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Characterization of the Binding of Delmopinol to Salivary Precipitates

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The aim of this study was to quantify the amount of delmopinol recovered in different molecular weight ranges of salivary proteins in the supernatant and pellet of delmopinol-saliva mixes. Unstimulated whole saliva was collected from 5 subjects and mixed with radiolabeled delmopinol to obtain a final drug concentration of 9.7 mM. The salivary-delmopinol solutions were incubated and then centrifuged. The resulting pellets and supernatants were studied in an electrophoresis assay. Each individual sample was run in three different electrophoretic gel lanes. The first lane was stained with silver dyes; the second lane was used for electrophoretic blotting and autoradiography; the third lane was cut in standard slices that were dissolved and analyzed with scintillation counting. The scintillation results demonstrated that higher radioactivity levels were detected at high molecular weight ranges (700–600 kDa). Furthermore, pellet samples were found to contain the highest amounts of delmopinol. The autoradiography results confirmed that delmopinol was bound to proteins of high molecular weight (700-600 kDa).

Key Words: salivary proteins, chemical plaque control, delmopinol, scintillation counting, autoradiography.

INTRODUCTION

Short-term clinical studies have shown that the surfactant agent delmopinol is capable of reducing and delaying dental plaque formation (1,2) as well as induce healing of gingivitis (1).

The clinical efficacy of delmopinol is proposed to be partly due to a reduction of surface-associated glucan synthesis that in consequence, lowers the cohesion of the dental plaque (3).

The presence of delmopinol in saliva mixtures has been shown to reduce the surface tension of saliva and to affect salivary film formation (4,5). Adsorption experiments showed that the addition of delmopinol to saliva solutions can increase the adsorbed amounts of salivary components on solid surfaces, probably due to the formation of delmopinol-protein complexes (5). Another study reported the capability of delmopinol to adsorb onto bacterial surfaces that had been previously coated with saliva (6). Recent electrophoresis analyses presented evidence that delmopinol is capable of precipitating salivary components, particularly proteins in the range of 300 to 45 kDa (7). The clinical relevance of such interactions are unknown, although, they may be important for the mechanism of action of this compound.

The objective of this investigation was to characterize the binding of delmopinol to salivary components in the precipitates.

MATERIALS AND METHODS

Saliva

Unstimulated whole saliva was collected from 5 healthy individuals (age, 28-47 years). The samples were clarified by centrifugation at 10,000 *g* for 15 min at 4°C, and the supernatants were the adjusted to pH 7

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by the addition of sodium hydroxide.

Delmopinol

Delmopinol hydrochloride (Biosurface Pharma AB, Askim, Sweden) solution was prepared in purified water (ELGA Ltd, High Wycombe, England) at pH 6. A 65 µCi ring labeled [^{14}C]-delmopinol (Amersham Int., Amersham, UK) was included for each ml of the cold solution.

Mixtures of Saliva and Delmopinol

Volumes of 2 ml of delmopinol and 2 ml of saliva or water were mixed to obtain a final concentration of 9.7 mM delmopinol (0.3%) . The mixtures were incubated on a rotating table at 150 rpm for 5 min. The samples were subsequently centrifuged in an Eppendorf centrifuge (Beckman microfuge E™, Palo Alto, CA, USA) at 17,000 *g* at 4ºC for 20 min. The pellets were resuspended with 700 µl of 32 mM delmopinol (1%) at pH 5. Finally, the supernatant and pellet or delmopinol in water for control samples (25 µl) were mixed with 125 µl of glycerol or 125 µl of glycerol and 10 µl bromphenol blue.

Electrophoresis

The protein patterns in supernatants and pellets were analyzed electrophoretically by using 7% separation polyacrylamide gels (8). The dimensions of the gels were 150 x 130 x 1.5 mm. Electrophoresis was carried out with a Bio-Rad Protein II (Bio-Rad Laboratories, Richmond, CA, USA) apparatus using a power supply at a constant voltage of 200 V, for 4-5 h at 4ºC. The assay was performed using electrophoresis buffer (0.025 M Tris and 0.192 M glycine) at pH 8.3. The HMW calibration kit of native standard proteins (Pharmacia Biotech, Sollentuna, Sweden) was used as the standard. Each individual sample was run in three different lanes. Immediately after the electrophoresis, the first lanes of the gel were stained with silver stain (Silver Stain Plus Kit, Bio Rad). The second lane of the electrophoretic gel was prepared for electrophoretic blotting and autoradiography; the third lane samples were prepared for detection of the radioactivity. One control lane contained delmopinol with ^{14}C-delmopinol and the other control lane contained saliva.

