AN IN VITRO STUDY ON COMPARISON OF BINDING OF STATHERIN LIKE PEPTIDES ON TO HYDROXYAPTITE SURFACES, USING SCANNING MICRORADIOGRAPHY

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ABSTRACT

Objective: *The objective was to compare the rate of mineral loss of hydroxyapatite disc treated with five different sequences to that of hydroxyapatite disc treated with StN21 using SMR.*

Materials and Methods: *The aim of this study was to identify the functional domain of statherin required for its cariostatic function by measuring the efficacy of STATHERIN-like peptides with five different amino-acid sequences (StN21 1A, StN21 2A, StN21 3A, StN21 4A, and StN21 5A) at the N-terminal respective, in vitro using scanning microradiography.*

Hydroxyapatite discs of known (20%) porosity, used as an ideal system for enamel. Discs were covered with acid-resistant varnish on all surfaces except for the surface to be tested, and then placed in SMR cells and fixed on the SMR horizontal stage. Discs were demineralized by 0.1 M acetic acid buffered of pH 4.0 for about 72 hours.

Results: *It was found that the effect exerted by the N–terminal of the StN21 peptide on the rate of Hap mineral dissolution was (44%) StN21 1A (53%), StN21 2A(31%), StN21 3A(49%), StN21 4A(46%) StN21 5A(48%),and finally phosphate buffer (1%).*

Conclusion: *Not only do the five amino acids in the N-terminal, i.e. aspartate, phosphated serine, and glutamic acid are involved in cariostatic activity, but also, there are other amino acids to play a vital role in inhibition of mineral loss. Moreover aspartate and phosphate serine are found to be least involved in inhibiting mineral loss when compared to another sequence*

Keywords: *Statherin, Peptides, Hydroxyapatite, Scanning microradiography, Amino acids*

INTRODUCTION

Dental Enamel

Dental Enamel is the protective covering of teeth, of epithelial origin. Matured enamel is the most highly mineralized tissue in our body[1] (Ten Cate, 1998). This acellular dental hard tissue consists of larger crystallites that are mineralized and more oriented.[2] These100µm long crystals are hexagonal, and around 50 nm in width.[3]

The inorganic content of tooth enamel is rich in Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$. It is present in a raw form of hydroxyapatite.[4] These immature, tightly packed microcrystalline apatite prisms are organized in a specific pattern similar to a honeycomb in appearance. The diameter of Enamel apatite is higher than any other crystal content present in dentine, cementum as well as bone. Enamel and Hap shares a physical properties hardness besides there mineral content and acellular matrix.

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The extracellular organic matrix of enamel consists of a diverse group of proteins containing ameloblastin, amelogenins, enamelin, tuftelin, numerous enzymes, serum albumin also at least single salivary protein.[5] The organic matrix is initially rich in amino acid residues like proline, histidine acid, leucine and glutamic. Mature enamel contains aspartic acid, serine, glycine and glutamic acid.[6] The acidic calcium-binding proteins of enamel matrix form parallel arrays of pleated sheets, separated by a width of about 100A. This distance is sufficient to accommodate a unit cell of HAp crystal.[7]

Control of Biomineralisation

The biomineralization in enamel is biologically controlled .using protein macromolecule outline needs three-dimensional delineation for regulation of size, shape, and position of budding crystals in a particular region. Diffusion– some degree flow of ion rules the composition of solution and biological regulation for moving ionic concentrations besides organic surfaces for well-organized nucleation. Natural control of all stages of crystallization of biominerals which include supersaturation, solubility, along with crystal nucleation and growth are coordinated by ion transport in addition to molecular-based inhibitors and promoters of crystal growth and phase transformation.[8]

Enamel Destruction Caries and Erosion

Dental diseases disturbing the stability of enamel include periodontal disease, caries, erosion, and developmental defects of enamel. The most prevailing disease affecting about 5 billion people of the whole world is dental disease.[9]

