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Incorporation of Caseinoglycomacropeptide and Caseinophosphopeptide into the Salivary Pellicle Inhibits Adherence of Mutans Streptococci

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Abstract. The protective effects of milk and milk products against dental caries have been demonstrated in many animal studies. We have shown that this effect was mediated by micellar casein or caseinopeptide derivatives. A reduction in the Streptococcus sobrinus population in the oral microbiota of animals fed diets supplemented with these milk components was consistently observed. A possible explanation for these findings is that milk components are incorporated into the salivary pellicle, thereby reducing the adherence of S. sobrinus. This hypothesis was tested in vitro by the incubation of bovine enamel discs with unstimulated saliva. The resulting pellicle was washed and incubated with caseinoglycomacropeptide (CGMP) and/or caseinophosphopeptide (CPP) labeled with 17- and 12-nm gold particles. All samples were prepared for electron microscopy by high-pressure freezing followed by freeze-substitution. It was demonstrated by high-resolution scanning electron microscopy with back-scattered electron imaging, as well as by transmission electron microscopy, that both peptides were incorporated into the pellicle in exchange for albumin, confirming previous findings. This protein was identified with a mouse anti-human serum albumin followed by goat anti-mouse IgG labeled with 25-nm gold particles. Incorporation of CGMP and/or CPP into salivary pellicles reduced the adherence of both S. sobrinus and S. mutans significantly. It is suggested that the calcium- and phosphate-rich micellar casein or caseinopeptides are incorporated into the pellicle. The resulting ecological shifts, together with the increased remineralization potential of this biofilm, may explain its modified cariogenic potential.

Key words: caseinophosphopeptides, pellicle, caries, milk, caseinoglycomacropeptide.

Introduction

The protective effects of milk and milk products against dental caries have been an early topic of research and speculation for more than 50 years. Ever since Lady May Mellanby (1929, 1930) proposed milk as an important nutritional factor affecting pre-eruptive tooth mineralization and post-eruptive caries resistance, the relationship between milk and caries has been examined from various angles [for a review, see Reynolds and Storey (1979) and Herod (1991, 1994)].

Although Mellanby's theory was not tenable, numerous animal studies have demonstrated that milk and milk products (primarily cheese) have a protective effect against the development of dental caries (Beveta and McClure, 1957; Shaw et al., 1959; Dreizen et al., 1961; Reynolds and Del Rio, 1984; Harper et al., 1986; Reynolds and Black, 1987; Bowen and Pearson, 1993; Mundorff-Shrestha et al., 1993).

The observed caries reductions gave rise to a number of interpretations. After enamel was treated topically with milk, reduction of enamel solubility was reported by Pearce and Bibby (1966), Weiss and Bibby (1966), and Jenkins and Ferguson (1966), and was attributed to milk's relatively high calcium and phosphate content. The latter authors also reported an adsorption of milk proteins onto enamel. In some animal experiments, milk or milk products were found to reduce the numbers of mutans streptococci (Harper et al., 1986), though others observed no effect on the numbers of these streptococci colonizing the oral cavities of rats (Rosen et al., 1984; Reynolds and Black, 1987; Bowen et al., 1991; Reynolds et al., 1995).

Krolicka and Bowen (1986) proposed that in rats the consumption of cheese altered the composition of saliva. In humans, it has been shown that chewing cheese not only increased salivary flow but also rapidly elevated the plaque pH if the cheese was consumed after a sugar-containing beverage or food item (Rugg-Gunn et al., 1975; Imfeld et al., 1978). Silva et al. (1986), using a human in situ model, demonstrated that cheese eaten immediately after a 10% sucrose rinse substantially reduced enamel demineralization. This effect seemed to be due to watersoluble compounds, as shown in a subsequent set of experiments (Silva et al., 1987). In a more comprehensive in...
situation study, Reynolds (1987) observed that two exposures of 20 minutes each per day for ten days of 2% (w/v) sodium caseinate, \( \delta_{41} \)-casein, or trypptic digest thereof applied in vitro in a 3% sucrose, 3% glucose-salt solution prevented subsurface enamel demineralization. It was demonstrated that the casein and casein derivatives were incorporated into plaque scrapings, did not produce a significant change in the amount or composition of plaque bacteria, and increased the calcium phosphate content. It was speculated that the prevention of demineralization was related to the increased plaque calcium phosphate content and buffering capacity by the release of basic amino acids and peptides indirectly through bacterial catabolism.