Electrophoretic Blotting

The second lane of the electrophoretic gel was cut and placed in contact with a Zeta-Probe blotting membrane (Bio-Rad) after being saturated in a buffer solution (0.025 M Tris, 0.2 M glycine and 20% methanol at pH 8.3) for 30 min. The gel was thus firmly and evenly pressed against the membrane. The assembly was put into an electrophoretic chamber (Trans-Blot, Bio-Rad), containing the buffer solution with the Zeta-Probe™ blotting membrane (Bio-Rad) facing the anode using a power supply at a voltage gradient of 5 V/cm for 20 min. The gels were subsequently stained as described above to assure that the proteins were transferred to the membrane. The dried membrane was placed in direct contact with an X-ray film (Hyperfilm-b max, Amersham Int.), clamped onto a radiographic Kodak cassette for one week at room temperature (20ºC). After the exposure, the film (Agfa Gevaert G 150, Leverkusen, Germany) was developed according to the manufacturer's instructions. The image obtained, after autoradiography, was compared with the stained protein pattern, as well as with the radioactivity amount in the polyacrylamide gel slices.

Detection of Radioactivity

The third lane of the electrophoretic gel was cut first in a vertical direction in 1-cm wide strips along the gel lanes, then in horizontal 1-cm pieces. Each piece was then transferred to separate scintillator vials and left to dry. Each gel slice was dissolved in 19 parts 30% H_2O_2 and 1 part ammonia at 37ºC, vortexing briefly with 5 ml scintillation liquid (4 g of PPO/l toluene) (9). The amount of radioactivity of each vial was counted twice in a Packard Tricarb 460 C (Zurich, Switzerland).

RESULTS

Figure 1 gives the mean and standard deviations of scintillation counts (dpm) found for 5 subjects in the supernatant and pellet samples of whole saliva and delmopinol mixtures. Determinations of the radioactivity counts in the control lanes showed that labeled delmopinol was also detectable in all pieces of the cut gel strips (Figure 1). High molecular weight bands (700-600 kDa) were found to incorporate more delmopinol molecules in both supernatants and pellets

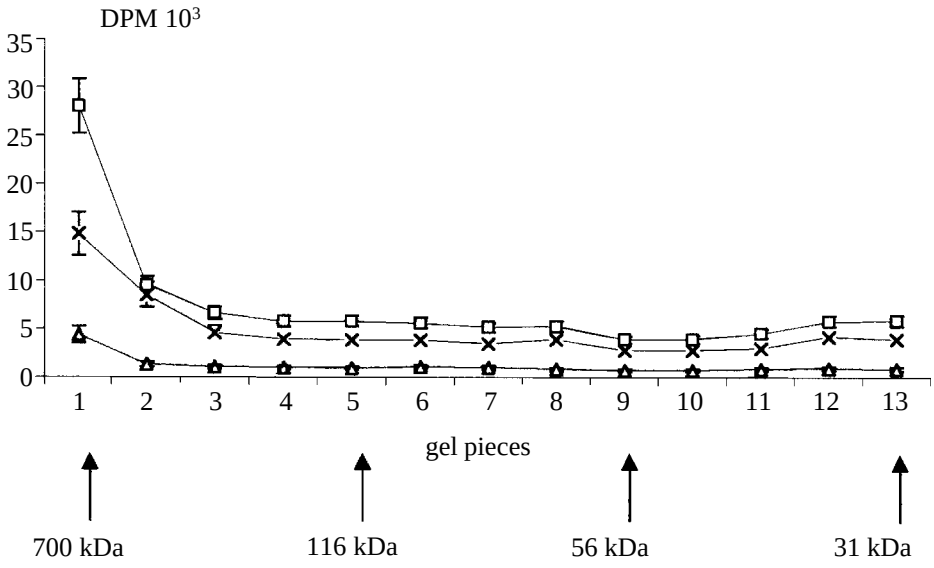

Figure 1. Mean and standard deviations of scintillation counts (dpm) found for 5 subjects in the pellet (square), supernatant samples of whole saliva and delmopinol mixtures (X) and for control labeled delmopinol without addition of proteins (triangle).

