CHAPTER 4

Identification and characterization of a salivary-pellicle-binding peptide by phage display.

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Submitted

Abstract

Dental biofilms are associated with oral diseases, making their control necessary. One way to control them is to prevent initial bacterial adherence to the salivary pellicle and thereby eventually decrease binding of late colonizing potential pathogens. The goal of this study was to generate a salivary-pellicle-binding peptide (SPBP) with antifouling activity towards primary colonizing bacteria. In order to achieve this goal we aimed to: (i) identify novel SPBPs by phage display. (ii) characterize the binding and antifouling properties of the selected SPBPs. A library of $2x10^9$ phages displaying a random sequence of 12-mer peptides was used to identify peptides that bound selectively to the *in vitro* salivary pellicle. Three rounds of panning resulted in the selection of 10 pellicle-binding phages, each displaying a novel peptide sequence. The peptides were synthesized and their binding to the *in vitro* salivary pellicle was characterized in the presence and absence of calcium ions and Tween-20. The antifouling property of hydroxyapatite (HA) and saliva-coated HA discs treated with and without SPBPs were evaluated against *Streptococcus gordonii*. Ten unique SPBPs were identified using the phage display. One of these peptides, SPBP 10 (NSAAVRAYSPPS), exhibited significant binding to the *in vitro* salivary pellicle which was neither influenced by calcium ions, nor affected by up to 0.5% Tween-20. Its antifouling property against *S. gordonii* was significantly higher on the treated surfaces than on untreated surfaces. Use of the phage display library enabled us to find a specific SPBP with antifouling property towards *S. gordonii*.

Introduction

In the oral cavity, the salivary proteins and peptides are selectively adsorbed to the enamel surface to form a proteinaceous film called the acquired salivary pellicle (Zaharadnik et al. 1976; Lendenmann et al. 2000). This pellicle is composed of peptides such as statherin and histatins; (glyco)proteins including proline-rich proteins and mucins; and lipids. Together these constituents make the pellicle dynamic in structure and function. Some domains of the salivary pellicle-forming proteins serve as receptors for adherence of early colonizing bacteria during dental-biofilm formation (Hay 1973; Jenkinson & Lamont 1997).

The development of a dental biofilm is a multi-step process which begins when oral Streptococcal species, the early colonizers, recognize and adhere to moieties of proteins in the salivary pellicle (Li et al. 2004). One of the early colonizers, *Streptococcus gordonii*, binds to the salivary amylase as part of the salivary pellicle via the protein A moiety on its cell wall (Rogers et al. 2001). When the pellicle is left untreated, the early colonizers aggregate with late-colonizing bacteria which finally will result in the development of a mature biofilm.

S. gordonii co-aggregates with other primary colonizer such as *Actinomyces naeslundii* and secondary colonizers such as *Porphyromonas gingivalis* which are both implicated in periodontitis (Egland et al. 2001; Lamont et al. 2002; Kuboniwa et al. 2006). *S. gordonii* also promotes adherence of opportunistic yeast pathogen such as *Candida albicans* (O'Sullivan et al. 2000). Besides periodontal infection, dental biofilms are also suggested to be associated with systemic diseases and preterm births, underlining the need for their prevention and control (Senpuku et al. 2003; Han et al. 2004). Biofilms can be controlled in various ways, example by preventing bacterial adherence to the enamel surface, or by removing established biofilms.

Oral care products currently used to control dental biofilms include antibacterial agents such as ethanol, sodium lauryl sulfate, triclosan, cetylpyridinium chloride, chlorhexidine digluconate, and nisin. Although *in vitro* studies with these antimicrobial agents on dental biofilm models showed antimicrobial efficacy, they did not completely remove the biofilms (Corbin et al. 2011).

