

ADHESION OF SALIVARY COMPONENTS TO *STREPTOCOCCUS MUTANS* PEPTIDES

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ABSTRACT

Streptococcus mutans is the main microorganism associated to dental caries; it adheres to the dental enamel by interacting with the acquired film's proteins and the cell surface adhesin, called variously antigen PAc. At least two distinct sites in PAc interact with salivary receptors in vitro, these are within residues 816-1213, the most conserved portion of PAc, and within residues 186-469, the alanine-rich sequence. Our purpose was to establish differences or similarities in PAc's peptides interactions with the salivary components of individuals with and without previous caries experience. 40 saliva samples were obtained from patients with (n=20) and without (n=20) caries. The acquired film's proteins were

extracted using hydroxyapatite, and subjected to interaction with three synthetic PAc peptides (PAc (301-319), PAc (365-377), and PAc (1025-1044)) synthesized from PAc's bonding sites to the salivary components. The results show low interaction between the acquired pellicle components and the peptides in all patients. This suggests that the examined PAc's are not relevant as far as the initial adhesion of *Streptococcus mutans* to the tooth's surface is concerned, as defined by the similarities in the results for healthy and affected individuals.

Key Words: salivary proteins, *Streptococcus mutans*, dental caries, PAc's peptides

ADHESIÓN DE COMPONENTES SALIVALES A PÉPTIDOS DE *STREPTOCOCCUS MUTANS*

RESUMEN

Streptococcus mutans es el principal microorganismo asociado a la caries dental, esta bacteria se une al esmalte a través de su interacción con las proteínas de la película adquirida y la proteína de superficie celular comúnmente denominada PAc. Por lo menos dos sitios de PAc interactúan in vitro con los receptores salivales, uno está dentro de la región más conservada de esta proteína que comprende los residuos de 816-1213 y el otro dentro de la secuencia rica en Alanina, residuos 186-469. El objetivo del presente trabajo fue establecer similitudes o diferencias en la interacción de péptidos de PAc con los componentes salivales de individuos con y sin experiencia de caries, para lo cual se tomaron muestras de saliva por salivación espontánea de 20 individuos con caries y 20

sin caries. A partir de las muestras de saliva se extrajeron las proteínas de la película adquirida (PA) utilizando hidroxilapatita sintética y fueron sometidas a la interacción con tres péptidos sintéticos de los segmentos de unión de PAc con los componentes salivales: PAc (301-319), PAc (365-377) y PAc (1024-1044). Los resultados muestran una baja interacción entre los componentes de la PA y los péptidos en todos los individuos, sugiriendo que con base en las similitudes entre los individuos sanos y los individuos con la enfermedad los péptidos de PAc estudiados no son relevantes en la adhesión inicial de *S. mutans*.

Palabras Clave: proteínas salivales, *Streptococcus mutans*, caries dental, péptidos PAc.

INTRODUCTION

Caries is an infectious disease of bacterial origin affecting a very large portion of the world's population (1). The adhesion of cariogenic microorganisms to the tooth's surface is one of the most important characteristics in the development of the disease (2). Initially *Streptococcus mutans* (*S. mutans*) adheres to the dental enamel by means of the PAc's surface cell antigenic protein through the interaction with the salivary components. PAc belongs to a family of surface polypeptides expressed in all oral strepto-

coccus species, exhibiting approximately 65 to 70% primary sequence identity (3). The gene codifying for this protein in *S. mutans* serotype c, has been cloned and entirely sequenced (4) and consists of 4695 base pairs (5). Based on these data, the deduced aminoacid sequence is constituted by 1561 residues (6). Each of these polypeptides has seven structural regions: a signal peptide, a highly charged N-terminal region, an alanine rich repetitive domain (residues 186 to 469) that interacts with salivary receptors (7), a central divergent region, a repetitive

region rich in proline, a highly preserved region, a C-terminal domain, and a sequence anchored in the wall (3). The second binding site for salivary proteins is located within residues 816 to 1213 (8).

PAC functions both *in vivo* and *in vitro* as an adhesin (9), forming hydrophobic links with the salivary film (10). It has been established that immunization with synthetic peptide PAC (301-319) suppresses *S. mutans* colonization on murine teeth and that the use of monoclonal antibodies directly applied to the teeth of non human primates prevents the colonization process and therefore the development of caries (8).

PAC (301-319), PAC (1025-1044), and PAC (365-377) peptides were synthesized (the last one as a trimmer) based on PAC's adhesion sequences to the salivary components described in the literature (8, 11, 12, 13). The purpose of this study was to characterize, by molecular weight, the acquired film's proteins that interact with *S. mutans*' adhesion peptides on patients' teeth (with and without caries).

