ORIGINAL ARTICLE

INFLUENCE OF N-TERMINALS STATHERIN PENTAPEPTIDE SEQUENCE RESIDUES ON CARIOSTATIC EFFICACY

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ABSTRACT

In vitro studies have shown that Statherin (StN43) and its shortened analogues containing only the N-terminal 21 amino acids (StN21) inhibit demineralization under artificial cariogenic challenges. This has been shown by experimentally and computationally to be associated with interactions of specific N-terminal residues with hydroxyapatite surfaces. The aim of this study was to identify which of the first 5 N-terminal residues are most important for reducing the rate of demineralization under artificial caries conditions, using scanning microradiography (SMR).

Five statherin21-like peptides were prepared using FMOC synthesis with each of the N-terminal five residues of StN21 replaced by the non-polar residue alanine and dissolved in phosphate buffer at pH 7.4 (0.188 mM). HA pellets (20% porosity) used as model substrates for enamel demineralization studies were sectioned and mounted in scanning microradiography (SMR) environmental cells and exposed to 0.1M acetic acid at pH4.0 for 72h. The sections were then treated with one of the StN21 solutions for 24h, then demineralization continued for a further 72h. SMR was used to measure demineralization rates (RDHA) before and after peptide treatment.

Treating with StN21 resulted in 43% reduction in RDHA. StN21 peptides in which the phosphorylated serine at position 2 was replaced only reduced RDHA by 32%. For StN21 peptide with the phosphorylated serine at position 3 replaced, RDHA was not reduced. Replacement at position 5 (glutamate) also reduced RDHA by only 32%. Replacements at residue positions 1 and 4 showed the maximum reduction in RDHA by 52% and 50% respectively.

In conclusion, the phosphorylated serine residue at N-terminal position 3 of Statherin is required to inhibit demineralization. Other important residues, but to a lesser extent, are the phosphorylated serine at position 2, and the glutamic acid at position 5. These studies demonstrate that the molecular architecture arrangements of Statherin are required for cariostatic efficacy.

Key Words: *Statherin, dentistry, hydroxyapatite, peptide surface interactions, rate of demineralization of hydroxyapatite (RDHA).*

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INTRODUCTION

The oral environment is a complex biological system, with many interacting processes between the oral hard tissues and the oral biofluids.[1,2] Therefore, it is incorrect to consider any single structure in isolation without recognition of these complex interactions. Though the literature contains many reports of in vitro studies of caries development in enamel, less consider the co-involvement of saliva. Likewise, there are many reports on the properties and functions of saliva without consideration of its action on enamel. However, there are considerably fewer more "holistic" studies on the role of saliva as part of the enamel homeostasis process, particularly in those using artificial caries systems.[2,3]

Saliva is a complex superfluid which serves to protect enamel from carious and erosive acidic challenges. Saliva's protective function of enamel operates at many different levels; from providing a source of calcium and phosphate ions to reverse the chemical equilibrium position that occurs at low pH and calcium concentrations, to the interactions of systems which serve to selectively destroy oral flora, thus protecting enamel from mineral tissue destruction. Saliva contains well over 2000 proteins and peptides, and although at low concentrations, many of these serve to protect enamel by a myriad of different mechanisms.[4] One such enamel homeostasis system mechanism recently reported is the action of Statherin in protecting enamel against acid attack, by binding at specific dissolution sites on hydroxyapatite surfaces.[5]

Enamel is an impure form of calcium hydroxyapatite (HA), a basic calcium orthophosphate.[6] These impurities include CO_3^{2-}, F^-, Mg^{2+}, and as well as many other metal ions which replace Ca^{2+} in the HA lattice structure. These substitutions significantly influence the mineral dissolution behavior.[7]

