Delmopinol hydrochloride- and chlorhexidine digluconate-induced precipitation of salivary proteins of different molecular weights

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Gel electrophoresis was used to analyze precipitates formed of delmopinol hydrochloride or chlorhexidine digluconate mixed with unstimulated whole saliva samples from five test subjects. Final concentrations of delmopinol (6.4 mM) or chlorhexidine (6.4 mM, 2.2 mM) mixed with whole saliva were incubated for 10 min at 37°C. The precipitates were pelleted by centrifugation and resuspended to a similar protein density. The protein patterns in the pellets were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, using 12.5% gels. The amount of pellet protein was determined by densitometry in four molecular weight ranges (10–21.5, 21.5–26, 26–45, and 45–300). The results indicated that high molecular weight (45–300) proteins dominated in the precipitate and that 2.2 mM chlorhexidine precipitated more salivary protein than 6.4 mM. At equimolar concentration (6.4 mM) delmopinol precipitated more high molecular weight salivary proteins than chlorhexidine. □ *Dental plaque; electrophoresis; saliva*

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Thin organic films have been shown to adsorb quickly to solid and semi-solid surfaces in the oral cavity (1–4). The composition of salivary pellicles, however, has been shown to depend on intrinsic characteristics of the surfaces (5–8). Several experimental methods have been used in vitro and in vivo to identify organic components of salivary pellicles, such as submandibular and sublingual mucins, -amylase, cystatins, salivary mucin, secretory IgA, and lipids (9–11).

Anti-plaque agents are often suggested as adjunct methods for mechanical control of dental plaque formation (12–14). Several factors may influence the efficacy of an anti-plaque agent. Factors related to the chemical agent itself are, for example, the concentration of the drug, exposure time, and the delivery system (13–15). Other factors considered to be important are related to individual oral characteristics such as morphologic, nutritional, and salivary composition.

Chlorhexidine is a cationic compound that interacts with salivary components (16, 17). Chlorhexidine has been shown to bind to salivary proteins and enamel through electrostatic interactions (18). Freitas et al. (19) have shown other evidence indicating that chlorhexidine also has an amphiphilic behavior leading to increased concentration of the drug at air–liquid (19, 20), solid–liquid (20), and bacterial interfaces (21). Such behavior of chlorhexidine may be an important mechanism for the inhibition of plaque build-up.

A clinical study has shown the capability of delmopinol hydrochloride to control dental plaque and promote healing of experimental gingivitis (22). The mode of action of delmopinol was suggested to reduce and control bacterial plaque formation by modifying the physicochemical characteristics of the tooth surface (12). Delmopinol has strong affinity for constituents of saliva adsorbed onto solid surfaces such as hydroxyapatite (23). Interactions of delmopinol and proteins of the salivary pellicles show that this drug is able to alter the cohesive and adhesive properties of such biofilms (24, 25)—particularly the cohesive forces in the glucan-containing plaque (26).

The consequences of the interactions between chemical agents for dental plaque control and salivary proteins are at present not well studied. However, an investigation of the affinity between chlorhexidine and salivary mucins showed the formation of precipitates (27). These findings indicate that interactions between plaque control agents and proteins may affect the substantivity of these agents in the oral cavity.

Bearing the above in mind, it was considered important to analyze the precipitates formed between unstimulated whole saliva and delmopinol hydrochloride or chlorhexidine digluconate.

Materials and methods

Saliva

Unstimulated whole saliva was collected from five healthy individuals (28–47 years old) at the same time of the day on three different days. Immediately after each saliva collection the samples were clarified by means of centrifugation at 10,000 *g* for 15 min at 4°C. The supernatants were adjusted to pH 7 by the addition of sodium hydroxide.

Delmopinol and chlorhexidine

The chlorhexidine digluconate (Sigma Chemical Co, St Louis, Mo., USA) and delmopinol hydrochloride (Biosurface AB, Malmö, Sweden) solutions were prepared in distilled and deionized water (ELGA Ltd, High Wycombe, England) at pH 6.

Mixtures of saliva and chemical agents

Volumes of 5 ml of saliva and 5 ml of delmopinol or 5 ml of chlorhexidine were mixed and incubated on a rotating table (KS 500, Janke & Kunkel Ika-werk, Staufen, Germany) at 150 rpm for 10 min at 37°C. Final concentrations were 6.4 mM for delmopinol and 6.4 mM and 2.2 mM for chlorhexidine. Thereafter, the samples were centrifuged at 10,000 *g* for 15 min at 4°C. The pellets were resuspended with 5 ml of 6.4 mM sodium acetate and 1% sodium dodecyl sulfate (SDS), mixed, and immediately heated at 90°C for 10 min. All the samples were stored in a freezer at −80°C.