Demineralization of enamel resulting in caries mainly caused by acid from bacteria.[10] Whereas erosion is a process regulated by local acid concentration and affected by the amount of supersaturation in relation to HAp/enamel and pH as well as a balance between pathological and protective factors.[10]

The equilibrium between the pathological and protective causes will control the chemistry of caries and decide whether demineralization will progress, stop itself, or back towards remineralization.[11]

Role of Saliva in enamel Protection

Proteins in saliva play a leading role in the protection of dental hard tissue by developing a selectively penetrable pellicle that delays the dispersal of acid towards tooth surface and inhibits mineral dissolution as well as encourages remineralization of early carious lesions of enamel. The biological train in plaque leads to pellicle formation of pellicle.[12]

Salivary proteins like statherin and acidic proline-rich peptides that are adsorbed on enamel surface owning a high affinity for particularly charged calcium can recruit the formation of calcium-protein complexes as well as rise the resistance of enamel to dissolution and prevent spontaneous primary and secondary precipitation escorted with a rise in the buffering capacity.

Statherin 43 is a 43 amino acid salivary protein by acinar cells of the parotid and submandibular salivary glands. It has high tyrosine residues and has vicinal phosphoserines in its principal structure.[13] Structural studies on statherin using CD, NMR, and various structure predictions by computer simulation/modeling show α-helix confirmation of the first twelve residues of N-terminal on the HAp surface. This peptide shows charged polarity that is the amino-terminal region holds all the ten charged amino acids with the exemption of one. Hydrophilic N-terminal (pS2-G12) undertakes a helical structure when binding to Hap. The first five negatively charged amino acids Asp-PSer2-Glu2) in the N-terminal of statherin bind strongly to HAp surface while the remaining helix is weakly bound. In α-helix pattern, explained by Pauling and Corey, all of the amino acids organized on the right side deliver stability as well as strength to the peptide.[12] The left-behind structure of statherin contains hydrophobic and uncharged polar molecules. The middle part has an unclear poly L-proline II (PPII). C-terminal which is hydrophobic is a flexible helical structure with uncharged polar molecules.[14]

Statherin(StN43) (DpSpSEEKFLRRIGRFGYGYGPYQPVPEQPLYPQPYQPQYQQYTF)

Statherin, mainly of its strong affinity to HAp is an initiator of enamel pellicle.[15] Statherin keeps the pellicle supersaturated over and above prevent crystal growth and of calcium phosphate in a supersaturated environment.[16] Statherin is the single salivary protein skilled in inhibiting primary precipitation.[17] In-depth the 18 remains of N-terminal prevent crystal growth and the first five amino acid

of the N-terminal confine crystal growth whereas inhibition of secondary precipitation requires N-terminal and hydrophobic C-terminal concurrently.[18] The microbial binding capability of the C-terminal improves primary bacterial migration in the newly formed enamel pellicle. Part of statherin in biomineralisation of enamel is to achieve calcium concentration with the determination of maintaining enamel homeostasis by inhibiting unwanted precipitation of calcium phosphates.[19]

N-terminal of StN43 (Asp-Pser-Pser-Glu-Glu-Lys-Phe-Leu-rg-Arg-Ile-Gly-Arg-Phe-Gly-)[16] is liable for binding of statherin to the HAp surface by taking on an α-helix structure on adsorption.

StN21

Kosoric *et al.* (2007) calculated the effects of a statherin equivalent with 21 amino acids alike to the statherin 43 molecules on the rate of demineralization using scanning microradiography under replicated carious conditions. This *in vitro* study disclosed that HAp coated with StN21 for 24 hours before exposing them to a replicated carious condition reduced the rate of mineral loss by 40 %.

The aim of this study was to identify the functional domain of statherin required for its cariostatic function by measuring the efficacy of STATHERIN-like peptides with five different amino-acid sequences (StN21 1A, StN21 2A, StN21 3A, StN21 4A, and StN21 5A) at the N-terminal respective, *in vitro* using scanning microradiography.

