Characterization of saliva proteins from 'stainers' and 'non-stainers' adsorbed to hydroxyapatite

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Stainers and non-stainers were selected on the basis of their individual tendency to develop extrinsic tooth discolorations from a chlorhexidine mouth rinse. Saliva proteins adsorbed to hydroxyapatite in vitro and in vivo pellicle from the participants were analyzed by gel filtration and ion-exchange chromatography. Two distinct anionic components were isolated. The elution patterns from stainers and non-stainers were identical. Amino acid analyses of the main peaks demonstrated a prevalence of serine, glycine, and glutamic acid. □ *Amino acid analyses; dental pellicle; ion-exchange chromatography; tooth discoloration*

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Extrinsic discoloration of teeth is commonly observed in connection with the use of chlorhexidine and other chemical plaque-inhibiting agents.

In the oral cavity a layer of salivary glycoproteins is adsorbed to the tooth surfaces, forming an organic pellicle (1). If the accumulation of bacterial plaque is inhibited, an extraordinarily thick consolidated pellicle may develop (2), increasing the possibilities for organic browning reactions to occur.

The discoloration tendency shows inter-individual variations. Some people invariably respond to oral rinsing with plaque-inhibiting agents with severe extrinsic tooth discoloration, whereas others do not develop such discolorations at all (3, 4). This observation has enabled the selection of stainers and non-stainers for experimental purposes (3–5). The etiology of these discolorations is not fully understood, but various etiological possibilities have been suggested (3).

The aim of the present experiments was to elucidate the biochemical basis for formation of extrinsic tooth discolorations. This has been approached through a characterization of salivary glycoproteins from stainers and non-stainers adsorbed to dental enamel in vivo and to hydroxyapatite powder in vitro.

Materials and methods

Selection of stainers and non-stainers

Three stainers and three non-stainers were selected among dental students on the basis of 14 days' rinsing with 0.2% chlorhexidine acetate twice daily. Testing was also performed with four stainers and four non-stainers, selected among military recruits. This selection was based on a previous plaque-inhibition study using 0.2% chlorhexidine (5).

Protein adsorption in vitro

Paraffin-stimulated whole saliva was collected individually in the morning from the participants. The saliva samples were then clarified by centrifugation at 12,000 *g* for 15 min at 4°C. The sediments were discarded. Test tubes with samples of 50 mg hydroxyapatite powder Bio-gel HTP (Bio-Rad Laboratories) with a surface area of 50–75 m^2/g and Ca/P ratio of 1.5 : 1 (six samples per individual) were washed consecutively

five times in 1-ml solutions of 1 mM K_2HPO_4, 1 mM $CaCl_2$, 0.1 mM MgCl, and 50 mM KCl, pH 6.5. Centrifuged saliva samples were added to the washed hydroxyapatite powder and allowed to interact for 2 h at room temperature with frequent shaking of the test tubes. The hydroxyapatite was then washed four times with the buffer solutions consecutively, dissolved, and separated from the adsorbed saliva proteins by dialysis against 0.2 M ethylenediaminetetraacetic acid (EDTA)' for 48 h, then against 0.9 M NaCl for 24 h, and finally against 1 mM Tris buffer, pH 7.5. This procedure has been described in detail by Hannesson Eggen (6).

Protein adsorption in vivo—collection of pellicle material

The same participants were also used for pellicle sampling in vivo. Their teeth were pumiced, and 2 h of adsorbed pellicle scraped off with a Coromant Scaler no. 4 and a suction apparatus (7) after being dried with compressed air. The pellicle material, pooled separately from stainers and non-stainers, was solubilized in 0.2 M EDTA for 24 h and dialyzed against 0.9 M NaCl for 24 h and against 1 mM Tris buffer, pH 7.5. Finally the suspensions were clarified by centrifugation at 12,000 *g* for 10 min at 4°C.

Gel filtration and ion-exchange chromatography

Salivary proteins adsorbed to hydroxyapatite in vitro and in vivo pellicle were examined by gel filtration and ion-exchange chromatography. The following columns were used: Sepharose Cl-6B (116 × 0.65 cm), CM-Sephadex (15 × 1 cm), and DEAE-Sephadex (15 × 1 cm) columns, with 1 mM Tris-buffer, pH 7.5, for elution. The flow rate was 0.2 ml/min, and 2-ml fractions were collected. The Sepharose Cl-6B column separates proteins on the basis of their molecular weight, and the present column had an operating range between molecular weights 10^4 and 10^6. One distinct peak was obtained from this column. This peak was further examined by ion-exchange chromatography. The columns used were CM-C-25-Sephadex, retaining cationic proteins that may be eluted by an NaCl gradient, here ranging from 0 to 1 M in 1 mM Tris buffer. The main peak that did not bind to the anionic bed material was further transferred to the DEAE-A-25-Sephadex column. Bound anionic material was eluted by an identical NaCl gradient. At each step the protein concentrations were estimated by spectrophotometric readings at 215 nm. The elution pattern was visualized by planimetry of concentration curves plotted in accordance with the spectrophotometric readings (Figs. 1–3).