Nyvad and Fejerskov (1984), basing their study on earlier work by Weiss and Bibby (1966) and Pearce and Bibby (1966), used transmission and scanning electron microscopy (EM) to show that milk of various fat contents could substantially modify the structure of the pellicle formed in vivo. They suggested that the pellicle was not a uniform protein layer, but rather had a distinct globular structure. This was confirmed by Relia and Rykke (1994), who found that the surface of recently formed pellicle was composed of micelle-like protein globules formed in the presence of saliva or milk. It was suggested that the globules were salivary micelles structurally closely related to the casein micelles occurring in milk.

In our laboratories, experimental rat caries studies showed that the caries-protective effect of milk is dependent on the presence of micellar casein; if these micelles are chemically denatured, the protective effect is almost eliminated (Guggenheim et al., submitted). Carionic diets containing micellar casein (Schmid et al., 1993; Guggenheim et al., 1994) or active fragments thereof significantly reduced the numbers of \( S.\ sobrinus \) colonizing the oral cavities of the test animals. In addition, in vitro experiments had shown that soluble caseinophosphopeptides adsorbed to saliva-coated hydroxyapatite beads release albumin in exchange (Neese et al., 1994). We reasoned, therefore, that micellar casein or the active sequences thereof were incorporated into the salivary pellicle, reducing the adherence properties for these streptococci.

In the present work, this hypothesis was verified in an in vitro study based mainly on scanning electron microscopy (SEM) and transmission electron microscopy (TEM) experiments performed after high-pressure freezing and freeze-substitution. Caseinoglycomacropeptide (CGMP) and caseinophosphopeptide (CPP) were found to exchange with albumin in the salivary pellicle. Salivary pellicle with incorporated CGMP and/or CPP had reduced adherence properties for \( S.\ mutans \) and \( S.\ sobrinus \) compared with an untreated control.

**Materials and methods**

**Preparation of enamel discs**

Enamel cylinders (3-mm diameter) were cut out of bovine teeth with a slowly rotating trepan bur. We prepared circular enamel discs (0.5 mm thick) by slicing the cylinders using a diamond wheel on a micro-sectioning machine (Hofer, Aathal, Switzerland). Each disc was controlled under reflected light with an Olympus stereomicroscope SZH to exclude those with dentinal surfaces. The outermost-located disc of each cylinder was discarded. The surfaces of the remaining discs were polished to a high gloss with wet silicon-carbide papers of, successively, 1200-, 2400-, and 4000-grit size. Discs were then washed with ethanol (70%), cleaned ultrasonically for 3 min for removal of remnants of polishing debris, and air-dried. The circumferential surface and the bottom plane of each disc were sealed with a 30-nm sputtered platinum layer (MED 010 sputter unit, Bal-Tec, Liechtenstein). Finally, the discs were allowed to equilibrate overnight in a 0.05 M KCl buffer containing 1 mM phosphate (pH 6.0), 1 mM CaCl\(_2\), and 0.1 mM MgCl\(_2\) (Clark et al., 1978).

**Formation of a salivary pellicle on enamel discs**

Whole unstimulated saliva was collected from five 24- to 48-year-old donors into tubes chilled in ice. The donors, all members of our institute, voluntarily agreed to provide us with saliva and were fully informed on the purpose of its further use. The saliva was held at 60°C for 30 min to inactivate degrading enzymes, then clarified by centrifugation at 10,000 g for 15 min (Gibbons and Spinell, 1970; Clark and Gibbons, 1977). Each enamel disc was immersed in 5 mL of clarified saliva for 3 h at 37°C under continuous gentle agitation (25 rpm) and subsequently dipped ten times in 0.05 M KCl buffer.

