Quantitative and qualitative analyses of human salivary micelle-like globules

Alix Young, Morten Rykke and Gunnar Rölla
Faculty of Dentistry, University of Oslo, Norway

Young A, Rykke M, Rölla G. Quantitative and qualitative analyses of human salivary micelle-like globules. Acta Odontol Scand 1999;57:105–110. Oslo. ISSN 0001-6357.

In the present study we examined the protein proportion and amino acid profile of the salivary micelle-like globules (SMGs) of human whole saliva and parotid saliva (HWS, HPS). Saliva and SMG samples from each subject (clarified HWS and HPS from 6 subjects, and unclarified HWS from 3 subjects) were analysed for amino acids using standard acid hydrolysis procedures. HPS, clarified HWS and the respective supernatant samples (remaining after removal of the SMGs) were also measured for protein using the micro-Kjeldahl method. SMGs from clarified and unclarified HWS made up 4.7% and 19.7%, respectively, of the total salivary protein based on amino acid analyses. With the micro-Kjeldahl method SMGs from clarified HWS made up 7.3% of the total saliva protein. SMGs isolated from HPS were found in only small amounts. The amino acid profile for the SMGs was strikingly similar to that known for the 2-h pellicle, and differed significantly from HWS or HPS. The results support previous morphological studies indicating that the SMGs represent a major component of the newly formed pellicle. □ *Amino acid analysis; micro-Kjeldahl; parotid saliva; salivary micelle-like globules; whole saliva*

Alix Young, Department of Pedodontics and Caries Prophylaxis, Faculty of Dentistry, University of Oslo, PO Box 1109, Blindern, N-0317 Oslo, Norway. Tel. +47 22 85 21 90, fax. +47 22 85 23 86, e-mail. alixr@odont.uio.no

Cows' milk contains protein in the form of casein micelles. Casein micelles consist of four different casein molecules, which are largely amphiphilic phosphoproteins exhibiting properties that allow the formation of very stable complexes with a relatively large amount of calcium phosphate (1–3). Transmission and scanning electron microscopic studies of human saliva have demonstrated globular structures similar to the casein micelles in milk (4–10). These structures have been termed salivary micelle-like globules (SMGs) due to their morphological similarity with the milk micelles (8, 9). Saliva contains amphiphilic proline-rich phosphoproteins with a structure similar to that of the amphiphilic caseins, and calcium is also thought to play an important role in maintaining the structure of the SMGs (9). Scanning and transmission electron microscopic studies and photon correlation spectroscopic studies of human parotid saliva (HPS) have indicated a diameter of 100–300 nm for the SMGs. Single units or 'submicelles' of 40–100 nm appeared to cluster (6–10 globules) with increasing time after sampling (8, 9). Zeta potential determinations have determined an overall surface potential of about −9 mV for parotid SMGs at physiological pH, and an isoelectric point at pH 2.9–3.1 (11).

It is suggested that many of the physiological properties of saliva are at least partly attributable to the presence of the SMGs acting as functional mixed-protein aggregates. Biological activities such as bacterial agglutination and a high affinity for hydroxyapatite surfaces have recently been reported associated with the SMGs (12, 13). In attempting to evaluate the importance of these structures, it would be of interest to determine how much of the salivary protein is associated with the SMGs.

The newly formed enamel pellicle has been seen to consist predominantly of globular structures (14, 15), or to have an uneven, knotted appearance (16), as observed by transmission and scanning electron microscopic morphological studies. Similar morphological structures have been shown in scanning electron microscopic studies of enamel exposed to milk or cream (17, 18). In a preliminary study performed on limited material, results indicated that the amino acid profile of SMGs isolated from human whole saliva (HWS) was similar to the profile for the 2-h pellicle collected directly from human teeth in vivo (8). Based on the earlier studies and these findings it is suggested that the SMGs form a major component of the acquired enamel pellicle (8).

