An *in vitro* evaluation of hydrolytic enzymes as dental plaque control agents

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The plaque-control potential of commercially available amylase, lipase and protease was evaluated by observing their effects on coaggregation and on bacterial viability within various plaque microcosms. A quantitative coaggregation assay indicated that protease significantly inhibited the extent of coaggregation of *Actinomyces naeslundii* and *Streptococcus oralis* (*P* <0.05) and of *Porphyromonas gingivalis* and *S. oralis*. Amylase significantly (*P* <0.05) increased the coaggregation of *A. naeslundii* versus *Fusobacterium nucleatum* and *A. naeslundii* versus *P. gingivalis*. Concomitant challenge of constant-depth film fermenter-grown plaques with the enzymes did not result in detectable ecological perturbations (assessed by differential culture and denaturing gradient gel electrophoresis). Similar dosing and analysis of multiple Sorbarod devices did not reveal increases in bacterial dispersion which could result from disaggregation of extant plaques. A short-term hydroxyapatite colonization model was therefore used to investigate possible enzyme effects on early-stage plaque development. Whilst culture did not indicate significant reductions in adhesion or plaque accumulation, a vital visual assay revealed significantly increased aggregation frequency following enzyme exposure. In summary, although hydrolytic enzymes negatively influenced binary coaggregation, they did not cause statistically significant changes in bacterial viability within plaque microcosms. In contrast, enzyme exposure increased aggregation within extant plaques.

**INTRODUCTION**

Hydrolytic enzymes with specificity for molecules involved in coaggregation and biofilm architecture have previously been evaluated for use as anti-plaque agents (Marsh, 1992). Whilst a small number of publications have focused on enzymic cleaning of dentures (Budtz-Jorgensen, 1977; Odman, 1992), other studies have assessed the potential of enzymic formulations as adjuncts for *in situ* oral hygiene (Hull, 1980; Kelstrup *et al.*, 1973, 1978; Robinson *et al.*, 1975). Few investigations have, however, systematically evaluated hydrolytic enzymes against complex, multi-species plaques or tested the effects at the cellular level against coaggregation, which has been defined as cell–cell adherence of genetically distinct bacteria (Kolenbrander, 2000) and is regarded as an important process in the development of multi-species biofilms (Hughes *et al.*, 1988).

Coaggregation was first observed in bacteria associated with dental plaque (Childs & Gibbons, 1990; Gibbons & Nygaard, 1970), and research activity related to the phenomenon remains largely focused on oral bacteria (Foster & Kolenbrander, 2004; Kigure *et al.*, 1995; Shen *et al.*, 2005). The importance of coaggregation in the formation of oral biofilms can be inferred from two types of evidence: (i) the incidence of coaggregative bacteria in the mouth is markedly higher than in most other environments (Rickard *et al.*, 2003); and (ii) structures indicative of coaggregative interaction can be visualized *in situ* (Bolstad *et al.*, 1996; Kolenbrander, 2000). An example of the latter is the characteristic corncob formations that are formed by *Fusobacterium nucleatum* and *Streptococcus sanguinis* (DiRienzo *et al.*, 1985). Coaggregation has been defined as the intergenic, intragenic or multigenic (Hughes *et al.*, 1988; Kolenbrander, 2000) interactions that are usually dependent on highly specific lectin–carbohydrate interactions occurring between the aggregating partners (Cisar *et al.*, 1979). These interactions can be reversed by the addition of sugars or chelating agents (Kolenbrander, 2000), and it is possible that disruption of coaggregative interactions *in vivo*, for example using hydrolytic enzymes, could be used to inhibit early colonization events on the tooth surface. Additionally, as some hydrolytic enzymes are

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**Abbreviations:** CDFF, constant-depth film fermenter; DGGE, denaturing gradient gel electrophoresis; HA, hydroxyapatite; MSD, multiple Sorbarod devices.
already licensed for human consumption and are non-toxic and biodegradable, they potentially offer a simple and cost-effective method of plaque control. As the dental plaque matrix is composed of water and extracellular polymeric substances including polysaccharides, lipids, proteins and cellular debris (Sutherland, 2001), it is logical to attempt to disrupt dental plaque physically by degradation of these bacterial molecules. Indeed, previous studies using enzymes to target biofilms were successful in removing bacterial molecules. Indeed, previous studies using substances including polysaccharides, lipids, proteins and effective method of plaque control. As the dental plaque and biodegradable, they potentially offer a simple and cost-efficient approach to plaque control as an adjunct to mechanical removal.