Synthetic combinatorial technologies have been used to develop new antimicrobial peptide libraries with potential antibiofilm activity. Such a library was used to identify a decapeptide (KKVVFKVKFK), named KSL, which has a broad range microbicidal activity, and also inhibits biofilm development (Concannon et al. 2003; Leung et al. 2005). Phage display is an alternative, established method for identifying novel peptides that have the selectivity to target biomolecules, such as enzymes, cell-surface receptors and biomaterials such as hydroxyapatite (HA) and titanium (Rousch et al. 1998; Roy et al. 2008; Liu et al. 2010).

The goal of this study was to generate a salivary-pellicle-binding peptide with antifouling activity towards bacteria. In order to achieve this goal we aimed to: (i) to identify peptide sequences with specific selectivity to the *in vitro* salivary pellicle using the phage display library. (ii) to determine the binding characteristics of the selected peptides and evaluate their antifouling property against *S. gordonii*.

Materials and Methods

Collection of saliva

Unstimulated human whole saliva samples were collected from 10 healthy volunteers, in tubes pre-cooled on ice. The saliva samples were vortexed for 2 min to reduce its viscosity and subsequently centrifuged (40 min, 3,000 *g*, 4 °C) to remove insoluble cellular debris. The clarified human whole saliva (CHWS) was stored in aliquots at -20 °C.

Bacteria and chemicals

Escherichia coli ER2738 was maintained on Luria Bertani (LB) medium agar plates containing 10 g/l Bacto-Tryptone (Difco, Detroit, USA), 5 g/l yeast extract (Difco, Detroit, MI, USA), 10 g/l NaCl (Sigma-Aldrich, St. Louis, MO, USA), 15 g/l agar (BD, Sparks, USA) and 45 µM Tetracycline (Sigma-Aldrich) under aerobic conditions at 37 °C. *S. gordonii* ATCC 1053 was maintained on brain heart infusion (BHI) agar plates containing 37 g/l BHI (BD bioscience, USA) and agar (BD) under anaerobic (10% CO_2, 10% H_2, and 80% N_2) conditions at 37 °C. Titration and amplification of eluted phages was done using LB agar plates supplemented with 0.2 mM isopropyl-β-D thiogalactopyranoside (Sigma-Aldrich) and 0.1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal, Sigma-Aldrich).

In vitro salivary pellicle on hydroxyapatite

Prior to surface panning with the phage display peptide library, a mixture of 1 mg of HA (surface area = 40 m^2/g, Biorad, Macro-Prep ceramic hydroxyapatite, Type I, 20µM, Hercules, CA, USA) with 0.5 ml CHWS was kept in suspension by end-over-end rotation at 37 °C for 2 h. The mixture was centrifuged at 14,000 *g* at room temperature (RT) for 15 min, the supernatant was removed by pipetting and the saliva-coated HA sediment was washed three times with artificial saliva buffer (2 mM K_2HPO_4, 50mM KCl, 1mM $CaCl_2$, 0.1mM $MgCl_2$, pH 6.8).

The saliva-coated HA for peptide binding assay was prepared by incubating 100 mg of HA with 3 ml of CHWS. The mixture was continuously mixed by end-over-end rotation at 4 °C for overnight, centrifuged and the saliva-coated HA was washed with artificial saliva buffer as mentioned above.

Surface panning of in vitro salivary pellicle using phage display peptide library

The Ph.D.-12 Phage Display Peptide Library from New England Biolabs (Ipswich, MA, USA) was used to select peptides binding to HA coated with an *in vitro* formed salivary pellicle. The phage library consisted of approximately $2x10^9$ independent clones with each phage displaying a unique 12-mer peptide. 1 µl of the phage library, representing $2x10^9$ phages was added to 1 mg of saliva-coated HA suspended in 200 µL of artificial saliva buffer and mixed continuously by end-over-end rotation at RT for 20 min. The suspension was centrifuged at 10,000 *g* for 1 min and the supernatant containing unadsorbed phages was discarded. The pellet was washed five times to remove non-specifically bound phages. Washing was done with 1 ml of artificial saliva buffer containing 0.1% Tween-20 (v/v), mixed end-to-end for 5 min and centrifuged at 10,000 g for 1 min at RT. The phages adsorbed to the salivary pellicle were eluted with 1 mL of 0.2 M Glycine-HCl, pH 2.2, supplemented with 1 mg/ml BSA, incubated at RT for 10 min and neutralized with 150 µl of 1M Tris-HCl, pH 9.1.

