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Effect on Pellicle Proteome upon Adsorption of Salivary Peptide on Hydroxyapatite and their effects on Enamel Demineralization

Rajesh Kr. Gupta, *The University of Western Ontario*

Supervisor: Dr.Walter L.Siqueira, *The University of Western Ontario*

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Abstract

According to WHO, oral health is an important indicator of overall health. Poor oral health cause serious health problems (cardiovascular, stroke, diabetes, liver, and respiratory related), in different age groups, costing billions of dollars. So, proper oral health care management has assumed top priority. Salivary antimicrobial peptides (histatin and statherin), are part of enamel pellicle, required for the oral homeostasis. Current work focused to study the demineralization protection and pellicle proteome modulation capabilities of novel peptide constructs (single and tandem duplicate histatin constructs DR9, DR9-DR9 and histatin-statherin hybrid construct DR9-RR14). For the first time, we provided evidence that, these peptide constructs (low concentration) modulated the pellicle proteome composition as analyzed through nLC-MS. We showed that DR9, and DR9-DR9 provided enamel demineralization (Ca^{2+} and PO_4^-) protection in presence of parotid saliva pellicle. It is important work from the point-of-view of enamel protection and inhibition of pathological biofilm formation by novel salivary peptides and hence infection in the oral cavity.

Keywords

Parotid saliva, antimicrobial peptides, salivary peptides, DR9/DR9-DR9/DR9-RR14, histatin, statherin, demineralization, enamel, acquired enamel pellicle, proteome.

Co-Authorship Statement

All chapters were written by Rajesh Gupta and edited by Prof. Walter L. Siqueira, DDS, PhD. Prof. Siqueira provided guidance throughout the whole study, including concept of the idea, experimental design, data interpretation, statistical analyses and technical advice for all parts of the study. Yizhi Xiao, MSc, PhD, provided technical support for all the experimental parts and offered generous knowledge and consult throughout the study.

All experiments were performed by Rajesh Gupta except the followings: Paula Karina Jorge (DDS, M.Sc.) and Vinicious Ausgustus (DDS) prepared the enamel discs for the demineralization experiments. Eduardo Moffa (DDS, M.Sc.) and David Zuanazzi (DDS, M.Sc.) helped with the collection of parotid saliva.

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(Rajesh Gupta)

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Chapter 1

1 Introduction

According to World Health Organization (WHO), today oral health has become important indicator of overall health, well-being & quality life (Sheiham 2005). It is one of the most important health management issue all over the world, but unfortunately highly neglected for various reasons including socio-economic & lack of awareness. Oral health in parts, is governed by the intricate and fragile balance of consortia of non-pathogenic and pathogenic microorganisms in the oral cavity. Poor oral health inevitably leads to the development of more serious, sometime deteriorating health complications oftentimes resulting in debilitating, and fatal health outcome. This includes direct/indirect involvement of poor oral health in precipitation of cardiovascular diseases, stroke, development of diabetes, liver problems, adverse respiratory complications, pregnancy complications & many more health problems with often extreme consequences (Health Canada, 2009; Scannapieco *et al.* 2003; Okuda *et al.* 2004). According to an estimate in Canada, oral health has become one of the major health concern economically, next to cardiovascular health in majority of population encompassing different age groups (Health Canada, 2009). According to the Canadian Institute for Health Information estimates, for the past more than two decades, total dental expenditure continues to rise by Billions of dollars, as more & more population is fast aging (Health Canada, 2009). Under such grim scenario, the proper oral health care management assumes nonetheless more importance & priority than ever before.

Oral cavity is one of the best example of diverse communities of different types of microorganisms living in peaceful co-existence. These consortia have different species of Gram-positive & Gram-negative bacteria, covering a whole multitude of cocci, bacilli, actinomycetes & other motile as well as non-motile forms (Kolenbrander and London 1993; Kolenbrander & Andersen 1986), different types of yeast, fungi & viruses as well. According to conservative estimates, based on *in vitro* cultivation, PCR amplification, 16 S rRNA molecular typing (Paster *et al.* 2006; Nasidze *et al.* 2009; Heller *et al.* 2016) & pyro sequencing technology (Zaura *et al.* 2009), there are > 1000 different types of

microorganisms, that coexist both as planktonic form, in the saliva, as well as multi layered, mixed species biofilms in highly specific collaborative partnership on dental & other mucosal surfaces in the mouth (Kolenbrander *et al.* 2010). These biofilms are formed by specific cell-cell interactions between genetically diverse microorganisms & their interactions with acquired enamel pellicle through the complicated processes of co-adhesion & co-aggregation (Kolenbrander *et al.* 2010). For example, intercellular interactions in *Capnocytophaga gingivalis* & *Actinomyces israelii* or between *Prevotella loescheii* & *Streptococcus sanguinis*, respectively (Bos *et al.* 1996).

Saliva is a complex aqueous mixture consisting of inorganic and organic components, that are derived from secretions of salivary glands, oral mucosa, the gingival fold, epithelial cells, blood cells, food, and microorganisms (Humphrey and Williamson 2001; Macarthur and Jacques 2003; Edgar *et al.* 2004; Huang 2004; Guo *et al.* 2006; Schipper *et al.* 2007; de Almeida Pdel *et al.* 2008; Siqueira *et al* 2008).

From the biochemical point of view, the salivary proteins represent a rich source biological information and at the same time offer unique opportunity to be of immense clinical relevance (Schipper *et al.* 2007).

Salivary proteome consists of thousands of salivary proteins (2300 plus proteins and peptides), that are secretory product of three major salivary glands (submandibular, sublingual, and parotid glands) and several minor salivary glands, including various non-salivary components (Dawes 2004; Bandhakavi *et al.* 2009; Helmerhorst and Oppenheim 2007; Siqueira *et al.* 2008). The salivary secretion to the tune of around ninety percent in the whole saliva is contributed by three pairs of major salivary glands (Pedersen *et al.* 2002). Remaining ten percent of the salivary fluid in the total saliva is contributed by minor salivary glands (estimated to be somewhere between 400-600) in the oral cavity. These minor glands are present in the oral mucosa coverings in the mouth cavity, including tongue (Lingual/Von Ebner glands), palate, cheeks, land lips (Schipper *et al.* 2007; Proctor and Carpenter 2007).

Some of the salivary protein secretory IgA (main salivary antibody) are present in the secretion from all major glands. There are other salivary proteins that are specific to a

particular gland (PRPs (proline-rich proteins)), that are secreted by parotid and minor salivary glands only (Dawes *et al.* 1963; Siqueira *et al.* 2008).

Saliva being one of the most crucial and vital fluids of the human body, performs multidimensional roles within the micro-environment of the oral cavity. These roles include, aid in the food digestion, and organoleptic detection of the taste and flavor of the food we eat. Additionally, saliva in the oral cavity, acts like a lubricant and antimicrobial agent. Saliva also provide protection against the acid induced teeth enamel demineralization. Besides this, one of the most important function of saliva is the formation, as well as continuous development and maturation of acquired enamel pellicle (AEP) on the enamel surface.

Enamel pellicle is principally composed of a thin organic, proteinaceous covering layer on the teeth enamel surface (Dawes *et al.* 1963). It is formed by highly selective adsorption of salivary proteins and peptides on to enamel surface (Hay 1967; Siqueira *et al.* 2007). Pellicle perform many diverse functions for example enamel surface lubrication (Tabak 1995; Douglas *et al.* 1991; Ramasubbu *et al.* 1993), enamel mineral homeostasis (Moreno *et al.* 1979; Hay *et al.* 1979; Bennick 1979), and microbial flora and biofilm modulation (Johansson *et al.* 2000; Gibbons and Hay 1989; Carlen *et al.* 1998; Gibbons 1996; Lindh *et al.* 2002).

The acidic proline-rich proteins and statherin, present in human parotid saliva, submandibular as well as sublingual saliva (Hay 1989; Bennick and Cannon 1978), are most important salivary proteins involved in the pellicle formation and its multidimensional functions (Hay 1973; Bennick 1987; Yao *et al.* 2001).

The processes of the formation of acquired enamel pellicle on enamel surface has been studied extensively for last 40 years to gain insight into the pellicle composition and structure. Hannig (1999), observed that with in one-minute exposure of the enamel surface, to the oral environment, a thin layer of enamel pellicle could be detected. Two hour period was found to be sufficient to complete pellicle formation as after 2 hours, no further increase in the protein/peptide could be observed. On the other hand, significant amount of microorganisms attach to the pellicle and initiate the formation of

biofilms/dental plaque. (Siqueira *et al.* 2012). The structure and architecture of the pellicle have been further probed with the help of exhaustive electron microscopic studies. The early pellicle precursor proteins selectively adsorb on to enamel surface via electrostatic interactions, resulting in the formation of a thin pellicle layer of 10-20 nm (Hannig and Joiner 2006). Within next 1.5 hours, late precursor proteins & their aggregates are selectively recruited and adsorbed to thin pellicle on the enamel surface. This process quickly leads to a mature pellicle of around 1000 nm thickness (Skjorland *et al.* 1995). Majority of these studies have helped to elucidate not only the detailed fine structure of pellicle the pellicle but also the pellicle thickness ranging from 30-100 nm. (Tinanoff *et al.* 1976; Hannig and Joiner 2006; Hannig 1989).