A control group was also tested by mixing 5 ml of unstimulated whole saliva with 5 ml of water. The assay described above was also applied to the control group.

The protein content of each sample was calculated with the Lowry assay (28), the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, Calif., USA), the phenol reagent method for biologic fluids (Diagnostics-micro protein determination, Sigma), and the Bio-Rad Protein Assay (Bio-Rad), based on the Bradford dye-binding procedure (29).

The absorbance of the supernatants and whole saliva was measured at 215 nm, using a U2000 spectrophotometer (Hitachi, Tokyo, Japan). The absorbance values were used for the calculation of a dilution factor for all samples to obtain equal protein amounts for SDS–polyacrylamide gel electrophoresis (PAGE).

Electrophoresis

The samples of saliva-anti-plaque agent and the saliva-water mixtures (100 µl) were treated with a solution containing 95% v/v glycerol (10 µl), 20% w/v SDS containing 250 mM dithiothreitol and 0.01% w/v bromphenol blue (10 µl) and heated at 90°C for 2 min (30). Samples of supernatant and pellets were then analyzed by SDS–PAGE by the method of Laemmli (31), using 12.5% gels and stacking gels of 4%. The gel dimensions were 150 × 150 × 1.5 mm. Standard broad-range SDS–PAGE proteins (Bio-Rad Laboratories, Richmond, Calif., USA) were used to determine four different molecular weight ranges (10–21.5, 21.5–26, 26–45, 45–300) in each lane, as shown in Fig. 1. These molecular weight ranges were used for video densitometry analysis. The gels were electrophoresed in parallel with a Bio-Rad Protein II apparatus (Bio-Rad Laboratories) at 190 V for 5 h at 4°C.

Staining procedure

The gels were fixed with 20% trichloroacetic acid in distilled water for 20 min. Thereafter, the gels were washed for 5 min with a destaining solution containing 70% methanol and 20% acetic acid in water. The gels were stained with Coomassie Blue R 250 in accordance with Gorg et al. (32). The gels were destained with several changes of destaining solution. Furthermore, all gels were silver-stained as described by Heukeshoven & Dernick (33). This treatment provided transparent gels that were adequate for video-densitometry measurements.

Video densitometry

The gels were photographed (Repro-copy outfit PF-4, Nikon, Japan), and the images were stored in a personal computer (Hewlett-Packard, USA). A video-densitometric system developed by Mr. Lars Kopp (Makab, Göteborg, Sweden) (34) was used to assess the relative density of stained protein bands on the basis of the molecular weight ranges defined earlier.

The proportion of pellet in all molecular weight ranges in relation to the total protein content was calculated for all subjects by dividing the pellet density value by the sum of the pellet and supernatant densities.

Statistical analysis

The difference in salivary protein precipitation induced by equimolar concentrations of delmopinol or chlorhexidine and the comparison of 2.2 mM and 6.4 mM chlorhexidine was evaluated with the two-sample t test. The null-hypothesis was rejected at $P < 0.0001$.

Results

Figs. 1 and 2 show the stained polyacrylamide gels containing salivary samples from five subjects with a final concentration of 6.4 mM of delmopinol or 6.4 mM and 2.2 mM chlorhexidine at three different occasions.

The mean pellet density values of each molecular weight range and their respective proportional representation of the total salivary protein content are shown in Fig. 3 for 6.4 mM delmopinol, 2.2 mM chlorhexidine, and 6.4 mM chlorhexidine.

The statistical analysis applied to the data comparing protein precipitation levels with 6.4 mM chlorhexidine and 6.4 mM delmopinol is shown in Table 1. In general, these results indicate that salivary samples presented more protein precipitates when mixed with 6.4 mM delmopinol than with 6.4 mM chlorhexidine.

The statistical analysis applied to the data comparing protein precipitation levels with 6.4 mM chlorhexidine and 2.2 mM chlorhexidine is shown in Table 2. The results show that salivary samples presented more protein precipitates when mixed with 2.2 mM chlorhexidine than with 6.4 mM chlorhexidine.

The control group showed no precipitation of salivary proteins.