than other molecular weight ranges (600-31 kDa). Since delmopinol is a cationic compound it might be argued that when it is ionized it will migrate in the opposite direction in the electric field and remain at the entrance of the electrophoretic gel lane. This is, however, not the case since scintillation counting clearly detected delmopinol in all parts of the gel also in the absence of proteins when the delmopinol was evenly distributed along the gel (Figure 1). A typical example of the pattern of protein separation for the pellet samples of whole saliva-delmopinol mixtures is shown in Figure 2. The staining of lane 1 shows the migration pattern of the proteins. This figure also shows the corresponding autoradiographic results in lane 2 and the binding of delmopinol to proteins of high molecular weight range 700-600 kDa. The electrophoretic blotting procedure showed that salivary components of the supernatant samples were transferred to the membrane, leaving the radioactive delmopinol bound to the gel. It is important to have in mind that the gels were also stained after blotting to assure that the proteins were transferred to the membrane.

Scintillation counting and autoradiography are considered complementary methods to detect ^{14}C in pure protein systems (9). However, this study observed that supernatant samples showed separation, but a corresponding autoradiography image was not detected in the film. These analytical techniques, however, were not used earlier with complexes of salivary components and surfactants. Moreover, the radioactivity counts from

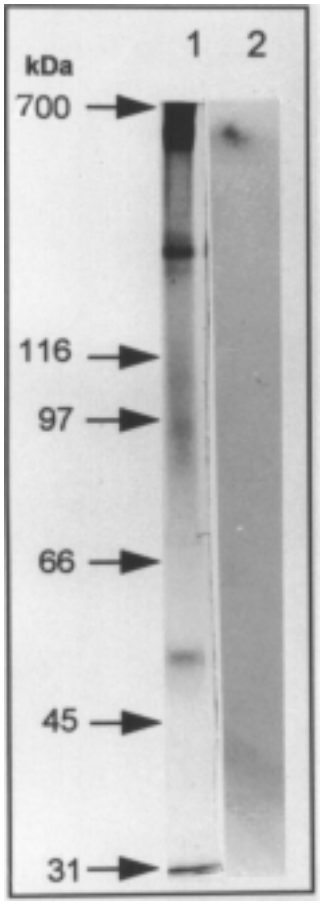

Figure 2. Lane 1, a representative sample of protein separation after electrophoresis and silver staining of a pellet sample from whole saliva-delmopinol mixture. Lane 2, a corresponding autoradiography result.

the pellet and supernatant lanes were found to be distributed along the gel as well as from the positive control lanes.

A number of control tests were performed to assure the reliability of the experiments. The buffers used for electrophoresis and electrophoretic blotting showed no radioactivity, indicating that there was no loss of radioactive delmopinol from the gels.

DISCUSSION

It is generally agreed that salivary proteins selectively adsorb to solid and semi-solid surfaces and consequently form a salivary film on surfaces in the oral cavity (10,11). High molecular weight mucins have been isolated *in vivo* as one of the most common components of salivary pellicles (12). In saliva, two groups of mucins have been identified: high molecular weight MG1 (>10^3 kDa) and lower molecular weight MG2 (200-250 kDa) (13,14). These glycoproteins fulfill functions such as pellicle formation, masticatory lubrication and mediation of bacterial adherence (12,14-16).

