

Protein adsorption to hydroxyapatite and to calcium fluoride in vitro and amino acid analyses of pellicle formed on normal enamel and on calcium-fluoride-covered enamel in vivo

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Fluoride treatment of enamel has been reported to result in the formation of a layer of a CaF₂-like material on the enamel surface. Protein adsorption to enamel is a specific process dependent on the nature of the surface, and little is known about protein adsorption to CaF₂. Albumin and lysozyme were adsorbed to hydroxyapatite (HA) and CaF₂ powder in vitro, and protein adsorption patterns constructed. In vivo pellicle was collected from three volunteers from fluoride-treated enamel and from normal enamel, and the amino acid compositions analyzed separately. The results showed that CaF₂ took up small amounts of proteins as compared with HA. When the CaF₂ was pretreated with a phosphate buffer, pH 6.8, the protein adsorption increased markedly. The amino acid analyses showed no major differences in the amino acid compositions between pellicle collected from CaF₂-covered enamel and pellicle collected from normal enamel. This lack of difference is presumably due to the adsorption of phosphate ions to the CaF₂ crystals and hence changed surface properties. □ *Electron spectroscopy (XPS analysis); in vitro and in vivo adsorption; protein analysis*

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Several studies indicate that the topical use of a fluoride solution on dental enamel results in the formation of a layer of globules on the enamel surface (1-6). This layer has been found to be alkali-soluble (7-11) and consists of nodules of a calcium-fluoride-like material (1, 2, 7-10, 12). Calcium fluoride has recently been shown to be more resistant in the oral environment than previously assumed, probably owing to the adsorption of phosphate ions to the crystal surfaces (13, 14). A calcium-fluoride-like material is thus probably always present on the tooth surface in populations in which topical fluorides are frequently used (for example, fluoride-containing dentifrices or fluoride rinses). Little is known about the protein adsorption patterns to calcium fluoride, although some information is present on the surface properties of the crystal (15).

Pellicle formation is thought to be a

specific reaction between salivary proteins and dental enamel. Small changes in the chemical properties of the solid surfaces have been shown to influence the adsorption behavior of salivary proteins to the surfaces (16-18). It has been suggested that the chemical composition of the acquired pellicle may be one of the factors that affect the adsorption and adhesion of bacteria, and hence the bacterial composition of the initial plaque (19-22). Topical treatment of the enamel surface with an acidulated fluoride solution may alter the chemical properties of the enamel surface by means of a CaF₂-like material covering the enamel surface (23). It was therefore thought of interest first to study the adsorption of some model proteins to CaF₂ as compared with hydroxyapatite (HA) in vitro and then to compare the amino acid composition of pellicle adsorbed to calcium-fluoride-covered enamel with that

of the acquired pellicle adsorbed to non-covered enamel *in vivo*.

Materials and methods

In vitro adsorption of proteins to hydroxyapatite (HA) and CaF₂

Bovine albumin and lysozyme powders (Sigma Chemical Co.) were used to make series of protein solutions of 3 ml with protein concentrations ranging from 0.25 to 5.0 mg/ml. To series of these protein solutions were added either 50 mg hydroxyapatite powder (HTP-Bio-Gel, Bio-Rad Laboratories; specific surface area, 50–75 m²/g), 100 mg CaF₂ powder (Merk Suprapur, art. no 2842; specific surface area not available from the manufacturers), or 100 mg CaF₂ powder pre-treated with a 10-mM sodium phosphate buffer (pH 6.8) for 20 h under stirring at room temperature, then washed twice with distilled water and dried. The protein suspensions were stirred and incubated for 20 h at room temperature. The amount of protein adsorbed to the mineral phases was determined by spectrophotometry at 280 nm, and adsorption isotherms were constructed. We chose 100 mg CaF₂ because pilot experiments indicated that CaF₂ took up markedly less protein than HA. CaF₂ pretreated with phosphate was introduced because phosphate is known to adsorb to CaF₂ and form a solubility-limiting layer (14, 15, 24, 25).

Pellicle collection

Pellicle material was collected from three volunteers with complete dentition, a firm healthy gingiva, and no buccal or lingual dental restorations. The amino acid compositions were determined separately after hydrolysis. All buccal and lingual surfaces were pumiced, rinsed with water, dried with air, and then treated with an acidulated fluoride solution (0.6%, pH 1.9) for 1 min. This treatment has been shown to induce the formation of a continuous layer of small globules of a CaF₂-like material on the enamel surface (23).