The aim of the present study was therefore to establish how much of the total salivary protein occurs in the form of SMGs. Furthermore, based on more comprehensive material, a second aim was to test the hypothesis that the amino acid profile of the SMGs is similar to that of the 2-h pellicle. Both clarified and unclarified HWS and HPS were used for the examinations. The protein content of the isolated SMGs was compared with the total protein content of saliva, and the ratio calculated. Amino acid analysis was chosen as a suitable method for calculating total protein content by summing up the amino acids present in a hydrolyzed sample of the protein mixtures. Amino acid analysis has been extensively used in the

analysis of the 2-h pellicle on tooth surfaces. The micro-Kjeldahl method for protein determination was also included because this method is extensively used in the analysis of milk protein, and, as mentioned previously, milk contains casein micelles which are both morphologically and chemically closely related to the SMGs (8, 11, 19, 20).

Material and methods

Saliva samples

Twelve milliliter samples of paraffin wax-stimulated HWS and sweet/sour-stimulated HPS samples were collected on ice from 6 healthy adult subjects (3 male, 3 female, age range 32–70 years). Subjects were asked not to consume food or drink following their normal morning oral hygiene routine, and saliva collected during the first minute was discarded. Three separate samples per subject of both HWS and HPS were collected on separate days between 0900 and 1100 h. The HPS samples were processed directly after collection at a flow rate of 0.9–2.3 ml/min by means of Curby cups or individually fitted bilateral appliances made of Provil®-P impression material (BayerDental, Leverkusen, Germany) as described previously (9). HWS samples were clarified immediately after collection at a flow rate of about 1.5–3 ml/min ($\sim$1,500 $\times$ *g* for 15 min). Three additional 6-ml HWS samples from 3 of the subjects were collected and not clarified (unclarified HWS). 25-µl aliquots of all HWS (unclarified and clarified) and HPS samples were pipetted directly into hydrolysis tubes. After freezing, the samples were freeze-dried in preparation for amino acid analyses. 6-ml aliquots of clarified HWS and HPS were immediately frozen until analysis by the micro-Kjeldahl method. Following gentle water-bath warming of the remaining 6-ml aliquots of chilled saliva to room temperature, SMGs were isolated in accordance with a procedure described previously based on acidification of the saliva to the isoelectric point (8, 11). The isolated SMG pellets obtained from 5-ml centrifuged saliva samples were washed twice with deionized water and freeze-dried ready for amino acid analysis. The supernatants remaining after isolation and removal of SMGs were frozen until analysis by the micro-Kjeldahl method.

Amino acid analysis

Unclarified and clarified HWS, HPS and the respective isolated SMG samples were subjected to quantitative amino acid analysis using standard procedures. The lyophilized samples were hydrolyzed in 6 M HCl, under pure nitrogen atmosphere for 24 h, at 108–110°C. The SMG samples were transferred from the test tubes in which they were isolated to the hydrolysis tubes first after the addition of the acid. Some unavoidable loss of material occurred during the transfer, although precautions were taken to minimize these losses, and all samples were treated similarly. Samples were run on a Model 421 Amino Acid Analyzer (Perkin Elmer Corp, Foster City, CA, USA) using phenyl isothiocyanate (PITC) as a detector reagent. The analysis is based on the Edman degradation and the use of high performance liquid chromatography (21). The results were obtained via connection to a Model 600 Data Module. Protein concentrations for the amino acid analyzed samples were calculated by adding together the products (concentrations of the individual amino acid residues) $\times$ (molecular weights minus one water molecule per residue).

Micro-Kjeldahl method

Clarified HWS, HPS and the respective supernatants remaining after isolation and removal of the SMGs were analyzed for their nitrogen content using a Kjeltec Auto Sampler System 1035 Analyzer (Tecator, Höganäs, Sweden). 2-ml triplicate samples of the defrosted samples were digested in H_2SO_4 with added K_2SO_4 and selenium (Kjeltabs Auto, Thompson & Capper Ltd, Cheshire, England). Recovery tests were carried out to ensure adequate yield using $(NH_4)_2SO_4$ and L-Cysteine (Sigma Chemicals). Water blanks and skimmed milk control samples were employed. Calculation of the percentage of SMGs by the micro-Kjeldahl technique was based on the amount of protein remaining in the supernatant. It was assumed that the total salivary protein concentration minus the protein concentration of the supernatant after isolation and removal of SMGs was equivalent to the SMG-related protein concentration. A Kjeldahl factor of 6.25 was used for all samples in the conversion of percentage nitrogen to percentage protein, as this factor has been employed in similar studies previously (22, 23).

