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Design and Characterization of an Acid-Activated Antimicrobial Peptide

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Abstract

Dental caries is a microbial biofilm infection in which the metabolic activities of plaque bacteria result in a dramatic pH decrease and shift the demineralization/ remineralization equilibrium on the tooth surface towards demineralization. In addition to causing a net loss in tooth minerals creation of an acidic environment favors growth of acid enduring and acid generating species, which causes further reduction in the plaque pH. In this study we developed a prototype antimicrobial peptide capable of achieving high activity exclusively at low environmental pH to target bacterial species like *Streptococcus mutans* that produce acid and thrive under the low pH conditions detrimental for tooth integrity. The features of clavanin A, a naturally occurring peptide rich in histidine and phenylalanine residues with pH-dependent antimicrobial activity, served as a design basis for these prototype “acid-activated peptides” (AAPs). Employing the major cariogenic species *S. mutans* as a model system, the two AAPs characterized in this study exhibited a striking pH-dependent antimicrobial activity which correlated well with the calculated charge distribution. This type of peptide represents a potential new way to combat dental caries.

Keywords

Targeted antimicrobial therapy; pH dependent antimicrobial activity; biofilm; *Streptococcus mutans*

1. Introduction

Dental caries (tooth decay) is one of the most prevalent infectious diseases affecting humans and despite decade long efforts to reduce the disease a recent survey revealed an increased incidence in young children (NIDCR – http://www.nidcr.nih.gov/). Certain bacterial species including the major oral pathogen *Streptococcus mutans* reside in the biofilms on the tooth surface and produce acids upon fermentation of dietary carbohydrates (1). Continued acid production eventually drops the pH below the critical threshold of 5.5 and triggers a shift in the enamel demineralization/ remineralization equilibrium towards demineralization (2). This decrease in pH favors the growth of acid-tolerant and acid-generating species which in turn accelerate the demineralization process and subsequent caries development.

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Current approaches to reduce caries include neutralization of plaque pH with sodium bicarbonate (baking soda) containing chewing gums and toothpastes (3). Also recommended are dietary changes to reduce the carbohydrate sources that are metabolized to acids by limiting sugar intake or substitution with sugar analogues (4). The effect of these approaches, however, is not permanent and requires repeated application or change of dietary habit for sustained effects. Other efforts have attempted to remove the major causative agent of the disease *S. mutans* from the biofilm community via replacement therapy, passive or active immunization attempts (5), and targeted antimicrobial agents (6).

In this study, we explore an alternative approach to target bacteria based on acid production, which is the cause for tooth demineralization. The basic concept of a pH-responsive antibiotic has been realized in nature by the marine organism *Styela clava* (7) in form of the 23 amino acid long peptide clavanin A that displays a significant increase in antimicrobial activity at low pH compared to neutral conditions (8). The pH-dependent activity of clavanin A requires the presence of multiple histidine (His) and phenylalanine (Phe) residues (8,9). Substitutions of these amino acids resulted in increased antimicrobial activity at neutral pH and thus reduced the differential activity of the peptide at low and neutral pH. Using the central features discovered for the pH-responsiveness of clavanin A as a design template, we constructed two 14 aa long peptides, rich in both histidine and phenylananine residues, and tested their antimicrobial activities under different pH conditions.

2. Methods and Materials

2.1 Strains and growth conditions

All streptococci and the *Streptococcus mutans* UA140 derivative JM11 (10) (UA140::*pldh-luc*, Spc^r) were grown anaerobically (N_2 85%, H_2 10%, CO_2 5%) in Todd–Hewitt (TH) medium (Difco) or agar plates at 37 °C.