The eluted phages were amplified by infecting 20 ml of 100 fold diluted overnight culture of *E. coli* in LB broth and incubated at 37 °C for 5 h in well aerated condition. The suspension was centrifuged at 3000 *g* at 4 °C for 30 min to remove bacteria. The supernatant, containing phages, was transferred to a new vial, and centrifuged again to remove any remaining bacterial debris. The supernatant, containing amplified phages, was precipitated with 20% (w/v) polyethylene glycol (PEG) 8000 and 2.5 M NaCl at 4 °C for overnight. The phage precipitate was recovered by centrifugation at 3,000 *g* at 4 °C for 30 min and the pellet was suspended in 200µl Tris buffered saline (TBS). 10 µL of the amplified phages was titrated as described above to confirm amplification of phages. The steps described above comprised one round of panning. Phages amplified in the first round of panning were used for the second round of panning and the steps were repeated as described above. In total three rounds of panning were performed. In the second and third round of panning the pellet, containing phages bound to saliva-coated HA, was washed ten times with saliva buffer containing 0.5% Tween-20.

DNA extraction and sequencing

After 3 rounds of panning, sixteen blue plaques, each representing a single phage clone, were selected and transferred to a single tube containing 1 ml of a 100-fold diluted overnight culture of *E. coli* in LB broth. The tubes were incubated for 5 h at 37 °C with vigorous shaking and subsequently centrifuged at 10,000 *g* for 1 min. The amplified, individual clones of phages were precipitated with 200 µL of PEG/NaCl to 500 µl supernatant, incubated for 20 min at RT and

centrifuged at 10,000 *g* for 10 min. The phage pellet was resuspended in 100 µl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 4 M Sodium iodide to extract DNA. The DNA was precipitated with 250 µl ethanol and incubated for 20 min at RT. The phage DNA was recovered by centrifugation at 10,000 *g* for 10 min, washed with 500 µl of 70% ethanol at -20 °C and air dried by laminar flow. The pellet was suspended in 30 µl 10mM Tris-HCl, pH 8.0, 1 mM EDTA and stored at -20 °C.

The DNA region of phage genome encoding for the peptide was decoded using primers -96 gIII (5'CCC TCA TAG TTA GCG TAA CG 3') and -28 gIII (5'GTA TGG GAT TTT GCT AAA CAA C 3'). The sequencing of phage DNA region and primer synthesis was done by ServiceXS (Leiden, The Netherlands).

The DNA sequences for the peptides were translated to amino acid sequences using appropriate software (CLC Main Workbench 5, Aarhus, Denmark). The peptides identified were designated as SPBPs.

Peptide synthesis and purification

The emerging SPBPs were synthesized with solid-phase peptide synthesis using Fmoc chemistry with a Syro II peptide synthesizer (Syro II, MultiSynTech, GmbH, Germany). The synthesized peptides were purified with reverse phase – high-performance liquid chromatography (RP-HPLC, Jasco Corporation, Tokyo, Japan), as described previously (Veerman et al. 2007). The peptide solutions, except for SPBP 15, were prepared in 0.1% trifluoroacetic acid (TFA) at a concentration of 10 mg/ml. SPBP 15 was first dissolved in dimethylformamide and then in 0.1% TFA. The individual peptide solutions were introduced in VYDAC C18-column (218TP, 1.0x25CM, 10µm particles, Vydac, Hesperia, CA) equilibrated with 0.1% TFA. Elution was performed with a linear gradient from 15-40% acetonitrile containing 0.1% TFA in 30 min at a flow rate of 4 ml/min. The eluate was monitored at 220 nm and the peak fractions were pooled and lyophilized. The purity of the peptides was determined with RP-HPLC and was at least 90%. The authenticity of the peptides was confirmed with matrix-assisted laser desorption/ionization-reflectron time-of-flight mass spectrometry (MALDI-TOF MS, Microflex, Bruker, Germany) as described previously (Khechine et al. 2011).