Amino acid analysis

Amino acid analysis of the main peaks of the in vitro-adsorbed salivary proteins eluted from the final DEAE-A-25-Spehadex column was performed after removing NaCl by dialysis against 1 mM Tris buffer. EDTA-solubilized in vivo pellicle pooled from stainers and non-stainers separately was analyzed for amino acid composition after dialysis. The amino acid analyses were performed in a Bio-Cal BC 200 analyzer after freeze-drying and hydrolyzing the samples for 24 h in 6 N HCl at 105°C in a nitrogen atmosphere (6).

Results

Gel filtration of hydroxyapatite-adsorbed saliva proteins from stainers and non-stainers on Sepharose Cl-6B showed one distinct peak eluted at 40 ml (Fig. 1). This was between the eluted fractions of albumin, mol.wt. 67,000, and lysozyme, mol.wt. 14,600, used as reference substances in the present gel filtration system (Fig. 1). No difference could be observed between stainers and non-stainers.

Ion-exchange chromatography of this main peak on anionic bed material (CM-Sephadex) showed one distinct peak eluted before application of the NaCl gradient and minor amounts of cationic material eluted with the salt gradient applied (Fig. 2). The elution pattern of cationic material was inconsistent. No differences between stainers and non-stainers could be observed

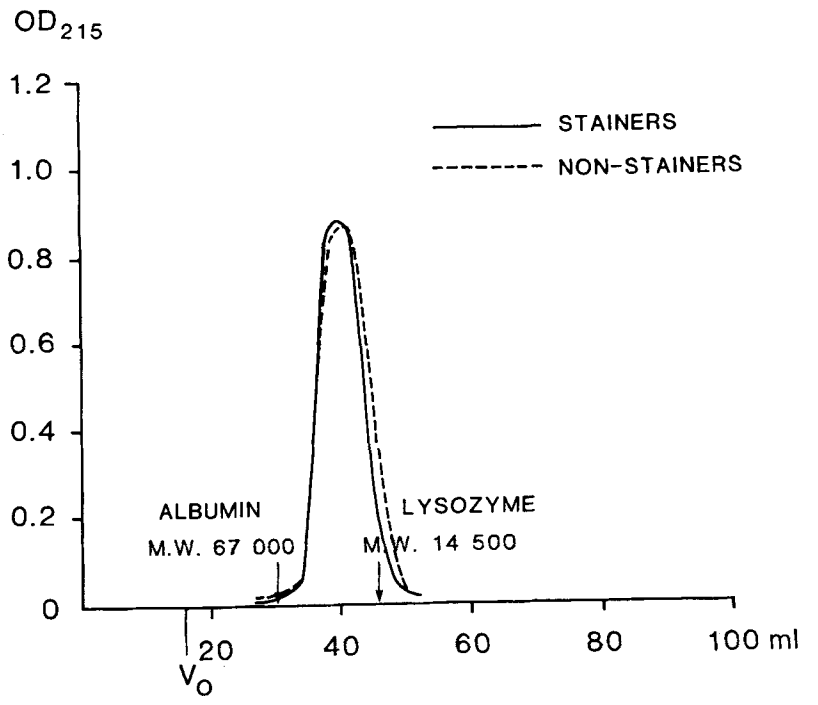

Fig. 1. Sepharose Cl-6B filtration of hydroxyapatite-adsorbed saliva proteins from stainers and non-stainers measured spectrophotometrically at OD_{215}. Elution volume indicated along the x-axis, in ml.

from this part of the ion-exchange chromatography.

When the peak was examined on cationic bed material (DEAE-Sephadex), one small peak was regularly eluted before application of the salt gradient and two anionic peaks eluted at 0.3 M and 0.5 M NaCl, respectively (Fig. 3). Minor variations were observed from individual measurements, but average values based on planimetric readings of the individual elution curves demonstrated similar elution patterns for stainers and non-