Effective control of dental plaque is necessary to minimize the development of caries, gingivitis and periodontitis. Physical oral hygiene measures alone may not be sufficient to prevent the progression of plaque-associated diseases. It is therefore often necessary to employ chemical plaque control as an adjunct to mechanical removal. Antimicrobial agents can be delivered to the oral cavity by formulation into either dentifrices or mouthwashes. Only a limited number of antimicrobial agents have to date demonstrated clinical efficacy in reducing dental plaque. These include bis-biquanides, e.g. chlorhexidine, phenolics such as triclosan, detergents, metal ions, essential oils and quaternary ammonium compounds. This limited range is largely due to the difficulty of maintaining substantivity of antimicrobials in the oral cavity, together with problems associated with their formulation into dental hygiene products (Marsh, 1992).

To date, the evaluation of any potential effects of hydrolytic enzymes on the oral microbiota has relied on traditional laboratory culture. Even based on the most optimistic estimates, up to half of the bacteria present in the mouth remain uncultured (Paster et al., 2001). The current study evaluated the effects of amylase, lipase and protease on dental plaque formation by determining their effects on binary bacterial coaggregation and on developing and extant plaques grown in oral microcosms. Complex consortia were characterized using differential culture, PCR and denaturing gradient gel electrophoresis (PCR-DGGE), and fluorescence microscopy.

METHODS

**Enzymes.** Amylase (glucoamylase; 333 amyloglucosidase units g⁻¹), lipase (120,000 U g⁻¹) and protease (papain; 939,000 milk clotting units g⁻¹) were obtained from Enzyme Development Corporation.

To ensure that the enzymes retained significant activity in situ and that combinatorial activities were not reduced, validator enzyme assays were conducted in the fluid phase and on treated surfaces. Hydroxylapatite (HA) discs were pre-conditioned in 0.04 % amylase, 0.03 % lipase or 0.23 % protease for 1 h. After this time, the treated discs were subjected to enzyme assays at 37 °C. Amylase activity was determined by incubating with 1 % soluble starch for 15 min (Bernfeld, 1955), after which the reducing sugar released was measured by the method of Miller (1959). Lipase activity was determined according to the methods of Pencrace & Baratti (1996) whereby 16.5 mM p-nitrophenyl palmitate solution dissolved in 2-propanol was added to the disc. Activity was determined by the change in A₄₅₀ of the assay against a blank without enzyme over 2 h. Protease activity was determined according to the methods of Charney & Tomarelli (1947). Discs were added to azocasein solution (25 mg ml⁻¹) at 37 °C for 30 min. After this time, digestion was stopped and undigested azocasein was precipitated from the solution by the addition of trichlororacetic acid. After centrifugation and the addition of 0.5 M NaOH, activity was observed colorimetrically.

**Bacterial strains and culture conditions.** The reference strains of bacteria used in this study were: Actinomyces naeslundii WVU 6627, Fusobacterium nucleatum subsp. polymorphum NCTC 10562, Lactobacillus rhamnosus AC 413, Neisseria subflava A 1078, Porphyromonas gingivalis NCTC 11834, Prevotella oralis NCTC 11459, Streptococcus mutans NCTC 10832, Streptococcus oralis NCTC 11427, Streptococcus sanguinis NCTC 7863 and Veillonella dispar ATCC 17745. All bacteria (except N. subflava) were maintained on Wilkins–Chalgren anaerobe agar and broth in a Mark 3 Anaerobic Work Station (Don Whitley Scientific) at 37 °C (gas mix: 80 % N₂, 10 % CO₂ and 10 % H₂). N. subflava was grown on nutrient agar and in nutrient broth and was incubated aerobically at 37 °C.

**Preparation of inocula for coaggregation assays.** Cultures were grown in 500 ml Wilkins–Chalgren broth and incubated anaerobically for 7 days at 37 °C (Bradshaw et al., 1998). N. subflava was grown at 37 °C under static aerobic conditions for 3 days.