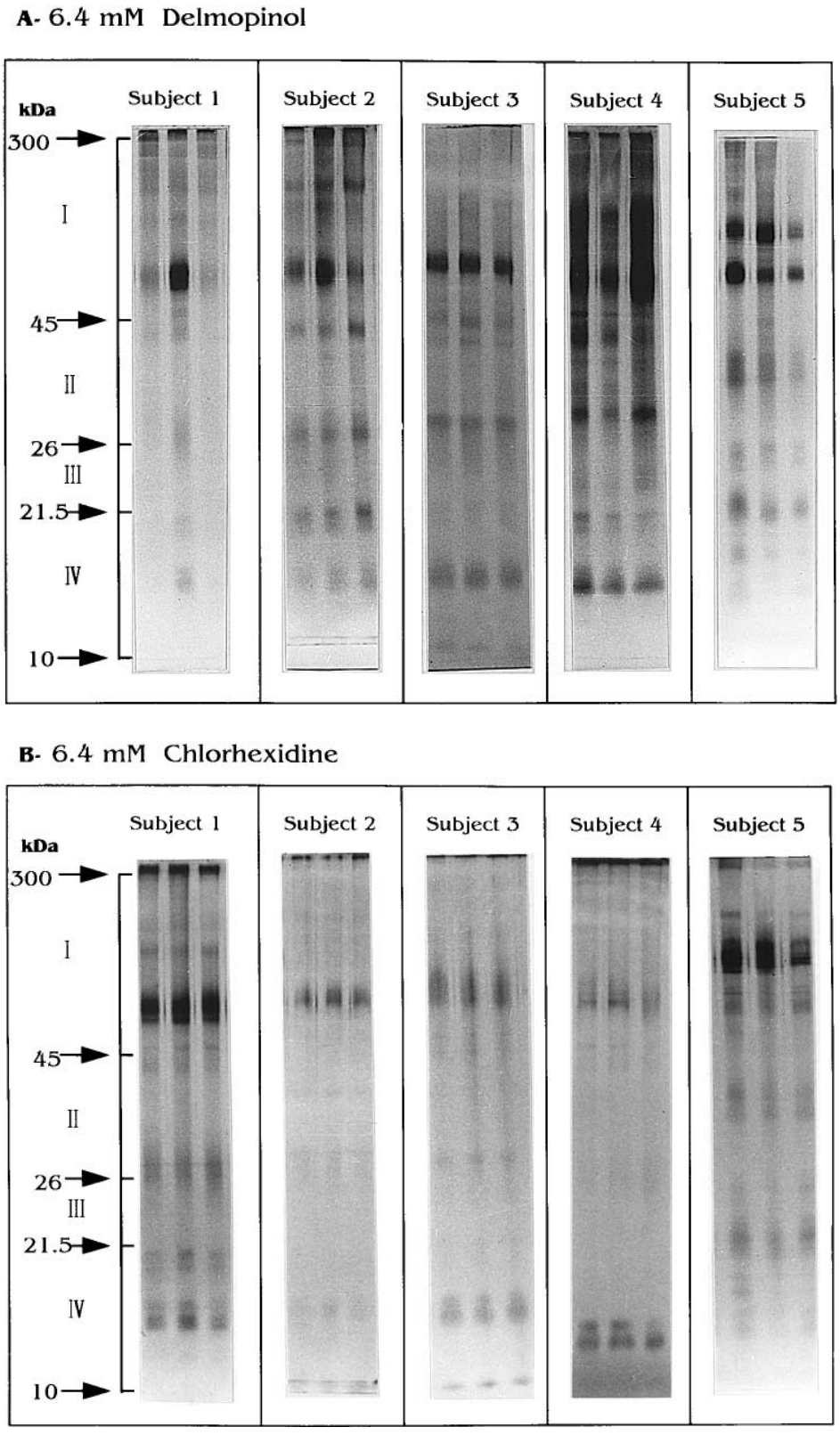

Fig. 1. A representative precipitation pattern of salivary protein from five subjects, mixed with delmopinol (A) or chlorhexidine (B) and having 6.4 mM of final concentrations on three different occasions. Molecular weight ranges: I, 300–45; II, 45–26; III, 26–21.5; and IV, 21.5–10.