**Incorporation of caseinoglycomacropeptide (CGMP) and caseinophosphopeptide (CPP) into the pellicle**

CGMP was isolated from a whey protein concentrate (Danmark Protein, Videback, Denmark) by protein precipitation with trichloroacetic acid (12% final, w/v). The resulting supernatant was extensively dialyzed against water until reaching pH 5.0 before being freeze-dried. This crude material was purified by gel filtration on a G-50 Sephadex column (Pharmacia, Uppsala, Sweden) with a 0.1-M acetic acid buffer. Pure CGMP was eluted with the void volume, and only fractions containing sialic acid residues (Jourdian et al., 1971) were collected, pooled, and freeze-dried. This procedure guaranteed removal of any molecular species smaller than 10 kDa and consequently any residual CPP that might still have been present.

CPP (Meiji CPP-III) was obtained from Meiji Seika Kaisha Ltd. (Japan). The absence of contaminants in CGMP derivatives was established by assay of sialic acid residues (Jourdian et al., 1971), which were not detected in this preparation.

Monodisperse colloidal gold of different sizes was prepared by reduction of a dilute solution of gold chloride with yellow phosphorus or sodium citrate, and labeled with CGMP or CPP as described previously (Horisberger and Rosset, 1977). The labeled compounds were suspended in a 0.15 M NaCl-0.02 M Tris-buffer, pH 7.4, containing 0.5 mg/mL of polyethylene glycol and 0.02% sodium azide. CGMP (mol. weight ~ 10 kDa), labeled with 17-nm colloidal gold particles (\( \Delta u_{17} \)), contained 2 x 10\(^{13}\) gold particles/mL (272 molecules of CGMP/particle). CPP (mol. weight ~ 3 kDa), labeled with 12-nm colloidal gold particles (\( \Delta u_{12} \)), contained 4.2 x 10\(^{13}\) gold particles/mL (115 molecules of CPP/particle).
Tests. Pellicle-coated discs were pre-washed by being dipped ten times in 0.05 M KCl buffer. The discs were then incubated under continuous gentle agitation (25 rpm) for 30 min at room temperature in colloidal-gold-labeled CPP (Au14) diluted (1:50) with 0.05 M KCl buffer or in colloidal-gold-labeled CGMP (Au17) diluted (1:50) with 0.05 M KCl buffer. Immediately following a post-wash in 0.05 M KCl buffer, the discs were cryo-immobilized by high-pressure freezing (Moor, 1987).

Controls. The same procedure as above was performed on enamel discs devoid of salivary pellicle.

Demonstration of proteins in the pellicle by SEM

Tests. Pellicle-coated discs were pre-washed by being dipped ten times in 0.05 M KCl buffer. The discs were then incubated under continuous agitation for 30 min at room temperature in the first antibody diluted (1:10) with 0.05 M KCl buffer. After being dipped ten times in 0.05 M KCl buffer, the discs were incubated under continuous agitation for 30 min at room temperature (RT) in diluted (1:50 with 0.05 M KCl buffer) secondary antibody labeled with 10-nm gold particles.

Controls. Pellicle-coated discs were pre-washed by being dipped ten times in 0.05 M KCl buffer. The discs were then incubated under continuous agitation (25 rpm) for 30 min at RT in the secondary antibody.

High-pressure freezing

Following the last wash steps, the discs were immersed in 1-hexadecene (Fluka, Buchs, Switzerland) (Studer et al., 1989). While thus immersed, each disc was placed in the specimen holder of a high-pressure freezer (HPM 010; Balzers Union, Liechtenstein) with the test side directed upward. A thin copper ring (inner diameter, 2 mm; thickness, 0.1 mm) was placed on the disc. A cylindrical aluminum plate (diameter, 3 mm; thickness, 0.5 mm) which had a cavity (diameter, 2 mm; depth, 0.2 mm) was placed on the copper ring. Thereby a small hollow space was formed over the test surface (Fig. 1). The sandwiches were then cryo-immobilized with a high-pressure freezer (HPM 010; Balzers Union, Liechtenstein) (Moor, 1987). After immobilization, the sandwiches were immediately transferred to liquid nitrogen and stored therein.

Freeze-substitution

Freeze-substitution was performed in anhydrous ethanol with 0.5% uranyl acetate (Harreveld and Crowell, 1964). Frozen specimen sandwiches were opened under liquid nitrogen, and the samples were kept at -90°C, -60°C, and -30°C for 8 h at each step (Müller et al., 1980), by means of a freeze-substitution unit (Balzers FSU 010), and finally were kept at -20°C. Half of the specimens were then washed three times with anhydrous ethanol and embedded stepwise in (30%, 70%, 100%) LR Gold. The infiltration times were 6 h at each resin concentration. The final polymerization was at -20°C under UV-light. The remaining unembedded specimens were prepared for high-resolution scanning electron microscopy.