2.2 Peptide Synthesis

Peptides were synthesized using Fmoc (9-fluorenylmethoxy carbonyl) solid-phase synthesis (431A peptide synthesizer; Applied Biosciences, Foster City, CA), as previously described (6), utilizing double coupling cycles at a 0.25 mmol scale. Briefly, N-terminal deprotection was conducted in 20% (v/v) piperidine/N-methylpyrrolidone (NMP) for 3 minutes followed by 8 washes with NMP. For the coupling cycle, amino acids were solubilized in 0.45 M N-hdroxybenzotrazole (HOBt)/HBTU (O-benzotriazole-N, N, N, N–tetramethyl-uronium hexafluoro-phosphate) in dimethylformamide (DMF) with 0.9 M diisopropyl ethylamine (DIEA) (1:1:1:2 ratio Fmoc-amino acid:HOBt:HBTU:DIEA) before being added to the resin for 30 min, followed by 10 NMP rinses. Peptides were N-terminally labeled with rhodamine through the addition of 4-molar excess rhodamine B to the resin in HOBt/HBTU/DIEA coupling solution (as described above) with shaking at ambient temperature for 24 hr followed by 10 rinses in dichloromethane (DCM). After synthesis, the resin was washed 4 × in DCM, 1 × in MEOH, and dried 24 hr under desiccant vacuum. Completed peptides were cleaved from the resin with 90% trifluoroacetic acid (TFA) and appropriate scavenging reagents. All amino acids, synthesis resins and reagents were peptide synthesis grade (Anaspec, San Jose, CA). All peptides were purified to 90 to 95% by reverse-phase high-performance liquid chromatography (ACTA purifier; Amersham, UK). Peptide mass was determined by electrospray ionization (ESI) mass spectrometry (3100 Mass Detector, Waters, Milford, MA).

2.3 Circular Dichroism (CD) measurements

CD spectra were recorded at room temperature on a J-715 spectropolarimeter (JASCO). The optical path cuvette was 1 mm and the CD signal was monitored in the wavelength range of

195–260 nm. The peptide concentration was 0.2 mM for AAP1 and 0.22 mM for AAP2 in 50% (v/v) TFE. The solvent pH was adjusted with HCl (1M) or NaOH (1M). For each experiment, the data were averaged over four scans, taken with a 2 s time constant and with the blank subtracted.

2.4 Determination of antimicrobial activity as minimal inhibition concentration (MIC)

Peptides antimicrobial activity was determined by broth microdilution (11) using ~1×10^5 CFU/ml of the streptococci to be tested (*S. mutans* strains UA140, UA 159, LT-11, MB-2148, NG8, NCTC 10449 as well as *S. sanguinis*, *S. mitis*, *S. gordonii* and *S. cristatus*) in TH adjusted to pH 7.5 and 5.0. Two-fold serial dilutions of peptides were added and the samples were incubated anaerobically at 37°C. MIC was determined as the peptide concentration that inhibited growth after 48 hr of incubation.

2.5 Determination of peptide bactericidal kinetics

The short-term killing rate of peptides was determined by time-kill experiments (6). In brief, *S. mutans* UA140 was diluted to ~1×10^5 CFU/ml in TH (adjusted to pH 7.5 or pH 5.0), 25 μM peptide was added to the cell suspension and incubated at 25°C. At each time point (0, 5, 30, 120 or 180 min), 10 μl of the cell suspension was removed, diluted in growth medium (1:50), and kept on ice prior to plating. CFU/ml were calculated after overnight incubation at 37°C under anaerobic conditions.

2.6 Determination of peptide activity at different pH

Exponentially growing *S. mutans* UA140 was harvested and resuspended to ~1×10^6 cells in 100 μl of fresh TH medium (pH as indicated in the figure). Cells were incubated with 25 μM peptide at each pH condition for 10 min using TH adjusted to the corresponding pH as controls. The treatment was stopped by immediate addition of PBS followed by two washes prior to resuspension in fresh medium and plating onto TH 1.5% agar plates. CFU/ml was calculated after overnight incubation at 37°C under anaerobic conditions.