**Determination of the effects of enzymes on coaggregation.** A quantitative spectrophotometric assay was used, as described previously (Ledder et al., 2008), to determine the effects of the enzymes on coaggregation activity when added before mixing the partner strains. Briefly, bacteria were harvested by centrifugation at 10 000 g for 20 min and resuspended in coaggregation buffer (Cisar et al., 1979). This buffer contained 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.15 M NaCl and 3.1 mM Na₂CO₃ dissolved in 1.0 mM Tris/HCl and was adjusted to pH 7.0. The strains were then washed three times in coaggregation buffer and resuspended to give a final OD₆₆₀ of 1.5. Equal volumes of each suspension were mixed and the OD₆₆₀ values of the mixtures were recorded. In order to test the effects of the enzymes, stock solutions of 1 % amylase, 1 % lipase and 10 % protease were prepared in sterile coaggregation buffer. Enzymes (5 μl) at concentrations of 0.04 % (w/v) amylase, 0.03 % (w/v) lipase and 0.23 % (w/v) protease, or sterile coaggregation buffer, were added to the resuspended pure cultures. Cultures were then mixed, after which the OD₆₆₀ was immediately recorded. Mixtures were incubated for 1 h at room temperature and the OD₆₆₀ was recorded again. In subsequent experiments, optical density values were recorded every 10 min for up to 1 h. The percentage coaggregation was calculated by the following equation: coaggregation=((pre-incubation value [OD₆₆₀]–test value [OD₆₆₀])/([pre-incubation value [OD₆₆₀]])×100).

**Constant-depth film fermenters (CDFFs).** CDFFs incorporating HA substrata were used to maintain oral microcosms as described previously (McBain et al., 2003a, b, c; Wilson, 1999). The temperature (36 °C) was maintained by locating the fermenters within incubation chambers (Stuart Scientific). CDFF plugs were set to a depth of 200 μm and the rotor speed was 3 r.p.m. A modified artificial saliva medium was used (McBain et al., 2003b) containing (g l⁻¹) in distilled
water): mucin (type II, porcine, gastric), 2.5; bacteriological peptone, 2.0; trypthone, 2.0; yeast extract, 1.0; NaCl, 0.35; KCl, 0.2; CaCl₂, 0.2; cysteine hydrochloride, 0.1; haemin, 0.001; and vitamin K₁, 0.0002. Saliva used for inoculation was obtained from a healthy adult male (aged 33). Prior to inoculation, the CDFD plug surfaces were conditioned for 24 h with culture medium, which was continuously added to each fermenter by a peristaltic pump (9.6 ± 0.2 ml h⁻¹, Minipuls 3; Gilson). The fermenters were inoculated with fresh saliva (2.0 ± 0.5 ml per fermenter per inoculation) on three separate occasions. Anaerobiosis was maintained for 48 h following inoculation within the CDFDs by constant addition of an anaerobic gas mixture (5:95, CO₂:N₂) at 1 litre h⁻¹.

Addition of enzymes to CDFD microcosms. After the establishment of a dynamic steady state (as evidenced by the congruence between separate colony counts over time), all three enzymes together [0.04 % (w/v) amylase, 0.03 % (w/v) lipase, 0.23 % (w/v) protease] or sterile phosphate buffer at pH 6.0 (1.5 ml) were added aseptically daily over 5 days to the test and control CDFDs, respectively. Samples were taken daily, 1 h after enzyme addition.

Sampling of CDFDs. Three CDFD plugs were transferred aseptically to 9 ml half-strength thioglycollate broth (USP) and homogenized by mechanical shaking in a bead beater (0.5 min, 240 oscillations min⁻¹). Multiple Sorbarod devices (MSDs).

Oral microcosms were conditioned for 24 h with culture medium, which was continuously added to each fermenter by a peristaltic pump (9.6 ± 0.2 ml h⁻¹, Minipuls 3; Gilson). The fermenters were inoculated with fresh saliva (2.0 ± 0.5 ml per fermenter per inoculation) on three separate occasions. Anaerobiosis was maintained for 48 h following inoculation within the CDFDs by constant addition of an anaerobic gas mixture (5:95, CO₂:N₂) at 1 litre h⁻¹.