Peptide binding assay

The SPBPs were evaluated for binding to HA and saliva-coated HA. 25 µM (~30 µg) of each SPBP was incubated with 100 mg of HA and saliva-coated HA in a final volume of 1 ml of artificial saliva buffer in eppendorf vials. Vials were incubated rotating end-over-end on an

orbital rotator for 90 min at RT. The HA was recovered by centrifugation at 10,000 *g* at RT for 3 min. The supernatant, containing unadsorbed peptides, was transferred to a new vial and centrifuged three times to ensure complete removal of HA from the supernatant. The amount of unadsorbed peptides in the supernatant was quantified with RP-HPLC under same conditions used for purification of the peptides as described above. The amount of peptide bound to the HA and saliva-coated HA was calculated by subtracting their peak areas from the peak area of the control peptide solution without HA. All experiments were conducted in triplicate and repeated at least two times.

Bacterial adherence assay

Adherence of bacteria to HA and saliva-coated HA discs treated with SPBP 10 peptide was evaluated using the active attachment model as described previously (Exterkate et al. 2010). HA discs (diameter: 9.7 mm diameter; thickness: 1.7 mm Himed, NY, USA) were incubated overnight at 4 °C with either 1.5 ml of artificial saliva buffer or 1.5 ml saliva for each disc in a 24 well plate. Subsequently the HA discs were washed 3 times by transferring the lid to a 24 well plate containing 1.6 ml of artificial saliva buffer and moved 10 times up and down to remove excess saliva. The uncoated and saliva-coated HA discs were treated with SPBP 10 peptide for 90 min and washed twice with tris buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.5). An overnight culture of *S. gordonii* grown in BHI medium was washed twice in TBS and final cell density was adjusted to approximately 10^7 cells/ml. The HA discs were incubated anaerobically with 1.5 ml of bacterial suspension for 2 h at 37 °C and subsequently washed twice with TBS to remove the non-adherent bacteria. Then the discs were transferred to vials containing 2 ml TBS and the attached layer of bacteria were dispersed by sonication for 1 min with 1 s pulses. The resulting suspension was plated in different dilutions on BHI plates and incubated anaerobically for 48 h at 37 °C before CFUs were counted.

Statistical analysis

The statistical analysis was performed using the statistical software package IBM SPSS version 20 (IBM, Armonk, NY, USA). The data for binding of the SPBPs to HA and saliva-coated HA were analyzed using Mann-Whitney U test. The Kruskal-Wallis test was used for the analysis of data of the dose response assays of SPBP 3 and SPBP 10, binding of SPBP 10 to HA and saliva-coated HA in presence and absence of calcium and Tween-20 and also for bacterial adherence assay. All levels of significance were set at $p \leq 0.05$.

Results

Surface panning of in vitro *salivary pellicle with phage display peptide library*

The phage display peptide library containing approximately $2x10^9$ clones, each displaying a unique 12-mer peptide sequence, was panned for three rounds against saliva-coated HA. After three rounds of panning, sixteen plaques were selected and the DNA and peptide sequence of the individual clone was determined. As a result 10 unique SPBP sequences were identified (Table 1). Out of the 16 phage clones, four clones had no peptide encoding DNA region, SPBP 9 and SPBP 13 occurred twice while the other peptides occurred once.

The theoretical isoelectric point (pI) and hydropathy values of all the peptides were determined *in silico*. Interestingly the pI values of the peptides are ≥ pH 7 and the hydrophobicity values show that all the peptides are hydrophilic. Analyses of amino acid composition of all the SPBPs reveal predominance of proline (P) and histidine (H) rich sequences. *In silico* analysis of the secondary structure of peptides showed that only one peptide, SPBP 10 (NSAAVRAYSPPS), has a tendency to adopt a α helical structure spanning amino acid sequence AAVR.