Theoretical pH titrations were calculated by determining the expected peptide charge at each pH based on the number of histidines and terminal charges using the equation:

$\sum_{N_{charged}} n_{res} \frac{(10^{(pKA-pH)})}{(10^{(pKA-pH)}-1)}$, where $N_{charged}$ refers to the total number of charged groups in the peptide, n_{res} to the number of each given charged group, pKa to the pKa of each individual charged group, and pH to the pH of the bulk solution. Calculations were performed using the charge calculation tool made available by Gale Rhodes (http://www.usm.maine.edu/~rhodes/Goodies/PeptChg.xls).

2.7 Peptide treatment of biofilm cells

Approximately 1×10^6 cells of *S. mutans* strain JM11 were inoculated in TH supplemented with 1% sucrose for biofilm growth. Biofilms were grown anaerobically for 3 hr and washed with PBS prior to treatment with 40 μM peptide in TH (pH adjusted to either 7.5 or 5) at 25°C for 10 min. The treatment was stopped by immediate addition of PBS and two additional washes with PBS. TH medium adjusted to pH 7.5 or pH 5.0 served as negative controls.

To determine sustained treatment effects, biofilms were washed after treatment, replenished with 100 μl of fresh medium and allowed to grow anaerobically at 37°C. At each time point (60, 120, or 180 min), cell viability was assessed by determining luciferase activity as previously described (10) with the following modification: 25 μl of 1 mM d-luciferin (Sigma, St. Louis, MO) suspended in 0.1 M citrate buffer (pH 6.0) was added to each

microfuge tube containing biofilm cells in 100 µl TH prior to measurements a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA).

2.8 Peptide binding to *S. mutans* biofilms

Rhodamine-labeled peptides were first tested for their antimicrobial activity by performing MIC test at pH 7.5 and pH 5.0 to ensure that addition of the label does not interfere with peptide activity. The labeled peptides were then utilized to quantify binding to exponentially growing planktonic or biofilms grown *S. mutans* cells. Planktonic cells binding assay was conducted as previously described (6). Biofilms were washed with PBS prior to treatment with 25 µM peptide in PBS (pH 7.5 or pH 5.0) for 10 min. The peptide was removed, and biofilm cells were washed with PBS (same pH as in the binding step) and imaged (Nikon Eclipse 80i, SPOT software suite, Diagnostic Instruments, Sterling Heights, MI) using the appropriate filter set for rhodamine imaging and the same exposure time for each sample. To quantify the level of bound rhodamine-labeled peptide, red pixels were selected from each image utilizing Gimp software (www.gimp.org), adjusted for any background red fluorescence and expressed as binding intensity units.

2.9 Measurement of extracellular ATP

S. mutans UA 140 was treated with AAP2 at pH 7.5 or pH 5.0 as described in section 2.5. The previously described membrane-disruptive *S. mutans* specific antimicrobial peptide C16G2 served as a control (6). After 10 min incubation, the reaction was stopped by immediate centrifugation for 10 min, and the cell pellet and supernatant were subjected to ATP quantization using CellTiter-Glo kit (Promega, Madison, WI) following manufacturer's instruction.

3. Results and discussion

3.1 Design of acid-activated antimicrobial peptides

In this study, we focused our efforts to develop antimicrobial peptides that target organisms such as the study model organism *S. mutans*, based on their ability to produce acids, the physiological condition leading to caries. The strategy of employing peptides with antimicrobial activity exclusively at low pH could be especially advantageous in dental caries prevention and treatment: cariogenic pathogens would be eliminated due to the local low pH environment surrounding these species, while these peptides should have minimal effect on commensal bacteria that do not produce acid. The resulting shift in microbial dental plaque composition would contribute to neutralizing plaque pH and drive the equilibrium towards tooth surface re-mineralization (2).

