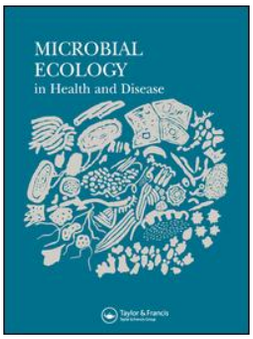

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# Effect of Sucrose Intake and Growth Conditions on Numbers of Dental Plaque Bacteria Expressing Proteolytic Activity

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# Effect of Sucrose Intake and Growth Conditions on Numbers of Dental Plaque Bacteria Expressing Proteolytic Activity

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Proteolytic enzymes in dental plaque are important elements in the pathophysiology of periodontal disease and are putative virulence factors. The effect of sucrose intake versus a sucrose-free diet (substituting glucose for sucrose) on numbers of isolates from early dental plaque expressing extracellular proteolytic enzymes able to hydrolyse gelatin and azocoll was studied. The bacteria were isolated from 0–3 d dental plaque formed on the buccal surface of a lower premolar in six subjects. A total of 7987 isolates were tested. Sucrose intake was associated with lower numbers of isolates expressing proteolytic activity at the start of plaque formation and higher numbers in 2 and 3 d plaque. Limitations of current methods for cultural studies of developing dental plaque are discussed, and the need for improved methods to study the nature and activities of all microorganisms colonising the teeth is stressed.

KEY WORDS: sucrose intake; dental plaque; proteolytic activity; virulence factors; validity; plaque ecology

## INTRODUCTION

Extracellular proteolytic enzymes are, like extracellular carbohydrate metabolising enzymes,[29] produced by many different microbial species and are important for plaque formation, ecology, and virulence in several ways. Proteolytic enzymes may affect the initial colonisation by exposing receptors (cryptitopes) and causing conformational changes in the acquired pellicle.[11] They may also interfere with binding between bacterial surface structures and components in the pellicle.[24,38] Further, proteolytic enzymes synthesised by plaque microorganisms may influence plaque formation by interfering with bacterial growth[12] and coadhesion,[9,27] inhibiting[17] and degrading glucosyltransferases,[17,26,30] degrading fructanase,[3] and by influencing the effect of bacteriocins.[13,20] Amino acids released during proteolysis serve as nutrients for bacteria.[8] Proteolytic enzymes are regarded as virulence factors in periodontal disease[35,36,39] and advanced stages of dental caries.[4,23]

The presence of sucrose may modify the expression,[15] amount,[14] and activity[5] of an enzyme. Proteolytic activity may vary with microbial growth rate[2,34] and pH.[7,34] Sucrose intake has been demonstrated to influence the growth of and acid production by dental plaque.[28] Consequently, sucrose intake any affect numbers of microorganisms in dental plaque expressing proteolytic activity *in vivo*.

The present investigation is part of a series of studies on the effect of sucrose intake on the microbial composition of early dental plaque. The aim of this part of the study was to investigate quantitatively, from a biological rather than a taxonomic point of view, the effect of a sucrose-containing versus a sucrose-free diet (substituting glucose for sucrose) on numbers of bacteria in early dental plaque expressing extracellular proteolytic enzymes.

## MATERIALS AND METHODS

Details of the diets used and the experimental design have been described previously.[28] The study plan was accepted by the local ethics committee, and the declaration of Helsinki in its 1975 revised form was followed throughout.

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*Microbiology*

Six experimental subjects consumed for 14 days a sucrose-free diet ( − S) and 3 to 6 months later a similar, but sucrose-containing diet (+S). After 7 days on the experimental diets, plaque samples were taken with a scaler along the gingival margin on the buccal surface of the right first premolar in the lower jaw at time zero, immediately after toothbrushing, and from the same area after 2 h, 4 h, 6 h, 12 h, 1 d, 2 d, and 3 d of plaque formation.