StN21 DS*S*EEKFLRRIGRFGYGYGPY

StN21 1A **A**S*S*EEKFLRRIGRFGYGYGPY (StN21 1Alanine)

StN21 2A **AA**S*EEKFLRRIGRFGYGYGPY (StN21 2Alanines)

StN21 3A **AAA**EEKFLRRIGRFGYGYGPY (StN21 3Alanines)

StN21 4A **AAAA**EKFLRRIGRFGYGYGPY (StN21 4Alanines)

StN21 5A **AAAAA**KFLRRIGRFGYGYGPY (StN21 5Alanines)

Alanine residues were used to replace aspartate, phosphorylated serine, and glutamic acid residues in order to identify which if any of the amino-acids at the N-terminal of the peptide is involved in preventing hydroxyapatite dissolution.

The objective of the study was to analyze the effects of statherin like peptides on the degree of mineral mass lost from Hap surface linearly connected to time. To compare the percentage of mineral lost from HAp discs treated with StN21 1A, StN21 2A, StN21 3A, StN21 4A, and StN21 5A to that of HAp disc treated with StN21 using SMR and then study and discuss the results in correlation with significant and relevant literature.

MATERIALS AND METHODS

Scanning microradiography (SMR) is a unique photon counting method which is delicate to small variations in the attenuation of X-ray of specimens is used to measure detect and measure the small structural changes in the experiment. Hydroxyapatite manufactured by Plasma–Biotal Ltd, UK was used as samples for this study as a tooth analog with related chemistry to natural enamel. Synthetic HAp discs with proved mineral composition and clarity were used for this study as an alternative of enamel because studies by Elliott, 1994, confirm structural abnormalities, dissimilarity in pore volume and chemical structure of tooth enamel both individually and collectively leads to unclear demineralization results whereas Hap discs act more reliable during *in vitro* studies that simulate carious and erosive conditions.[20]

A total of seven SMR cells with HAp discs of even size and thickness were set and fixed on the horizontal scanning stage. With the peristaltic pump, deionized water was circulated through cells for 24 hours at a rate of 2.20 rpm. Line and area scans for all HAp discs were done using the software designed by Dr. Paul Anderson. The prepared demineralizing solution was formerly circulated for 72 hours for first demineralization. HAp discs were then washed with deionized water to make them ready for peptide adsorption. All five peptides plus StN 21 were prepared, and HAp discs were coated for 24 hours by injecting five peptides, StN21, and phosphate buffer in the cells. Acetic acid was circulated again through all these cells for 72 hours to record the decrease in rate of demineralization in each sample. Line scans continued till the end of the experimentation, and data of the rate of HAp surface mineral loss was studied.

RESULTS

The effects of statherin like peptides**StN21 1 A, StN21 2A, StN213A, StN21 4A, StN21 5A, and StN21** on Hap discs are represented in the following figures' 'B' 'C' 'D' 'E,' and 'F' respectively. Similarly the figure'G' represents the single point in the HAP disc effects of Phosphate Buffer. It is a part of the test group. In these graphs the changes in mineral mass of the HAp pellet in real-time over 72 hours before coating with statherin like peptides and for 72 hours after coating with statherin like peptides were observed. The HAp discs were exposed to demineralising solution comprising of acetic acid buffered to pH 4.0 before a 24 hour before introduction of statherinlike peptide mentioned above. The rate of mineral loss recorded from line scans during demineralization before introduction of statherin like peptides**StN21 1 A,StN21 2A, StN213A, StN21 4A, StN21 5A, and StN21**is represented by the blue dots in the above-mentioned figures. After the HAp specimen was exposed to peptides demineralizing solution was reintroduced, and the rate of mineral loss was scanned along the same points on the line as before. This rate is represented by the pink dots in the above-mentioned graphs. A comparison of gradients acquired from the line scans across the HAp discs surface shows a rate of mineral loss per hour (gm./h-2) before and after the introduction of statherin like peptides **StN21 1 A,StN21 2A, StN213A, StN21 4A, StN21 5A, and StN21**.