To achieve the desired properties of pH-dependent antimicrobial activity, we resorted to the well-studied features of clavanin A, a natural antimicrobrial peptide that exhibits a significant increase in killing efficiency (~10-fold reduction of MIC) upon pH decrease (9). In brief, the pH-responsiveness of clavanin A has been attributed to the presence of multiple His which enable the conformational flexibility necessary to transition from the less effective more general membrane disruptive α-helical form at close to neutral pH to its substantially more active random coil conformation at low pH (12). The increase in peptide charge upon His protonation is not essential for membrane interaction of clavanin A which was found to be driven by hydrophobic forces (12). The hydrophobicity necessary for membrane interactions is provided by Phe groups which in contrast to other hydrophobic amino acids allow for the conformational flexibility needed for the pH-dependent antimicrobial activity of clavanin A (9).

To test if the organization of the amino acids His and Phe into an amphipathic helix is sufficient to provide pH-responsive antimicrobial activity, we took these key features of clavanin A to the extreme by designing two 14 amino acid long peptides, AAP1 and AAP2, that were exclusively comprised of His and Phe residues (Fig. 1A–B).

In addition to AAP1 and AAP2, we have designed longer peptides with additional amino acid sequences to either N-term or C-term of AAP1 or AAP2, We have found that additions to N-term of the peptides resulted in loss of activity in both pH 7.5 and pH 5.0, while additions to C-term of the peptides did not enhance the antimicrobial activity at either pH tested. Thus we carried out further experiments with peptides of the minimal length that demonstrated the highest pH-dependent differential activities.

In the arrangement of His and Phe in these peptides as amphipathic helices AAP1 would have a smaller hydrophobic interface (6 Phe and 8 His) compared to AAP2 (8 Phe and 6 His). Circular dichroism spectra were obtained for both peptides correspond to a predominantly helical conformation at pH 7.5, while acidic pH values result in spectra consistent with a random conformation (Fig. 1D–E). Interestingly, this behavior is very similar to observations for clavanin A that was reported to undergo a similar pH-dependent transition from α-helix at neutral pH to a random coil at pH 5.5 (12).

3.2 AAP1 and AAP2 exhibit pH-dependent antimicrobial activity

For a basic characterization of anti-*S. mutans* activity MIC tests were performed at pH 7.5 and pH 5.0 against a panel of six different *S. mutans* strains (UA 140, UA 159, LT-11, MB-2148, NG8, NCTC 10449). At pH 7.5 both peptides did not exhibit any growth inhibition at the highest concentration tested (125 μM) against any of the strains tested, while the MICs at pH 5.0 were ~12 μM (AAP1) and 3 μM (AAP2), corresponding to a minimum 10 to 40-fold increase in antimicrobial activities, respectively. This “switch” in antimicrobial activity from a complete lack at pH 7.5 to significant activity at pH 5.0 (especially for AAP2) renders these artificial peptides promising candidates for targeting acid generating species while leaving the benign flora intact.

Based on the greater increase in antimicrobial activity under acidic conditions, AAP2 was chosen for a more detailed characterization of its time-kill kinetics. At pH 5.0, AAP2 was able to reduce *S. mutans* viability for nearly two orders of magnitude upon a 5 minute treatment (Fig 2). Maximum killing of *S. mutans* was achieved after 2 hr of treatment. Consistent with the MIC data, at pH 7.5 AAP2 had no effect on cell viability during this incubation period. This significant killing effect in a short time period would be consistent with a membrane-active antimicrobial mode of action (13).

S. mutans is known to induce an acid tolerance reaction (ATR) upon prolonged exposure (> 2hr) to sublethal pH values of 5.5. It has been demonstrated that ATR enhanced survival of *S. mutans* when pH further decreased to 3.5 (14). Since the time-killing experiments in this study were performed for a maximum of 2 hr and therefore shorter than the time period indicated for ATR activation, it is unlikely that this mechanism played a significant role in the outcome of the observed acid-activated killing of *S. mutans*. The influence of ATR on AAP activity therefore remains to be evaluated.