Addition of enzymes to MSD microcosms. After the establishment of a dynamic steady state (as evidenced by the congruence between separate colony counts over time), all three enzymes together [0.04 % (w/v) amylase, 0.03 % (w/v) lipase, 0.23 % (w/v) protease] or sterile phosphate buffer at pH 6.0 (1.5 ml) were added aseptically daily over 5 days to the test and control CDFDs, respectively. Samples were taken daily, 1 h after enzyme addition.

Sampling of MSDs. MSD perfusate samples (~5 ml) were collected by disconnecting the waste tube at the bottom of the MSD and placing a sterile plastic Universal bottle directly underneath. For biofilm analysis, MSDs were opened aseptically and a single Sorbarod filter was removed and immediately replaced with a new, sterile filter. Filter biofilms were divided longitudinally into two equal portions by cutting the outer sheaf with a sterile scalpel. One portion was then used for immediate differential viable counting following maceration and dilution by adding 9 ml half-strength thioglycollate medium, along with 1.5 g 3.5–5.5 mm sterile glass beads (BDH) followed by mechanical shaking as outlined above. Perusates (1 ml) were also serially diluted. The remaining portion of the filter and perusates (2 ml) were stored at −60 °C for subsequent PCR-DGGE analyses.

Addition of enzymes to MSD microcosms. After the establishment of a dynamic steady state (as evidenced by the stability of colony counts), sterile phosphate buffer (pH 6.0) was added to the control MSD, and 0.04 % amylase, 0.03 % lipase and 0.23 % protease were added to the test MSD. Enzymes or buffer were added daily for 2 days by removing the upper section of the fermenter and depositing 10 ml of the appropriate mixture of enzymes or buffer directly into the upper chamber. Samples were taken daily, 1 h after enzyme addition.

**HA disc models.** Batch cultures of oral microcosms were maintained in an HA biofilm model. Enzymes (0.04 % amylase, 0.03 % lipase and 0.23 % protease) were used to pre-condition sterile HA discs (9.6 mm diameter) for 1 h. The control discs were treated with sterile phosphate buffer. Saliva (1 ml) from a volunteer (female, aged 27) was then dispensed into each of eight wells of a sterile 24-well tissue culture plate. The discs were transferred aseptically into the wells. Inoculated plates were mounted onto an orbital shaker and continuously mixed gently at 144 oscillations min⁻¹ in an anaerobic workstation at 36 °C for 1 h. For enumeration, an HA disc was aseptically removed from the tissue culture plate before being passed through an air–liquid interface (sterile PBS).

**Differential bacteriological analysis.** Samples (derived from CDFD plugs, MSD filters, MSD perfusates and HA discs) were serially diluted using pre-reduced, half-strength thioglycollate medium. Appropriate dilutions (100 ml) were plated in duplicate onto selective and non-selective media as follows: Wilkins–Chalgren agar (for total anaerobes); Wilkins–Chalgren agar supplemented with haemin (5 mg 1⁻¹), menadione (0.5 mg 1⁻¹), nalidixic acid (10 mg 1⁻¹), vancomycin (10 mg 1⁻¹) and sodium succinate (2.5 g 1⁻¹) (for gramm-negative anaerobes); Rogosa agar (for total lactobacilli); trypctase yeast extract/cysteine/sucrose agar (van Palesen Helderman et al., 1983) (for Streptococcus spp.); and nutrient agar (for total aerobes). All except for the nutrient agar were immediately placed in an anaerobic chamber (80 % N₂, 10 % H₂, 10 % CO₂) and incubated at 37 °C for up to 5 days. Nutrient agar plates were incubated aerobically in a standard incubator at 37 °C for 3 days.

**Community analysis by PCR-DGGE.** DNA was extracted from the archived MSD samples using a DNA stool mini kit (Qiagen) in accordance with the manufacturer’s instructions and analysed by PCR-DGGE as described previously (McBain et al., 2003a).

**Dendrogram construction for cluster analysis.** Gel images were aligned using Adobe Photoshop Elements Version 7. Gel images were then analysed using the Bionumerics software package (Applied Maths). The bands present in each lane were detected automatically and then checked manually. A reference lane was created using bands present in each lane to generate matching profiles. The matching profiles for each lane were used to produce an unweighted pair group method with arithmetic mean (Sneath & Sokal, 1973) dendrogram so that clustering patterns could be determined.