Pellicle was collected after 2 h, during

which intake of food or beverages was avoided. The buccal and lingual surfaces of all teeth were then isolated with cotton wool rolls, rinsed with water, dried with air, and scraped carefully with a 3M no. 311–312 scaler. The whitish material that was scraped off was collected in a tube with distilled water by means of a bent Pasteur pipette connected to a suction device (26). To ensure enough material for one analysis, the collecting procedure was done twice. Pellicle material was collected three times from each volunteer and analyzed separately. The tubes with the collected pellicle material were kept frozen until analyzed.

All fluoride treatments were then withdrawn for 12 weeks. The test persons used a non-fluoride-containing dentifrice during this period. The drinking water in Oslo contains 0.01 ppm fluoride. During this period the teeth were pumiced twice, after 6 weeks and after 9 weeks. After 12 weeks pellicle was collected as described above.

Amino acid analyses

The frozen samples of pellicle material were evaporated to dryness and suspended in 3 ml 6 N HCl, evacuated, and sealed for vacuum hydrolysis. Before evacuation, highly purified nitrogen was added. Hydrolysis was performed for 24 h at 108°C. The amino acid analyses were performed with a Biotronic Auto Amino Analyzer LC 5000, using ninhydrin as detector reagent, and equipped with an automatic integrator (27).

XPS analysis of enamel surfaces

To ascertain that pellicle material was collected from different enamel surfaces—fluoride-treated enamel and normal enamel—the nature of the enamel surfaces was investigated by electron spectroscopy (XPS analysis).

Two enamel fragments (approximately 3 × 5 mm) were prepared from unerupted, surgically removed third molars. Both were, after rinsing and drying, treated with an acidulated fluoride solution (pH 1.9, 0.6%) for 1 min, rinsed, and dried. One of the fragments was then brushed for 20 min with

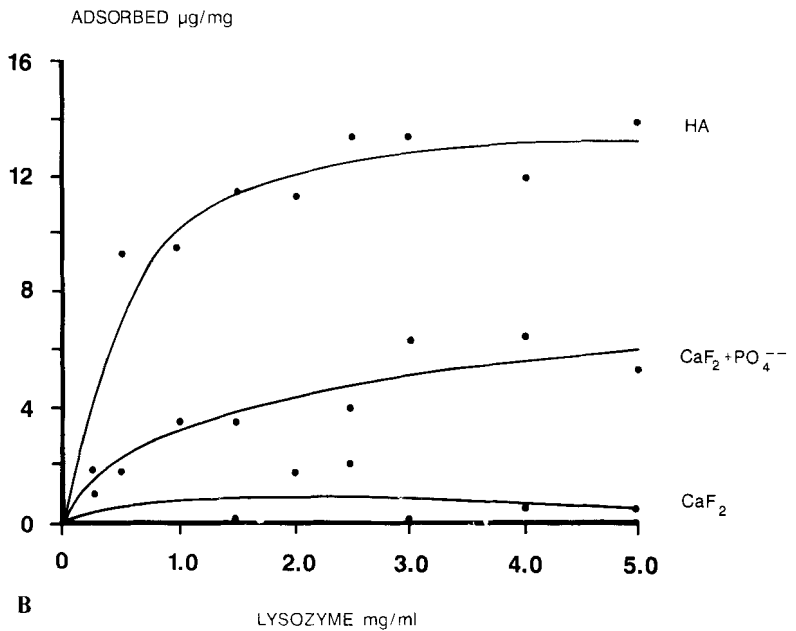
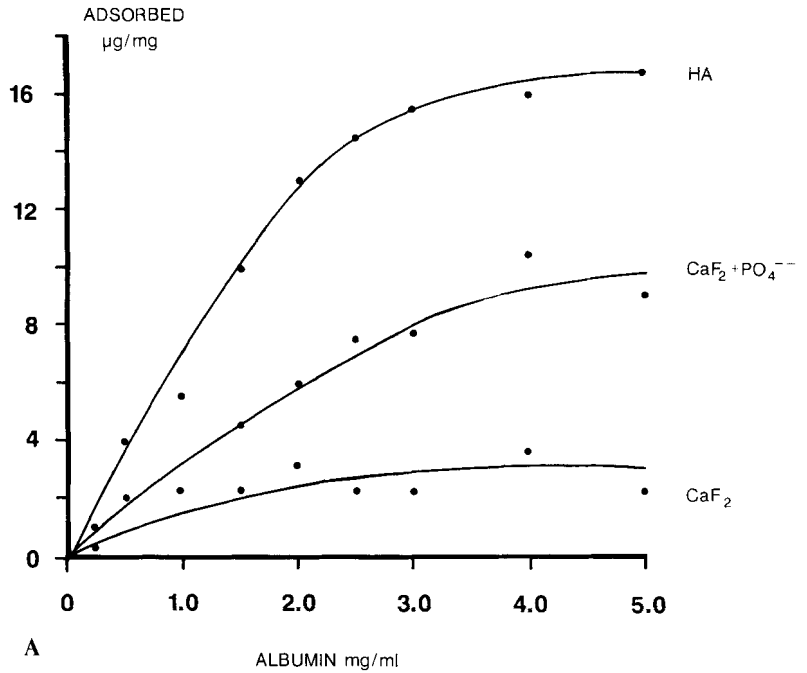