The general formula of enamel calcium orthophosphate mineral is $Ca_{10}(PO_4)_6X_2$, where X can be F, OH, Cl, forming fluorapatite, hydroxyapatite or chlorapatite respectively or it can be replaced by CO_3, whereas (PO_4) can be substituted by CO_3, HPO_4, SiO_4 or VO_4.[8] Though there is chemical variation, the structural pattern remains the same and ionic exchange occurs only when the relative size of ions is the same and thus biominerals also serve as a reservoir of mineral ions.[9] The F ion is readily incorporated in the hydroxyapatite lattice of enamel giving more stability to the lattice and reducing the solubility of the mineral content of enamel.[10] Enamel and HA have many similarities. The physical properties of enamel such as color, translucency, hardness and density are similar to HA. In addition, enamel solubility is closely related to the solubility of HA, an important consideration in both dental caries and erosion.

Enamel has been shown to show enormous variety in composition between not only different subjects, but between different teeth from the same subject and within the same tooth. For this reason, HA is often used as an enamel substitute.

Caries is a multifactorial disease in which three factors play the fundamental role; the saliva and teeth, the dietary refined carbohydrates, and the microflora.[11] It has been reported that "It is the demineralization of enamel, dentine or cementum caused by organic acids produced by acidogenic bacteria in plaque which feed upon fermentable carbohydrates".[12] The process of demineralization can be described as a chemical interaction between an acid and the hydroxyapatite mineral, although a variety of both kinetic, and thermodynamic factors influence the overall dissolution rate. The kinetic factors include the accessibility and porosity of the enamel and are influenced by transport within the inter-prism and inter-crystalline space as well as the rate of provision of acids to the demineralization surfaces through any biofilms that overlay the enamel surface. The thermodynamic factors include the solubility of hydroxyapatite, which is modified by substitutions within the lattice.

Statherin is a multifunctional salivary protein produced by the acinar cells of the parotid and submandibular salivary glands, and is a 43-residue peptide (molecular weight 5380 Daltons).[6] It has a typical range of concentration in human saliva of 10-40 μmol^{-1}. It contains tyrosine rich residues and phosphoserines in its primary structure. It has a role in inhibiting primary as well as secondary precipitation of calcium phosphate.[13]

The peptide was first described by Hay[14] who described its ability to stabilize calcium phosphates in the oral environment by inhibiting primary and secondary precipitation and named the protein Statherin, derived from the Greek statherio, to meaning stabilize. The first five amino acid residues of the anionic negatively charged N-terminal inhibit crystal growth.[15] The remaining 19-43 residues have no charge and do not inhibit either primary or secondary precipitation.[16] The C-terminal of Statherin binds to bacteria, and is involved in the selective initial bacterial colonization of newly acquired enamel pellicle.[17] The basic charges at the N-terminal bind to phosphates[14] and act to reduce protein repulsion on HA surfaces, thus increasing the packing density of the protein onto HA surfaces.[18] It is thought that the HA surface is stabilized by adsorbed proteins which reduce the rate of enamel dissolution.[19] Statherin has a strong affinity for HA so it is a precursor in forming a protective salivary biofilm (the enamel pellicle) and is a major component.[20] The pellicle covers the possible precipitation sites on the HA surface, and also serves as a semi-permeable membrane which slows the diffusion of calcium and phosphate ions into the surrounding fluid upon acid attack.[21]

The aim of the study was to identify the functional domain of Statherin required for its cariostatic function by measuring the efficacy of Statherin-like peptides (StN21-X) with first five amino acid sequences having residues replaced by alanine, at the N-terminal (containing only 21 amino acids) in simulated caries condition in vitro by using scanning microradiography (SMR) which provided precise and repeatable measurements. The primary sequence of StN21 is DS*S*EEKFLRRIGRFGYGYGPY(22)

METHODOLOGY

Materials used in the study were hydroxyapatite discs, SMR cells and scanning system, demineralizing solution, phosphate buffer solution (PBS) with pH 7.4, peptides with amino acid sequence of StN21 and StN21-like peptides with any first 5 residues replaced with alanine, as shown in Table 1. HA was purchased as pellets from Plasma-Biotal Ltd. (UK). These pellets were made of sintered powder (Captal® S, D50), approximately 4µm particle size with a porosity of 20%. HA powder was pressed uniaxially in a standard tablet dye (diameter 26mm) under 6895kPa. The pellets were then sintered in an atmospheric electric furnace at 1250-1300°C for 2 hours. The demineralizing solution used was 0.1M acetic acid, buffered to pH 4.5 with 1M NaOH. Acetic acid has been previously used as a caries simulating agent.