The samples were transferred to nutrient broth with 0·1 per cent L-cysteine hydrochloride, homogenised, serially diluted, and inoculated into roll tubes containing a prereduced, anaerobically sterilised, non-selective agar medium containing tryptone, yeast extract, glucose, starch, $K_2HPO_4$, $MgSO_4$, cysteine, menadione and haemolysed calf blood,[18] and on agar plates containing an identical medium. Roll tubes were flushed with oxygen-free gas containing 10 per cent $CO_2$ and incubated at 37°C for 7 d. Agar plates were incubated microaerophilically in 10 per cent $CO_2$ and 90 per cent $N_2$ for 4 d. One hundred colonies, or as many as possible, were isolated from the lowest dilutions having fewer than 300 colonies in roll tubes and on agar plates. The isolates were transferred to a liquid medium similar in composition to the agar except that blood was substituted by haemin. Media were sterilised at 121°C for 20 min. All isolates able to grow in the first subculture were investigated for enzyme activities which were performed on the first and second subcultures.

The number of isolates demonstrating each of the enzyme activities was calculated for every plaque sample, and the highest number obtained from either anaerobic or microaerophilic incubation was log-transformed. To enable values below detection level to be included, these were represented in the calculations by a value corresponding to one isolate.

*Determination of enzyme activities*

All isolates were tested for their ability to hydrolyse gelatin and azocoll, a cow hide powder preparation rich in denatured collagen which is coupled to an azo dye.[33] Both proteins are suitable for detection of non-specific proteolytic activities.[6,16]

The ability to hydrolyse gelatin was detected with the method described by Whaley *et al.*[40] Gelatin agar medium was Todd Hewitt broth supplemented with agar and 0·4 per cent (wt/vol) gelatin. The plates were inoculated in the center of each quadrant with liquid cultures using cotton swabs and incubated at 37°C for 14 days. Afterwards, the plates were flooded with Frazier's solution[10] which precipitated gelatin and hydrolysis was detected as clear zones around the cultures.

The ability to hydrolyse azocoll was detected as described by Söder and Frostell.[37] One hundred mg azocoll was suspended in 10 ml nutrient agar in a Petri dish. Liquid cultures were inoculated into wells and the plates incubated at 37°C for 2 d. A positive reaction appeared as a zone without azocoll grains around the inoculated well.

Isolates from roll tubes were incubated anaerobically in the first test. In repeated tests all isolates which did not require anaerobic growth conditions were incubated in 10 per cent $CO_2$ and 90 per cent $N_2$. In azocoll tests the isolates were considered positive if they demonstrated a positive reaction in a test inoculated from either the first or the second subculture or from both of them. Although the gelatin test was optimised as suggested by Whaley *et al.*,[40] it was difficult to distinguish between weak positive and negative reactions. Therefore, two positive tests were required for a positive result. Consequently, the test was repeated a third time if the first two results differed.

## RESULTS

A total of 7987 colonies were subcultured. The numbers tested from each subject ranged from 987 to 1422. Gelatinase activity was demonstrated by 1510 isolates and 1135 were able to hydrolyse azocoll. Numbers of isolates which demonstrated hydrolysis of gelatin and azocoll increased with time. Generally, the numbers of isolates demonstrating these activities (Figures 1 and 2) were equal or higher at 2 h in − S than in +S samples and highest in +S samples at the end of the experimental period. Most isolates able to hydrolyse azocoll also hydrolysed gelatin.

The percentages of isolates able to grow in culture until they were tested at least once for their ability to hydrolyse gelatin and azocoll are given in Table 1. With a few exceptions, the mean and median percentages of isolates from anaerobic incubation and those from plates incubated microaerophilically which were tested for azocoll hydrolysis ranged from 85 to 100. On the other hand, the percentage of isolates from microaerophilic incubation which could be tested for