Fig. 1. Protein adsorption isotherms of albumin (1A) and lysozyme (1B) to hydroxyapatite (HA), CaF_2 , and CaF_2 pretreated with phosphate. The ordinate expresses the amount of protein adsorbed per milligram solid matter and the abscissa the amount of protein available in the solutions.

a non-fluoride-containing dentifrice, then rinsed and dried.

The XPS analyses were performed with an ESCA-LAB Mark 2 (Vacuum Generators, VG Scientific). AlK-alpha was used as X-ray source, with 12.5 KV and 10 mA current.

Tablets made from the CaF_2 powder were used as standards. The analyses were performed at the National Laboratory for Surface Studies, NOLAS, ELAB, the Technical University of Trondheim, Trondheim, Norway.

Results

Protein adsorption to CaF₂ and hydroxyapatite

The adsorption of albumin and lysozyme to hydroxyapatite and CaF₂ are shown in Fig. 1. The adsorption is shown as micrograms protein adsorbed per milligram CaF₂ or hydroxyapatite on the ordinate. The abscissa expresses the amount of protein available in the solutions. The results showed that both albumin and lysozyme adsorbed to hydroxyapatite and reached a point of saturation of approximately 12–16 µg protein adsorbed per milligram powder, when more than 2 mg protein per milliliter was present in the solution. In contrast, the proteins adsorbed only sparingly to CaF₂; that is, less than 2 µg protein adsorbed per milligram powder.

When the CaF₂ powder was treated with a sodium phosphate buffer, the adsorption of protein increased markedly, to approximately 6–8 µg protein adsorbed per milligram powder. The particle sizes of the HA and the CaF₂ powder were comparable as judged by scanning electron micrographs.

XPS analyses

The XPS analyses of the two different treated enamel fragments are shown in Fig. 2. The widescan spectrum of sample A (fluoride-treated) shows the characteristic fluoride peak at 685 eV binding energy. This fluoride peak at 685 eV binding energy is not found in the widescan spectrum of sample B (fluoride-treated and pumiced). This indicates that the mechanical brushing removes the fluorides from the enamel surfaces.

When the widescan spectrum of the fluoride-treated enamel fragments is compared with the widescan spectrum obtained from analysis of the CaF₂ standard tablets, the corresponding fluoride peak at 685 eV binding energy is found in both spectra. This binding energy at 685 eV supports the contention that fluoride is bound to calcium as CaF₂ on the enamel surface. The peak at 878 eV is an Auger peak for fluoride. This peak is not connected to binding energy but reflects the detection of fluoride on the enamel surface.

Amino acid analyses of in-vivo-collected pellicle material

The results of the amino acid analyses of the collected pellicle material are shown in Fig. 3. The results are the mean of the three analyses of pellicle material collected from three volunteers. There seem to be no major differences in the amino acid compositions between pellicle collected from normal enamel and pellicle collected from enamel covered by a CaF₂-like material. In both cases the acidic and neutral amino acids were the most abundant, constituting approximately 60% of the total amount of the amino acids. Glutamic acid together with glycine and alanine comprise some 26% of the total amount. Only traces of the sulfur-containing amino acids cysteine and methionine were found. Small amounts of galactosamine and glucosamine indicated the presence of glycoproteins in the pellicle material.

Discussion

The study comparing the in vitro adsorption of proteins to calcium fluoride and to hydroxyapatite showed that the adsorption of proteins by CaF₂ was markedly lower than the uptake by hydroxyapatite. This corresponds with the observations of Eggen & Rølla (28). They showed that fluoride-treated hydroxyapatite took up less acidic proteins, and they suggested that the hydroxyapatite then was covered by a layer of a CaF₂-like material. When we treated the CaF₂ powder with a phosphate buffer, the protein adsorption increased markedly. Kanaya et al. (24) showed that calcium fluoride took up a layer of phosphate when phosphate was present in the solution, and Chander et al. (15) have shown that the CaF₂ crystal changes the charge from positive to negative on exposure to phosphate ions. This obviously increases the protein-binding capacity of the CaF₂. It is known that HA as such is also negatively charged when no divalent counterions are present on the surface.