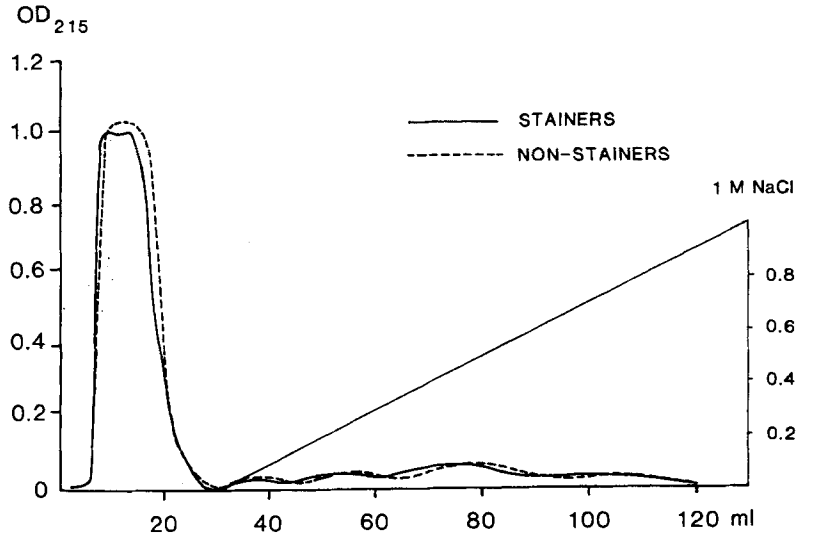

Fig. 2. CM-Sephadex chromatography of the main peak eluted by Sepharose filtration. Anionic or non-polar material is eluted before application of the NaCl gradient measured spectrophotometrically at OD_{215}. Elution volume indicated along the x-axis, in ml.

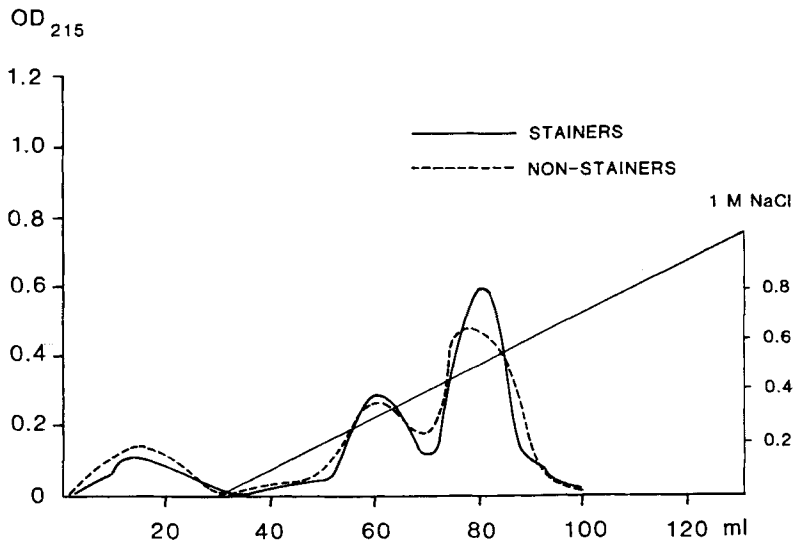

Fig. 3. DEAE-Sephadex chromatography of the main peak from the CM column measured spectrophotometrically at OD_{215}. Elution volume indicated along the x-axis, in ml. Anionic material is desorbed by the NaCl gradient.

stainers. Gel filtration and ion-exchange chromatography of in vivo pellicle demonstrated similar elution patterns.

Amino acid analyses of the main peaks obtained from the final DEAE-Sephadex separation of in vitro pellicle material demonstrated similar compositions for the neutral and the two anionic peaks (Fig. 4). The readings were based on mol/100 mol, and serine, glycine, and glutamic acid were most prevalent, adding up to 55% of the total amount of amino acids. Again, no substantial differences between stainers and non-stainers were observed.

Amino acid analyses of in vivo pellicle from stainers and non-stainers, dissolved in EDTA, dialyzed, and freeze-dried, showed a composition similar to that of the peaks retrieved from the DEAE-Sephadex separation (Fig. 4).

Discussion

Extrinsic tooth discoloration in connection with oral use of chlorhexidine shows marked inter-individual variations. The present experiments were initiated to elucidate possible constitutional differences in the composition of natural pellicle material or hydroxyapatite-adsorbed saliva proteins from stainers and non-stainers to explain this variation in discoloration tendency.

Gel filtration and ion-exchange chromatography of in vitro-adsorbed saliva proteins demonstrated similar compositions from stainers and non-stainers. The elution pattern confirmed previous results from analyses of in vitro (6) and in vivo (7) pellicle material.

Two closely related anionic fractions seemed to be the major components of the hydroxyapatite-adsorbed saliva proteins, whereas a non-polar component was also eluted before application of the NaCl gradient. The detection of anionic components supports the theory of selective adsorption of anionic polar groups onto the tooth surfaces by electrostatic interactions (8).