MATERIALS AND METHODS

The study and informed consent was approved by the Institutional Board of Ethics. Salivary samples were obtained via spontaneous salivation from a total of 40 individuals (18-30 years old), who voluntarily agreed to participate in the study. Subjects were distributed in two groups based on an oral exam and the DMTS index (D: decayed, M: missing (due to caries), F: filled, S: surfaces). The control group consisted of 20 individuals without caries (caries-free individuals) and the experimental group comprised the same number of individuals but with caries (with restored lesions and multiple lesions in enamel and dentin). Patients with permanent dentition, without periodontal disease and no fluoride and sealants therapy participated in the study. Except for the presence of caries, all individuals were clinically healthy. Subjects were then instructed to hold saliva in their mouths for a period of time, and spit into an ice-chilled test tube. The samples were clarified by centrifugation at 10.000 *xg* for 10 minutes and then 100 μ l of a protease inhibiting solution (pH 7.5): Tris (0.1 M) (Pharmacia), Na₂EDTA (2%) (Merck), n-propanol (10%) (Mallinckrodt), phenylmethylsulfonyl fluoride (2 mM) (Sigma) (14) were added for each millilitre of sample and stored at -20°C until use.

Extraction of the proteins adsorbed by hydroxyapatite

5 mg of HA (particle size 300 μ m) were saturated with 1 ml of pH 7.2 buffer: KCl (50 mM) (Sigma), CaCl₂ (1 mM) (Mallinckrodt), MgCl₂·6H₂O (0.1 M) (Merck), KH₂PO₄ (0.78 M) (Mallinckrodt) and K₂HPO₄ (1.22 M) (Mallinckrodt) and stirred constantly for 16 hr. The HA was then separated by centrifugation and 1 ml of saliva was added; the saliva-hydroxyapatite suspension was stirred for 2 hr at room temperature. The suspension was then centrifuged for 15 min at 10.000 *xg* and 4°C. The sediment containing the adhered proteins was then rinsed twice with NaCl (0.1 M) (Sigma). Next, EDTA (0.2 M, pH 7.5) (Merck) was added to dissolve the HA, stirred for 16 hr at room temperature, centrifuged, and the supernatant was stored. EDTA was added to the residue to dissolve leftover HA and the supernatants were blended. The adsorbed fractions of salivary proteins were then dialyzed with cellulose membranes (10.000 Daltons cut point) (Sigma), first against NaCl (0.1 M) for 48 hr, changing the solution every 12 hr; next, against NaCl (0.01 M), and finally against deionized water for the same amount of time used during the first dialysis, changing the solution every 12 hr. The dialysates were lyophilized (Labconco, freezezone 4.5) and stored at -20°C until use (15).

Peptide synthesis

Three segments were synthesized, two of them found in the alanine rich region: PAC (301-319) which corresponds to the ANAANEADYQAKLTAYQTEL sequence, PAC (365-377), TYEAALKQYEADL, used as a trimmer; and one, PAC (1024-1044), QLK-TADLPAGRDETTSFVLV found in the proline rich region. The selected peptides were synthesized in the presence of biotin at *Fundación Instituto de Inmunología de Colombia* (FIDIC). A peptide sequence found in *Plasmodium falciparum*, (TKETMKDHFIEASKKESQLL) was used as negative control.

Western blot

The proteins adhered to the HA were separated by electrophoresis, both in denaturant (SDS-PAGE) and native conditions (NATIVE-PAGE), and transferred into 0.45 μ m nitrocellulose filters (NC) at 150 mA (constant) for three hours. The NCs were rinsed three times, for 10 min, with a TTBS buffer

(Tris (10 mM), NaCl (150 mM) and Tween 20 (0.1%) (Sigma) pH 7.5). After rinsing, they were blocked with skimmed milk (3% in TTBS) for two hours under constant stirring, rinsed once, and stored between 0.5 cm wide filter paper strips at 4°C until use. To incubate the peptides with the NCs, the former were diluted in PBS (Sigma), pH 7.4 (10 mM) with dimethyl sulfoxide (250 µM) (Sigma) at a 10 µg/ml concentration. The NCs with the transferred proteins were incubated under constant agitation for 16 hr at room temperature with each of the three synthetic peptides and the negative control peptide. After incubation, the NCs were rinsed 3 times with TTBS for 10 min each time, incubated with alkaline phosphatase streptavidin (Vector) for two hours, and finally the interactions were identified by means of a color reaction with the NBT/BCIP (Sigma). The approximate molecular weight of the exposed strips was calculated by interpolation within the calibration curve obtained for each gel, based on its relative mobility versus the logarithm of the molecular weight of each of the proteins contained in the corresponding molecular weight marker used specifically according to whether the technique was denaturing or not.