In addition to our target organism *S. mutans*, we explored the effect of AAPs on representatives of the commensal oral flora including the oral health associated species *S. sanguinis* as well as *S. mitis*, *S. gordonii* and *S. cristatus*. None of these oral streptococci was susceptible to AAP1 or AAP2 at neutral pH even at the highest concentration tested of 125 μM. MICs at the acidic pH 5.0 could not be determined since these strains exhibited severe growth inhibition at this low pH independent of the presence of AAPs. These

findings suggest that AAPs have the potential to selectively reduce acid-producing *S. mutans* while leaving the non-cariogenic flora intact.

3.3 The pH-dependent antimicrobial activity of AAP2 against *S. mutans* biofilm cells

Acid-producing bacteria like *S. mutans* cause demineralization when they are tightly associated with the tooth surface as dental biofilms. When tested against *S. mutans* biofilm cells, AAP2 was found to significantly reduce viability at pH 5.0, while no antimicrobial effect was observed at pH 7.5 (data not shown). In order to address the long-term impact of AAP2 treatment on *S. mutans* biofilms – an important parameter in the efficacy of antimicrobial treatments - we monitored the recovery of treated biofilms for up to 3 hr using the luciferase expressing *S. mutans* strain JM11. The medium-treated control samples at both test pHs as well as the sample exposed to AAP2 at pH7.5 exhibited a comparable increase in luciferase activity over time reflecting uninhibited continuous biofilm growth after treatment (Fig 3). In contrast, the samples that were incubated in the presence of AAP2 at pH 5.0 for 10 min did not recover from this treatment. These findings confirmed the ability of AAP2 to induce irreversible cell damage upon activation under low pH conditions against biofilm cells. The observed sustained effect indicates that AAP2 could have the potential to reduce growth of the acid producing population within the biofilm community.

3.4 pH-responsive antimicrobial activity of AAP2 is not mediated by differential binding to *S. mutans* or membrane disruption

The initial step in peptide binding to bacterial membranes is influenced by cationic and hydrophobic interactions (15). Therefore, the increase in antimicrobial activity of AAP2 at low pH could be enhanced binding to the target cells due to the shift from an almost neutral to a highly positively charged peptide. To explore this possibility, we examined binding of rhodamine labeled AAP2 to *S. mutans* UA 140 planktonic and biofilm cells at pH 7.5 and pH 5.0. Since no significant difference in binding at either pH was observed (Fig. 4a), the pH-sensitive antimicrobial activity of AAP2 against *S. mutans* is likely not the result of pH-dependent differential binding. This behavior is similar to clavanin A, which has been described to display a pH-independent binding to cell membranes (12).

We also examined if the AAP2 antimicrobial activity was correlated with membrane disruption by testing for ATP-leakage. The previously described membrane disrupting *S. mutans* specific antimicrobial peptide, C16G2, served as a control (6). Unlike C16G2 which caused significant ATP-leakage after 2 min exposure, treatment times of up to 10 min with AAP2 at pH 5.0 did not lead to a significant increase in extracellular ATP (Fig. 4b) despite resulting in cell death of more than 95 % of the *S. mutans* cells (data not shown). Interestingly, the absence of membrane leakage during antimicrobial action at low pH is again consistent with observations reported for clavanin A, which functions at low pH by interfering with proton-translocating proteins without disturbing the membrane barrier (12). Even though both clavanin A and AAP2 undergo a similar transition from a helical conformation to random coil as the pH drops to the acidic range (Fig 1D–E) (12), they do not appear to function exactly alike since clavanin A displays an albeit greatly reduced antimicrobial activity at neutral pH by causing general membrane disruption. AAP2, in contrast, did not exhibit any antimicrobial activity at neutral pH which changed to a low MIC of ~3 μM at pH 5.0 that significantly exceeded the observed 10-fold decrease in MIC for clavanin A under similar conditions. Interference with proteins involved in maintaining the membrane potential could be a mechanisms employed by the pH-responsive antimicrobial peptides designed in this study but further detailed characterization will be necessary to fully understand the mode of action of AAP2.