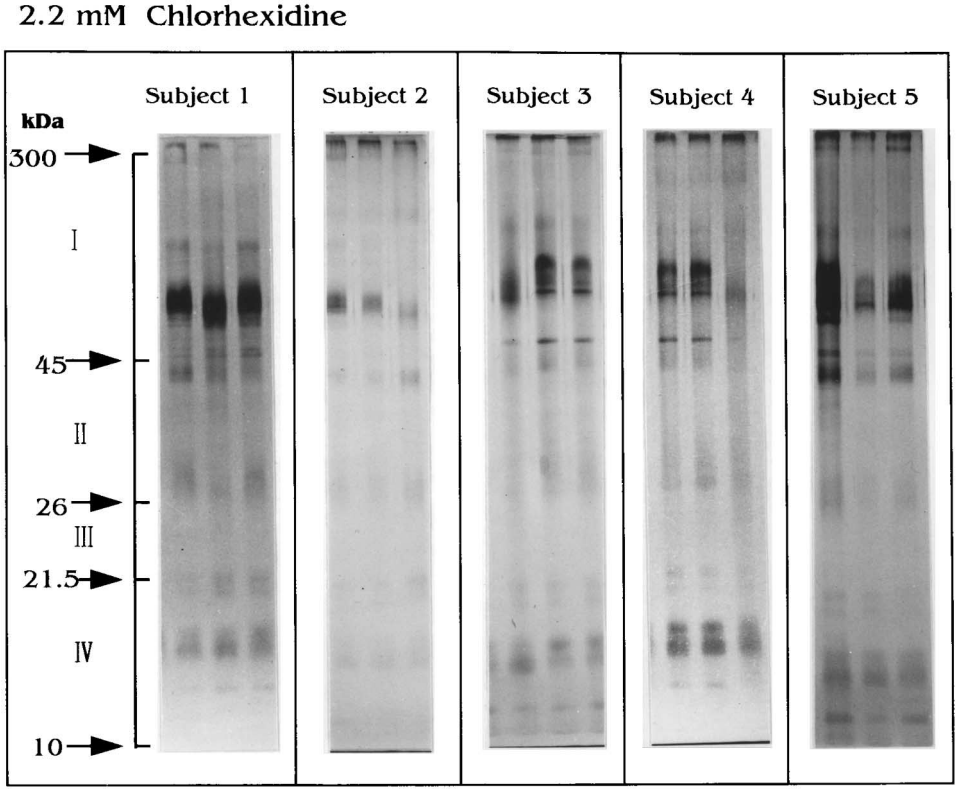

Fig. 2. A representative precipitation pellet pattern of salivary protein from five subjects mixed a final chlorhexidine concentration of 2.2 mM on three different occasions. Molecular weight ranges: I, 300–45; II, 45–26; III, 26–21.5; and IV, 21.5–10.

Discussion

Chlorhexidine and delmopinol are able to interact with salivary components at solid–liquid (19, 25) and air–liquid interfaces (19). The formation of complexes between these compounds and salivary proteins is, however, not fully understood. Inter-individual variations with regard to salivary components are often reported in the literature (30, 35–37). This study confirms such findings and also showed a high inter-individual variation in the salivary protein patterns.

The methods used in this study, such as spectrophotometry, electrophoresis, and densitometry, are routinely used in biochemical laboratories. The experimental tests were set up in such a manner as to ensure high reproducibility of the tests and validity of the overall experiments. Collection of unstimulated whole saliva was preferred, to prevent changes in salivary composition which are commonly found in actively stimulated saliva samples. Saliva samples were collected on three different occasions, to detect variations in saliva composition. The results have confirmed the reproducibility of the samples, as it was shown that the electrophoretic pattern of the salivary proteins was highly consistent (Figs. 1–3). The salivary samples were centrifuged to eliminate large particles as, for example, bacterial and/or epithelial cells. Such large particles would constitute a confounding factor in the experiments. The samples used to control the experiments showed no salivary protein precipitation at electrophoretic analyses, indicating that the results found were indeed due to the action of the chemical agents.

Standardization of the saliva samples with regard to the level of proteins is important to determine the proportion of protein precipitates in relation to a total protein value. Estimation of the protein content of the mixtures of saliva and delmopinol or chlorhexidine was attempted with several protein assay methods but was found to give non-reproducible results. A possible reason for such findings is that both chlorhexidine and delmopinol interfered with the assay reagents. Spectrophotometric readings were used as an alternative method to standardize the protein level of the salivary samples. Although spectrophotometric readings are unable to detect the amount of proteins in each sample, they give the optical density of the samples, indicating the total level of proteins in solution. The precision of this experiment turned out to be good, as shown by the similar patterns of the electrophoreses gels and the low standard deviations found intra-individually by video densitometry.