**Statistical analyses.** The Student’s t-test was used to determine whether the effects of the enzymes on coaggregation assays and in microcosms were significant. Data were arranged in tables and subjected to a Microsoft Excel macro.

**Light microscopy.** After the 1 h pre-conditioning period described above, HA discs were removed aseptically from the tissue culture plate and transferred to 9 ml sterile half-strength thioglycollate broth and subjected to gentle inversion for 30 s. The broth was then filtered through a 0.2 µm pore-size, black polycarbonate filter (Whatman) and stained using a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) in accordance with the manufacturer’s instructions. Finally, the filter was mounted in LIVE/DEAD immersion oil and a coverslip was added before visualizing using an epifluorescence microscope (Axioskop 2; Zeiss). Cells were scored as live (green) or red (dead) and either c.f.u. (single cells or up to three cells in a microcolony) or aggregates (greater than three cells in a microcolony).

**Confocal laser-scanning electron microscopy.** After incubating the HA discs for 1 h as described above, they were removed aseptically from the tissue culture plates and stained in situ as
follows: 100 μL LIVE/DEAD BacLight stain (Molecular Probes), prepared according to the manufacturer’s instructions in 0.01 M PBS (pH 7.0), was added to the disc. The samples were then incubated for 10 min at room temperature in the dark. The HA discs were then transferred to a microscope slide using sterile forceps. A coverslip was placed on top of the biofilm samples and sealed into place using molten candle wax to enable inversion. Biofilms were examined using a Zeiss Combi LSM 510 META/Confocor II inverted microscope. Excitation wavelengths of 488 and 633 nm were used to image the live and dead bacterial cells, respectively. Confocal images were obtained using a ×40 1.3NA DIC oil-immersion objective. Each biofilm was scanned at randomly selected positions. Image acquisition and analysis were carried out with the software Combi LSM (version 3.2; Zeiss).

RESULTS AND DISCUSSION

The aim of the current study was to investigate the anti-plaque potential of amylase, lipase and protease. These enzymes were selected because they exhibit broad specificity towards major biomolecules responsible for the physical integrity of dental plaque. In order to evaluate the effects of enzymes on early stages of plaque development as well as on extant plaque, we included binary and in situ aggregation assays, with oral microcosms, designed to simulate mature and developing plaque.

The application of hydrolytic enzymes with specificity for both bacterial surface and matrix components was originally considered as a possible non-biocidal approach to dental plaque in the 1960s (Gorbach et al., 1967). Enzymes have several theoretical advantages over chemical formulations, including the possibility of combining bactericidal activity with highly specific plaque disruption and disaggregation. Additionally, a range of hydrolytic enzymes of differing specificity (amylase, lipase and protease) are already licensed for oral administration and food use. Despite considerable theoretical potential, studies carried out to evaluate the efficacy of enzymes to inhibit the formation of or reduce extant plaque have given inconclusive results. For example, whilst one early study reported significant anti-plaque activity of a dextranase-containing dentifrice (Kitamura et al., 1980), others did not (Keyes et al., 1971; Lobene, 1971). Proteolytic formulations have also not been unequivocally successful (Odmann, 1992; Robinson et al., 1975). Such variability can be attributed to a lack of standardization in experimental approach, the use of different enzymes and their variation in formulation together with the fact that even optimally deployed enzymes may not always offer an effective means of plaque removal. Determination of enzyme-mediated effects on complex plaques using colony counting may be complicated by the fact that enzymes will not necessarily cause significant cell death, but rather are likely to influence biofilm integrity through degradation or alteration of the biofilm matrix or possibly by affecting early colonization through inhibition of coaggregation and coadhesion. As the efficacy of enzymatic formulations for plaque control is currently unclear and potential mechanisms of action are poorly understood, an experimental evaluation should ideally cover the main possible areas of action, which include modulation of cell–cell interactions and effects on developing and mature plaques.