Various researchers have employed diverse experimental setup like hydroxyapatite (HA) discs and HA powder, with whole saliva to understand the enamel pellicle composition. Such experimental models led to the detection, identification of important and abundant pellicle proteins such as amylase, albumin, carbonic anhydrase VI, cystatins, histatins, lysozyme, proline-rich proteins (PRPs) & statherin (Hannig 1999; Li, *et al.* 2004; Jensen *et al.* 1992; Oppenheim *et al.* 1988, 2007; Oppenheim *et al.* 1986).

The current knowledge about the AEP ultrastructure is the result of various electron microscopic and AFM studies in the last more than 15 years, (Schüpbach *et al.* 2001; Hannig *et al.* 2005; Deimling *et al.* 2004; Siqueira *et al.* 2012), through ultra-microscopic examinations via AFM, TEM, and SEM. Even though, important insights about the pellicle structure and composition have been gained in last forty years, still a complete understanding of the 3-D architecture of AEP is eluding so far.

Within the last decade, the sensitive techniques of LC-ESI-MS/MS (Siqueira *et al.* 2007) and MALDI-TOF-MS (Vittorino *et al.* 2008) were employed to elucidate the pellicle proteome composition. These studies resulted in the identification of 130 proteins in the salivary pellicle composition (Siqueira *et al.* 2007). Out of these 130 identified proteins, 113 were found to be novel proteins. Histatins, statherin, cystatins, and PRPs, are the major constituent of pellicle making up the bulk of salivary proteins (Oppenheim *et al.* 1986, 1971; Hay 1975, 1973). In addition, these technical advances in the proteomic

instrumentation, resulted in the identification of 78 naturally occurring pellicle peptides, derived from 29 different in vivo pellicle proteins (Siqueira and Oppenheim 2009).

Multiple peptides identified in pellicle peptidome were the proteolytic products of statherin and histatins salivary proteins. The enamel pellicle acts as lubricant in the oral cavity, improving the speech and chewing efficiency (Tabak *et al.* 1982). Statherin, aPRP1, and mucins, are pellicle constituents, that are involved in these processes (Tabak 1995; Berg *et al.* 2004; Vukosavljevic *et al.* 2014). Due to lubrication properties, pellicle also play a role to protect enamel from mechanical damage (Joiner *et al.* 2008). Through various studies, it has been demonstrated that multiple pellicle components protect enamel from acid-induced demineralization (Hannig and Balz 2001; Cheaib and Lussi 2011; Siqueira *et al.* 2010).

Statherin is a salivary phosphoprotein that contain phosphorylated serine residues at 2nd and 3rd position. It has very important physical properties, crucial for proper saliva functioning. In the saliva, statherin is the highly enamel surface-active component with important lubrication function on the enamel surface. (Proctor *et al.* 2005; Douglas *et al.* 1991; Harvey *et al.* 2011). Additionally, statherin and aPRPs play a clinically crucial role in inhibition and prevention of crystal deposition on enamel surfaces (Hay *et al.* 1979). Statherin is the most important calcium phosphate precipitation inhibitor, that can display inhibitory effect at physiological concentrations (Tamaki *et al.* 2002). Moreover, recently our group has found that the N-terminal domain phosphate groups of statherin oligopeptides inhibit the growth of hydroxyapatite crystal (Xiao *et al.* 2015). Additionally, statherin quickly disappear from whole saliva due to proteolytic activity and its affinity for tooth surfaces.

Enamel pellicle contain histatins protein family members, which are secretory product of major and minor salivary glands. Histatins consists of higher amount of histidine residues (Oppenheim *et al.* 1986) and display significant antifungal activity against *Candida albicans*, the major pathogen of oral candidiasis. Histatin 1, 3 and 5 are the major members of the histatin family out of the 12 members histatin family, and account for 80% of this family (Oppenheim *et al.* 1988). Histatin 1, similar to statherin, contain

phosphoserine at N-terminal 2nd residue. In whole saliva, histatins is found at lower concentrations in comparison to glandular salivary secretions. This happens most likely because of salivary proteolytic activity from various proteolytic enzymes of host as well as microbial origin in the oral cavity (Siqueira *et al.* 2010). This results in the rapid breakdown of these salivary proteins, as soon as they are secreted into the oral cavity. Additionally, histatins are involved in multitude of processes, ranging from modulation of enamel mineralization, antibacterial activities, and saliva buffering (Castagnola *et al.* 2004; Groot *et al.* 2006).

In the light of this background, we hypothesized that novel constructs of the functional domain of histatin and statherin salivary peptides could provide protection against the acid induced enamel demineralization. This novel approach of synthetically constructing salivary peptides could possibly help us leverage and reap the immediate benefits, ahead of its time of millions of years of natural evolution, that it would otherwise follow naturally over a long period of time, as these salivary proteins could undergo evolutionary pathways that they will eventually take.

Therefore, the main aims of this study were to:

1) Investigate the demineralization protection capabilities of the novel constructs of histatin and statherin peptides functional domains compared to parotid saliva

2) Additionally, to investigate, if these novel constructs could modulate the acquired enamel pellicle proteome composition, which in turn could modulate the biofilm composition on the enamel pellicle surface.

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Chapter 2

2 Role of saliva and AEP in oral and overall health

Saliva is one of the very versatile, heterogenous fluid of human body, very essential for the normal health and functioning of the oral cavity. It protects the teeth against the continuous mechanical, biochemical, and microbial assaults through a protective covering, salivary pellicle. Additionally, the pellicle helps with the remineralization of dental enamel apart from minor repairs of teeth and other tissues in the oral cavity against small injuries or wounds. Disturbances in the normal production of saliva not only affect the pellicle composition but also impact the oral and overall health of the individual (Sheiham 2005; Edgar *et al.*, 2004; Dawes 2004).

Several studies have shown that there are many proteins, especially indicators of various serious pathological conditions as well as infections, that are present in both blood plasma and saliva (Humphrey and Williamson 2001; Macarthur and Jacques 2003; Edgar *et al.* 2004; Schipper *et al.* 2007; de Almeida Pdel *et al.* 2008; Siqueira *et al.* 2008; Boackle *et al.* 1999 and Kajisa *et al.* 1990). These disease markers offer very good opportunities for saliva as well as pellicle to be a very reliable, robust, and sensitive diagnostic tool for the early diagnosis of the diseases and infection.

2.1 Oral cavity

Oral cavity is one of the best example of multicultural communities of different types of microorganisms living in, harmonious, peaceful co-existence. These consortia have different species of Gram-positive & Gram-negative bacteria, covering a whole multitude of cocci, bacilli, actinomycetes & other motile as well as non-motile forms (Kolenbrander *et al.* 1993; Kolenbrander & Andersen 1986), including different types of yeast, fungi & viruses as well.

According to conservative estimates, based on *in vitro* cultivation, PCR amplification, 16 S rRNA molecular typing (Paster *et al.* 2006; Heller *et al.* 2016) & pyro sequencing technology (Zaura *et al.* 2009), there are > 1000 different types of microorganisms, that coexist both as planktonic form, in the saliva, as well as multi layered, mixed species

biofilms in highly specific collaborative partnership on dental & other mucosal surfaces in the mouth. These biofilms are formed by specific cell-cell interactions between genetically diverse microorganisms & their interactions with AEP through the complicated processes of co-adhesion & co-aggregation. (Kolenbrander *et al.* 2010).

In the oral cavity, Streptococci recognizes pellicle proteins. Most of these proteins like statherin, proline rich proteins (PRPs), salivary α-amylase, salivary agglutinin, and mucins, are part of host derived pellicle. Actinomyces attached itself to phosphorylated statherin and PRPs. On the other hand, Fusobacteria selectively and specifically attaches to statherin only, but not to PRPs (Kolenbrander *et al.* 2010).

2.2 Saliva

The saliva is a fluid of various structural and functional proteins, various minerals, inorganic ions, (calcium, phosphate, bicarbonates, and urea), immunoglobulins, hormones, cytokines, and other components whose abundance is dependent upon the gland from which it is secreted. Three pairs of major salivary glands, (parotid, sublingual and submandibular) and hundreds of minor salivary glands, distributed throughout the oral cavity, produce it (Thylstrup and Fejerskov 1994).

Whole saliva is a heterogenous mix fluid, (Huang 2004), that contain secretions from many source, primarily the extrinsic glands, epithelial cell secretions, and the gingival crevicular fluid. Blood capillaries, passing through the salivary glands, also contribute the entry of analytes from the blood circulation into saliva (Farnaud *et al.* 2010).