The demineralizing solutions were prepared to include 1.0 mM CaCl2 and 0.6 mM KH2PO4 giving a partial saturation to the demineralizing solutions with respect to HA To simulate erosive conditions, 0.1M acetic acid (pH 4) was used.

TABLE 1

Amino-acid Sequence	Missing Amino-acid	
AS*S*EEKFLRRIGRFGYGYGPY	aspartic acid	Polar charged
DAS*EEKFLRRIGRFGYGYGPY	phosphorylated serine	Polar neutral
DS*AEEKFLRRIGRFGYGYGPY	phosphorylated serine	Polar neutral
D S * S * A E K F L R R I G R F GYGYGPY	glutamic acid	Polar neutral
D S * S * E A K F L R R I G R F GYGYGPY	glutamic acid	Polar neutral

Experiment

A total of 7 SMR cells containing a single HA disc of 20% porosity, uniform size and thickness, in each cell were prepared and mounted on the SMR stage. Deionized water was circulated through each cell for 24 hours through a peristaltic pump which allowed flow of water in and out of SMR cells at a speed of 2.20 RPM. Initial SMR area scans for all HA discs were obtained.[8]

Once the precise location of specimens through area scan was determined, demineralizing solution was prepared and circulated through each cell for initial demineralization period of 72 hours, and SMR line scans were performed. HA discs were then rinsed with deionized water to provide an acid free environment for subsequent peptide adsorption. StN21 and 5 other StN21-like peptides with amino acid residues replaced with alanine at each of first five positions of N terminal of Statherin were prepared (Shown in Table 1), each at a concentration of 1.88 x 10 -5mol L-1 in phosphate buffer.[22] Each HA disc was treated for 24 hours by injecting the peptides and phosphate buffer solutions into the cells. After 24 hours, these peptides were removed from each cell and acetic acid was re-circulated again through all these cells for a further period of 72 hours in order to measure the reduction in rate of demineralization in each HA disc following treatment. Each SMR cell had its own circulation of demineralizing solution from 1 L glass bottles. SMR line scans continued until the end of the experiment. The rates of HA demineralization, before and after treatment, was measured. All experiments were performed at a room temperature of 20ºC +/- 20ºC.

Data Acquisition: Area Scan and Line Scan

Area scans of each HA discs fixed in each SMR cell was carried out using the SMR system.

Data Analysis

Standardization points were selected outside each specimen line scan as standard measurement points to allow compensation for any X-ray source or detector instabilities.[23]

According to the Beer's Law for a homogenous material, assuming the X-ray beam is monochromatic, the intensity of the transmitted beam is given by:

$$I = I_0 \exp(-\mu_m m) \quad (1)$$

where I0 is the incident intensity, µm is the mass absorption coefficient and m are the projected mass per unit area.

This equation can be rearranged as:

$$m=1/\mu_m [\log_e 1/N - 1/N_0] \quad (2)$$

where N is the number of transmitted photons and N_0 is the number of incident photons obtained from the first point of the line scan outside the specimen.

The mass of HA disc per unit area (g cm^{-2}) is obtained using the mass attenuation coefficient of HA (4.69 cm^2 g^{-1}) calculated for AgKα radiation[8] and the attenuated X-rays transmitted count at each point in SMR line scan.