Results

The results for the protein quantification and calculations of the percentage of SMGs are given in Table 1. The results for clarified HWS from one of the donors as determined by the micro-Kjeldahl method were excluded for technical reasons. Quantitative amino acid analyses indicated that the SMGs isolated from unclarified whole saliva contributed on average four times as much to the total salivary protein compared with the SMGs isolated from clarified whole saliva (4.7% and 19.7%, respectively). The average ratio of SMGs isolated from clarified HWS was similar with the two methods. HPS collected directly from the ducts contained only small amounts of protein in the form of the SMGs.

The amino acid profile of SMGs isolated from unclarified HWS is represented diagrammatically according to Robinson et al. (24) (Fig. 1), and showed striking resemblance to the previously determined amino acid profile for the 2-h pellicle (Fig. 2) (8). These profiles were markedly different from the profile for unclarified HWS

Table 1. Protein concentration (mg/ml), and ratio of salivary micelle-like globules (% SMGs), with mean and standard deviation (sd), as measured by amino acid analyses and micro-Kjeldahl determinations of unclarified and clarified human whole saliva and human parotid saliva.

Protein content	Saliva (mg/ml) Mean (sd)	SMGs (mg/ml) Mean (sd)	% SMGs # Mean (sd)
By amino acid analysis			
Clarified HWS §	1.543 (0.61)	0.072 (0.04)	4.7 (2)
Unclarified HWS ¶	2.092 (0.77)	0.413 (0.13)	19.7 (13)
HPS §	2.224 (0.80)	small amounts	
By the micro-Kjeldahl method			
Clarified HWS §§	2.629 (0.91)	0.197 (0.11)*	7.3 (3)
HPS §	3.311 (0.98)	0.069 (0.05)*	2.1 (1)

HWS = human whole saliva; HPS = human parotid saliva.
§ Data based on 3 samples from each of 6 subjects.
§§ Data based on 3 samples from each of 5 subjects.
¶ Data based on 3 samples from each of 3 subjects.
# % SMGs calculated from the mean for saliva and SMGs.
* SMGs calculated from HWS minus supernatant.

(Fig. 3). Neutral and acidic amino acids accounted for about 70–72% of the total for the SMGs and the 2-h pellicle. In comparison, these amino acids accounted for about 57–59% of salivary amino acids. Proline formed about 6–8% of the total for the SMGs and the 2-h pellicle, whereas saliva contained about 20–27% proline.

Discussion

The present study showed that the ratio of SMGs was highest in unclarified whole saliva (~20%, as given in Table 1). HPS contained only small amounts of SMGs. Furthermore, the amino acid profile of the whole SMGs was strikingly similar to that of the acquired enamel pellicle, confirming the results of a previous study that consisted of only one sample (8). Owing to the relatively small number of subjects, the results cannot be analyzed for statistical significance. Instead, the distinct patterns seen diagrammatically allow for direct visual comparisons. As parotid saliva collected directly from the ducts contained few SMGs, the results could indicate that a major part of the formation of the SMGs occurs outside the ducts after the individual secretions have become mixed in the oral cavity. The possible contribution of secretions other than parotid saliva to whole saliva, with regard to the SMGs, requires further investigation (42).

Clarified whole saliva and parotid saliva samples were tested because these fluids have been extensively used in studies relating to the physiology of human saliva, including studies on pellicle formation in vitro. In the present study, unclarified whole saliva was also included because this fluid is the secretion encountered in the

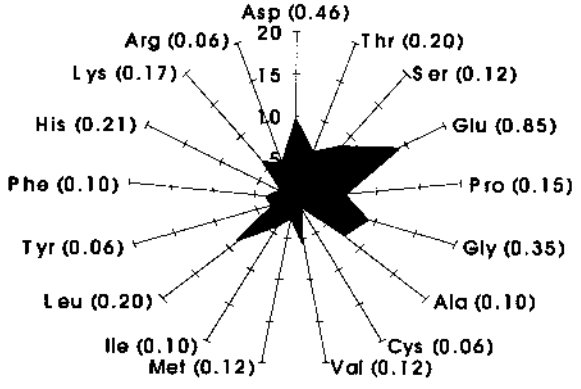

Fig. 1. Amino acid profile of salivary micelle-like globules isolated from unclarified human whole saliva, based on the mean values for 3 subjects. The amounts of the different amino acids are calculated as mol/100 mol. Standard deviations for each of the amino acid residues are shown in parentheses. This profile shows a striking resemblance to the profile for 2-h pellicle (Fig. 2), and differs significantly from the profile for unclarified human whole saliva (Fig. 3).