Whole saliva mainly consists of 99.5% water, and 0.2% inorganic substances (calcium, phosphate, bicarbonate, urea), and 0.3% proteins (Humphrey and Williamson 2001; Amerongen *et al.* 2004).

From human saliva, as of today, > 2300 salivary proteins have been identified using various analytical techniques (2-D PAGE, HPLC and MS) (Bandhakavi *et al.* 2009).

Unstimulated and stimulated saliva substantial differ in their composition. Under resting conditions, submandibular/sublingual glands contribute around 70% of the whole saliva,

whereas about 15-20% saliva comes from the parotid glands and minor salivary glands contribute around 5-8% saliva. Under stimulated conditions, the parotid gland can contribute up to 50% of the total saliva (Sreebny 2000).

2.3 Salivary glands

The salivary glands vary greatly in the type of secretion they produce, which corresponds to the proportion of serous to mucous cells within the respective glands. Parotid glands contain serous cells that produce a watery fluid, that is free of mucins. On the other hand, submandibular, labial, palatal, sublingual, and lingual glands, contains substantial number of mucous cells, that produces mucin rich saliva (Turner and Sugiya 2002).

2.4 Salivary composition

The saliva composition and amount is dictated by many variables like the circadian rhythm (Zheng *et al.* 2012), age (Denny *et al.* 1991), gender (Inoue *et al.* 2006), physiological status (Fábián *et al.* 2007), and flow rate (Sreebny 2000).

Low protein and ion concentration in the saliva, modifies the physiological properties of water into an aqueous solution of very different physical and physiological properties. These modulated properties of saliva help immensely due its multidimensional roles in lubrication, speech, food digestion, and most importantly in the pellicle formation, all of which collaborate to maintain oral health directly or indirectly (Carpenter 2012).

2.5 Acquired enamel pellicle

In the oral cavity, acquired enamel pellicle (AEP) is nonembryonic, extracellular, organic integument or bacteria free thin film of heterogenous proteins, that not only acts as a protective covering of the tooth enamel and modulator of bacterial biofilms, but also acts as a semipermeable membrane in caries development. (Dawes *et al.* 1963; Hannig and Joiner 2006).

AEP is a complex biological, multilayer, heterogeneous, 3-D architecture of specific salivary proteins fragments, small peptides, intact native proteins, glycoproteins, enzymes, mucin and their derivatives, lipids, carbohydrates & food components (Siqueira

and Oppenheim 2009; Slomiany *et al.* 1986; Slomiany *et al.* 1990; Kautsky and Featherstone 1993; Sonju 1975).

Hay (1967) in a classical study demonstrated that the hydroxyapatite adsorbed proteins had same electrophoretic mobility whether harvested from freshly extracted teeth *in vivo* or salivary proteins harvested from hydroxyapatite and enamel powder *in vitro*. This was significant finding as to hypothesize that the pellicle that forms on tooth surfaces originates from salivary protein present in saliva.

Enamel pellicle functions as an interface between enamel surface & first layer of microorganism biofilm in the oral cavity, with diverse protective functions in the oral cavity. For example, the AEP helps in teeth lubrication, protecting enamel against mechanical damages during mastication and erosion (Hannig *et al.* 2004), and modulating the early microbial colonizer composition on AEP (Zahradnik *et al.* 1978; Lendenmann *et al.* 2000; Siqueira *et al.* 2007, 2010, 2012; Hanning & Joiner 2006; Vitorino *et al.* 2007).

Additionally, it acts like a semipermeable barrier helping to promote tooth re-mineralization, at the same time protecting acid induced teeth demineralization in the oral cavity (Hannig *et al.* 2007; Kosoric *et al.* 2007; Kosoric *et al.* 2010).

Interestingly, the AEP is double edged sword like a kind of necessary evil, since, not only it protects the enamel against various mechano- physical and chemical damages, but also acts as the basic platform for microbial attachment and subsequent build-up of microbial biofilms, which can augment the dental caries risk many fold (Hannig and Hannig 2009).

Despite so much importance and direct involvement of the AEP in oral physiology and pathological processes, still a complete understanding and elucidation of AEP structure, functions, and underlying physico-chemical mechanisms is lacking to great extent. The major hurdle to achieve this has been due to very small quantity formed pellicle, that could be harvested from tooth surfaces (Yao *et al.* 2001).

2.6 AEP composition

AEP is a complex mix of various proteins and peptides, and there have been many technical challenges in elucidating its composition. Despite these technical challenge, several researchers (Gocke *et al.* 2002: Li *et al.* 2004; Hannig *et al.* 2005; Hannig *et al.* 2009; Yao *et al.* 2003; Siqueira *et al.* 2007; Vitorino *et al.* 2007; Vitorino *et al.* 2008; Yao *et al.* 2001) have employed various methods and tools to gain understanding of the AEP composition.

2.7 AEP salivary proteins

The major salivary protein families associated with AEP include acidic proline rich proteins (aPRPs) (Oppenheim *et al.* 1971), basic PRP, amylase, MUC5B, agglutinin, cystatins (Larkin *et al.* 1991), histatins (Hay 1975; Oppenheim *et al.* 1986) and statherin (Hay 1975; Helmerhorst and Oppenheim 2007; Messana *et al.* 2008; Schipper *et al.* 2007).

Many salivary proteins, like statherin, proline-rich glycoproteins, mucins, acidic proline-rich proteins, and amylase, have been proposed to be associated with the lubricating properties of pellicle (Proctor *et al.* 2005; Berg *et al.* 2004; Hannig *et al.* 2007).

Previous work discovered that mature AEP proteome consists of different proteins and peptides (Siqueira *et al.* 2007; 2009). Siqueira *et al.* (2007) employed LC-ESI-MS/MS to identify the salivary protein components of *in vivo* AEP. Through MS following a shotgun approach, they identified 130 proteins and peptides ranging in size from >250 kDa to <5 kDa. Most of them were without any known function. About 50% were small molecular weight peptides in the *in vivo* formed AEP from a single subject as well as from pooled samples of number of volunteers.

Siqueira *et al.* (2009) in previous study discovered and identified small molecular weight salivary proteins and peptides from the *in vivo* AEP employing MS. They showed for the first time that 50% of AEP protein composition is made up of small proteins and peptides ranging in size from 0.8 kDa to 3.9 kDa. In all, they found that there were 78 naturally occurring peptides in AEP which originated from 29 different salivary proteins.

Zimmerman *et al.* (2013), for the first time did the comprehensive analysis of the proteome and peptidome composition of AEP from deciduous teeth. They identified 76 pellicle proteins and 38 naturally occurring peptides from 10 salivary proteins using MS.

Based on the possible role of these proteins in AEP development, Siqueira *et al.* (2007), classified these pellicle proteins into 3 major groups; Ca^{2+} binding proteins, PO_4^{3-} binding proteins & proteins interacting with other salivary proteins. Pellicle proteins have also been classified according to their putative biological functions; inflammatory responses, immune defense, antimicrobial activity & remineralization potential.

Furthermore, in a recent study, Hannig *et al.* (2009), found that alanine amino transferase and amino transferase enzymes were present after 3 minute of pellicle formation, in active form. These enzymes, by complexing adsorbed molecules, might influence pellicle formation (Iontcheva *et al.* 1997) or they may influence pellicle proteins cross-linking enzymatically (Yao *et al.* 2000). So far, a complete role, that these active enzymes may play, in pellicle formation or its structure is yet to be elucidated.

Many different studies (electrophoretic, chromatographic, immunological) have been undertaken to investigate and elucidate the pellicle composition. Most of these studies have shown that proteins, enzymes, mucins, and glycoproteins are the major components of the pellicle (Lendenmann *et al.* 2000). For example, amylase, IgA, S-IgA, PRPs, mucins MG1 and MG2, albumin, carbonic anhydrase, cystatins, histatins, statherin, lactoferrin, and lysozyme, shave been identified as major components of pellicle by different researches (Schüpbach *et al.* 2001; Li *et al.* 2004; Vitorino *et al.* 2004, Hey 1975; Oppenheim *et al.* 1986; Siqueira *et al.* 2007; Siqueira *et al.* 2009; Siqueira *et al.* 2010).

Many indirect techniques like immunological detection has immensely contributed in further identification of pellicle components and the composition of *in vivo* pellicle (Yao *et al.* 2003). The strong evidence for the presence of statherin, lysozyme, and alpha-amylase, as major components of the acquired enamel pellicle were also provided by these indirect methods of detection. (Hannig *et al.* 2007; Li *et al.* 2004).

Lately the development in analytical tools like mass spectrometry and its algorithms has given a major impetus to the high throughput discovery and characterization of salivary proteins and peptide due to its extreme sensitivity up to femtomole level. For example, Siqueria *et al.* (2007) for the first time identified 130 proteins pellicle through LC-ESI-MS/MS, of which 89 proteins were reproducibly present in three out of the five independent experiments, demonstrating high degree of inter sample uniformity.