Chlorhexidine (897.8 Da) has a molecular weight almost

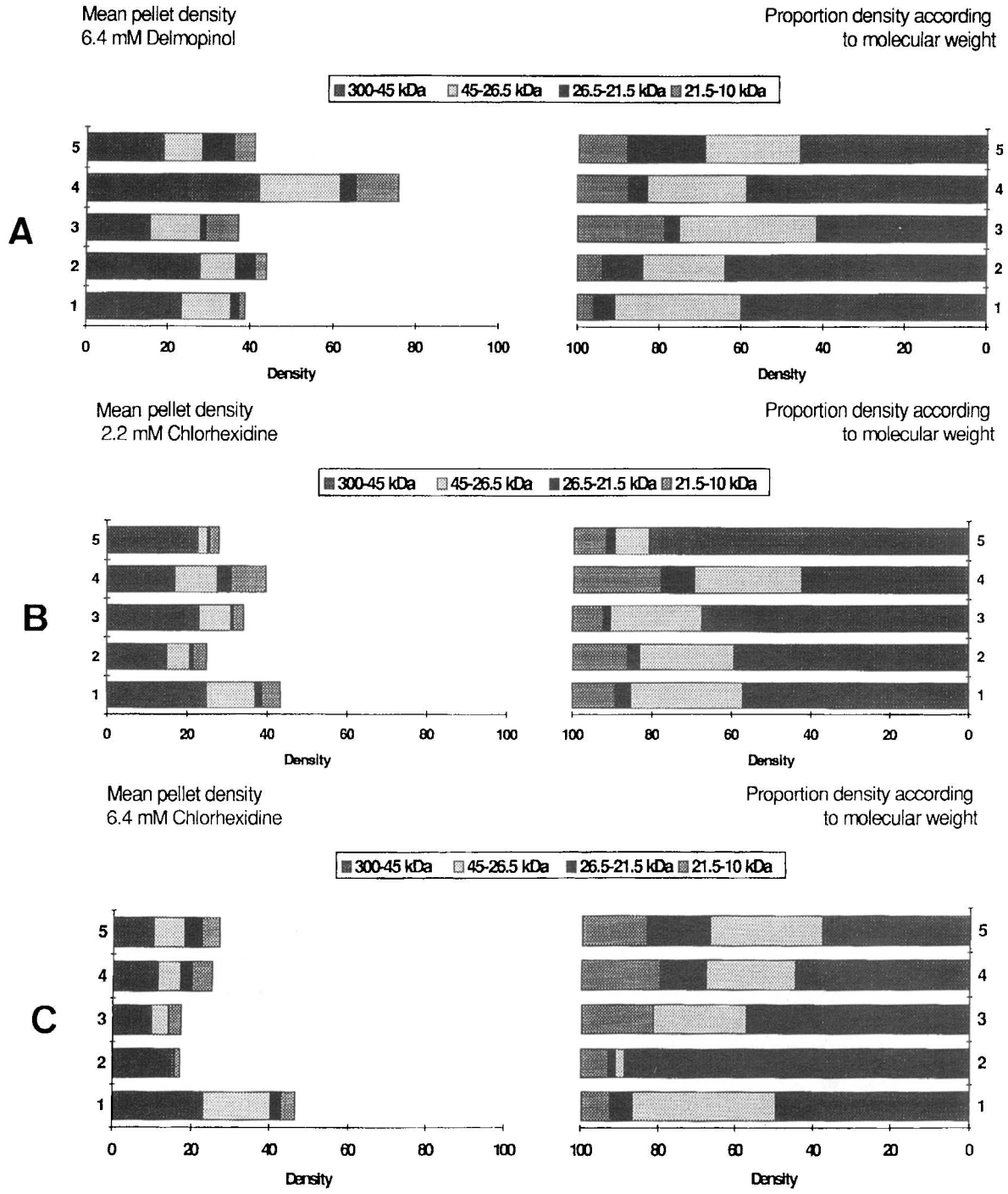

Fig. 3. Proportion and mean pellet density on the basis of the molecular weight of salivary proteins from five subjects, mixed with a final delmopinol concentration of 6.4 mM (A) (standard deviations range from 0.6×10^{-3} to 0.25) and final chlorhexidine concentrations of 2.2 mM (B) (standard deviations range from 0.5×10^{-3} to 0.17) and final chlorhexidine concentrations of 6.4 mM (C) (standard deviations range from 0.5×10^{-3} to 0.26).