High-resolution scanning electron microscopy

The specimens were critical-point-dried (CPD 030; Bal-Tec, Liechtenstein), mounted on copper specimen-holders, and 6-nm sputter-coated with carbon by means of a MED 010 sputter device (Bal-Tec, Liechtenstein). The specimens were examined with a Hitachi S-900 high-resolution field emission scanning electron microscope (FE-SEM) at magnifications of 80,000x and 150,000x. Secondary imaging for morphological examinations as well as back-scattered imaging for demonstration of the gold-labeling in corresponding areas were used.

Microtomy and transmission electron microscopy

Following removal from the beam capsules, the flat surfaces of the polymerized resin blocks were ground with wet silicon-carbide paper (1200-grit size) until the enamel was reached. Resin blocks were immersed in a solution containing 0.15 M EDTA and 0.2 M sucrose for 2 wk. Following dissolution of the enamel, the blocks were re-embedded in Epon 812. Thin sections were prepared on a Reichert Jung Ultracut E microtome equipped with a diamond knife (Diatome, Biel, Switzerland), stained for 10 min with uranyl acetate and lead citrate (Reynolds, 1963), and examined in a transmission electron microscope (Philips 400) at an accelerating voltage of 80 kV.

Experiment to test the effect of CGMP/CPP on the adherence of Streptococcus mutans OMZ 7 and Streptococcus sobrinus OMZ 176 to hydroxyapatite

Pellicle-coated discs (10 mm x 0.5 mm) of synthetic hydroxyapatite (HY-APATITE®, Euro-Crystals, Landgraaf, The Netherlands) were washed by being dipped ten times in 0.05 M

![Figure 1](https://example.com/figure1.png)

Figure 1. Schematic illustration of the sample preparation for high-pressure freezing. A copper ring was placed over the pellicle-covered enamel disc, and a cylindrical aluminum platelet which had an inner cavity (diameter, 2 mm; depth, 0.2 mm) was placed on this copper ring. The hollow space formed over the pellicle surface was filled with 1-hexadecene to facilitate the cooling of the specimen surface.
Figure 2. (a,b) SEM micrograph of a pellicle formed on a bovine enamel surface. Note the globular appearance of the surface by individual globules with a diameter of 50 to 100 nm. (c,d) TEM micrographs through a pellicle subjected to high-pressure freezing and freeze substitution. Note the heterogeneous composition of the pellicle with multiple layers of irregularly shaped patches of various sizes (c). Also note globules at the surface (arrow in d). Bars: (a,c) 500 µm; (b,d) 100 µm.

KCl buffer. Half of the discs were incubated under continuous gentle agitation (25 rpm) for 30 min at RT in a solution containing 5% CGMP and 5% CPP, then washed twice in 0.05 M KCl buffer. The remaining discs had a salivary pellicle without incorporation of CGMP and CPP. Discs were immersed in a suspension of either S. mutans OMZ 7 or S. sobrinus OMZ 176 (3 mL, optical density = 0.2, λ = 650 nm) for 30 min at 37°C. The streptococci had been grown overnight in fluid universal medium (Gmür and Guggenheim, 1983), then centrifuged and washed in 0.05 M KCl buffer. Discs were agitated for five minutes. Following an additional three washes with 0.05 M KCl buffer, the discs were subjected to chemical fixation (2% glutaraldehyde in 0.1 M N-acacodylate buffer for 3 h), washed with buffer, and post-fixed in 1.33% osmium tetroxide (2 h). Following three wash steps with 0.05 M KCl buffer, the samples were critical-point-dried by means of a Balzers CPD-030 (Bal-Tec, Liechtenstein) critical point apparatus. Following sputter-coating with gold in a MED-010 sputtering device (Balzers), the specimens were examined with a Cambridge S-180 Stereoscan SEM. The experiment as well as the control were performed in triplicate. Counting of streptococci was performed in the S-180 SEM at a magnification of 3000x. One hundred twenty fields, each measuring 24 x 36 µm, were examined on each disc. In a total area of ~ 10^5 µm² per disc, all adhering streptococcal cells were counted.