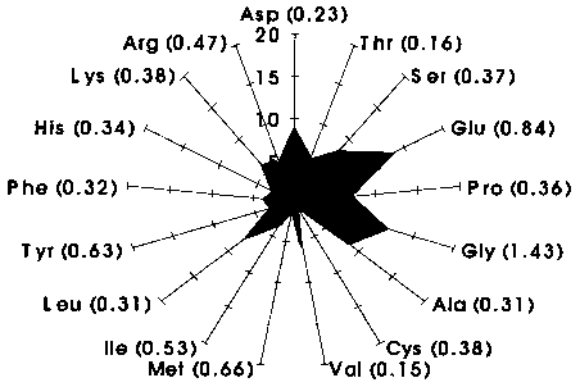

Fig. 2. Amino acid profile of 2-h pellicle based on the mean of 6 subjects as measured in a previous study (41). The amounts of the different amino acids are calculated as mol/100 mol. Standard deviations for each of the amino acid residues are shown in parentheses.

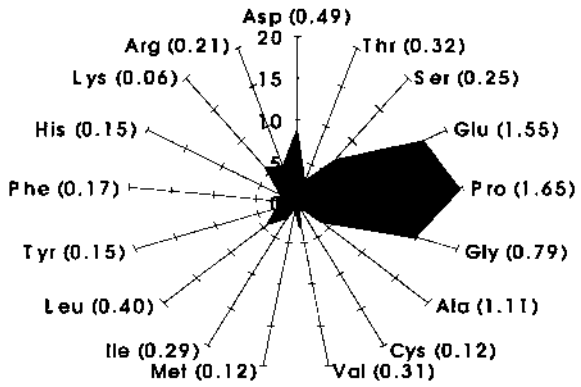

Fig. 3. Amino acid profile of unclarified human whole saliva based on the mean values from 3 subjects. The amounts of the different amino acids are calculated as mol/100 mol. Standard deviations for each of the amino acid residues are shown in parentheses. This profile differs markedly from the profile for salivary micelle-like globules isolated from unclarified human whole saliva (Fig. 1).

mouth, and as such could be considered more important in oral physiology, for example by participating in pellicle formation. Pellicle formation on tooth surfaces occurs in an oral cavity constantly bathed in saliva which is for the most part stimulated, and collection of saliva was therefore carried out following stimulation. Clarified whole saliva has been used extensively in many salivary experiments, probably partly to minimize the likelihood of contamination and also for convenience purposes, and was therefore included in the present study. However, it appears likely that centrifugation for clarification removes at least part of the high-density fraction of salivary components containing SMGs. The ratio obtained for the contribution of salivary micelle-like structures from clarified whole saliva to total salivary protein most likely represents an underestimate. Concurrently, the above-mentioned ratio of about 20% for unclarified whole SMGs may represent an overestimate due to possible contamination with cells and bacteria. Previous transmission and scanning electron microscopic studies of isolated SMGs, however, demonstrated a minimal contamination and the amino acid analyses did not reveal amino acids commonly originating from bacterial cell walls such as diaminopimelic acid and muramic acid (8, 25).

The isolation procedure is based on adjusting the saliva to the isoelectric point of the SMGs (pH 3.1), centrifugation to spin down the high-density SMGs (the structures not repelling each other at this pH), followed by washing twice in deionized water (8). This procedure is thus based on 2 properties: the low isoelectric point of the SMGs, and their high density. Initially, lipid-soluble dye was used in the isolation of the SMGs in order to visualize their presence in the saliva (8). The pellet formed as a result of centrifugation of the acidified saliva, assumed to contain the SMG fraction, also contained all the dye, except a layer on top of the supernatant that presumably represented the salivary lipids (8). It has also been shown that more than 90% of radioactive triclosan (which is lipid-soluble) added to HWS was found in the SMG component after the isolation process (26). This indicated that the isolation process resulted in sedimentation of close to all the SMGs.