Coaggregation has been proposed as a fundamental process in the development of complex dental plaque through the formation of aggregates in the fluid phase and through coadhesion where cells aggregate with cells immobilized in biofilms (Kolenbrander, 2000). In the current investigation, preliminary studies were therefore designed to elucidate the effects of the three enzymes in combination on this process. Binary assays using ten commonly isolated oral bacteria (Bradshaw et al., 1996; McKee et al., 1985) were carried out (Table 1). Treatment with amylase, lipase and protease in combination significantly (P < 0.05) reduced coaggregation after 1 h of treatment for 13 pairs; most notably for the pairs A. naeslundii and N. subflava, L. rhamnosus and Porphyromonas gingivalis, Porphyromonas gingivalis and S. oralis, S. mutans and V. dispar, and S. oralis and S. sanguinis.

Based on significant inhibition of coaggregation with the combined enzyme mixture, four human oral bacteria were selected for further analyses to determine the effects of the enzymes individually. Neither amylase nor lipase exhibited any significant effects on the coaggregation of these bacteria (data not shown). Table 2, however, shows that protease significantly (P < 0.05) reduced the percentage aggregation for A. naeslundii and A. naeslundii (autoaggregation), and for A. naeslundii and F. nucleatum. Interestingly, A. naeslundii is recognized as a primary colonizer of the teeth (Socransky, 1970) and has been suggested as significant in the development of eubacterial diversity in the oral environment (Yeung & Cisar, 1990). Promiscuous coaggregation of this bacterium has been linked to the expression of cell-surface fimbriae (Cisar et al., 1988). Targeting of this bacterium with an enzyme treatment might therefore enable the disruption of biofilm formation.

Fig. 1 shows the effects of individual enzymes on coaggregation between A. naeslundii, F. nucleatum, Porphyromonas gingivalis and S. oralis in all possible combinations by scoring percentage coaggregation every 10 min for 1 h. Amylase significantly (P < 0.05) increased coaggregation scores for the pairs A. naeslundii and Porphyromonas gingivalis, and A. naeslundii and F. nucleatum. Amylase binding by many types of oral bacteria has been shown to promote colonization (Rogers et al., 2001; Scannapieco et al., 1994; Tseng et al., 1992). Protease significantly (P < 0.05) reduced coaggregation scores of Porphyromonas gingivalis and S. oralis, and of A. naeslundii and S. oralis, which suggests that Papain-sensitive proteins are involved in mediating the interaction of these bacteria. As lectins, which are proteinaceous, have been shown to be involved in coaggregation (Cisar et al., 1979), it seems likely that their degradation by proteases would significantly interfere with coaggregation. Assuming that coaggregation is a significant moderator of plaque development.
Table 1. Effects of amylase, lipase and protease on the percentage coaggregation scores of pairs of ten human oral bacterial species

Control coaggregation scores are indicated in the upper left half of the square and scores after enzyme treatment in the lower right. The percentage coaggregation was measured by OD₆₆₀ change over 1 h. AN, *A. naeslundii*; FN, *F. nucleatum*; LR, *L. rhamnosus*; NS, *N. subflava*; PG, *Porphyromonas gingivalis*; PO, *Prevotella oralis*; SM, *S. mutans*; SO, *S. oralis*; SS, *S. sanguinis*; VD, *V. dispar*. Data are means ± SD (in parentheses) from three separate experiments. Significant differences are indicated by light-grey (*P* < 0.05) and dark-grey (*P* < 0.001) shading.

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<th>AN</th>
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Table 2. Effect of protease on aggregation index after 1 h by spectrophotometric assay

Data are means ± SD (in parentheses) from three separate experiments. Control coaggregation scores are indicated in the upper left half of the square and scores after enzyme treatment in the lower right. The percentage coaggregation was measured by OD₆₆₀ change over 1 h. Significant differences (*P* < 0.05) are indicated by grey shading.

<table>
<thead>
<tr>
<th>Species</th>
<th><em>A. naeslundii</em></th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
<th><em>S. oralis</em></th>
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and maintenance, then its inhibition should affect the extent of plaque accumulation, and the cohesive strength or the ecological balance of plaques over time. To determine whether such effects could be reproduced within extant, complex oral microcosms, amylase, lipase and protease were added to steady-state CDFF salivary microcosms. The data in Fig. 2 show the results of enumeration of culturable aerobes and anaerobes within the CDFFs. Anaerobic counts of 6–7 log_{10} c.f.u. mm^{-2} were obtained from the fermenters with lower numbers of aerobes, streptococci and Gram-negative anaerobes (data not shown). A dynamic steady state was achieved in the microcosms within 4 days of inoculation. The addition of all three enzymes to the test fermenter led to a decrease in the aerobic and anaerobic species that was non-significant and transient.