Fast Protein Liquid Chromatography (FPLC) with Mono Q Resin FPLC was performed by means of equipment obtained from Pharmacia LKB which contained an HR 5/5 column (analytical scale). The method used has already been described in detail (Juillerat et al., 1989). Briefly, for analytical chromatography, two solutions were used. Solution A consisted of 0.02 M Tris-HCl buffer (pH 8.0), whereas solution B contained 1 M NaCl in the same buffer. These were applied to the column as follows: From time 0 to 7 min, solution A was applied; this was followed (7 to 22 min) by a linear gradient of B in A up to 50% (v/v) of solution B, and then by solution B (22 to 26 min). Finally, the column was washed with solution A (26 to 30 min). The flow rate was 1 mL/min, and detection was based on absorbance at 214 nm. Sample load was 1 to 2 mg dry solids/run.

The sequence described above was used in most cases; however, to optimize the separation of CGMP and CPP, we used a different sequence: A mixture of solution B in A (26%) was applied (time 0 to 2 min), followed by another mixture of solution B in A (28%) from 2 to 6 min. Then, solution B was applied (6 to 9 min), and finally, the initial mixture of solution B in A (26%) from 9 to 12 min. During these two last steps of the sequence, the flow rate was increased to 2 mL/min.

Statistics

Total cell numbers per pellicle-coated-disc with or without incorporated CGMP/CPP were compared by Student's t test. The significance level was set at 95%.

Results

High-power SEM and TEM micrographs of a salivary pellicle formed on a bovine enamel surface are shown in Fig. 2. The surface of the pellicle had a globular appearance, with individual globules having a diameter of 50 to 100 nm (Figs. 2a, 2b). Sections through the pellicle subjected to high-pressure freezing and freeze-substitution showed the heterogeneous composition of the pellicle, which consisted of multiple layers of irregularly shaped patches of various sizes. The sections also showed single globules at the surface (Figs. 2c, 2d).

Fig. 3 demonstrates a pellicle after incubation with CGMP-Au17. The secondary SEM micrograph (s-SEM) (Fig. 3a) as well
Figure 4. (a) Secondary SEM micrograph and (b) corresponding back-scattered SEM micrograph of a pellicle treated with CPP labeled with 12-nm gold particles. Note evidence of gold particles also in the s-SEM micrograph (arrows in a). (c) TEM micrograph of such a pellicle showing gold labeling near a globule and at the surface (arrows). Bars: 100 nm.

as the back-scattered SEM micrograph (b-SEM) (Fig. 3b) were performed at a magnification of 80,000x. Globular structures on the surface of the pellicle were visible in the s-SEM micrograph (Fig. 3a). The distribution of 17-nm gold particles, visible in the b-SEM micrograph in this same area, shows that CGMP-Au$_{17}$ was incorporated into the pellicle (Fig. 3b). Some of the gold particles could also be identified in s-SEM micrographs (arrows in Fig. 3a). TEM micrographs also showed globules with attached gold particles. This indicated that CGMP-Au$_{17}$ was incorporated in a globular form into the pellicle (Fig. 3c).

Corresponding observations occurred following incubation of a pellicle in CPP-Au$_{12}$ (Fig. 4). Both b-SEM and TEM micrographs (Figs. 4b, 4c) revealed the incorporation of CPP-Au$_{12}$ into the pellicle in the form of globules as well as in the form of geometrically irregular structures (Fig. 4a).

A globular structure was also evident on the surfaces of pellicles used to demonstrate the presence of albumin (Fig. 6a). That these molecules formed part of the pellicle was evident in both b-SEM and TEM micrographs in which gold particles (Au$_{58}$) of the secondary antibody were distinctly visible (Figs. 5b, 5c). The TEM micrographs also indicated that albumin was present at the pellicle surface in a non-globular form (Fig. 5c). Halo-like areas, visible around gold particles in s-SEM micrographs suggesting the presence of globules, were formed by the 6-nm-thick carbon sputtered onto the surface to avoid charging of the specimen (Fig. 5a).