Vittorino *et al.* (2008) employed MALDI-MS/MS to analyze human AEP and identified more than 90 pellicle peptides/proteins. Most of the identified peptide and proteins were part of statherin, histatins, cystatins basic PRPs, and aPRPs.

2.7.1 AEP formation

Initially, Alexander Nasmyth described it as persistent dental capsule of embryonic origin (Nasmyth 1839). Later, it was accepted that embryologic integuments are lost after teeth eruption. Dawes *et al.* (1963) introduced term acquired enamel pellicle to denote non-cellular, bacteria-free, protein rich membrane integument that covers the dental enamel in the oral cavity.

Pellicle formation is a highly selective process, since only a limited number of the salivary proteins are a part of AEP (Yao *et al.* 2003; Siqueira *et al.* 2007). The amino acid profiles of the saliva samples were different from the pellicle profiles, clearly indicating that pellicle was composed of selective adsorption of salivary proteins. (Siqueira *et al.* 2007). Phosphorylated proteins like, histatin, statherin, and acidic proline rich proteins, are the main salivary proteins, speculated to play an important role as pellicle precursor proteins. Therefore, these proteins have been extensively characterized in terms of their ability to adsorb calcium phosphate rich enamel surfaces and influencing formation of pellicle. (Yin *et al.* 2006; Smith and Bowen 2000).

Consensus among oral biologist is that the acquired enamel pellicle forms in two phases. In the first phase, first few layers of initial pellicle forms through non-covalent interactions of the calcium and phosphate binding proteins, with very high affinity to

hydroxyapatite (HA), adsorb the tooth surface almost instantaneously. This process occurs within few milliseconds to few minutes. (Hannig 1999).

Hannig (1999) observed that the initial stage of pellicle formation can be visualized as an electron dense basal layer observed via transmission electron microscopy. He found that the dense basal layer of the AEP starts to form within seconds, taking between 2 - 3 minutes to complete, reaching an estimated thickness of 10 - 20 nm, where it remains for about 30 minutes. A slower second phase follows the rapid first stage of AEP formation, whereby pellicle thickness increases by continuous and selective adsorption of salivary proteins from saliva.

This first phase then paves the way for a secondary slower phase, where the continuous and selective adsorption of salivary proteins and other biomolecules present in saliva continues. These two phases of protein adsorption have been observed as two distinct zones through electron microscopy, of which the first phase, adjacent to hydroxyapatite surface is primary electron dense basal layer, and the second phase is an outer globular, porous layer. (Baek *et al.* 2009; Lendenmann *et al.* 2000; Hannig 1999; Hannig *et al.* 2004; Hannig *et al.* 2005; Deimling *et al.* 2007). It is further speculated that mainly PRPs constitute the AEP dense basal layer, while the combination of mucins and protein aggregates constitute outer globular, porous structure.

Clasen *et al.* (2000), showed that pellicle thickness differences could be more likely attributed to differences in the salivary secretion from different parts of the oral cavity, as secretions from different salivary glands contain various sizes and types of proteins in varying amounts and concentrations, which differ significantly between glands Walz *et al.* 2006).

It was demonstrated in previous studies, that low molecular proteins present in parotid saliva, like statherin, make an initial adsorbed layer of pellicle (Proctor *et al.* 2005; Santos *et al.* 2008), which will potentially follow monolayer adsorption behavior, and adsorption will be self-limiting when the surface is saturated (Rabe *et al.* 2011).

Taking advantage of recent advances in ultrastructural visualization tools, most of the efforts have been geared toward the elucidation of structure in the *in vitro* and *in vivo* AEP using several techniques, like scanning electron microscopy (SEM), transmission electron microscopy (TEM) confocal laser scanning microscopy (CLSM), and atomic force microscopy (AFM) leading to in-depth understanding of AEP structure (Siqueira *et al.* 2012; Deimling *et al.* 2004, 2007; Hannig *et al.* 2004; Hannig *et al.* 2005; Schwender *et al.* 2005; Baek *et al.* 2009; Ash *et al.* 2013; Veeregowda *et al.* 2012).

Hannig & Hannig (2009), elucidated that the pellicle formation mechanism is most likely mediated through electrostatic and hydrophobic interactions during adsorption of the salivary proteins on to hydroxyapatite surface. The smaller molecular weight, surface active phosphor-serines rich proteins like histatins and statherin, have a strong affinity for the hydroxyapatite and potentially have a higher surface diffusion coefficient, which allowing them to adsorb more quickly and form the primary layers in both parotid and whole saliva in the oral cavity.

Ash *et al.* (2013) found that subsequent adsorption of late pellicle proteins depends on the attractive interactions between the proteins present in the early pellicle layer, and these attractions perhaps, can be enhanced by specific calcium mediated interactions.

Pellicle precursor proteins appear to undergo conformational changes and increase their cross-sectional area to cover the enamel surface, when they adsorb to tooth surface (Norde 1995). Santos *et al.* (2010) also observed and confirmed earlier observations that after initial protein adsorption, conformational changes occur in the pellicle structure.

During the last 40 years in many studies, a variety of electrophoretic techniques have been employed to examine salivary protein adsorbed on enamel surfaces. All these techniques resulted in the identification of different salivary protein and classification of individual members to different protein families (Al-Hashimi and Levine 1989; Kousvelari *et al.* 1980; Oppenheim *et al.* 1986).

All these studies using different techniques have firmly established that the pellicle is composed of selective adsorption of salivary proteins on to the enamel surface (Siqueira *et al.* 2007; Siqueira and Oppenheim 2009; Siqueira *et al.* 2010).

Several *in vitro* (Fujikawa *et al.* 2008; Joiner *et al.* 2006), *in vivo* (Siqueira et al 2007; Siqueira and Oppenheim 2009), and *in situ* (Santos *et al.* 2008; Deimling *et al.* 2007) studies have been done with an intent to gain additional insight about the pellicle structure. These studies have generated diverse, in depth understanding about the pellicle composition, formation, structure, and function.

In a previous study, Douglas *et al.* (1991), demonstrated that salivary statherin, due to its amphipathic nature, most likely acts as an important boundary lubricant as a constituent of the pellicle. Reeh *et al.* (1996) provided another piece of evidence, that the amphipathic nature of molecules was important for lubrication, as amphipathic molecules reduced friction between opposing tooth surfaces.

Several systematic approaches have been adopted to get deeper insight into pellicle formation. Various *in vivo*, studies where the pellicle is harvested from the enamel surface (Li *et al.* 2003), *in situ* studies, where bovine enamel, is exposed in the oral cavity (Hara *et al.* 2006), or *in vitro* studies, where different dental components are exposed to collected saliva outside oral cavity (Vitorino *et al.* 2004), with a view to further understand the pellicle formation process.

Many studies have been performed using the saliva incubation with hydroxyapatite plaques (Deimling *et al.* 2004; Smith and Bowen 2000), or hydroxyapatite powder (Vitorino *et al.* 2004; Lamkin *et al.* 2001), to simulate pellicle formation in oral cavity.

Studies have demonstrated that, statherin, acidic PRPs, and histatins, also known as pellicle precursor proteins, that show preferential, selective enamel adsorption compared to other salivary proteins (Vitorino *et al.* 2008; Svendsen *et al.* 2008).

These *in vitro* studies led to invaluable clues why some salivary proteins display differential adsorption affinities for hydroxyapatite surfaces and provide evidences which proteins may bind to enamel surfaces.

It is consensus among oral biologists, that there are 4 fundamentals steps in AEP formation: 1). Diffusion of salivary proteins towards the enamel surface, 2). Selective protein adsorption on the enamel surface, 3). Conformational changes and cross-linking of adsorbed salivary proteins, 4). Detachment of salivary protein from the pellicle and diffusion from the enamel surfaces. The protein adhesion is the outcome of Van der Waals, acid–base, and electrostatic interactions taken together. (Demodarshan *et al.* 2008).

In several studies, it has been observed that the composition of pellicle differs significantly in different locations, from where it is collected in the oral cavity. Smith and Bowen (2000), demonstrated that the overall protein composition of *in vivo* formed pellicles displayed location specific characteristics, prevailing in the different locations within oral cavity, from where the pellicles were harvested.

Amaechi *et al.* (1999), and Hannig (1999) through independent investigations of *in situ* AEP with confocal laser-scanning microscopy and transmission electron microscopy found that AEP in different areas of oral cavity differ in the thickness. Palatal site pellicle is thinner than that of buccal sites pellicle and are therefore more likely to have different pellicle composition.