3.5 pH-dependent bacterial killing of AAP2 is correlated with the theoretical changes in peptide charge

For a more detailed characterization of its pH-responsiveness, the AAP2 antimicrobial activity was assessed at various pH, ranging from 5.0 to 7.5 in increments of 0.5 pH units. To account for possible peptide independent pH effects, the antimicrobial activities were normalized as the percentage surviving cells relative to the medium control at the corresponding pH (Fig. 5). Consistent with above MIC data and killing kinetics (Fig. 1) at pH 7.5, only a 5 % reduction in cell viability was observed in the presence of AAP2. With decreasing pH, AAP2 activity increased, and maximum killing was reached already at pH 6.0 (93% reduction) when the peptide should carry 6 to 7 positive charges. Theoretical charge calculation confirmed that although the mechanism through which AAP2 is activated at low pH remains unclear, its activity was well correlated with the increase in positive charges (Fig 5). It was reported that the critical pH for shifting the equilibrium towards demineralization is 5.5 (2). We have shown here that AAP2 activity is pH dependent, and at the critical pH of 5.5, its activity is at maximum level.

4. Conclusions and future directions

In conclusion, this limited proof of concept study suggests that targeting acid production could be developed into a new direction for dental caries treatment and prevention. Further studies will be necessary to determine if AAPs can remove acid-producing bacteria from mixed biofilm communities. Cariogenic acid-producers in dental plaque create “micro-environments” of low pH around them which could be exploited by AAPs and lead to the targeted elimination of *S. mutans* or other cariogenic pathogens in a pH-dependant manner. This would greatly enhance the survival of non-pathogenic normal flora, whose preservation could benefit the caries status and long-term health of the host.

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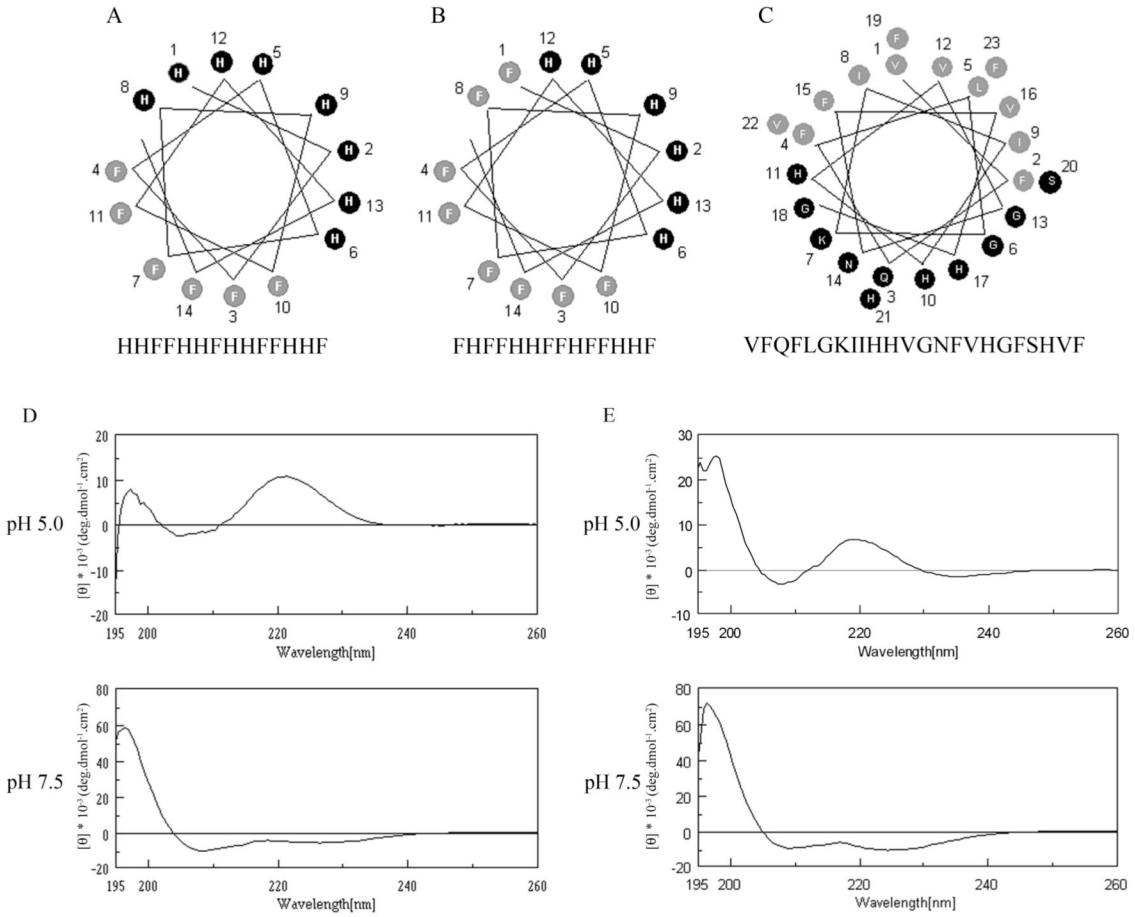