An adsorption of phosphate to the CaF₂ powder may therefore change the surface

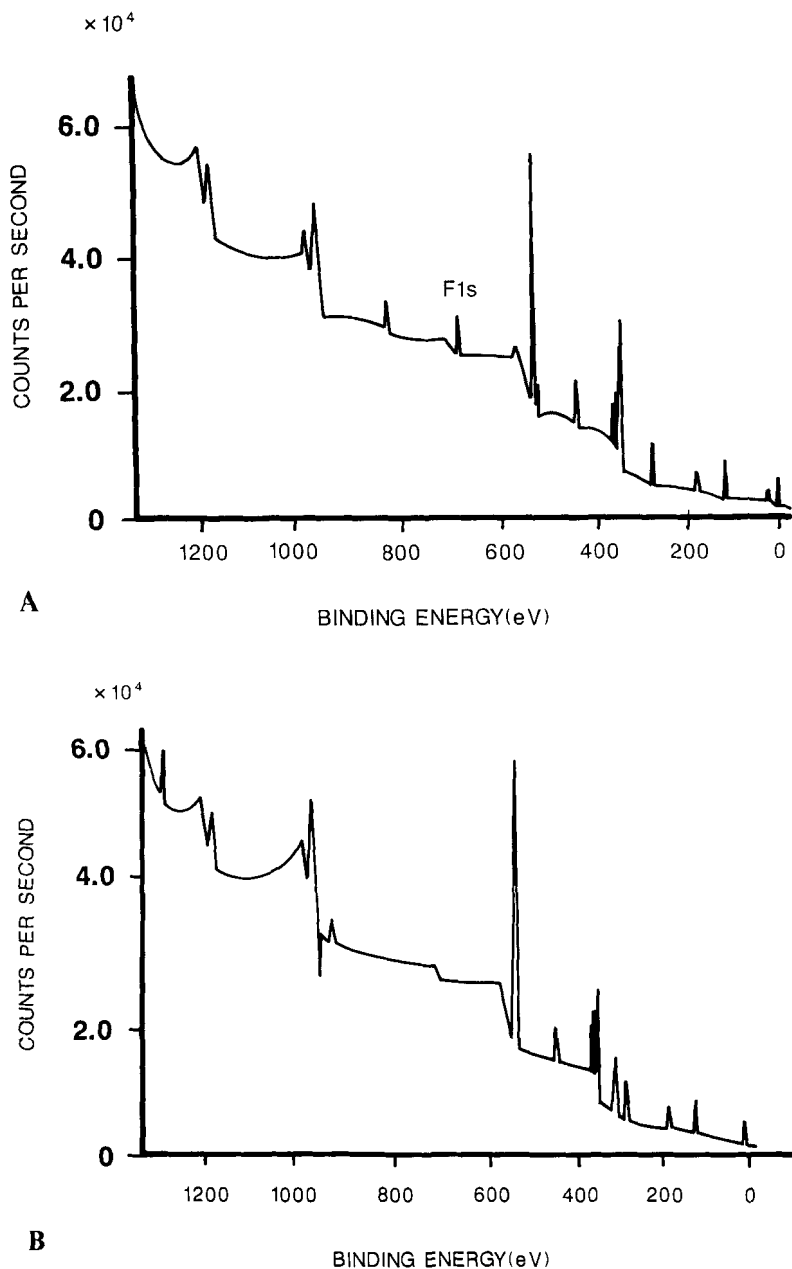


Fig. 2. Electron spectroscopy analyses of enamel fragments: widescan spectrum from fluoride-treated fragment (2A); widescan spectrum from fluoride-treated and then pumiced fragment (2B). The fluoride peak at 685 eV binding energy (F1s) is observed in the spectrum from the fluoride-treated enamel fragment (A) and not in the other.

characteristics of the calcium fluoride globules, and hence the protein adsorption capacity towards that of HA. This may explain the increased adsorption of albumin and lysozyme to the CaF₂ powder when the CaF₂ powder was pretreated with a sodium phosphate buffer.