Phosphoproteins contain calcium-binding domains, that may provide a region of high calcium concentration in close vicinity to the enamel surface, thus augmenting the teeth mineralization. The importance of the pellicle in preserving oral health cannot be underestimated in Xerostomia patients, suffering from dry mouth syndrome (Tabak 2006; Vissink *et al.* 2010; Turner and Ship 2007). In the absence of sufficient saliva in the oral cavity, as effective salivary pellicle cannot be produced, these patients suffer from higher incidences of dental caries and damages to the mucous membrane. (Vissink *et al.* 2010)

In last few years, different researches tried to investigate the relationship between saliva ionic composition and the pellicle structure, for which the driving force was to dissect and understand the role of calcium ions in pellicle structure. (Vassilakos *et al.* 1992; Tanizawa *et al.* 2004; Macakova *et al.* 2010; Kandori *et al.* 2008; Harding *et al.* 2005).

Calcium ions have been implicated to modify the attraction between enamel surfaces and salivary proteins, to which these proteins attach during pellicle formation (Hannig *et al.* 2009; Macakova *et al.* 2010).

For example, Tanizawa *et al.* (2004), demonstrated that pellicle formation onto hydroxyapatite surfaces was enhanced by calcium ions through calcium bridging of proteins.

Proctor *et al.* (2005), found that saliva calcium ion chelation resulted in a drastic loss of mechanical properties in adsorbed pellicle, indicating the pellicle structure breakdown. Therefore, investigations of physical structure of the pellicle under different calcium concentrations may help to dissect the pellicle formation mechanisms.

2.8 AEP functions

2.8.1 Enamel demineralization protection

AEP provide protection to teeth surface from mechanical and abrasive forces. It can regulate enamel calcium phosphate demineralization and remineralization and neutralize acid produced by bacteria in the oral cavity (Amerongen *et al.* 2004).

AEP behaves like a semipermeable membrane, and hence by preventing demineralization and/or augmenting enamel remineralization, it helps maintain the enamel surface structure. In terms of calcium and phosphate ionic concentrations, saliva is supersaturated at pH 7.0. (Larsen and Pearce 2003).

When the salivary pH in the microenvironment of the tooth turns acidic, it is no more supersaturated with respect to calcium phosphate ions. Since the H^+ ions react with phosphate (PO_4^{3-}) or hydroxyl ions (OH^-) in the saliva, their concentration gets reduced in

saliva. So, the loss of calcium and phosphate ions from teeth is directly related to the loss of calcium and phosphate ions in saliva following law of mass action. It means that due to acidic pH, as the saliva becomes less saturated with calcium phosphate, the enamel hydroxyapatite starts demineralizing to counter the change in concentration of calcium phosphate ions in saliva. So, longer the tooth surface encounter acidic environment, under saturated with respect to calcium phosphate ions, the enamel start demineralizing or dissolving. Surprisingly enough, instead of acid, is water that breaks the crystal lattice bonds of hydroxyapatite structure due to loss of attraction between calcium and phosphate ions (Pearce 1988).

In the oral cavity, when bacteria encounter fermentable sugars, they produce formic acid, acetic acid, lactic acid, and propionic acid as metabolic by-products. These small chain acids, cause enamel demineralization (Garcia-Godoy and Hicks 2008).

In a recent study, Siqueira *et al.* (2010), provided first evidence that intact histatin peptides when adsorbed to hydroxyapatite were not only resistant to the protease degradation, but also able to protect the hydroxyapatite demineralization under acidic challenge.

Since, pellicle acts like semipermeable membrane, it controls the downward acid diffusion from biofilm to enamel surface and decelerate calcium and phosphate ion movement into and out of enamel surface. This results in slowing down the enamel demineralization in hostile low pH environments and in basic or neutral pH promotes enamel remineralization (Lussi *et al.* 2006, Hara *et al.* 2006).

Rios *et al.* (2008) and Meurman and Frank (1991) through independent ultramicroscopic studies demonstrated, that the acidic soft drinks induced enamel demineralization was significantly reduced by salivary pellicle.

In a previous study, Amaechi *et al.* (1999) showed that just one hour old *in situ* acquired pellicle could protect enamel surface to certain extent against the orange juice induced demineralization. Hannig *et al.* (2004), in another study demonstrated that even a 3-min

old *in situ* pellicle could provide some protection against enamel surface demineralization induced by citric acid.

In a recent study, Hannig *et al.* (2009), concluded that orange juice caused less damage than low pH carbonated beverages to the *in situ* pellicle coated bovine enamel.

Hannig *et al.* (2003) in previous *in vivo* pellicle formation study, demonstrated, that 2 hour old AEP was as efficient as 24 hours old AEP in protecting against acid induced demineralization of the enamel surface without any statistically significant difference.

Hannig *et al.* (2004) in another study demonstrated there was no significant difference, in the acidic challenge induced demineralization protection, provided by 3 minutes *in situ* pellicle versus 2 hour *in situ* pellicle.

2.8.2 AEP as a platform for biofilms

The pellicle performs dual function of protecting tooth enamel besides providing sites for the initial attachment of bacteria to the tooth surface, which is the first step in plaque formation. Thereafter, AEP plays a major role in the interactions, that take place at the tooth-saliva interface which are important both physiologically and pathologically (Wolff and Larson 2009).

Several structural components of AEP like, mucin MG 2, fibrinogen, and PRPs acts as specific bacterial receptors (Hannig and Joiner 2006; Bonifait *et al.* 2009). These pellicle proteins contain peptide sequences or epitopes on their surface, which are detected and utilized for attachment, by a variety of pili, fibrils, and fimbriae appendages on bacterial cell surfaces. Eighty percent of the early colonizers constitute Streptococcus mitis, S. soralis, and S. sanguis (Cassels *et al.* 1995).

Early colonizers adhere to the pellicle through a reversible and weak binding and subsequently other bacteria attach themselves over early colonizers through irreversible attachment (Meurman and Stamatova 2007). Over a period, as biofilm start maturing, these initial colonizers are slowly replaced by anaerobic *S. sobrinus*, *S. mutans*, and *L. actobacilli* (ten Cate 2006). These anaerobes cause the carbohydrate fermentation in

foods and produce organic acids in the oral cavity. It can reduce pH of oral cavity and ultimately leads to tooth demineralization.

On the contrary, some micorbial biofilm bacteria may counter the effects of acidogenic bacteria. For example, lactic acid produced by some bacteria is metabolized by *Veillonella*. In other case, ammonia compounds are synthesized by *S. salivarius* urease, which can potentially raise the saliva pH, leading to delayed initiation of tooth demineralization process (Garcia-Godoy and Hicks 2008).

2.9 AEP modulation

Besides differences in secretions from different salivary glands, the circadian cycle, age, sex, diet, and general health of a person, also known to modify the salivary composition, all of which are also likely to modulate the structural differences in the pellicle samples (Castagnola *et al.* 2004).

In the oral cavity, during pellicle formation, the saliva protein adsorption behavior on dental enamel, has been shown to influenced by several dietary components (Smith and Bowen 2000; van der Mei *et al.* 2002).

Additionally, certain processed food components, like calcium in dairy products or the low pH of soft drinks, may modify the pellicle structure (Ash *et al.* 2013; Hannig *et al.* 2009; Proctor *et al.* 2005).

Besides, other nonprotein salivary components may also exert influence over the pellicle formation and structure. For example, it is thought that the use of certain oral hygiene products and/or even drinking and eating certain foods may modulate the pellicle structure. Some components of toothpaste (sodium lauryl-sulfate and sodium tri-polyphosphate) are known to dislodge certain proteins from the pellicle (Shellis *et al.* 2005; Hannig *et al.* 2009).

Comelli *et al.* (2002), observed that bacterial strains *S. thermophilus* and *Lactobacillus lactis* ssp. lactis used in the processed dairy foods integrated into an *in vitro* grown biofilm. They interfered with development of *S. sobrinus*, a cariogenic species. This

study showed, that the pellicle composition could be modulated by saliva and other components in the oral cavity. Besides, this modulation could also be achieved through food components.

Yao *et al.* (2001) demonstrated that under *in vivo* conditions, the AEP appeared to undergo extensive enzymatic changes as *in vitro* pellicles contained greater number of intact proteins compared to *in vivo* pellicles. This clearly demonstrate that under *in vivo* conditions AEP is a very dynamic structure, in a dynamic equilibrium with the surrounding saliva. Therefore, the AEP can be seen as dynamic protein film, that is under continuous adsorption and desorption mode in the oral cavity, and is highly sensitive to micro environmental changes in its vicinity (Rosan and Lamont 2000).

Smith and Bowen (2000), demonstrated that during *in vitro* pellicle formation, rinsing the hydroxyapatite disc with solutions of sucrose, xylitol, sorbitol, or milk, led to composition modulation in the pellicle. Siqueira *et al.* (2012) also confirmed their results and observed that when the hydroxyapatite discs were coated with different concentration solution of Sodium fluoride, this led to pellicle composition modulation.