Data analysis

The results were analyzed as relative frequencies of the number of individuals who expressed positive proteins for peptide linkage in each of the molecular weight ranges. Chi-square and exact Fisher non parametric tests were applied in order to define the association between the interaction of the acquired film's proteins and the examined peptides, both in the presence or absence of the disease, using the Statistica program version 5, with a 0.05 significance level.

RESULTS

The acquired film proteins obtained from the saliva covered with hydroxyapatite and then separated by SDS-PAGE, ranged from 20 to 130 kDa (Fig. 1). To simplify the analysis, they were grouped in four molecular weight ranges: 20-39, 40-69, 70-100 and 110-130 kDa. The isolated proteins of all the patients in both study groups showed low linkage (%) with values from 2 to 27%; the most frequent interaction occurred with the PAC (365-377) peptide. No significant differences were found in the

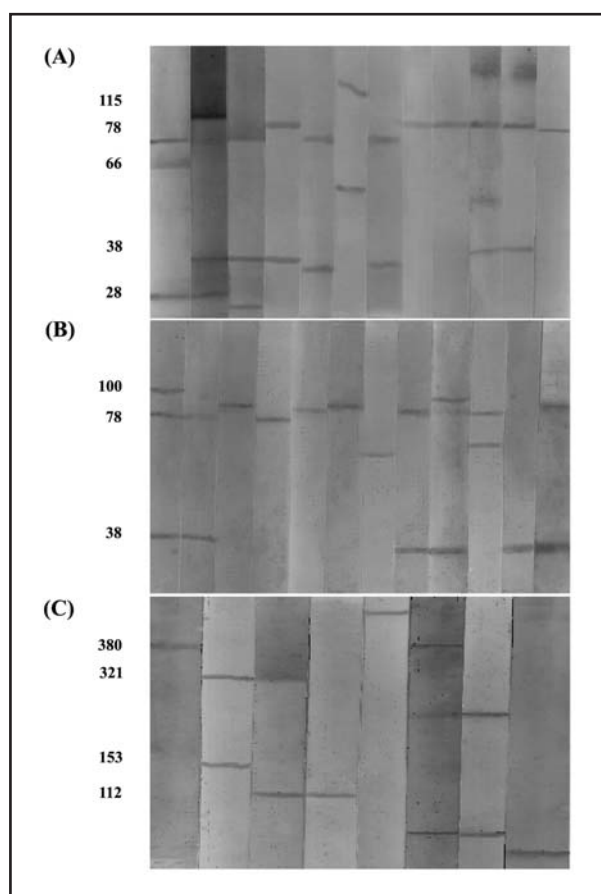


Fig 1: Interaction peptides with salivary components of the acquired pellicle subjected to electrophoresis. (A) Proteins subjected to SDS-PAGE. Lanes 1-4: proteins with PAC 301-319 in the control group (samples 1, 13, 14, 19 respectively). Lanes 5-8: proteins with PAC 301-319 in the cases group (samples 21, 23, 25, 38 respectively). Lanes 9-12: proteins with PAC 365-377 in the control group (samples 1, 3, 10, 19 respectively). (B) Proteins subjected to SDS-PAGE. Lanes 1-4: proteins with PAC 365-377 in the cases group (samples 30, 21, 38, 37 respectively). Lanes 5-8: proteins with PAC 1025-1044 in the control group (samples 1, 5, 6, 15 respectively). Lanes 9-12: proteins with PAC 1025-1044 in the cases group (samples 30, 28, 36, 31 respectively). (C) Proteins subjected to NATIVE PAGE. Lanes 1-4: proteins with PAC 365-377 in the control group (samples 4, 12, 20, 19 respectively). Lanes 5-8: proteins with PAC 365-377 in the cases group (samples 35, 27, 26, 30 respectively).

association rates among both groups or in the molecular weight ranges within each group.

Even if there is no sufficient statistical evidence to ensure that the proportion of individuals associated to each molecular weight range in both study groups is different, some interesting tendencies were observed, i.e., with PAC (301-319) peptide, the proportions in ranges 40-69 and 110-130 kDa, were

lower in the control group than in the case group; PAc (365-377) peptide showed the highest interaction frequencies even if such frequencies were similar for both groups; as for PAc (1025-1044) peptide, the interactions were similar, regardless of the existence of caries experience; the lowest peptide interaction frequency in the studied cases was shown by PAc (1025-1044) peptide in the 110-130 kDa range in both groups.