(4.1) HAp disc coated with Statherin like peptideAS*S*EEKFLRRIGRFGYGYGPY(StN21 1A)

This result, therefore, is indicative of the fact that the StN21 1A does offer a protective role in limiting the rate of demineralization of HAp around 53 % when exposed to acidic medium undersaturated with respect to HAp. This graphic representation was repeated for each point along the lines for each peptide. This graphic representation was repeated for each point along the lines for each peptide. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

(4.2) HAp pellet coated with Statherin like peptide AAS*EEKFLRRIGRFGYGYG-PY(StN21-2A)

This result, therefore, is indicative of the fact that

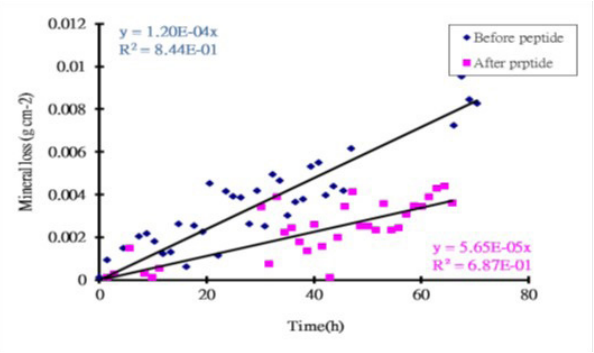

Figure A: Rate of mineral loss at the same point on HAp pellet coated with StN21 1A.

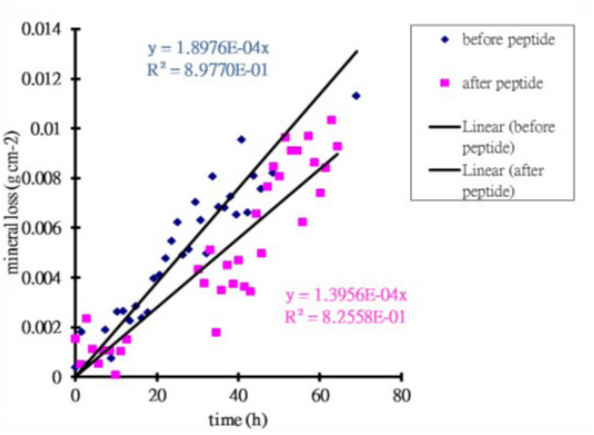

Figure B: Rate of mineral loss at the same point on HAp pellet coated with StN21 2A

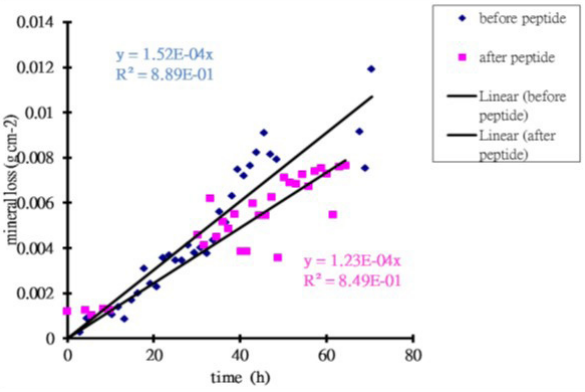

Figure C: Rate of mineral loss at the same point on HAp pellet coated with StN21 3A

the StN21 2Alanines does offer a protective role by limiting the rate of demineralization of HAp around 31% when exposed to acidic medium undersaturated with respect to HAp. This graphic representation was repeated for each point along the lines for each peptide. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

