the interaction with buccal cells by 'dissecting' crude EP preparations chemically or enzymically, and then determining the effect of these

treatments on the ability of EP to inhibit yeast adhesion *in vitro*. The advantages of this indirect approach as compared with direct chemical or enzymic treatment of yeast cells are that the effect is a specific one, limited to EP, and that yeast viability is not affected. Pretreatment of EP with heat, dithiothreitol or proteolytic enzymes (except papain) either partially or completely destroyed its ability to inhibit adhesion, whereas pretreatment with sodium periodate or α-mannosidase had little or no effect. Moreover, the protein-rich fraction obtained by incubating EP with endoglycosidase H inhibited adhesion to a greater extent than did the carbohydrate-rich fraction. These results seem to indicate that the predominant interaction between yeasts and buccal cells is one involving the protein portion of the mannoprotein adhesin. Such a mechanism would be analogous to that found in many Gram-negative bacteria where adhesion to mucosal surfaces is mediated by proteinaceous, carbohydrate-binding adhesins (Jones & Isaacson, 1983).

Adhesion of *C. albicans* to vaginal cells can be severely inhibited by subjecting the yeasts to direct treatment with a variety of different proteolytic enzymes (Sobel *et al.*, 1981; Lee & King, 1983*a*) or reducing agents such as 2-mercaptoethanol and dithiothreitol. By contrast, glycosidases have little effect (Lee & King, 1983*a*). Papain treatment of *C. albicans* has been reported to release a small mannoprotein which inhibits yeast adhesion to vaginal cells (Lee & King, 1983*b*). Incubation of EP with papain enhanced its ability to inhibit adhesion to buccal cells, while a combination of papain treatment followed by dilute alkali resulted in notably increased inhibition. Exposure to dilute alkali causes β-elimination of manno-oligosaccharides attached to serine and threonine residues in yeast mannoproteins (Sentandreu & Northcote, 1968). Presumably, digestion of EP with both papain and alkali produces peptide fragments which can more easily bind to epithelial cell receptors.

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