Subsequent experiments utilized MSDs, which are specialized model systems that allow extant plaque microbiology to be assessed but, importantly, also enable bacteria eluted from the biofilm within perfusates to be visualized and cultured (Ledder et al., 2006; McBain et al., 2005). Data from both culture (Fig. 3) and PCR-DGGE (data not shown) indicated that the MSD microcosms achieved a dynamic steady state within 4 days of inoculation. PCR-DGGE analysis revealed that the eubacterial community present in the salivary inoculum was accurately reproduced in the MSD (data not shown), confirming maintenance of a significant portion of salivary microbial diversity. Fig. 3 indicates that the addition of all three enzymes in combination had no significant effect on either the number of recoverable oral bacteria from the biofilm or, importantly, in the perfusates. This suggests that: (i) enzyme-related inhibition of coaggregation, demonstrated in binary

![Graphs showing time-course effects of amylase, lipase and protease on the percentage coaggregation between human oral bacteria. Data are means±SD from three separate experiments. ○, Control; ○, amylase; ▼, lipase; ▼, protease; *, significant (P ≤0.05) change.](http://jmm.sgmjournals.org)
assays, was not similarly manifested in mature, complex consortia; (ii) in situ inhibition of coaggregation did not markedly influence numbers of major culturable bacterial groups; or (iii) coaggregation may be more important in the early stages of plaque development, as suggested previously.

In the CDFF and MSD experiments, enzymes were added to extant plaques. Thus, in order to observe potential enzyme effects on the early stages of plaque development, a short-term HA model was used. Amylase, lipase and protease, both singly and in all possible combinations, were used to pre-condition the HA discs, which were then used to support the growth of salivary-derived microcosms. The results for the addition of all three enzymes in combination are shown in Fig. 4. No significant effects in terms of the reduction of culturable bacteria were observed for the enzymes singly (data not shown), in binary combination (data not shown) or combined. As validation experiments have demonstrated that no significant intra-enzyme antagonism occurred, this suggests that the coaggregation interactions that are inhibited by protease are (i) not essential in the early stages of development of salivary-derived plaques; (ii) not inhibited by the enzymes in situ; or (iii) not important under the particular physicochemical conditions within the model system. Fig. 5 shows data for numbers of individual live and dead cells together with live and dead bacterial aggregates developed in control and enzyme-exposed environments with salivary bacterial consortia derived from HA discs. Enzyme exposure significantly increased aggregation frequencies amongst viable cells (Figs 5 and 6). This observation is in apparent agreement with the data presented in Fig. 1, where amylase significantly increased the strength and rapidity of coaggregation. Protease exposure, however, increased...
consortium aggregation (Fig. 5) but decreased binary coaggregation (Fig. 1), which could indicate a change in the physical integrity of the plaque. Measurements of the frequency of aggregation, however, as indicated in Fig. 5, do not necessarily correlate with strength and rapidity of interaction between two species (Fig. 1), as these data could represent different phenomena. Furthermore, addition of each of the individual enzymes to oral consortia caused decreases in individual viable cell numbers that were statistically significant only for lipase (Fig. 5). Similar decreases in bacterial viability did not, however, occur following addition of lipase in combination with amylase and protease. This observation cannot be explained based on simple enzyme antagonism or inactivation, as we have demonstrated in validation experiments that this does not occur (data not shown). Some other form of combinatorial effect must be responsible, possibly related to the increases in rapidity of coaggregation or bacterial attachment such as that demonstrated for amylase exposure in binary assays (Fig. 1), or action in a different aspect of biofilm formation.

Biofilms have evolved to exist under a range of inimical physico-chemical conditions, and significant enzymic activity will in any case normally be expressed within saliva and plaque. For enzymic formulations to be effective, their deployment must either add significantly to those activities already expressed or manifest different specificity. In the current investigation, the former is more likely, as
the specificity of the enzymes does not differ from that of enzymes already present in the mouth and the effects that were observed on coaggregation in salivary microcosms indicate a supplementary effect.

In conclusion, whilst protease inhibited coaggregation in binary assays, protease, amylase and lipase treatments did not significantly alter consortium population dynamics.

REFERENCES