It is widely agreed that the determination of salivary protein is difficult (27). Many of the commonly used methods for protein determination are based on different characteristics of proteins. However, in biological fluids, such as saliva, that contain mixtures of proteins, not all proteins have the same proportion of a given characteristic (22). Furthermore, it is not easy to find a suitable standard for establishing protein content of mixtures of proteins, as demonstrated in one study that showed that the choice of protein standard affected the result (28). The methods chosen for the calculation of protein content in the present study are independent of a protein standard. The micro-Kjeldahl method is independent of the use of standards because it is based on the determination of the nitrogen content of the sample and has been widely used in the determination of milk protein (19, 20, 29, 30). Amino acid analysis involves minimal manipulation of the samples. The method has a reproducibility of 2% and avoids dependence on an external protein standard (28, 31). The protein content ranged from about 0.9 to 4.3 mg/ml for all the different saliva samples (with parotid saliva registering higher concentrations than whole saliva), and was in accordance with previous studies (22, 28, 32, 33), and gives credibility to both methods. Efforts were made to minimize the possible loss of SMG-related protein during clarification of the whole saliva by avoiding excessively high centrifugal forces in the isolation process ($\sim$1,500 $\times$ *g* for 15 min). Previous studies have reported the use of 10,000 $\times$ *g* or more for clarification of saliva (28, 34). The results of the present study indicate that about 25% of the total salivary protein was removed by clarification, thereby adding strength to the above suggestion that the ratio for clarified whole SMGs is rather an underestimate (see Table 1). Furthermore, the ratio for clarified whole SMGs was in the same order of magnitude in samples analyzed by both amino acid analysis and the micro-Kjeldahl method (4.7% and 7.3%, respectively).

A Kjeldahl factor of 6.25 was used in this study for conversion of percentage nitrogen in the samples to percentage protein. This factor has been used previously in similar studies using this method for measuring the protein content of saliva, and is derived from an average nitrogen content for the amino acids of 16%. However, a previous study indicated that both whole saliva and dialyzed parotid saliva have a lower percentage nitrogen than is commonly assumed, this being ascribed to the high amount of carbohydrate in salivary proteins (23). Researchers working with milk proteins have concluded that the only proper way of calculating the Kjeldahl factor for milk is from the amino acid content (20, 30). The present study showed that the saliva and supernatant samples had very similar amino acid profiles (results not shown),

indicating that the same Kjeldahl factor could be used when calculating percentage protein from percentage nitrogen for the two different samples.

Many authors have shown that the acquired enamel pellicle contains many different proteins (35, 36). This has posed a problem, because whereas it is known that certain proteins are selectively adsorbed to hydroxyapatite, it has not been understood how so many different proteins could be adsorbed to the tooth enamel at the same time. It was suggested in a previous paper that acidic proteins adsorbed to the tooth mineral first, while other proteins were then adsorbed via protein–protein interactions (37). However, the present concept that the acquired enamel pellicle consists largely of SMGs (containing several different proteins) may partly explain the problem outlined above. The SMGs express many exposed negatively charged protein terminals at their surfaces, as indicated by their low isoelectric point (11), thus conferring a high affinity for the tooth enamel. This is a well-known mechanism based on interaction between calcium ions in the hydration layer on the hydroxyapatite surface and negatively charged groups with high affinity for this cation (37, 38). The significance of this model is strongly supported by the observation that agents such as sodium lauryl sulphate and sodium pyrophosphate, which both exhibit high affinity for calcium, inhibit pellicle formation in vivo (39, 40). The SMGs at the same time consist of several other proteins making up the complete structure, and this allows many proteins to become concentrated on the tooth surface by only one mechanism.

It appears reasonable to conclude that the SMGs contribute at least 10% to the total whole salivary protein—a figure which represents a compromise between the values obtained for unclarified and clarified whole saliva. However, the unclarified whole saliva of one of the donors consisted of SMGs contributing as much as 32% of the total salivary protein. The study also confirmed the hypothesis that the amino acid profile for the SMGs is strikingly similar to the well-established profile of the 2-h pellicle. The amino acid profile of the SMGs was totally different from that of whole saliva, demonstrating that the selection of proteins for the formation of the SMGs is very specific in relation to salivary proteins available. Together, these findings suggest that the biological activities shown to be associated with the whole SMGs *are* of physiological significance in the oral cavity. Furthermore, these results confirmed previous morphological studies that have shown globular structures (the SMGs) representing a major component of the newly formed enamel pellicle (14, 15). This is reasonable also from a teleological point of view. The globules have high affinity for the tooth mineral, as discussed above, and their selective adsorption allows a rapid establishment of a fairly thick protein layer. The protein layer may reduce the friction between the teeth, and between the teeth and the soft tissues of the oral tissues. The build-up of a similarly thick pellicle by selective adsorption of monolayers of single proteins would presumably be very time consuming, supplying little protection, and would scarcely provide for the selectivity and precision which is an integrated process in pellicle formation (41).