Schupbach *et al.* (1996), in an interesting study found that some milk casein derivatives can displace albumin by adsorbing onto the enamel surface and get incorporated into the pellicle. Therefore, some of the dietary components may also be considered integral components of the pellicle. This has important oral implications, since the pellicle display bacterial binding receptors for several bacteria. So, any pellicle compositional modulation, that is induced by dietary modifications, can potentially change its microbial binding behavior, thus leading to changes in the biofilm composition.

Hannig *et al.* (2009), in an *in situ* study, observed that a significant amounts of the outer loose, globular layer of the AEP was removed by consumption of acidic beverages, while the basal dense layer was not affected.

Rykke and Sonju (1991), concluded that food component can modulate the pellicle composition. They found that there were significant differences between 24 hours old pellicle formed with and without intake of food and beverages.

Moreover, the proteolytic activity of proteases in saliva also results in modulated pellicle composition, and provide protein receptor sites for bacterial attachment on the pellicle (Douglas 1994).

Furthermore, in the oral cavity, enzymes from dead bacterial cells as well mucosal tissues, blood and from consumed foods can also get incorporated into the pellicle and may also modulate the pellicle structure (Lendenmann *et al.* 2000; Kajisa *et al.* 1990). But, the effects of these nonstructural external factors on the pellicle functional properties and characteristics of AEP is still unknown.

Cardenas *et al.* (2007), demonstrated that the resilience of the basal electron dense of pellicle layer may be attributed to Mucin MUC5B, present in pellicle. Delecrodea *et al.* (2015) in a recent study supported earlier studies and demonstrated that, even after acid challenges to the salivary pellicle on hydroxyapatite surface, there were certain acid resistant proteins like Cystetin-B and mucin-3A, which were still retained on the hydroxyapatite surface and could not be removed easily from the surface.

Tanizawa *et al.* (2004), demonstrated that Ca^{2+} ions through calcium bridging of proteins, could enhance pellicle formation onto hydroxyapatite surfaces. On the other hand, Proctor *et al.* (2005), found that Ca^{2+} ion chelation from saliva caused a significant reduction in the mechanical properties of salivary pellicle, indicating the potential pellicle structure breakdown.

Ash *et al.* (2013), found that when the calcium concentration was increased from 0-10 mM, there were significant changes in the thickness, fresh weight, protein mass, and concentration of the pellicle proteins for both parotid and whole saliva derived pellicles.

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Chapter 3

3 AEP peptide mediated enamel demineralization protection studies

The acquired enamel pellicle is a protective covering on the enamel and other surfaces of the oral cavity, containing around 130 proteins and peptides. Around 50% of the pellicle proteins and peptides are smaller in size (<30 kDa), having posttranslational modifications. Salivary peptides are products of salivary proteins, resulting from host and bacterial origin proteases enzymatic digestion, which may either retain or enhance the functional properties of their native proteins. Statherin and histatin are the major pellicle component, highly effective in inhibiting the precipitation of primary and secondary calcium phosphate and having antimicrobial activities, respectively. Our group has identified N-terminal 9 amino acids residue peptide, DpSpSEEKFLR (DR9), from statherin, and a 14 amino acid synthetic analog of antimicrobial functional domain RKFHEKHHSHRGYR (RR14), from histatin, which are early components of in vivo pellicle, displaying a significant effect on the hydroxyapatite growth inhibition and antimicrobial activities, respectively (Xiao *et al.*, 2015; Tayebeh *et al.*, 2017). In the current study, we demonstrated that DR9 and its tandem duplicate DR9-DR9 peptides, exhibited significant protection against enamel demineralization, whereas DR9-RR14, the hybrid construct showed moderate protection against demineralization when compared to control.

3.1 Introduction

Several workers and studies have elucidated and reached a global consensus, that acquired enamel pellicle (AEP), is a protective, organic integument on the enamel and other surfaces in the oral cavity, consisting of around 130 proteins and peptides, through the selective adsorption of, salivary proteins and peptides, and other molecules including phosphoproteins, lipoproteins, glycoproteins, lipids and carbohydrates (Dawes *et al.* 1963; Vitorino *et al.* 2007; Siqueira *et al.* 2007; Hannig and Joiner 2006; Sonju 1975; Slomiany *et al.* 1986; Slomiany *et al.* 1990). The main source of these pellicle proteins and peptides, is salivary glands in the oral cavity, oral mucosa, gingival crevicular fluid,

and bacterial products (Siqueira *et al.* 2012; Hannig *et al.* 2009). Several studies have shown that pellicle proteome appears to be a derived from a subpopulation of salivary proteome through selective adsorption of salivary proteins.

The acquired enamel pellicle plays an extremely important role in the overall teeth homeostasis, by being a semipermeable membrane integument for demineralization protection, as well as to neutralize organic acids, produced as by product of bacterial metabolism (Hara *et al.* 2010; Siqueira *et al.* 2010; Siqueira *et al.* 2012; Zahradnik *et al.* 1977; Hannig *et al.* 2007; Kosoric *et al.* 2007; Kosoric *et al.* 2010). Pellicle also determines the early colonizers composition, that ultimately results in the microbial biofilm formation on various surfaces throughout the oral cavity. (Gibbons *et al.* 1988; Gibbons and Hay 1989; Li *et al.* 2004; Chaudhuri *et al.* 2007).

One of the important major component of pellicle proteins is statherin, which is highly effective in inhibiting the precipitation of primary and secondary calcium phosphate (Oppenheim *et al.* 2007; Schlesinger and Hay 1977) leading to supersaturation of saliva, that helps in the enamel surface remineralization. The N-terminal of statherin's peptide contain the functional domain (Long *et al.* 1998; Raj *et al.* 1992). A naturally occurring peptide, from this N-terminal region of statherin, was identified as an early component of the pellicle (Siqueira and Oppenheim, 2009). This small peptide, called DR9, has demonstrated a significant ($p<0.05$) effect on the hydroxyapatite growth inhibition effect in all concentrations tested in comparison to other native statherin peptides (Xiao *et al.* 2010; Xiao *et al.* 2015).

Yang *et al.* (2017), constructed a statherin N-terminal bioinspired version containing first six-amino acid residues DpSpSEEK (pS corresponds to phosphorylated serine). They demonstrated that, when the peptide sequence was cysteine-labeled, DpSpSEEKC, it strongly adsorbed to HAP and induced remineralization on the demineralized tooth enamel surface. In comparison to control, this new peptide coating significantly improved the mechanical properties of the coated samples.

Salivary proteins are known to have existed for millions of years in the nature. During a period of time, over evolutionary time scale, they counter the evolutionary pressure by

duplicating the functional domains that lie within their primary structures, in an effort to improve their functional capabilities to batter match the evolution. (Oppenheim *et al.* 2012; Troxler *et al.* 1997).

This protein diversity may be achieved at different hierarchical levels either at genome level through gene duplication, allelic variation, and recombination events, or at transcriptome level through alternate splicing events, and at proteome level through post-translational modifications. (Helmerhorst and Oppenheim 2007; Oppenheim *et al.* 2007).

Since statherin is very young protein in terms of evolutionary time scale and could not get the opportunity to evolve naturally (Oppenheim *et al.* 2012; Troxler *et al.* 1997), therefore, DR9 is the only known natural functional domain as far as the primary and secondary calcium phosphate precipitation inhibition is concerned (Xiao *et al.* 2015; Xiao *et al.* 2010). So, one possible way to test and observe the effect of evolution could be to artificially duplicate or triplicate DR9 sequence in tandem. It may result in the expected effects, we would like to see in the due course of natural evolution, thereby constructing a salivary protein with augmented dental enamel demineralization protection.

Besides functional domain multiplication, natural evolution processes over a period may also recombine the functional domains of different proteins due to gene shuffling/recombination processed to create a natural hybrid protein. Hence, another AEP protein, histatin is best candidate to be studied. Histatin has very strong activities of antimicrobial, demineralization protection, buffering, and regulation of mineral formation (Oppenheim *et al.* 2012; Edgerton and Koshlukova 2000; Siqueira *et al.* 2010; Helmerhorst *et al.* 2006; Vukosavljevic *et al.* 2012).

Histatins are known to degrade immediately in the hostile oral cavity, upon secretion from salivary glands, due to enzymatic proteolysis. Despite the degradation, histatin fragments still function like parental proteins (Helmerhorst, *et al.* 2007; Groot, *et al.* 2006, Castagnola, *et al.* 2004). A peptide RR14, a synthetic analog of antimicrobial functional domain RKFHEKHHSHRGYR, derived from histatin 3, was identified as member of the *in vivo* pellicle. It was connected with DR9 derived from statherin to create a hybrid peptide, DR9-RR14. Earlier studies have shown that the functional

domain of histatins could have the same antimicrobial effect as of the original protein. By synthetically combining RR14 with DR9, we hypothesize the creation of a novel protein with dual functionality of superior antimicrobial qualities and enhanced mineral homeostasis in one molecule. It will represent promising new molecular methodologies, with an immense potential for the oral health maintenance.