Figure 1.

Helical wheel projection of AAP1 **(A)**, AAP2 **(B) and clavanin A (C)**. The one letter code for amino acids is used, and the numbers indicate the position of the amino acid in the peptides. Hydrophobic residues are shown in grey circles and Hydrophilic residues in black circles. CD spectra of AAP1 (peptide concentration = 0.2 mM) **(D)** and AAP2 (peptide concentration = 0.22 mM) **(E)** at both pH 5.0 and 7.5 in 50% (v/v) TFE.

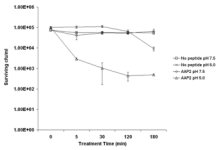

Figure 2.
Killing kinetics of AAP2. *S. mutans* cells were treated with AAP2 solutions at pH 7.5 (open diamond) and 5.0 (open triangle), for 5 min to 3 hr (specified in the figure). TH adjusted to pH 7.5 (open square) and pH 5.0 (cross) were used as controls. Each data point represents average of three independent experiments. Standard deviation is shown.

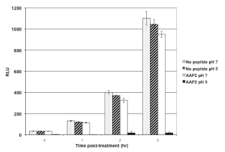

Figure 3.
Sustained inhibitory activity of AAP2 against *S. mutans* biofilms measured by luciferase activity. JM11 biofilms were grown and then exposed for 10 min to 40 μM AAP2 at pH 7.5 or pH 5.0 (as indicated in the figure). Biofilm recovery in fresh media was monitored over time by luciferase activity. Data points represent averages results from three independent experiments. Standard deviation is shown.

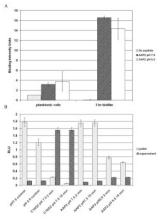

Figure 4.

AAP2 binding to *S. mutans* cells and effect on membrane membrane permeability at different pH. **(A)** Rhodamine-labeled AAP2 was used to bind planktonic and biofilm *S. mutans* cells at pH 7.5 and pH 5.0 and subject to fluorescent microscopy. The intensity of the bound fluorescent signal was quantified and data points represent averages of the results from three independent experiments. Standard deviation is shown. **(B)** Effect of AAP2 treatment on cell membrane permeability. Planktonic *S. mutans* cells were treated with AAP2 at pH 7.5 and pH 5.0 respectively. The known membrane disruptive *S. mutans* specific antimicrobial peptide C16G2 as well as TH medium at pH 7.5 and pH 5.0 were served as control. The level of ATP was measured in the cell pellet as well as the supernatant. Treatment times are indicted.