Table 1 – Ten different salivary-pellicle-binding-peptides (SPBPs) were selected against *in vitro* salivary pellicle using phage display peptide library.

Peptide	Amino acid sequence	pI	Charge at pH 7	Histidine / Proline content (%)	hydropathy values (Kyte & Doolittle)	Secondary structure
SPBP 2	RPLTISSAADHF	6.74	0	8.3 / 8.3	-0.033	n/a
SPBP 3	EAHVMHKVAPRP	8.86	+1	16.7 / 16.7	-0.633	n/a
SPBP 4	SEPPKAHGVLSS	6.47	0	8.3/16.7	-0.567	n/a
SPBP 6	LPSPPRIPGHKL	11	+2	8.3/33.3	-0.592	n/a
SPBP 9	TLHDLTRGQRTT	9.29	+1	8.3/0	-1.233	n/a
SPBP 10	NSAAVRAYSPPS	8.75	+1	0/16.7	-0.442	α helical structure
SPBP 11	QHANHQAWNNLR	9.76	+1	16.7/0	-1.825	n/a
SPBP12	SHNNSPLSYKPS	8.33	+1	8.3/16.7	-1.5	n/a
SPBP 13	ANPYSSTAKPAG	8.63	+1	0/16.7	-0.767	n/a
SPBP 15	NFMESLPRLGMH	6.75	0	8.3/8.3	-0.275	n/a

The underlined residues have a tendency to form α helical structure.

Peptide binding assay

All the 10 novel SPBPs were analyzed for binding to HA and saliva-coated HA. The peptides showed a great variation in binding. SPBP 10 exhibited higher adsorption to saliva-coated HA than to uncoated HA (Fig. 1). SPBP 3 showed equal binding to HA and saliva-coated HA, whereas, SPBP 4 exhibited less binding to the saliva-coated HA than to HA. Compared to SPBP 10, SPBPs - 6, 9, 11, 12, and 13 bound less to saliva-coated HA.

The interaction of SPBP10 with HA and saliva-coated HA was further characterized using higher amounts of HA and saliva-coated HA (100 and 200 mg). SPBP 3 was used as a negative control. At increasing amount of either HA or saliva-coated HA, SPBP 10 binding increased. Also in these experiments SPBP 10 bound better to saliva-coated HA than to uncoated HA, confirming the affinity of SPBP 10 for the *in vitro* salivary pellicle (Fig. 2).

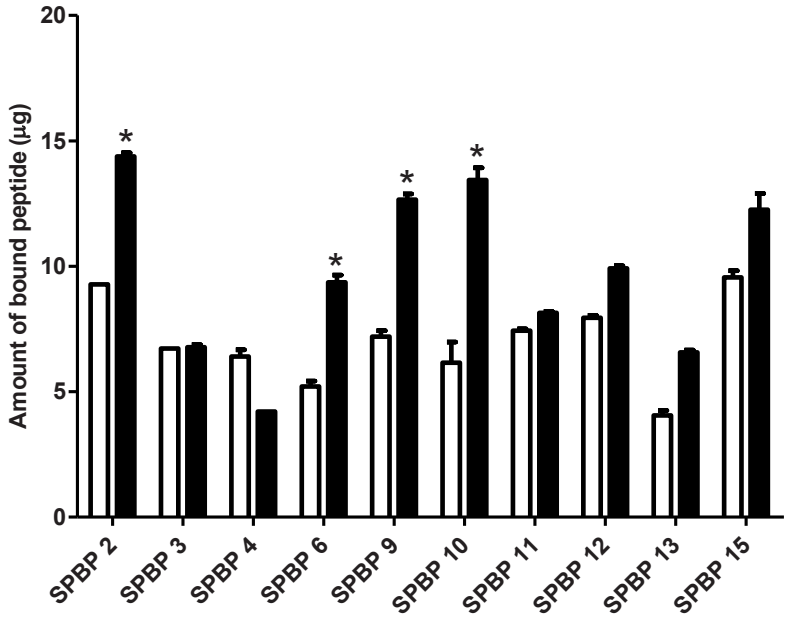

Figure 1 - Binding of SPBPs with HA (white bars) and saliva-coated HA (black bars). The bar graph represents the amount of bound peptide. N=3, *P ≤ 0.05.