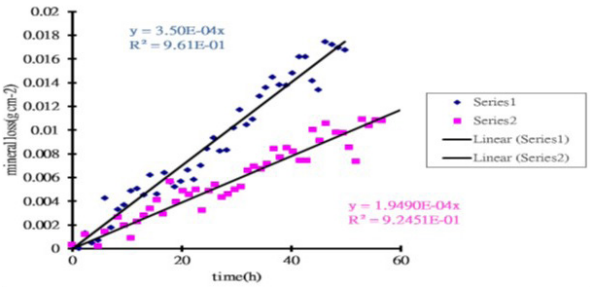

Figure D: Rate of mineral loss at the same point on HAp discs coated with StN21 4A

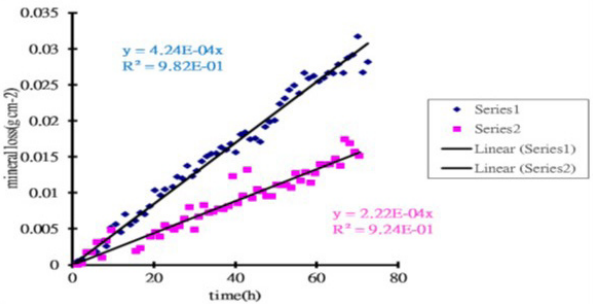

Figure E: Rate of mineral loss at the same point on HAp discs coated with StN21 5A

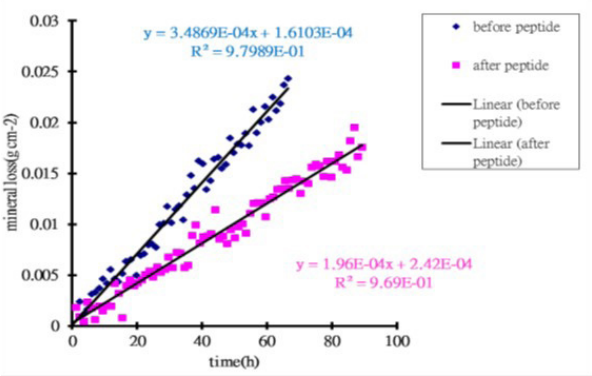

Figure F: Rate of mineral loss at the same point on HAp discs coated with StN21

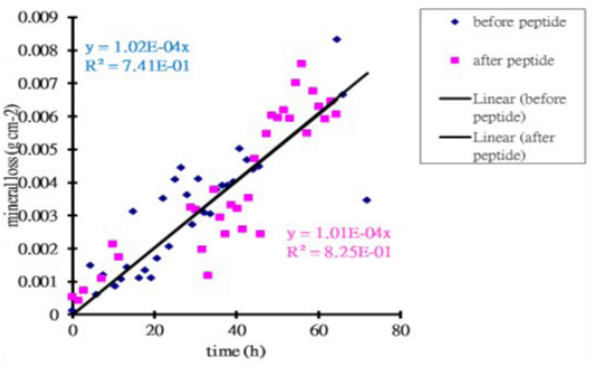

Figure G: Rate of mineral loss at the same point on Hap disc coated with PBS.

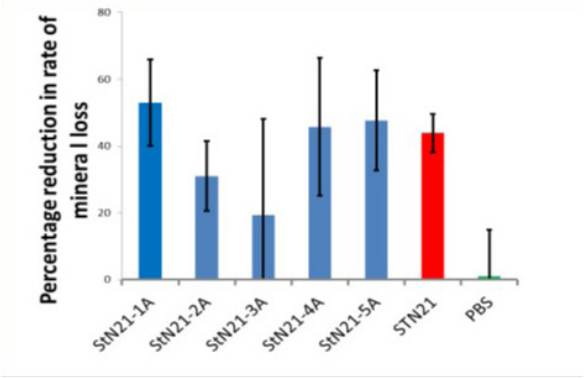

Figure H: Comparison of the rates of mineral loss in all 5 HAp samples.