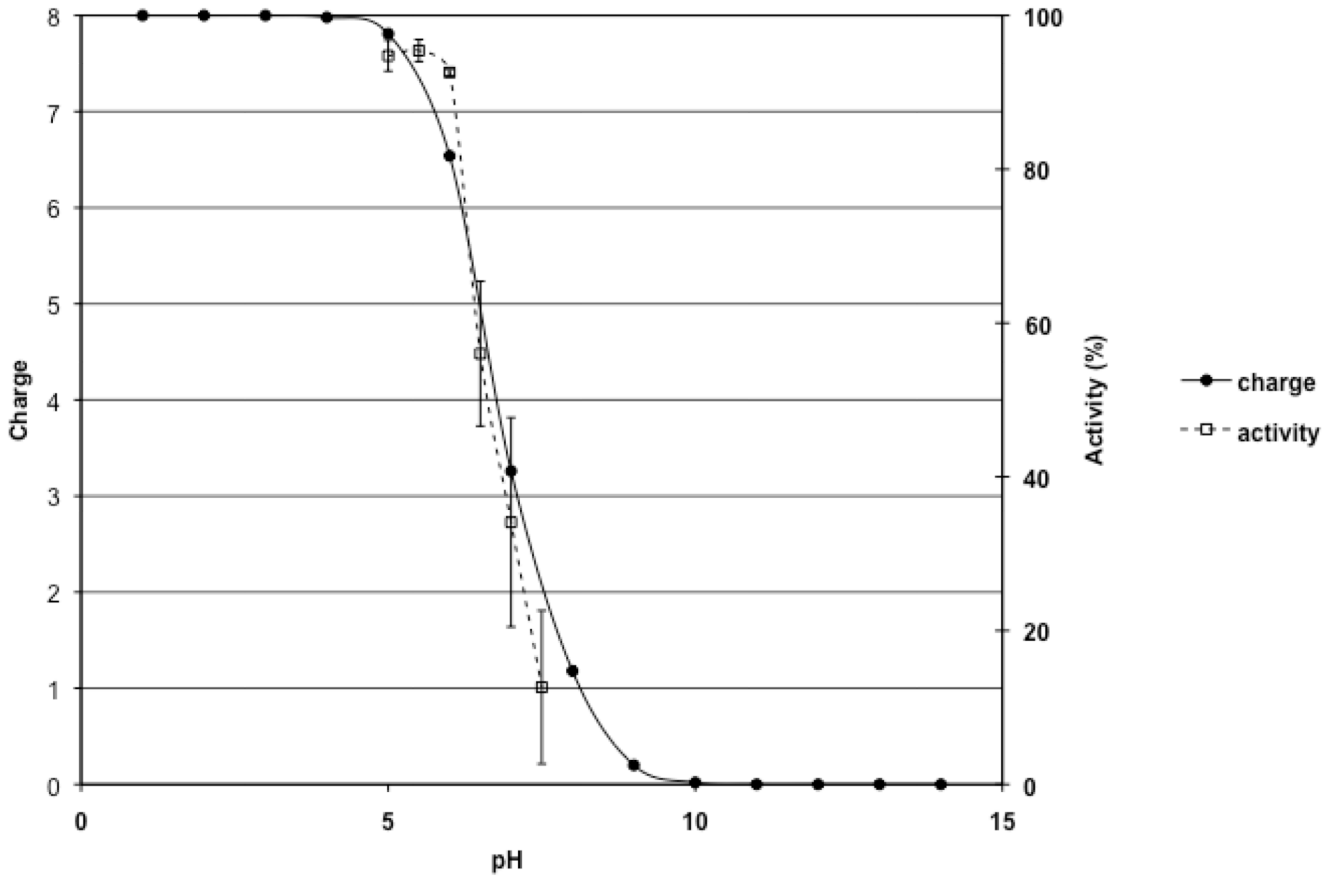

Figure 5.
Charge and antimicrobial activity correlation of AAP2. Theoretical charge of AAP2 was calculated as described in Methods and Materials. AAP2 was used to treat planktonic *S. mutans* cells at pH indicated in the figure. Cell viability was assessed by colony counting. AAP2 activity was presented as percentage reduction in viability relative to media control at each pH. Data points represent averages of three independent experiments. Standard deviation is shown.