S-SEM micrograph (Fig. 6a) and corresponding b-SEM micrograph (Fig. 6b) of a control pellicle treated only with goat anti-mouse IgG/25 nm gold revealed very few gold particles. This was also the case in both s- and b-SEM micrographs of control samples following treatment with CGMP as well as CPP on enamel discs without pellicle (Figs. 6c, 6d).

S-SEM (Fig. 7a) and b-SEM micrographs (Fig. 7b) of discs with pellicles that had been incubated with both CGMP-Au$_{17}$ and CPP-Au$_{12}$ revealed remaining albumin molecules by 25-nm gold particles (outlined by squares) and the incorporated CGMP-Au$_{17}$ globules (outlined by circles). Incorporated CPP-Au$_{12}$ globules are marked by triangles (Fig. 7b). The gold labeling in corresponding TEM micrographs verified the presence of remaining albumin and CPP as well as globular CGMP incorporated into the surface of the pellicle (Fig. 7c). When the number of gold particles per 20 $\mu$m$^2$ was counted, a mean of 48 ± 5 (n = 8) 25-nm gold particles derived from albumin was found in an untreated pellicle. After incubation with CGMP and CPP, this number was reduced to 23 ± 4 (n = 14).

The effect of incorporated CGMP and/or CPP on the adherence of mutants streptococci is shown in Figs. 8 and 9. Adherence of $S$. sobrinus to pellicle-coated hydroxyapatite discs treated with these caseinopeptides was significantly reduced. In the presence of CGMP, CPP, or CGMP + CPP, adherence of $S$. sobrinus was reduced by 49%, 75%, and 81%, respectively (Fig. 8). Similar observations were made for $S$. mutans adherence, whereby adherence was reduced 64%, 83%, and 84% by CGMP, CPP, and CGMP + CPP, respectively (Fig. 9).

Finally, the results presented so far are valid only if it can be shown beyond any doubt that the casein fractions utilized were of sufficient purity and, in particular, were not cross-contaminated. With respect to the preparation procedures of both these polypeptides, only contamination of CGMP by one CPP subcomponent, namely, peptide $\beta$(1-28), merited theoretical consideration. To check this, we subjected CPP, CGMP, and a mixture (1:1) to FPLC. The results are compiled in Figs. 10a-10d. Fig. 10a shows the elution pattern of the CPP preparation used, including identification of component $\beta$(1-28). In Fig. 10b, the elution pattern of our CGMP preparation using the same conditions is depicted. It is clearly seen that the peptide $\beta$(1-28) eluted later than the CGMP preparation. The large peak yielded by CGMP is due to the fact that many glycoforms occur in this
preparation, and that chromatographic conditions were not refined for resolution of CGMP subcomponents, but rather for analyzing the CPP preparation. Fig. 10c shows that a mixture (1:1) of CGMP and CPP allows for the complete separation of the peptide β(1-28) from CGMP. Finally, when the elution conditions were fine-tuned, CGMP and peptide β(1-28) were totally separated. Fig. 10d shows that our CGMP preparation contained less than 2% of a contaminant eluting with the same retention time as CPP β(1-28).

**Discussion**

High-pressure freezing is presently the only method whereby native samples approx. 500 μm thick can be cryo-immobilized without chemical pre-fixation or the addition of cryo-protectants (Moor, 1987). This technique preserves the ultrastructure close to its native state (Studer et al., 1989). It has been shown that pre-fixation of cellular and non-cellular structures with glutaraldehyde leads to denaturation and re-arrangement of molecular structures in addition to changing the antigenic properties of the macromolecules (Brandtzaeg, 1974). While the globular micellar structure of the surface of the salivary pellicle on enamel has been previously reported (Lie, 1977; Nyvad and Fejerskov, 1984; Rolla and Rykke, 1994), our TEM micrographs offer new insights into the ultrastructure of this biofilm. The pellicle is composed of distinct, most probably macromolecular, units forming a multi-layered patchwork composed of irregular and globular entities of different sizes. When the overall structure is examined carefully, it is obvious that this biofilm was created by a successive adsorption of salivary proteins. Only molecules in the first innermost layer had direct interaction with the hydroxyapatite surface, while subsequent accumulation must be governed by protein-protein interactions. In this context, it is interesting that Rolla and Rykke (1994) described such micelle-like globules exhibiting the same size and structure in whole saliva.