(4.3) HAp pellet coated with Statherin like peptide –AAAEEKFLRRIGRFGYGYGPY (St-N213A)

This result, therefore, is indicative of the fact that the StN21 3Alanines does offer a protective role by limiting the rate of demineralization of HAp around 19% when exposed to acidic medium undersaturated with respect to HAp. This graphic representation was repeated for each point along the lines for each peptide. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

(4.4) HAp pellet coated with Statherin like peptide **AAAA**EKFLRRIGRFGYGYGPY (StN21 4A)

This result, therefore, is indicative of the fact that the StN21 4Alanines does offer a protective role by limiting the rate of demineralization of HAp around 46% when exposed to acidic medium undersaturated with respect to HAp. This graphic representation was repeated for each point along the lines for each peptide. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

(4.5) HAp pellet coated with Statherin like peptide **AAAAA**KFLRRIGRFGYGYGPY (StN21 5A)

This result, therefore, is indicative of the fact that the StN21 5A does offer a protective role by limiting the rate of demineralization of HAp around 44% when exposed to acidic medium undersaturated with respect to HAp. This graphic representation was repeated for each point along the lines for each peptide. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

(4.6) HAp pellet coated with Statherin DS*S*EE KFLRRIGRFGYGYGPY (StN21)

This result, therefore, is indicative of the fact that the StN21 does offer a protective role by limiting the rate of demineralization of HAp around 44%when exposed to acidic medium undersaturated with respect to HAp. This graphic representation was repeated for each point along the lines for each peptide. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

(4.7) HAp pellet coated with Phosphate Buffer

This result demonstrates that the phosphate buffer does not offer any defensive role against demineralization of HAp when exposed to acidic fluids undersaturated with respect to HAp. Hence 6 points on HAp specimen were similarly scanned and analyzed for loss of mineral.

It is a characteristic graph at a distinct point in the HAp slice representative of the effects of phosphate buffer used as a negative control in this experiment. It shows the change in mineral mass of the HAp disc in real time over a period of 72 hours before coating with PBS and for 72 hours after coating with PBS. The HAp pellet was exposed to demineralising solution comprising of acetic acid buffered to pH 4.0 prior to introduction of phosphate buffer. The rate of mineral loss recorded from line scans during demineralisation prior to a 24 hour. The rate of mineral loss recorded from line 52 scans during demineralisation prior to a 24 hour introduction of phosphate buffer is shown by the blue dots. After the HAp specimen was bathed in phosphate buffer the demineralising solution was reintroduced and the rate of mineral loss was scanned along the same points on the line as before.

DISCUSSION

Overall Result

In this research rate of demineralization was linear for all cases both before and after the application of peptide. The above figure shows a comparison of the rates of mineral loss after coating of the HAp samples as a percentage of the rate before coating for the phosphate buffer and StN21 1A, StN21 2A, StN21 3A, StN21 4A StN21 5A, and StN21 peptides. The bar chart summarizes the results obtained in this study. The protective effect exerted by the N–terminal of the StN21 peptide on the rate of Hap mineral dissolution was (44%) StN21 1A(53%), StN21 2A(31%), StN21 3A(49%), StN21 4A(46%) StN21 5A(48%)and finally phosphate buffer (1%). From the bar chart it is clear that StN21 reduced the rate of mineral loss by 44 %,and we got the same range of percentage for StN21 1A, StN21 4A, and StN21 5A. This means that theses sequence of amino acid even after replacing with alanine showed to have the same result as that of positive control .from which it can be concluded that there are other amino acids that play a vital role which should be studied again with different sequence in future work.But on the other hand StN21 3A and 2A showed the least remarkable results of all which means that this sequence does not have much to do with inhibiting mineral loss

CONCLUSION

Not only do the five amino acids in the N-terminal, i.e. aspartate, phosphated serine, and glutamic acid are involved in cariostatic activity, but also, there are other amino acids to play a vital role in inhibition of mineral loss. Moreover aspartate and phosphate serine are found to be least involved in inhibiting mineral loss when compared to another sequence

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