3.2 Materials and methods

3.2.1 Characterization of peptide constructs

Synthetic peptides, derived from histatin 3 or statherin (DR9, DR9-DR9 and DR9-RR14), were synthesized by China peptide, Shanghai, China. Each peptide was verified by HPLC and MS analysis for purity (> 95%) and M^r values. For all the experiments, peptide solutions were prepared in water, pH 6.8, at a concentration of 1 mg/ mL. All the peptides, tested in this study, are listed along with their sequence and other physical characteristics in Table 3-1.

3.2.2 Calculation of pI values of peptide constructs

The isoelectric points (pI) of histatin 3 and statherin peptides were calculated using the online calculator developed by Gauci and coworkers using Scansite option (Azzopardi, *et al.* 2010). This approach let user define the specific pK values at a particular pH to calculates the pI of a given peptide. The calculation was performed in repetitive manner, till the pH, at which the net charge of peptide was zero, achieved. (Gauci, *et al.* 2008).

3.2.3 Enamel demineralization assay

3.2.3.1 Enamel sample preparation

Enamel sample preparation was done following the previous study by Siqueira, *et al.* (2010). Human permanent first molars, free of any physical defects, were cleaned, rinsed, and sectioned. After the removal of roots, the crowns were sliced vertically into 4 sections (with a 300 µm thickness each) through a diamond saw, followed by sandpaper grinding to a uniform, final thickness of 150 µm. Each section was coated with a layer of

lightcured dental adhesive (3M ESPE Scotchbond^{TM} Universal) and nail varnish paint, leaving aside a naked, uncoated 2 mm x 150 µm window on the enamel surface.

3.2.3.2 Enamel coating and demineralization treatment

Enamel sections were divided randomly into 4 groups (N = 10 per group; Table 4-2) and were incubated for 2 hours with either parotid saliva (PS; 10 ug/section) at 37°C or peptide constructs (DR9/DR9-DR9/DR9-RR14 at the rate of 20 nmol/section). After incubation, the sections were gently rinsed in distilled water to remove any loosely bound parotid saliva or peptide to the exposed window (150 µm x 2mm). Then sections were submitted for demineralization assay by incubating them at 37°C in demineralization solution (2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM Acetic acid pH 4.5) for 12 days. The pH of the demineralization solution was checked every 72 hours with pH strip to ensure that the pH changes was not significant. Following experimental treatments and groups were used in this set of experiments:

Group	**Treatment**
1	PS coated enamel section (Control)
2	DR9 coated enamel sections
3	DR9-DR9 coated enamel sections
4	DR9-RR14 coated enamel section

In another set of experiments, enamel sections were divided randomly into 4 groups (N = 10 per group; Table 4-3) and were incubated for 2 hours with PS (10 µg/section) at 37°C. After incubation, the sections were gently rinsed in distilled water to remove any loosely bound parotid saliva to the exposed window (150 µm x 2mm). After rinsing, they were again incubated with peptide (DR9/DR9-DR9/DR9-RR14 - 20 nmol/section) for 2 hours at 37°C. Following experimental treatments and groups were used in this set of experiments:

Group	Treatment
1	PS coated enamel section (Control)
2	PS coated enamel section + DR9 coating
3	PS coated enamel section + DR9-DR9 coating
4	PS coated enamel section + DR9-RR14 coating

After incubation, the sections were briefly rinsed with distilled water to remove loosely bound peptide. Then sections were submitted for demineralization assay by incubating them at 37°C in demineralization solution (2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 50 mM Acetic acid pH 4.5) for 12 days. The pH of the demineralization solution was checked every 72 hours with pH strip to ensure that the pH changes was not significant.

3.2.3.3 Calcium and phosphate estimation

After 12 days in the demineralization solution, the enamel sections were removed, and the supernatant was analyzed for the calcium concentration through quantitative colorimetric calcium determination assay at 595 nm and phosphate concentration using vanadomolybdate UV-Visible spectrophotometric assay at 415 nm. All the samples were analyzed in duplicate.

3.2.3.4 Statistical analyses

Statistical procedures were performed with the software package Minitab 13.1. After checking for normal distribution, we subjected the data to analysis of variance (ANOVA) followed by Tukey's test. The level of significance was set at a value of $p < 0.05$. Means and standard deviations of calcium and phosphate released from human enamel sections first exposed to parotid saliva (control) or peptide construct or parotid saliva + pellicle peptide constructs, followed by exposure to the demineralizing solution (n = 10 per group) for 12 days. Different letter superscripts indicate statistical difference, and same letter superscripts indicate no statistical difference within the same column, according to Tukey's test ($p<0.05$).

3.3 Results

Isoelectric points were calculated for each peptide construct at pH 6.8. For DR9, the statherin peptide construct, pI value was 3.62, with a net charge of -1 (at pH7.0) while for DR9-DR9, tandem repeat construct of statherin, lowest pI value of 3.44 was observed with a net charge of -2 (at pH7.0). In case of DR9-RR14 hybrid construct of statherin and histatin, intermediate pI value of 7.16 was observed with a net charge of +3 (at pH7.0). Results are summarized in Table 3-1.

Table 3-1: Sequences and other physical characteristics of histatin 3 or statherin peptide constructs.

Amino Acid Sequence	Amino residues	M^r (kDa)	Net charge at pH 7.0	pI
DSSEEKFLR (DR-9)	9	1.270	-1	3.62
DSSEEKFLRDSSEEKFLR (DR-9-DR-9)	18	2.522	-2	3.44
DSSEEKFLRRKFHEKHHSHRGYR (DR-9-RR-14)	23	3.127	+3	7.16

To have further insight and functional understanding of new synthetic constructs, derived from histatin and statherin pellicle peptides, in terms of demineralization protection, when adsorbed on the salivary pellicle, we decided to test the effects of those peptides on *in vitro* enamel demineralization. The effect of these peptide construct on the amounts of phosphate and calcium released from the enamel specimens after 12 days in demineralization solution are summarized in Tables 3-2 and 3-3.

Table 3-2: Means and standard deviations of Ca^{2+} and PO_4^{3-} released from human dental enamel sections, coated with either control (PS) or peptide constructs.

Treatment	N	Ca^{2+} (mM/mm^2) mean ± SD	PO_4^{3-} (mM/mm^2) mean ± SD	Ca^{2+}/PO_4^{3-} Ratio
Control (PS)	10	13.71 ± 1.35^{A}	3.22 ± 0.74^{A}	4.26
DR9-RR14	10	15.77 ± 1.46^{A}	$4.04\pm0.29^{C, D}$	3.90
DR9-DR9	10	14.38 ± 2.5^{A}	$4.1\pm0.82^{C, D}$	3.51
DR9	10	14.58 ± 1.7^{A}	$3.91\pm0.51^{A, C, D}$	3.73

3.3.1 Ca^{2+} and PO_4^{3-} demineralization protection with peptide coated

In case of calcium demineralization protection, different peptide constructs (DR9, DR9-DR9, DR9-RR14), could not provide any better protection against calcium demineralization in comparison to PS control. Because, there was no statistically significant difference between different treatments, compared to parotid saliva coated control.

In case of the phosphate demineralization protection, control (PS coated only) provided better protection (lowest level of demineralized phosphate) in comparison to either of DR9-DR9 and DR9-RR14 peptide constructs as there were significant differences between control and DR9-DR9, and DR9-RR14 constructs. On the other hand, there were no statistically significant differences between Control and DR9 as well as between any of the three peptide constructs (DR9, DR9-DR9 and DR9-RR14).

The overall trend was that, for calcium and phosphate demineralization, none of the peptide construct (DR9, DR9-DR9, DR9-RR14) exhibited any significant protection when compared to control PS group (Table 4-2). Under the current experimental conditions, none of the peptide construct provided any advantage or significant demineralization protection function against calcium and phosphate from enamel surface, when directly coated on to the enamel surface.

For each of the tested group, the Ca/PO_4 ratio was calculated. Among the groups, a % variation of less than 7.5 percent was observed. The lowest ratio was that of DR9-DR9 coated group (3.51) while PS coated control group showed the highest ratio of 4.26.

Table 3-3: Means and standard deviations of Ca^{2+} and PO_4^{3-} released from human dental enamel sections, coated with control (PS) or PS coated + peptide construct coated.