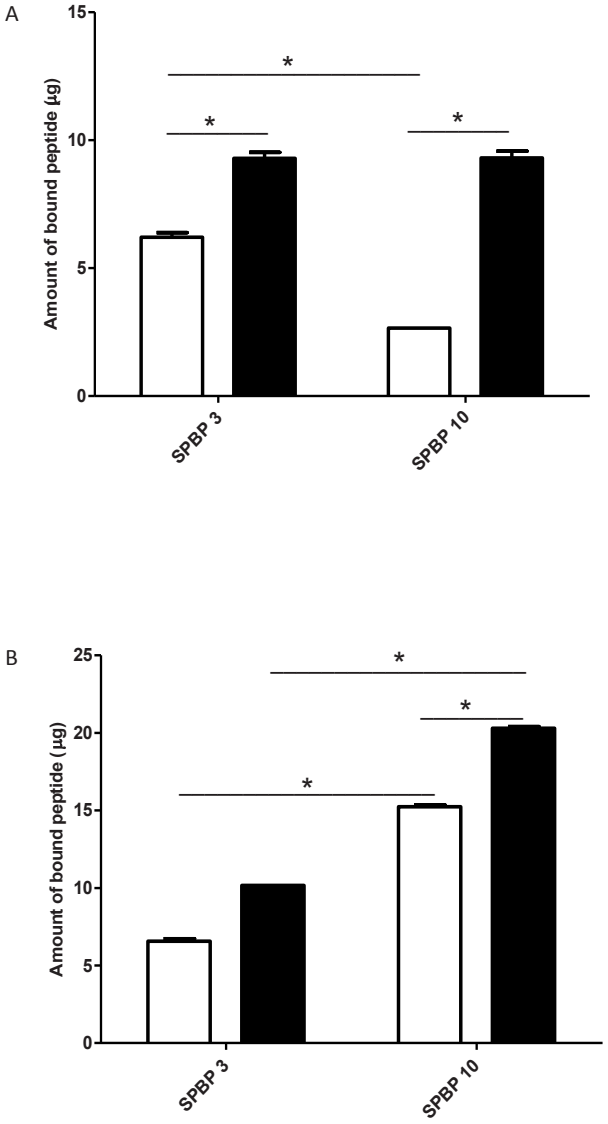

Figure 2 - Dose response assay of peptides SPBP 10 and SPBP 3 (a negative control). A) the binding assay was performed with 100 mg of HA (white bars) and 200 mg of HA (black bars). B) the binding assay with 100 mg of saliva-coated HA (white bars) and 200 mg of saliva-coated HA (black bars) N=3, *P ≤ 0.05.

Effect of $CaCl_2$ and Tween-20 on binding of SPBP 10

Binding of SPBP10 to HA was evaluated in artificial saliva buffer, containing 1 mM $CaCl_2$. To determine whether calcium ions were involved in the interaction between SPBP10 and (saliva-coated) HA, we repeated the assay in saliva buffer without calcium. Essentially the same result was found for binding of SPBP 10, to HA and to saliva-coated HA (Fig.3a). To examine if the SPBP 10 and HA interaction was stabilized by hydrophobic interactions, the effect of Tween-20 at 0.1% and 0.5% concentrations was tested. Binding of SPBP 10 to saliva-coated HA in the presence and absence of Tween-20 was virtually identical where as binding to HA in the presence of Tween-20 was somewhat decreased (Fig. 3b).