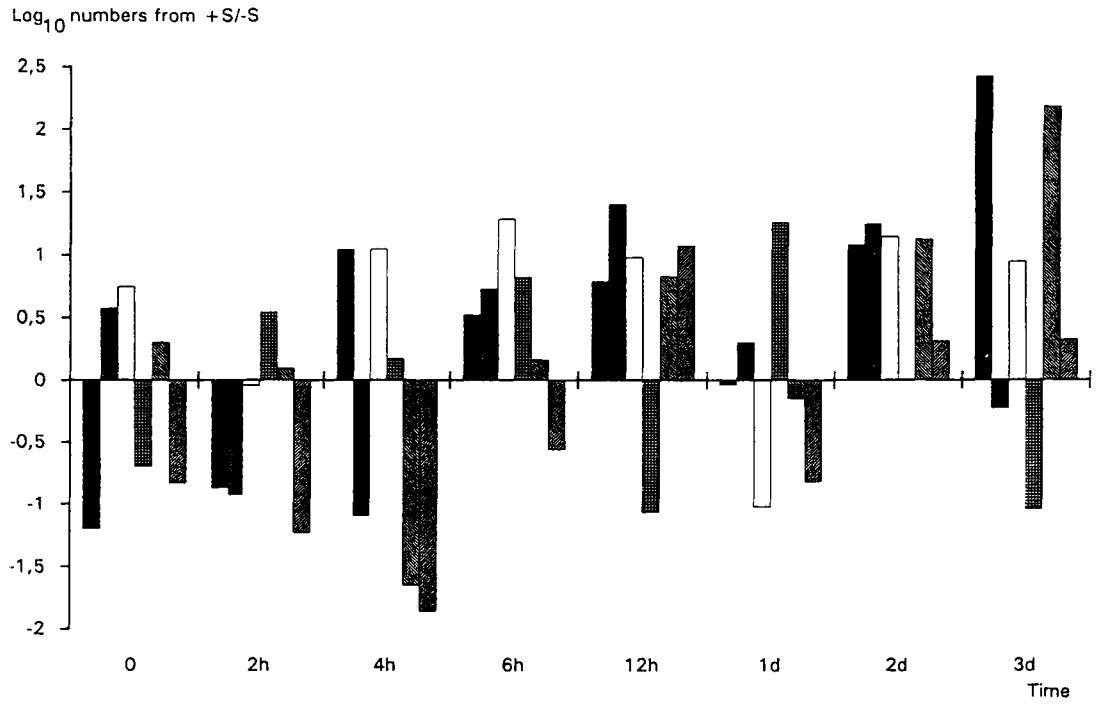


Figure 1. Ratios between numbers of bacteria able to hydrolyse gelatin in 0 to 3 d dental plaque from six experimental subjects on a diet containing sucrose (+S) and without sucrose ( − S). The horizontal line indicates identity between +S and − S numbers. Each pattern represents one subject.

hydrolysis of gelatin decreased with increasing plaque age. When the diet contained sucrose (+S), the mean and median percentages decreased after the 4 h sample to 65–79 in the 6 h to 2 d samples, and further decreased to a mean of 58 and a median of 57 for the 3 d isolates. When the diet was without sucrose ( − S), the mean and median percentages tested were high (84–96) for the time zero to 2 d plaque samples while it decreased to 67 and 70 respectively in the 3 d samples.

## DISCUSSION

In the present study and in the report[29] on carbohydrate metabolising bacteria in the same samples the isolates are grouped according to enzyme activities because these are biological factors essential for colonisation, metabolism and virulence of dental plaque bacteria. The identity of the isolates is of secondary interest in understanding plaque formation and polymicrobial infections like periodontal disease and dental caries. Grouping according to function rather than microbial species will better clarify the potential enzyme activities because these have been observed in a number of species while they may not be demonstrated in all strains within a species.[19,21]

Although the conventional substrates gelatin and azocoll used for the proteolytic tests are not identical to proteins found in the oral cavity they are structurally similar to some of these. Gelatin has a high content of proline[31] which is a major constituent of new acquired pellicle.[1] Further, gelatin has a similarity to collagen IV[39] which is the major protein of basement membranes as found in gingiva.[39]

The proteolytic activities demonstrated in the tests may be both extracellular and intracellular hydrolases, although it is likely that the bulk of the enzyme activities represented extracellular enzymes. The cultures used in the azocoll test did not have optimal pH values.[22] An adjustment could have given higher numbers of isolates able to hydrolyse azocoll. Glucose in the gelatin test medium may have caused a decrease in proteolytic activity[32] while the addition of cysteine in the pretest medium may have induced an increase.[25] Consequently, both glucose and cysteine in the