References

1. Swaisgood HE. Characteristics of edible fluids of animal origin: milk. In: Fennema OR, editor. Food chemistry, vol. 2. New York: Marcell Dekker; 1985. p. 791–828.
2. Holt C. Structure and stability of bovine casein micelles. Adv Prot Chem 1992;43:63–151.
3. Rollema HS. Casein association and micelle formation. In: Fox PF, editor. Advanced dairy chemistry: proteins, vol. 1. London: Elsevier Applied Science; 1992. p. 111–40.
4. Schmidt DG. Colloidal aspects of casein. Neth Milk Dairy J 1980;34:42–64.
5. Caldwell RC, Shackleford JM. A chemical, immunological and electron-microscopic study of centrifuged human submaxillary saliva. Arch Oral Biol 1967;12:333–40.
6. Glantz P-O, Wirth SM, Baier RE, Wirth JE. Electron microscopic studies of human mixed saliva. Acta Odontol Scand 1989;47:7–15.
7. Glantz P-O, Friberg SE, Wirth SM, Baier RE. Thin-section transmission electron microscopy of human saliva. Acta Odontol Scand 1989;47:111–6.
8. Rölla G, Rykke M. Evidence for the presence of micelle-like protein globules in human saliva. Colloids and Surfaces B: Biointerfaces 1994;3:177–82.
9. Rykke M, Smistad G, Rölla G, Karlsen J. Micelle-like structures in human saliva. Colloids and Surfaces B: Biointerfaces 1995;4:33–44.
10. Rykke M, Young M, Rölla G, Devold T, Smistad G. Transmission electron microscopy of human saliva. Colloids and Surfaces B: Biointerfaces 1997;9:257–67.
11. Rykke M, Young A, Smistad G, Rölla G, Karlsen J. Zeta potentials of human salivary micelle-like particles. Colloids and Surfaces B: Biointerfaces 1996;6:51–6.
12. Young A, Smistad G, Karlsen J, Rölla G, Rykke M. Zeta potentials of human enamel and hydroxyapatite as measured by the Coulter DELSA 440. Adv Dent Res 1997;11:560–5.
13. Young A, Rykke M, Smistad G, Rölla G. On the role of human salivary micelle-like globules in bacterial agglutination. Eur J Oral Sci 1997;105:485–94.
14. Lie T. Scanning and transmission electron microscope study of pellicle morphogenesis. Scand J Dent Res 1977;85:217–31.
15. Hannig M. Transmission electron microscopic study of in vivo pellicle formation on dental restorative materials. Eur J Oral Sci 1997;105:422–33.
16. Busscher HJ, Uyen HMW, Stokroos I, Jongebloed WL. A transmission electron microscopy study of the adsorption patterns of early developing artificial pellicles on human enamel. Arch Oral Biol 1989;34:803–9.
17. Nyvad B, Fejerskov O. Experimentally induced changes in ultrastructure of pellicle on enamel in vivo. In: tenCate JM, Leach SA, Arends J, editors. Bacterial adhesion and preventive dentistry. Oxford: IRL Press; 1984. p. 143–51.
18. Guggenheim B, Nesser J, Golliard M, Schupbach P. Salivary pellicle modified by milk components mediates caries protection. Caries Res (ORCA Abstracts) 1994;28:182.
19. Pomeranz Y, Meldan CE. Nitrogenous compounds. In: Pomeranz Y, Meldan CE, editors. Food analysis. New York: Chapman & Hall; 1994. p. 733–57.
20. Karman AH, van Boekel MAJS. Evaluation of the Kjeldahl factor for conversion of nitrogen content of milk and milk products to protein content. Neth Milk Dairy 1986;40:315–36.
21. Stryer L. Protein structure and function. Exploring proteins. In: Stryer L, editor. Biochemistry, vol. IV. New York: Freeman & Co, WH; 1995. p. 22–70.