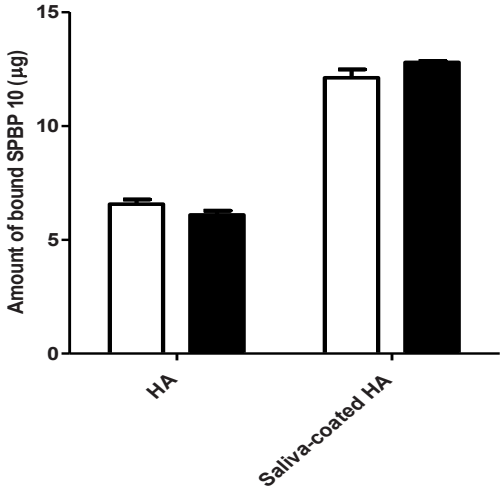

Figure 3a. Binding of SPBP 10 to HA and saliva-coated HA in the presence (white bars) and absence (black bars) of 1mM $CaCl_2$. N=3, *P ≤ 0.05.

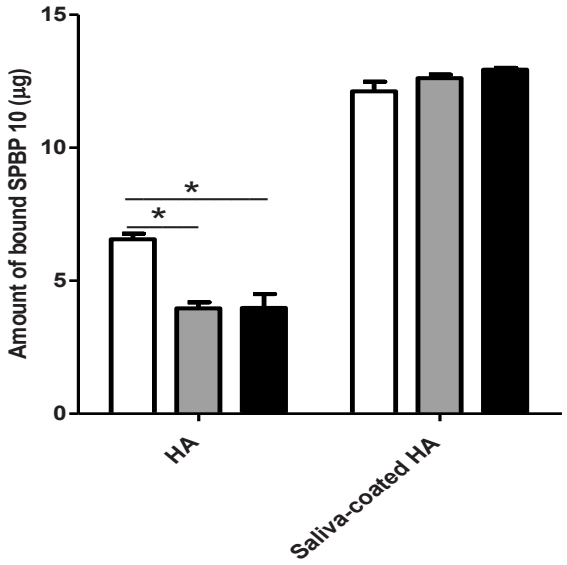

Figure 3b. Binding of SPBP 10 to HA and saliva-coated HA in absence (white bars) and presence of 0.1 % Tween-20 (grey bars) and 0.5% Tween-20 (black bars). N=3, *P ≤ 0.05.

Antifouling activity of SPBP 10

To determine whether SPBP 10 exerts antifouling activity, the effect of treatment of HA with SPBP 10 on the adherence of *S. gordonii* was evaluated in an *in vitro* adherence model. In the absence of SPBP10, approximately 7.7±5.9x10^5 CFU/ml *S gordonii* adhered to the surface of the HA discs. After treatment of HA discs with SPBP 10, the number of adhered bacteria decreased significantly to approximately 3.8±1.5x10^5 CFU/ml ($p \leq 0.05$). Considerably fewer bacteria adhered to saliva-coated discs than to HA discs. After treatment with SPBP 10 the number of adhered bacteria was even lower, but no statistical significance was reached (Fig. 4).

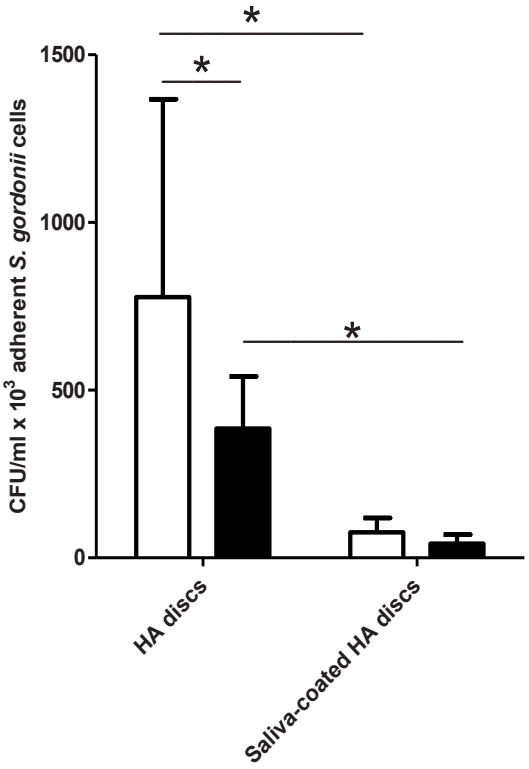

Figure 4. Antifouling activity of SPBP 10 against *S. gordonii*. The cells adherent to HA discs without peptide treatment (white bars) and with peptide treatment (black bars). N=6, *P ≤ 0.05.