Several studies have shown that delmopinol molecules have affinity for salivary pellicles on solid surfaces as well as salivary components in solution (5,7). The results of this study have confirmed previous observations that delmopinol precipitates high molecular weight proteins (7). Consequently, the effect of delmopinol on the availability of high molecular weight components could influence some of their functions in the oral cavity. The results obtained for pellet samples using both methods of scintillation counting and autoradiography showed evidence for delmopinol affinity to high molecular weight proteins. Such results are in agreement with the findings obtained for salivary film coating hydroxyapatite beads (17) and adsorption experiments on solid surfaces (5). In the latter, the authors found the addition of delmopinol to submandibular and sublingual saliva samples increased the adsorbed amounts of salivary films to solid surfaces to a higher extent than found for parotid and whole saliva. These findings indicate interaction of delmopinol with salivary high molecular weight components.

A concentration of 9.7 mM delmopinol, which is above the critical micelle concentration, was chosen for this investigation. A higher concentration of cold delmopinol solution was chosen in order to dissolve the pellets, instead of surfactants that could disturb the system. However, it should be noted that the analytical methods used are able to detect only labeled compounds.

Radioactive countings have shown that delmopinol was also found in the supernatant part of the saliva samples in the range of 700-600 kDa. These results were, however, not confirmed in the autoradiography tests. Although the amount of detectable radioactive delmopinol in the supernatant samples was smaller than those in the pellet samples, the levels of dpm countings should have been sufficient to produce an image on the autoradiography films.

The binding of surfactants to salivary proteins are influenced by factors, such as the protein concentration levels, the distribution of amino acid residues and their structure (18). High molecular weight glycoproteins are also reported (13) to have numerous exposed hydrophobic binding sites and negative charges. Delmopinol is an amphiphilic substance due to a polar head and a hydrophobic tail (4,5) which might bind to several salivary proteins in solution through electrostatic and hydrophobic interactions.

Association of surfactants in a cooperative process takes place above a critical concentration for most proteins. The forces related to the hydrophobic interaction (18) are probably one of those responsible for the interaction delmopinol/high molecular weight proteins. The binding described in this paper is in line with the behavior at interfaces where ellipsometric studies have shown that delmopinol was reversibly bound to salivary components and/or the substrate structure (4,5).

The binding affinity of delmopinol to high molecular weight proteins may have important implications on salivary glycoproteins in the oral cavity. Investigations showed a relationship between the level of the high molecular weight mucin in saliva and caries status (19). It was found that high molecular weight mucin (MG1) is predominate in saliva of caries-susceptible subjects, while the level of the low molecular weight (MG2) form appears to be higher in saliva of caries-resistant individuals (20). The formation of delmopinol-protein complexes has also been attributed to increased adsorbed amounts of salivary films on solid surfaces (5). Furthermore, the precipitation of high molecular weight proteins by delmopinol may thus influence salivary film composition, bacterial adherence to intra-oral surfaces as well as inter-bacterial adherence.

In conclusion, delmopinol binds to salivary protein precipitates which may change availability of some proteins in saliva and probably influences proteins adsorption on oral surfaces.

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RESUMO

Freitas-Fernandes LB, Rundegren J, Arnebrant T, Glantz P-O. Caracterização da associação de delmopinol com precipitados salivares. Braz Dent J 2001;12(3):173-177.

O objetivo do trabalho é determinar a quantidade de delmopinol associado com cada componente salivar de diferente peso molecular. Saliva não estimulada foi coletada com cinco indivíduos e misturados com delmopinol radioativo obtendo uma concentração final de 9,7 mM. As misturas de saliva e delmopinol foram analisados com electroforese tanto para o pelete como para o supernatante. Cada amostra foi analisada três vezes em electroforese. A primeira foi corada com corante de prata. A segunda amostra foi preparada para fazer auto-radiografia. A terceira fileira foi cortada em pedaços iguais dissolvidos e analisados com cintilografia. O resultado de cintilografia demostrou que um grande nível de radioatividade foi detectado em alto peso molecular (600-700 kDa). Todas as amostras dos peletes foram encontrados contendo grande quantidade de delmopinol. O resultado da auto-radiografia confirmou que o delmopinol interagiu com proteinas de alto peso molecular (600-700kDa).

Unitermos: proteinas salivares, controle químico de placa, delmopinol, cintilografia, auto-radiografia.

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