22. Wolf RO, Taylor LL. A comparative study of saliva protein analysis. Arch Oral Biol 1964;9:135–40.
23. Dawes C. Some characteristics of parotid and submandibular salivary proteins. Arch Oral Biol 1965;10:269–78.
24. Robinson C, Lowe NR, Weatherell JA. Amino acid composition, distribution and origin of "tuft" protein in human and bovine dental enamel. Arch Oral Biol 1975;20:29–42.
25. Wheat RW. The classification and identification of bacteria. In: Joklik WK, Willet HP, Amos DP, editors. Zinsser microbiology. New York: Appelton-Century-Crofts; 1980. p. 10–25.
26. Rölla G, Wåler SM, Kjærheim V, Rykke M. The salivary micelle-like globules (SMGs) are the major retention site for Triclosan. J Dent Res (IADR Abstracts) 1996;75:93.
27. Soderling E. Practical aspects of salivary analyses. In: Tenovuo JO, editor. Human saliva: clinical chemistry and microbiology, vol. I. Boca Raton, Florida, USA: CRC Press; 1989. p. 1–19.
28. Jenzano JW, Hogan SL, Noyes CM, Featherstone GL, Lundblad RL. Comparison of five techniques for the determination of protein content in mixed human saliva. Anal Biochem 1986;159:370–6.
29. Barbano DM, Clark JL. Kjeldahl method for determination of total nitrogen content of milk: collaborative study. J Assoc Off Anal Chem 1990;73:849–59.
30. van Boekel MAJS, Ribadeau-Dumas B. Addendum to the evaluation of the Kjeldahl factor for conversion of the nitrogen content of milk and milk products to protein content. Neth Milk Dairy J 1987;41:281–4.
31. Jakube H-D. Analysis of amino acids. In: Jakube H-D, Jeschkeit H, editors. Amino acids, peptides and proteins: an introduction. London: Macmillan Press; 1977. p. 22–83.
32. Mandel ID. Relation of saliva and plaque to caries. J Dent Res (Suppl) 1974;53:246–66.
33. Levine MJ, Ellison SA. Immuno-electrophoretic and chemical analyses of human parotid saliva. Arch Oral Biol 1973;18:839–53.
34. Hogg SD, Embery G. The isolation and partial characterization of a sulphated glycoprotein from human whole saliva which aggregates strains of *Streptococcus sanguis* but not *Streptococcus mutans*. Arch Oral Biol 1979;24:791–7.
35. Kraus FW, Ørstavik D, Hurts DC, Cook CH. The acquired pellicle: Variability and subject-dependence of specific proteins. J Oral Pathol 1973;2:165–73.
36. Rölla G, Ciardi JE, Bowen WH. Identification of IgA, IgG, lysozyme, albumin, α-amylase and glucosyltransferase in the protein layer adsorbed to hydroxyapatite from whole saliva. Scand J Dent Res 1983;91:186–90.
37. Rölla G, Ciardi JE, Bowen WH. Ionic exchange reactions on hydroxyapatite surfaces studied by the use of radioactive counterions (^{45}Ca and $^{32}PO_4$). In: Frank RM, Leach SA, editors. Surface and colloid phenomena in the oral cavity: methodological aspects. London: IRL Press; 1982. p. 203–10.
38. Gorbunoff MJ. The interaction of proteins with hydroxyapatite. II: Role of acidic and basic groups. Analyt Biochem 1984;136:433–9.
39. Rykke M, Rölla G, Sönju T. Effect of pyrophosphate on protein adsorption to hydroxyapatite in vitro and on pellicle formation in vivo. Scand J Dent Res 1988;96:517–22.
40. Rykke M, Rölla G. Effect of sodium lauryl sulfate on protein adsorption to hydroxyapatite in vitro and on pellicle formation in vivo. Scand J Dent Res 1990;98:135–43.
41. Rykke M, Sönju T, Rölla G. Interindividual and longitudinal studies of amino acid composition of pellicle collected in vivo. Scand J Dent Res 1990;98:129–34.
42. Young A. Co-aggregation of micelle-like globules from submandibular/sublingual and parotid saliva. Colloids and Surfaces B: Biointerfaces 1999. In print.

Received for publication 9 July 1998
Accepted 1 March 1999