We undertook the present study to demonstrate the incorporation of milk components into salivary pellicle and to elucidate the mechanism of the caries-protective effects of milk products noted in animal studies (Schmid et al., 1993; Guggenheim et al., 1994). It was observed that this effect is mediated not only by micellar casein, which externally exposes peptides of the β-casein fraction, namely, caseinoglycomacropeptide (CGMP), but also by peptides of the β-casein fraction, such as CPP, which are not exposed externally in the undigested casein molecule (Holt, 1992). In *vitro* studies had previously demonstrated a restriction of adherence of mutans streptococci to saliva-coated hydroxyapatite beads by milk casein derivatives (Neeser et al., 1994; Vacc- Smith et al., 1994).

It is now clear that both CGMP and CPP are incorporated into salivary pellicle in exchange with albumin. While CGMP was detected on the surface of the pellicle only in a globular-micellar form (φ 100 to 120 nm), CPP was found in microglobular (Fig. 4a) as well as in geometrically irregular structures (Fig. 7c). The globules formed by CPP (Au₁₂)
beads (S-HA) were studied by incubation of salivary molecules at the surface was reduced by about 9,000 kDa, smaller in diameter (20 Å) than the CGMP globules (~10 kDa). The globular structures seen in the pellicle are certainly not formed by single molecules of the caseinopeptides. A single 3-kDa peptide in a spherical configuration has a calculated diameter of ~20 Å, and that of a 10-kDa peptide ~30 Å. The globular structures visible are therefore molecules autoaggregated by the gold-labeling procedure or an accretion of labeled caseinopeptides with salivary proteins forming part of the pellicle. Although albumin was regularly detected in the pellicle by the 25-nm gold label, it was not possible to associate a distinct molecular arrangement with the labeled surface components (Fig. 7c). After incubation with CGMP and CPP, the density of albumin molecules at the surface was reduced by approximately 50% (Neesse et al., 1994). Although we have shown this in the present paper only histometrically, a very sensitive assay has been previously published by Neesse et al. (1994). In that study, the effects of these caseinopeptides on the protein composition of saliva-coated hydroxyapatite beads (S-HA) were studied by incubation of S-HA prepared with radiiodinated saliva samples with several caseinopeptides (CGMP, CPP, and asialo-CGMP). All these peptides displaced a unique protein band with a molecular weight of ca. 67 kDa. This single band was identified by Western blotting with a polyclonal antibody to human-salivary albumin. No other proteins were released. The hypothesis that salivary pellicle with incorporated CGMP and/or CPP substantially reduces the adherence of S. sobrinus and S. mutans was clearly proven. The adherence reduction experiments with CGMP and/or CPP were performed in triplicate in separate experiments. It is evident from Figs. 8 and 9 that the controls in these experiments showed much variance which we were unable to reduce in spite of great efforts. Nevertheless, there is no doubt that these compounds substantially reduced the numbers of adhering streptococcal cells (p < 0.001).

Bovine albumin mediated no preferential adherence for S. mutans and S. sanguis in vitro (Gibbons and Etherden, 1985). The observed adherence inhibition for S. sobrinus and S. mutans on the surface of salivary pellicle treated with CGMP and/or CPP can therefore not be explained by the removal of a ligand-carrying molecule. It is suggested that CGMP and CPP adsorb to the surface of the pellicle and mask receptors on salivary molecules for these streptococci. This had already been proposed in a more general form by Nyvad and Fejerskov (1984).

It had to be shown that the CGMP and CPP fractions were of sufficient purity, and in particular were not cross-contaminated. CPP β(1-28) is a well-known component of the proteose peptone fraction. It has been reported that, during renneting of milk, three-quarters of the proteose-peptone fraction coagulated with paracasein and consequently was removed from whey (van Boekel and Crijns, 1994). In addition, as described in "Materials and methods", CGMP was isolated from a whey protein concentrate by protein precipitation with trichloroacetic acid (TCA). It has previously been demonstrated that by such a precipitation with TCA, the components of the proteose-peptone fraction remaining with whey proteins are quantitatively precipitated (Pâquet, 1989); consequently, contamination of our CGMP preparation by the CPP β(1-28) was a priori very unlikely. To remove any lingering doubts, we subjected CPP, CGMP, and mixtures of the two caseinopeptide fractions to FPLC. The results confirmed that, if there was any contamination of CGMP by CPP, it was <2%. In addition, our CPP fraction was found to be free of sialic acid, further evidence that contamination of CPP by CGMP did not occur (data not shown).