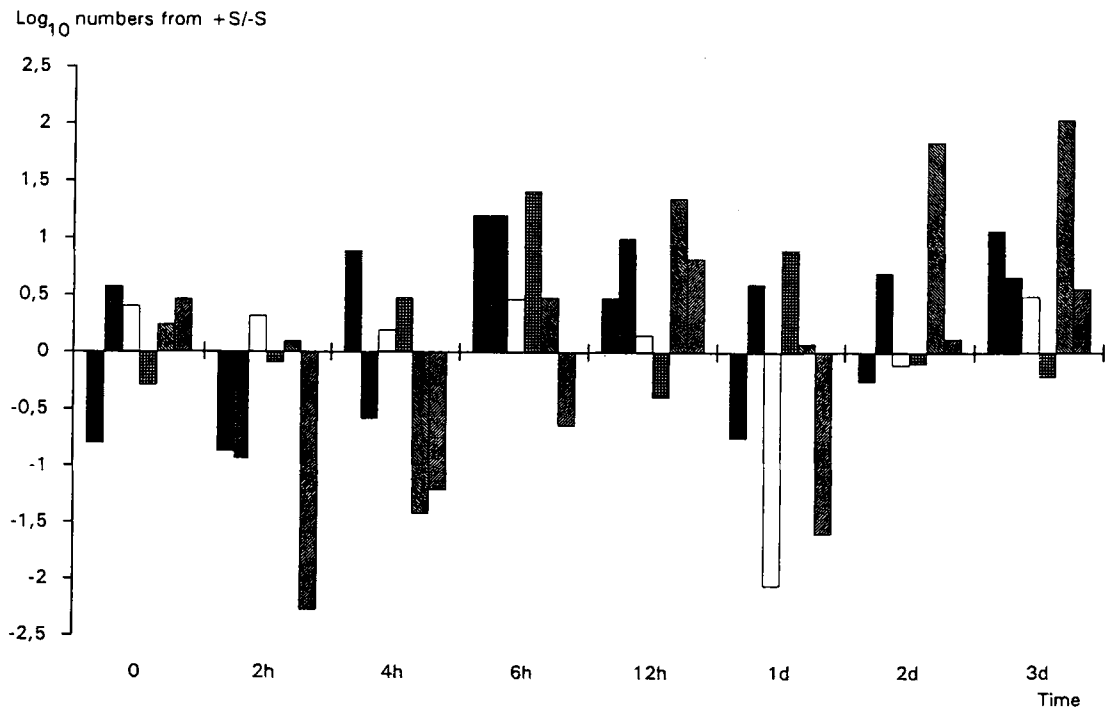


Figure 2. Ratios between numbers of bacteria able to hydrolyse azocoll in 0 to 3 d dental plaque from six experimental subjects on a diet containing sucrose (+S) and without sucrose ( − S). The horizontal line indicates identity between +S and − S numbers. Each pattern represents one subject.

media could have affected the numbers of isolates expressing proteolytic activity.

Sucrose intake was associated with lower numbers of isolates expressing proteolytic activity at the start of plaque formation and higher numbers in 2 and 3 day plaque (Figures 1 and 2). In two previous papers,[28,29] data on viable counts and on extracellular carbohydrate metabolising isolates of the same samples showed a similar pattern. Therefore, it may be speculated that total viable counts set some limits for the counts obtainable for each enzyme activity. The total viable counts[28] generally demonstrated logarithmic growth curves showing stationary level or even a decrease during part of the experimental period. On − S diet these stationary or decreasing counts occurred at about 4 hours and 6 hours resulting in relatively low numbers in 6 hour and 12 hour − S samples. On +S diet the stationary or decreasing counts started about 6 hours and 12 hours which resulted in relatively low numbers in 12 hour and 1 day samples.[28] This difference between − S and +S viable counts may have resulted in the changeable values between equal or higher numbers of isolates with proteolytic activity in − S samples early during the experimental period and higher numbers in +S samples at the end (Figures 1 and 2).

In the present study a high proportion of the isolates could be tested for hydrolysis of gelatin and azocoll (Table 1). In the gelatin test a decrease in proportions of isolates from microaerophilic incubation conditions started earlier on +S than − S diet (Table 1). The gelatin test required growth on the test medium, unlike the azocoll test. In the azocoll test high proportions of isolates from microaerophilic incubation demonstrated that the isolates were able to grow in the first subculture in the medium used for subculturing. This indicates that an increasing proportion of isolates from microaerophilic incubation are unable to grow on the gelatin test medium. Similar problems may arise in other tests requiring growth. In the case of isolates obtained from anaerobic incubation the decreasing proportions mentioned above were not observed.