The proteins separated by NATIVE-PAGE were contained within the 70-430 kDa interval and grouped in the following molecular weight levels: 70-100, 110-160, 250-330 and 380-430 kDa. Only low frequency interaction (3-13%) of these proteins with the PAc (365-377) was observed with no statistically significant differences between the study groups or between the different molecular weight ranges.

The negative control peptide used did not exhibit any adhesion capability to the examined proteins, both for the denaturing and non denaturing techniques.

DISCUSSION

The effectiveness of molecular targets in the search for an anti-caries vaccine has been explored by different means, which include intact protein, synthetic peptides, recombinant peptides, and conjugated peptides assays (2) of the virulence factors involved in *S. mutans* tooth adhesion. This adhesion takes place mostly due to the interaction between the salivary proteins and *S. mutans* PAc proteins.

Due to the well known bonding capacity of the salivary components to hydroxyapatite, as clearly seen *in vivo* when observing the rapid acquisition of salivary film by the dental surface (16), the model employed in the present study, i.e. synthetic hydroxyapatite covered with saliva, is the most widely used when researching bacterial adherence to the tooth's surface.

PAc (301-319), PAc (365-377) and PAc (1025-1044) peptides used in this study have been widely identified in the literature as adhesion sequences to salivary components (11, 17). Furthermore, some of the salivary components recognized for their interaction with the *S. mutans*' PAc, are: two pro-

line rich proteins 28 and 38 kDa (16), a salivary 74 kDa receptor (18), lysozyme (19), α -amylase (16), agglutinin (20), secretory IgA (15), mucin (21), Statherin (22) and some proline rich glycoproteins (23). However, their interaction frequencies were rather low and they showed no statistically significant differences among the two study groups or between the molecular weight ranges established in both groups. These findings confirm once more the complex nature of the salivary components interactions with adhesion proteins of all microorganisms.

Some of these trends show that these peptides play a role in the bonding. PAc (301-319), which was the first peptide recognized for its bonding capacity with the salivary components (6, 9), showed the highest frequency rates among the group of patients with previous experience of caries. It is likely that the rates are affected by the presence of some proteins in the 40-69 kDa range, which have been proposed as *S. mutans*' bonders, such as α -amylase (24), and a 60 kDa glycoprotein (25), and low molecular weight mucins (26) in the 110-130 kDa range. PAc (365-377) has been widely recognized both as an adhesion epitope and a B and T cellular epitope, which makes it a good candidate when designing an anti-caries vaccine (11, 27). Although it shows the highest interaction frequency with the salivary proteins, these events take place similarly in both healthy and diseased patients, thus its reactivity does not differentiate between healthy and diseased patients. In spite of the proven fact that the direct application of peptide PAc (1025-1044) on teeth prevents recolonizing by *S. mutans* (17), the scope of the study enabled us to visualize some interactions which, even if small in number, did not differentiate the (healthy/diseased) condition of the patient. Only in one of the molecular weight ranges the interaction was almost nil; presumably within this range there are no salivary proteins capable of bonding with this peptide.

The adhesion rate found among the acquired pellicle's peptides and proteins separated by means of the NATIVE-PAGE technique, were lower than those previously reported.; The presence of molecular complexes which are frequently formed in the salivary proteins and are not broken after implementing this technique, probably didn't allow for the exposure of the peptide adhesion regions. On

the other hand, when dealing with peptide PAC 365-377, we found frequencies that, although not statistically significant, do show that they have some relation with the bond, particularly in the 70-100 and 380-430 kDa ranges, where the frequency found in the healthy group was slightly lower than in the caries group. The diminished recognition might also occur *in vivo*, favouring the natural caries resistance shown by individual members of this group.

The low interaction values obtained in this study could be affected by physical interferences if we consider that the interactions involve stereospecific reactivity, such as hydrophobic and electrostatic forces (21). Obtaining the salivary components *in vitro* might prompt them to adopt structural conformations different to those of the native protein. These changes in conformation could occur when they are removed from the natural medium where they are secreted, thus creating unique conditions that allow for the acquisition of certain particular

folding. Furthermore, this effect might occur when the proteins are subjected to a series of adhesion and rinsing processes during experimental securing of the acquired pellicle. However, these findings contribute to the knowledge at a molecular level of these interactions, and may be relevant in terms of therapeutic potential. For example, the use of synthetic peptides as additives in mouthwashes or toothpastes could prevent *S. mutans* from adhering to the dental enamel. Any mechanism that interferes with the colonization of this microorganism could very well be an alternative to considerably reduce the impact of caries in humans (28).

In conclusion, the present study shows that the capacity of peptides to bind to the proteins of the acquired film is low, possibly due to the limitations of the model used. However, it does confirm the presence of interactions between peptides and the salivary components. It also confirms that these interactions are similar in healthy and diseased individuals.

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