The XPS analyses supported the contention that treatment of enamel surfaces with an acidulated fluoride solution results in the formation of a layer of globules of a CaF₂-like material. The widescan spectrum from the fluoride-treated enamel fragment showed the fluoride peak at 685 eV binding

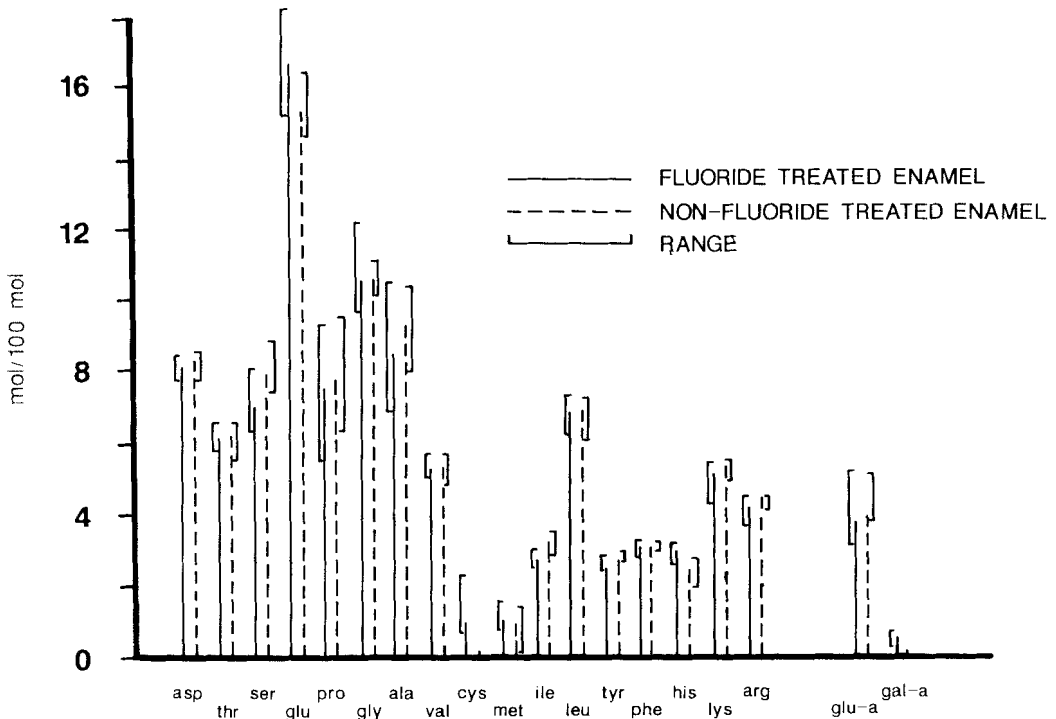


Fig. 3. Amino acid compositions of pellicle collected from CaF_2 -covered enamel and pellicle collected from normal enamel. The results are the mean, with ranges of three analyses of pellicle material collected three times from each of three volunteers.

energy, which corresponds with the fluoride peak in the standard wide-scan spectrum obtained from scanning of the CaF_2 powder. This fluoride peak was not found in the wide-scan spectrum from the fluoride-treated and pumiced enamel fragment. It therefore seems evident that pellicle collected after fluoride treatment was collected from an enamel surface covered with a CaF_2 -like material and that pellicle collected after withdrawal of fluoride treatment was collected from a non- CaF_2 -covered enamel surface. It should be noted that the teeth *in vivo* had been exposed to a much greater mechanical wear than the *in vitro* samples.

The adsorption of proteins to CaF_2 and HA concerned quantitative aspects of protein adsorption, whereas amino acid analyses of pellicle collected *in vivo* concerned qualitative aspects. The *in vitro* adsorption showed only minor adsorption of proteins to CaF_2 , but pellicle collection from fluoride-treated enamel showed abundant pellicle formation. Topical treatment of enamel sur-

face with an acidulated fluoride solution will induce a layer of a CaF_2 -like material on the surface (23). This may alter the chemical properties of the enamel surface and hence the adsorption of salivary proteins to the enamel.

The present study showed no major differences in the amino acid compositions between pellicle collected from CaF_2 -covered enamel and pellicle collected from normal enamel. The results are the mean of three collections and analyses of pellicle material from each volunteer. The pellicle material collected from each person was analyzed separately. The presence of phosphate in the saliva and its adsorption to the CaF_2 crystals on the enamel surface may explain the similarity between pellicle collected from fluoride-treated (CaF_2 -like-covered) enamel and pellicle collected from normal enamel.

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