These data indicate a difference in the microorganisms cultured from a plaque sample when microaerophilic or anaerobic incubation is used. Collectively, the present and a previous study[28] of 0–3 day dental plaque samples have shown that (i)

Table 1. Percentage of isolated microorganisms tested for proteolytic activity at least once (+S=sucrose-containing diet; −S=sucrose-free diet)

| | +S | | | | | | | | −S | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling time | 0 | 2 h | 4 h | 6 h | 12 h | 1 d | 2 d | 3 d | 0 | 2 h | 4 h | 6 h | 12 h | 1 d | 2 d | 3 d |
| *Gelatin* | | | | | | | | | | | | | | | | |
| Microaerophilic | 95[a] | 96 | 90 | 79 | 76 | 69 | 65 | 58 | 90** | 84 | 94* | 89** | 84 | 90 | 86 | 67 |
| Incubation | 100[b] | 98 | 94 | 77 | 78 | 71 | 76 | 57 | 91** | 91 | 96* | 89** | 87 | 92 | 88 | 70 |
| | 80–100[c] | 86–100 | 65–100 | 64–96 | 35–98 | 54–85 | 30–92 | 28–88 | 69–100** | 63–97 | 88–97* | 71–100** | 64–100 | 78–100 | 70–100 | 39–100 |
| Anaerobic | 87 | 97** | 94 | 78** | 77 | 89 | 90 | 84 | 83* | 93 | 77 | 91** | 80 | 91 | 95 | 93 |
| Incubation | 89 | 97** | 95 | 86** | 88 | 88 | 92 | 91 | 95* | 93 | 90 | 95** | 90 | 94 | 96 | 94 |
| | 59–100 | 94–100** | 90–98 | 24–100** | 48–98 | 78–98 | 76–98 | 40–100 | 84–97* | 79–100 | 24–100 | 70–100** | 33–95 | 76–96 | 87–100 | 83–100 |
| *Azocoll* | | | | | | | | | | | | | | | | |
| Microaerophilic | 95 | 96 | 93 | 93 | 97 | 84 | 91 | 89 | 91** | 89 | 96* | 97** | 95 | 97 | 92 | 95 |
| Incubation | 100 | 100 | 94 | 97 | 98 | 85 | 90 | 93 | 94** | 91 | 97* | 100** | 97 | 96 | 95 | 93 |
| | 80–100 | 86–100 | 69–100 | 73–100 | 88–100 | 68–100 | 80–90 | 63–98 | 69–100** | 68–98 | 88–97* | 89–100** | 57–100 | 88–100 | 77–100 | 74–97 |
| Anaerobic | 97 | 97** | 95 | 78** | 79 | 92 | 92 | 95 | 95* | 95 | 84 | 92** | 92 | 94 | 98 | 94 |
| Incubation | 98 | 97** | 95 | 86** | 88 | 91 | 96 | 93 | 95* | 96 | 99 | 97** | 90 | 97 | 97 | 94 |
| | 82–100 | 94–100** | 90–98 | 20–100** | 50–100 | 84–100 | 78–100 | 46–100 | 90–97* | 79–100 | 24–100 | 70–100** | 33–98 | 76–100 | 87–100 | 85–100 |

[a]Mean; [b]median; [c]range.
*$n$=4; **$n$=5.

one dietary factor, in this case sucrose, can affect the cultivable proportions of total microscopic counts; (ii) the ratio between cultivable microorganisms (colony forming units) and microscopic counts increased during the experimental period; (iii) proportions of isolated microorganisms able to grow on a test medium, in this case gelatin, decreased during the experimental period; (iv) microaerophilic and anaerobic incubation of isolation media expressed different microbial compositions of the same plaque; and (v) in plaque suspensions the number of microorganisms per clump, representing one colony forming unit may be affected by the presence or absence of dietary sucrose.

Consequently, to increase the validity of observations from studies on colonisation of tooth surfaces and ecology in early dental plaque, there is a need to develop improved methods of study.

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