The rate of demineralization of each HA disc is

based on the assumption that the mineral loss at each point is essentially linear with time. It is calculated as:

$m = at + b$

Where m is the projected mass of HA per unit area, t is the time since start of experiment, a is the rate of demineralization, and b is the intercept on y axis.

Each specimen was SMR scanned along two horizontal lines, and the mean value was calculated, along with the standard error.

Statistical analysis was done by analyzing and calculating data using Excel and Table curve CD (Systat Inc, USA).

RESULTS

Typical plots of the loss in projected mineral mass of hydroxyapatite with time at a single scan point throughout the acid challenge period, both before, and after exposure to each peptide, are shown in Figures 1a-e. In total, measurements were taken at 6 points along two SMR tracks in each HA block, and therefore 12 similar plots were obtained for each peptide tested. This data showed that the mineral loss was approximately linear with time for every case, both before and after treatment with peptide. A linear least squares fitting could therefore be used to calculate the rates of mineral loss at each scan point, both before and after treatment for each peptide tested.

a) StN21-Alanine1. Rate before: 1.20 (0.12) $x10^{-4}$ $g.cm^{-2}h$-1 , after 0.57 (0.04) $x10^{-4}$ $g.cm^{-2}$ h^{-1}.

b) StN21-Alanine2. Rate before: 2.30 (0.17) $x10^{-4}$ g. cm^{-2} h^{-1}, after 1.56(0.13) $x10^{-4}$ $g.cm^{-2}$ h^{-1}.

c) StN21-Alanine3. Rate before: 1.17 (0.07) $x10^{-4}$ g. cm^{-2} h^{-1} , after: 1.17 (0.05) $x10^{-4}$ $g.cm^{-2}$ h^{-1}.

d) StN21-Alanine4. Rate before: 5.84 (0.6) $x10^{-4}$ $g.cm^{-2}$ h^{-1}, after: 3.31 (0.29) $x10^{-4}$ $g.cm^{-2}$ h^{-1}.

e) StN21-Alanine5. Rate before: 2.30 (0.12) $x10^{-4}$ g. cm^{-2} h^{-1}, after: 1.56 (0.09) $x10^{-4}$ $g.cm^{-2}$ h^{-1}.

f) StN21. Rate before: 3.85 (0.18) $x10^{-4}$ $g.cm^{-2}$ h^{-1}, after: 2.00 (0.06) $x10^{-4}g.cm^{-2}$ h^{-1}.

g) PBS. Rate before: 0.99(0.1) $x10^{-4}g.cm^{-2}$ h^{-1}, after: 1.01(0.08) $x10^{-4}$ $g.cm^{-2}$ h^{-1}.

The mean percentage changes in the rates of mineral loss (calculated as the rate of mineral loss after peptide treatment compared to the rate of mineral loss before peptide treatment) from the 12 similar plots was calculated for each peptide length (Figure 2). The positive control STN21 peptide treatment shows an approximately 45% decrease, as previously established, and the PBS treatment shows no change in rate.

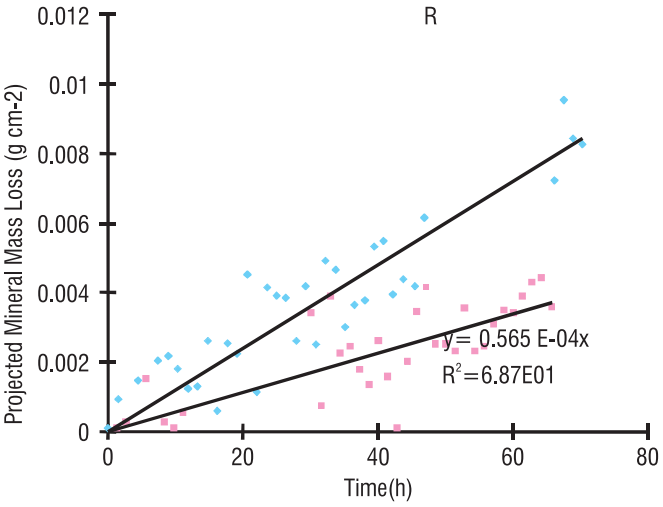

Fig 1a:

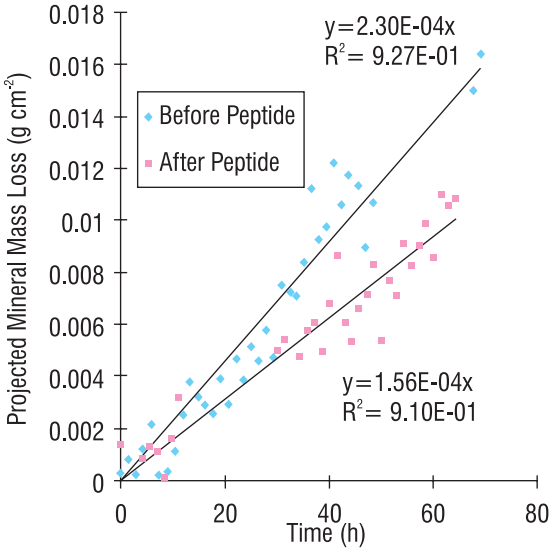

Fig 1b:

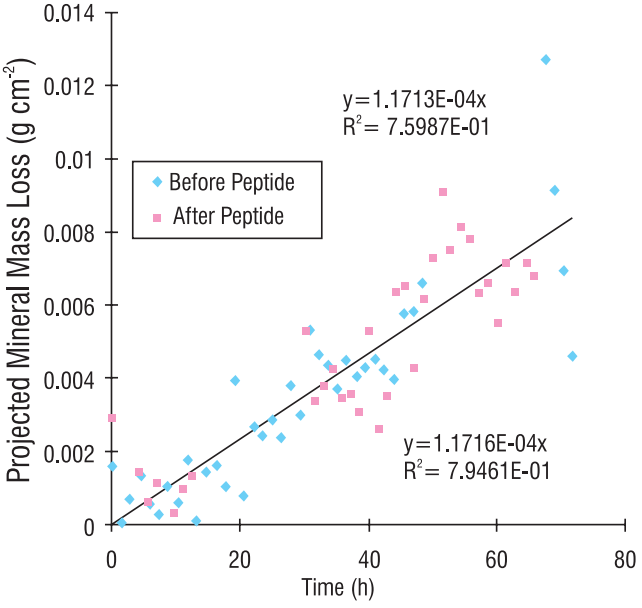

Fig 1c:

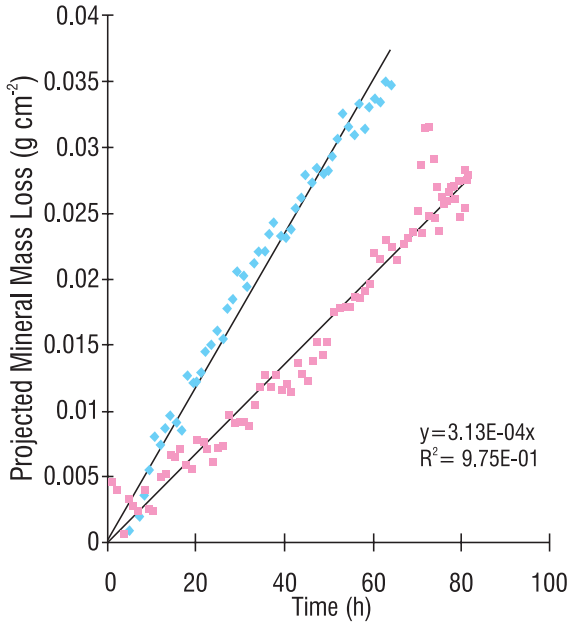

Fig 1d:

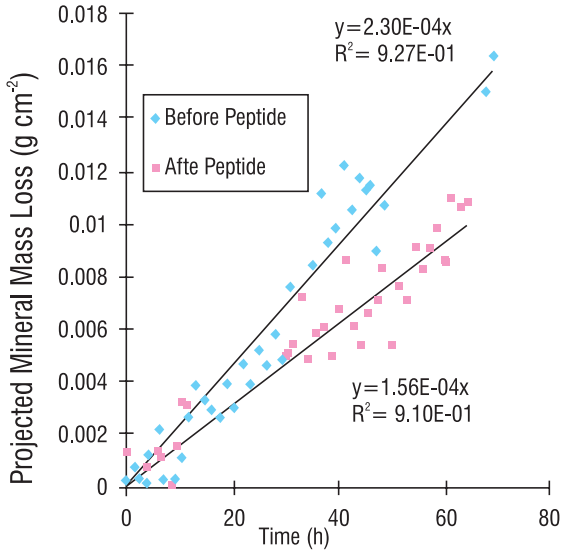

Fig 1e:

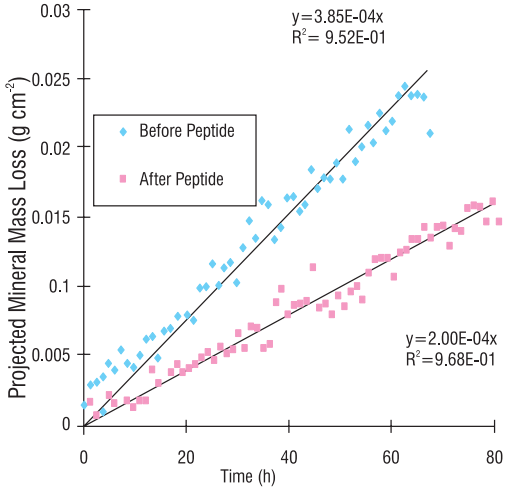

Fig 1f:

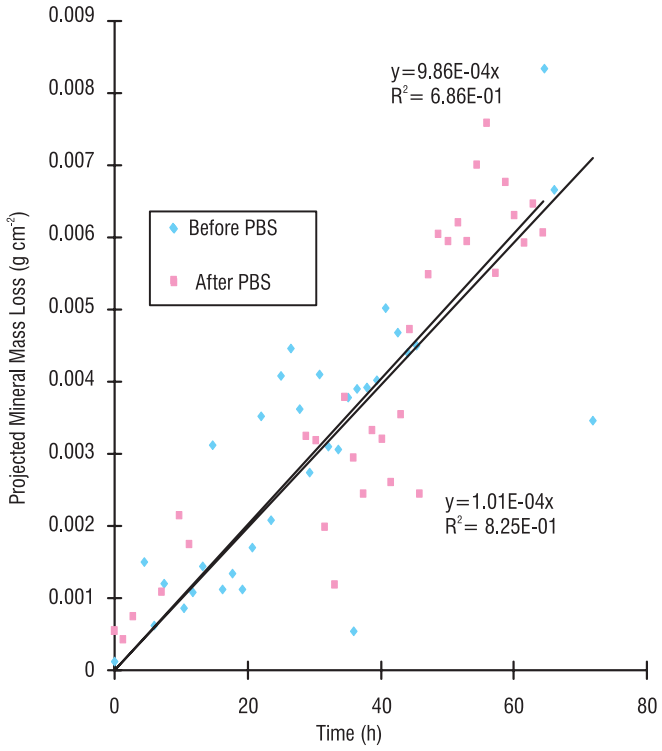

Fig 1g:

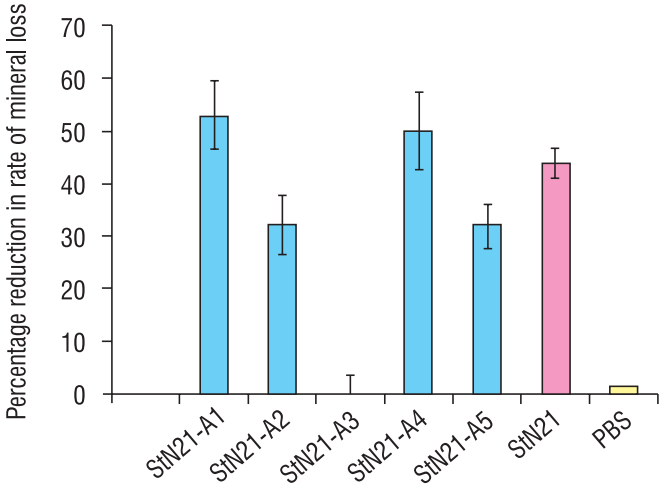

Fig 2: Comparison between the rates of mineral loss in all 7 HA specimens

The alanine substituted peptides show substantially differing effects on the reduction in the rate of demineralization, indicating significantly different influence of each substituted residue on the mode of action of Statherin in the protection of enamel. The HA discs coated with StN21 Alanine1(52%) and StN21 Alanine4 (50%) demonstrate even better cariostatic influence than unchanged StN21(43%). Both these peptides contain two phosphorylated serines. Clearly the phosphorylated serine residues at positions 2 and 3 are particularly significant for the cariostatic activity. Whereas, for StN21 Alanine2 and StN21 Alanine3,

only one of the phosphorylated serine is present their dissolution inhibition is only 32%, less than that for unchanged STN21.

DISCUSSION

The results demonstrate considerable variation in the influence of replacing individual N-terminal residues in STN21 with alanine on HA mineral demineralization protection. This demonstrates the importance of individual residue-mineral interactions, either within the molecule, or with the hydroxyapatite surface, which in turn affects the enamel demineralization inhibition action of Statherin, as suggested from calculations carried out by Makrodimitris et al.[24]

All results showed that the demineralization change was linear with respect to time, indicating that the treatment of the peptide does not influence the chemical kinetic order of the dissolution reaction, because, in those cases where alanine replacement did influence the protective mechanisms, the impact was immediate, and did not increase with time. This demonstrates that where there was demineralization inhibition, the binding occurs rapidly and totally.

The StN21-Alanine1 and StN21-Alanine4 peptide treatments demonstrated an increased inhibition compared with that seen for unchanged StN21. Both these peptides contain the two phosphorylated serines of the original STN21 peptide thought to be important for binding the molecule onto the HA surface. Whereas, the StN21-Alanine2 treatment and the StN21-Alanine3 treatment demonstrated much lower demineralization inhibition. In both these cases, the peptides only contain one phosphorylated serine residue. This suggests that the cariostatic function of Statherin, presumably by binding of the peptide onto a HA surfaces, requires the presence of both phosphorylated serine residues for maximal efficacy. StN21-Alanine3 treatment shows no reduction in the mineral loss at all, suggesting that the phosphorylated serine residue at position 3 is crucial for dissolution inhibition. For the StN21-Alanine5 treatment, the reduction in the rate of mineral loss is only 32%, although both phosphorylated serines are present. In this case, there is an absence of the E5 (glutamate) residue which is adjacent to K6 (lysine), which according to Makrodimitris et al[24] has a high residue-surface interaction energy. Thus, the replacement of the E5 residue may interfere with the binding of the lysine at residue position 6.

CONCLUSION

In this study, a non-polar amino acid has replaced various amino acids in the Statherin-like peptide and resulting cariostatic efficacy measured. It is concluded that both the phosphorylated serine residues in the N-terminal pentapeptide sequence of StN21 are required for optimum cariostatic function.

Understanding the mode of action of statherin provides clues for the improvement of the chemical design of chemically engineered molecules which can be included in oral health care saliva substitutes in order to provide additional protection, particularly for xerostomia patients.

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5 Jaffer Zaidi:	Proof reading, result compilation.
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