Results published by Hannesson Eggen & Rölla (7) demonstrate that the composition of salivary proteins adsorbed to hydroxyapatite in vitro closely resembles the natural in vivo pellicle. The present investigations confirm these findings and indicate that differences in extrinsic tooth discoloration tendency cannot be explained by differences in the composition of hydroxyapatite-adsorbed saliva proteins or in vivo pellicle material at the level detected by the techniques used here.

Analyses of the amino acid composition of the various peaks of in vitro pellicle eluted by DEAE-Sephadex chromatography indi-

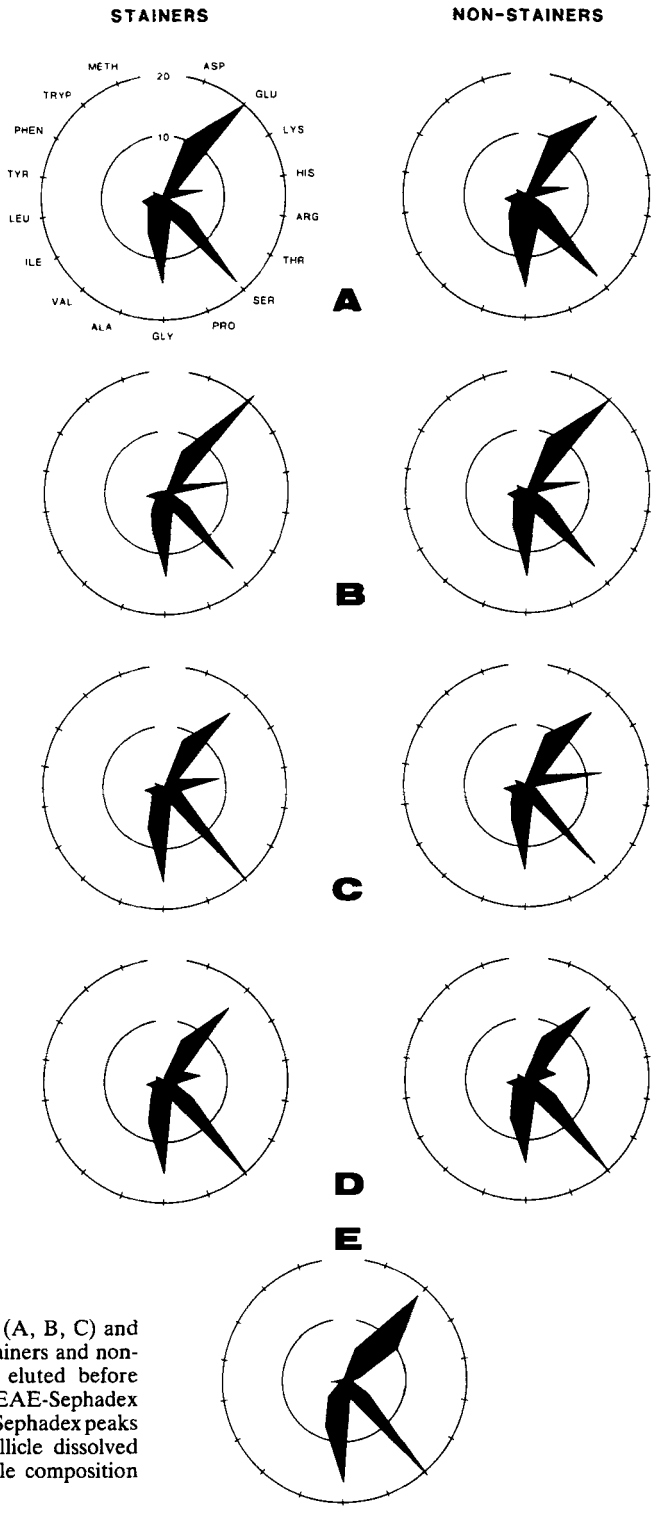

Fig. 4. Amino acid analyses of in vitro (A, B, C) and in vivo (D, E) pellicle material from stainers and non-stainers: (A) DEAE-Sephadex peaks eluted before application of the NaCl gradient; (B) DEAE-Sephadex peaks eluted at 0.3 M NaCl; (C) DEAE-Sephadex peaks eluted at 0.5 M NaCl; (D) in vivo pellicle dissolved in EDTA and dialyzed; and (E) pellicle composition published by Hannesson Eggen (8).

cate that both the non-polar and the two anionic components closely resemble the purified component isolated from in vitro (6) and in vivo pellicle (7). Further analyses of in vivo pellicle material from stainers and non-stainers dissolved in EDTA show an amino acid composition similar to that of the previously described in vitro pellicle.

The present analyses have not demonstrated any constitutional differences in pellicle composition to explain the observed inter-individual differences in discoloration tendency. More detailed analyses of pellicle components with more discriminating techniques may shed light on the etiology and constitutional background for these discolorations.

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