three times larger than delmopinol hydrochloride (308 Da). On the basis of molar concentrations, 6.4 mM delmopinol and chlorhexidine correspond to a concentration of 0.2% (w/v) delmopinol and 0.57% (w/v) chlorhexidine. The 2.2 mM chlorhexidine corresponds to a concentration of 0.2% (w/v). The choice of concentrations (w/v) used in this study was based on concentrations routinely used in clinical therapy such as 0.2% chlorhexidine and 0.2%

Table 1. Statistical evaluation of the level of protein precipitation in salivary samples from five subjects, mixed 6.4 mM delmopinol and 6.4 mM chlorhexidine. Comparisons resulting in statistically significantly higher precipitation values are marked for delmopinol (del) or chlorhexidine (chx) within four molecular weight ranges

	Molecular weight ranges			
	300–45	45–26.5	26.5–21	21.5–10
Subject 1	—	chx	—	—
Subject 2	del	del	del	del
Subject 3	del	del	del	del
Subject 4	del	del	—	del
Subject 5	del	del	del	—

Table 2. Statistical evaluation of the level of protein precipitation in salivary samples from five subjects, mixed 2.2 mM chlorhexidine and 6.4 mM chlorhexidine. Comparisons resulting in statistically significantly higher precipitation values are marked for 2.2 mM chlorhexidine (2.2) or 6.4 mM chlorhexidine (6.4) within four molecular weight ranges

	Molecular weight ranges			
	300–45	45–26.5	26.5–21	21.5–10
Subject 1	—	6.4	6.4	2.2
Subject 2	—	2.2	2.2	2.2
Subject 3	2.2	2.2	2.2	6.4
Subject 4	2.2	2.2	—	2.2
Subject 5	2.2	6.4	6.4	6.4

delmopinol. However, it is also important to compare samples with the same molarity, and therefore 6.4 mM chlorhexidine was used.

The results of the electrophoretic experiments showed that delmopinol and chlorhexidine precipitate salivary proteins. Both agents precipitated mainly proteins in the high molecular weight ranges, even though some lower molecular weight ranges were also affected (Fig. 3). These findings were to a certain extent expected, since high molecular weight proteins are the commonest fraction in human saliva. Other studies have, however, reported similar observations about chlorhexidine's affinity for high molecular weight components (27). In general, the electrophoretic results showed that delmopinol at equimolar concentrations promoted more protein precipitation than chlorhexidine (Table 1). Delmopinol is a cationic surfactant of high surface activity which presents a hydrophilic head and a hydrophobic hydrocarbon tail (24, 25) and which may favor surfactant–proteins interaction.

The reason that 6.4 mM chlorhexidine precipitated less salivary protein than 2.2 mM chlorhexidine (Table 2) may be that 6.4 mM is in the range of the critical micelle concentration of chlorhexidine. At this point the cooperative binding of chlorhexidine to protein should be expected to be established, thereby forming highly charged soluble complexes. At lower concentrations the positively charged chlorhexidine should be expected to neutralize the predominantly negative charge of the protein and thereby decrease the solubility of complexes formed. This result is in line with most studies that showed clinical efficacy of 2.2 mM chlorhexidine (13).

The clinical relevance of the precipitation of whole saliva components by anti-plaque agents was not studied in this research. A previous report, however, showed that delmopinol binds to salivary proteins in solution and increases the amount of salivary films adsorbed onto solid surfaces (25). The binding of chlorhexidine to protein has also been reported to lead to structural changes of proteins adsorbed to mucous membranes, with consequent reduction of the thickness of the mucin layer (27). Salivary proteins such as mucin, agglutinins, and proline-rich glycoproteins have been reported to influence the adherence of microorganisms to intra-oral solid surfaces and other bacteria (38–42). Therefore, considering that salivary proteins are involved in fouling events in the oral cavity, it is possible to speculate that complex formations between anti-plaque agents and salivary components may influence the availability of these components for pellicle formation. Further studies are currently being conducted to identify whether anti plaque agents are bound to the protein precipitates.

It is thus concluded that delmopinol and chlorhexidine induce precipitation of salivary proteins. Furthermore, at equimolar concentration (6.4 mM) delmopinol precipitated more high molecular weight salivary proteins than chlorhexidine.

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