How do these in vitro findings relate to in vivo experiments? It has been mentioned that, in our laboratories, the number of S. sobrinus in the oral cavity of rats had been reduced consistently if micellar casein as well as CGMP and/or CPP formed part of the diet (Schmid et al., 1993; Guggenheim et al., 1994). In contrast, in the same experiments, it was also shown that micellar casein or CGMP incorporated into the diet promoted colonization of Actinomyces viscosus highly significantly. Although we have not yet reproduced this in vitro, it appears that incorporation of these molecules into the surface of the pellicle may offer selective receptors for these micro-organisms. Micelles of casein and CGMP bear glycosylated moieties on their outer...
surfaces, in particular short disaccharide Galβ 1-3 GalNAc chains with sialic acid termini (Van Halbeek et al., 1980). Since *A. viscosus* Ny1 produces a neuraminidase (unpublished observation), it is assumed that this enzyme unmasks the Galβ 1-3 GalNAc sequences, which are known to act as ligands for the *Actinomyces* type 2 fimbriae (McIntire et al., 1983; Strömberg and Borén, 1992; Strömberg et al., 1992). A reduction of mutans streptococci has not been consistently observed in all experimental rat caries studies (for a review, see Herod, 1991; Reynolds et al., 1995). This, however, is no surprise. Due to the high variation between animals, such a reduction becomes evident only if the skim milk in the Keyes 2000a (Keyes, 1958) diet is replaced by a non-dairy protein substitute. In addition, the use of 56% sucrose in the diet may overwhelm the protective capacity of the casein fractions. In our experiments, we consistently replaced skim milk with soybean protein and reduced the sucrose content of the diet to 30 to 40%.

The present results should not be interpreted to mean that inhibition of adherence by mutans streptococci by casein fractions is the only caries-protective mechanism of milk and milk products. The calcium- and phosphate-rich casein fractions incorporated into the pellicle or into plaque may also increase the remineralization potential (Jenkins and Ferguson, 1966; Weiss and Bibby, 1966; McDougall, 1977; Reynolds, 1987). Since pellicle starts to be colonized by bacteria quite early after its formation, the caries-protective effects of casein fractions are difficult to explain by an exchange with albumin at the surface of the pellicle only. It therefore seems likely that these molecules also interact with already-colonized plaque bacteria in a quite non-specific manner, as demonstrated by Reynolds (1987) *in situ* and Neeser et al. (1994) and Vacca-Smith et al. (1994) for streptococci *in vitro*. Although there is no direct evidence for this hypothesis, it would be the missing link which could explain both the increase in remineralization potential of plaque and the continued restriction of colonization by mutans streptococci on the tooth surface.

The conservation of micellar casein in different milk products varies. Casein micelles are differently destroyed during cheese processing. It seems possible, however, that active components such as CGMP and/or CPP-containing sequences are liberated by enzymatic hydrolyses. The findings of Silva et al. (1987) suggest such a mechanism.
It becomes more and more doubtful whether the fat content of milk or milk products contributes to the observed caries-protective effect. At present, no solid evidence for such a mechanism is available. Furthermore, it seems unlikely that the buffering effects of basic amino acids and O-phosphoserine in milk products contribute substantially to the caries-protective effect as suggested by Reynolds (1987). It seems improbable that the additional buffering capacity fully available only after proteolytic breakdown of milk proteins would change the inherent buffering capacity of saliva considerably. This is in accordance with previous findings (Jenkins and Ferguson, 1966; Frostell, 1970; Edgar et al., 1975).

Whether micellar casein or the active enzymatically-produced fractions CGMP or CPP will have a future as caries-preventive compounds in oral care products or as food additives remains to be proven. What is of basic interest is the observation that dietary compounds may exchange with salivary molecules on the surface of a pellicle which is not yet confluent covered by growing bacterial cells or adhering to already-colonized bacteria in plaque. Such modifications may increase or decrease the adherence properties of pellicle and plaque for distinct microbial species. This may result in ecological shifts which might affect the pathogenicity of plaque in either direction.

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References