Discussion

Using the phage display peptide library, we identified 10 salivary-pellicle-binding phages displaying novel peptide sequences (Table 1). Among the 10 peptides that were characterized, SPBP 10 exhibited highest binding to the saliva-coated HA (Fig. 2). Sequence analysis of SPBP 10 reveals the presence of 5 hydrophobic amino acids that occur consecutively (NSAAVRAYSPPS), forming a hydrophobic region, which might be the driving force for binding to the *in vitro* salivary pellicle. Also this is the only peptide which shows a tendency to adopt α helical structure (residues spanning AAVR). Possibly this unique conformational characteristic within the full set of peptides, plays an important role in pellicle binding. The affinity of SPBP 10 to HA is probably due to the positively charged arginine that binds to negatively charged phosphates (Table 1). SPBP 4 exhibited more binding to the HA than to saliva-coated HA, suggesting that the protein pellicle masks SPBP 4 binding sites on the HA surface (Fig. 1).

Both saliva and the salivary pellicle contain calcium ions which play a vital role in remineralization of enamel *in situ* (Wolf & Larson 2009). Here it was found that binding of SPBP 10 to HA and saliva-coated HA was not affected by calcium ions (Fig. 3a), suggesting that SPBP 10 binding to the HA is not mediated by calcium ions in the surface. This is in line with the fact that SPBP 10 does not contain negatively charged residues which potentially interact with positively charged calcium ions, as example occurs in acidic proline rich proteins. (Moreno et al. 1979). The presence of 0.1% and 0.5% Tween-20, used during panning experiments, did not abolish the affinity of SPBP 10 to the salivary pellicle (Fig. 3b). These assays confirm the selectivity of SPBP 10 to the *in vitro* salivary pellicle and hence signifies successful panning using phage peptide library.

A protein blast analysis of all peptides revealed only for SPBP 10 a homology of 73% to the chain A of the N-terminal Ig1-2 and Ig1-3 of human receptor protein tyrosine phosphatase sigma (RPTPσ) which plays a role in glandular morphogenesis (Wang & Laurie 2004). RPTPσ has also been demostrated to bind to heparan and chondroitin sulfate proteoglycans (Coles et al. 2011). Since sulfated glycans also occur in the salivary mucins and these are present in salivary pellicle, it is tempting to hypothesize that mucins in the *in vitro* salivary pellicle might function as receptor for SBP10 (Veerman et al. 1997). Future experiments need to confirm this hypothesis.

In a previous study binding of phages, displaying pentavalent peptides on its protein coat, to HA was examined (Roy et al. 2008). In this way binding can be stabilized by multivalent interactions between the phage and the mineral surface. In contrast with the present study, the

peptides that emerged from three rounds of panning were synthesized and tested for their HA-binding properties. This favors selection of peptides with moderate to high affinity for the HA surface, and likely resulted in a different set of HA binding peptides. The limitation of using an *in vitro* salivary pellicle is that the pellicle composition can be different from that of the in situ formed pellicle. For instance, comparative analysis of protein composition of *in vitro* and *in vivo* salivary pellicle has revealed difference in the amount of acidic proline-rich proteins (Yao et al. 2001). Further studies will be required to explore the binding behavior of the peptide such as SPBP 10 to the *in situ* formed pellicle.

This study is first of its kind in which using phage display a novel peptide was selected, SPBP 10, which binds to the *in vitro* salivary pellicle on HA. This peptide also showed significant antifouling activity against the early colonizing bacteria, *S. gordonii*. This suggests that this approach shows potential for design of compounds that can be applied for controlling the early stage of bacterial adherence on biomaterials.

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