Treatment	**N**	**Ca^{2+} (mM/mm^2) mean ± SD**	**PO_4^{3-} (mM/mm^2) mean ± SD**	**Ca^{2+} / PO_4^{3-} Ratio**
Control (PS)	10	14.12 ± 1.52^{B}	2.10 ± 0.28^{A}	6.72
PS+DR9-RR14	10	16.77 ± 1.88^{A}	2.49 ± 0.36^{A}	6.74
PS+DR9-DR9	10	9.93 ± 1.58^{C}	1.70 ± 0.44^{B}	5.84
PS +DR9	10	9.17 ± 1.77^{C}	1.60 ± 0.67^{B}	5.73

3.3.2 Ca^{2+} and PO_4^{3-} demineralization protection with PS coated + peptide construct coated

The PS coated + DR9-RR14 group and control group (only PS coated) showed the highest levels of calcium and phosphate demineralization. In terms of calcium loss from the enamel sections, it was significantly different from each other. Compared to control group, both PS coated + DR9 and PS coated + DR9-DR9 groups clearly showed statistically significant reduction in the demineralization of the calcium from the enamel sections. Similarly, in comparison to PS coated + DR9-RR14 group, both PS coated + DR9 and PS coated + DR9-DR9 groups, clearly provided statistically significant protection against enamel demineralization of calcium. This imply that both these peptides, DR9 and DR9-DR9 could provide protection against calcium demineralization in comparison to both control as well as PS coated + DR9-RR14 groups. Even though both PS coated + DR9 and PS coated + DR9-DR9 groups did provide protection against demineralization of calcium, but the level of protection between these two groups was not statistically significant.

The PS coated + DR9-RR14 group and control group (PS coated) showed the highest levels of demineralization in terms of phosphate loss from the enamel sections, but were non-significantly different from each other. It means DR9-RR14 was no better than PS in protecting against phosphate mineralization from the enamel surface. Compared to control group, both PS coated + DR9 and PS coated + DR9-DR9 groups showed statistically significant reduction in the demineralization of the phosphate from the enamel sections. Similarly, in comparison to PS coated + DR9-RR14 group, both PS coated + DR9 and PS coated + DR9-DR9 groups, provided statistically significant protection against enamel demineralization of phosphate. This imply that both these peptides DR9 and DR9-DR9 could provide same level of protection against phosphate demineralization in comparison to control as well as PS coated + DR9-RR14 groups. Even though both PS coated + DR9 and PS coated + DR9-DR9 groups did provide protection against demineralization of phosphate, but the level of protection between these two groups was not statistically significant.

The calcium and phosphate demineralization results in terms of calcium and phosphate loss as well demineralization protection showed a similar trend. Overall, calcium and phosphate demineralization assay showed that compared to PS (control), PS+DR9 (Ca 9.17 ± 1.78; PO_4 1.60 ± 0.67) and PS+DR9-DR9 (Ca 9.93 ± 1.58; PO_4 1.70 ± 0.44) provided better protection, even though the DR9-DR9 and DR9 were non-significant from each other.

The overall trend was that, for calcium and phosphate demineralization, DR9 and DR9-DR9 peptides exhibited significant protection when compared to control PS group (Table 3-3). Under the current experimental conditions, the tandem duplication of DR9 neither did help in the augmentation of the demineralization protection function against calcium and phosphate losses from enamel surface, nor it provided any advantage, when the peptide construct coated the PS pellicle on the enamel surface.

For each of the tested group, the Ca^{2+} and PO_4^{3-} ratio was calculated. Among the groups, a % variation of less than 10 percent was observed. The lowest ratio was that of PS coated + DR9 group (5.73) while PS coated + DR9-RR14 group showed the highest ratio of 6.74.

3.4 Discussion

In this study we demonstrated, that when bound directly to the enamel surface, at lower concentration (nmol), the synthetic peptide constructs, did not provide any significant demineralization protection advantage over the control. It was contrary to the results, our group got in previous studies by either using full length histatin peptides or even using the peptide constructs at higher concentration (µg/ml) (Siqueira, *et al.* 2010; Tayebeh 2015 (MSc Thesis research); Tayebeh *et al.* 2017).

Besides tandem duplication of the functional domains, construction of salivary pellicle with augmented functionality is another potentially useful approach via hybrid molecule preparation. Tandem functional domain duplication helps in the enhancement of single functional property, the hybrid construct approach results in combining two or more different functional features in a single construct, displaying multiple functionalities. The

hybrid approach is promising strategy for novel pellicle constructs, as some of the pellicle peptides exhibit unique functionality like antimicrobial properties and or hydroxyapatite crystal inhibition properties, while other peptides display a high binding affinity for hydroxyapatite, (Vukosavljevic *et al.* 2011; Siqueira *et al.* 2012). Statherin-osteopontin (Raj *et al.* 1990) and cystatin-histatin (Bobek *et al.* 1993) hybrid constructs are best examples of such synthetic bi-functionality.

Natural functional domain of statherin, DR9, was tandem duplicated to construct DR9-DR9 as well as synthetically joined to a functional domain of histatin 3, RR14, to construct DR9-RR14 hybrid construct. Our *in vitro* results from this study demonstrated that at very low concentration of all constructs (nmol/disc), when peptide constructs directly coated the enamel surface, they were unable to provide any protection against acid induced demineralization as the functional differences were non-significant in comparison to control (PS only coating). Similar kind of observations were recorded by Kielbassa *et al.* (2005), with albumin and casein at lower concentration in terms of demineralization protection of bovine enamel. Kosoric *et al.* (2010) in another study demonstrated BSA concentration dependent modulation in the rate of hydroxyapatite demineralization.

This failure of peptide constructs to protect against demineralization, could be attributed to thin layer of peptide coating on enamel surface, formed as a result of monolayer formation on enamel surface because of low concentration (nmol) of the peptide. Recent studies by Tayebeh (2015; M.Sc. thesis research; Teyebeh, *et al.* 2017), have however, shown that all the peptides were able to provide significant demineralization protection to enamel surface against acid induced erosion, when peptides were used at higher concentration (µg/ml), possibly leading to formation of multi-layer peptide pellicle on enamel surface, that was able to not only neutralize the acid in the near vicinity of the enamel surface but also acted as semipermeable barrier, thereby further reducing the enamel demineralization.

Siqueira *et al.* (2010), in another study demonstrated that full length histatins provided demineralization protection to enamel surface. This protection could be attributed to high

concentration of full length peptides (mg/ml) used in that study, that might have resulted in multilayer pellicle on enamel surface, leading to acid neutralization and being semipermeable in nature, leading to demineralization protection against acid injury.

In case of DR9 and DR9-DR9, they carried a net negative charge due to presence of 2 and 4 phosphate groups respectively, which resulted in tight binding to the positively charged enamel surface. But during the acid challenge, as the pH of demineralization buffer was 4.5, which was above the pI of DR9 and DR9-DR9, it is very likely that, this might have resulted in further net negative charge on the adsorbed peptides and hence resulting in their gradual desorption from the enamel surface due to charge repulsion induced weakening of the interaction between peptide and enamel surface, leading to slight demineralization over a period of time, even though it was statistically non-significant compared to PS pellicle coating of enamel surface.

Our *in vitro* results from further experiments in this study demonstrated that even at very low concentration of all constructs (nmol), when peptide constructs coated the pre-coated PS pellicle enamel surface, they were able to provide some level of protection against acid induced demineralization, as the functional differences were significant in comparison to control (PS coating). Both DR9 and DR9-DR9 provided calcium and phosphate demineralization protection compared to control. DR9-RR14 did not provide any protection against calcium and phosphate demineralization. This failure to protect against demineralization by DR9-RR14 peptide could be attributed to thin layer of peptide coating on enamel surface, formed as a result of monolayer formation because of very low (nmol) peptide concentration, which was not efficient in providing a buffering action to neutralize the acidic demineralization solution as well as having very little or no semipermeable control of the pellicle in preventing the acid from coming in direct contact with the enamel surface.

Kosoric *et al.* (2010), suggested that BSA modifies the hydroxyapatite demineralization kinetics either by modulation of demineralization solution properties or by surface energy of hydroxyapatite. In a previous study, Juriaanse *et al.* (1981) demonstrated that proline rich proteins after adsorption to bovine enamel resisted the calcium and phosphate

demineralization thereby suggesting that PRP may provide protection against demineralization. We can speculate that at such lower concentration (nmol) of peptides, the charge differences due to number of phosphorylated sites, at 2^{nd} & 3^{rd} amino acid residues in DR9 and at 2^{nd}, 3^{rd}, 10^{th}, & 11^{th} amino acid residues in DR9-DR9 did not to have much influence to provide demineralization protection. As observed in previous studies, the presence or the number of phosphorylation sites can significantly influence the adsorption of these peptide constructs to the hydroxyapatite and the process of protection against enamel demineralization as well as growth inhibition of calcium phosphate crystal under *in vitro* conditions, when used at high concertation. (Siqueira *et al.* 2010; Hunter *et al.* 2009; Hunter 2013; Xiao *et al.* 2010; Xiao *et al.* 2015). Apart from phosphorylation, DR9-DR9 has the lowest pI when compared to DR9 in pH 6.8, the physiological salivary pH. It can facilitate the adsorption of this peptide on to PS pellicle surface, which in turn can provide protection against demineralization (Moreno *et al.* 1982). It was found to be true in presence of multicomponent PS pellicle in this study, where DR9-DR9 could provide better protection against demineralization of both calcium and phosphate in comparison to control parotid saliva.

Based on the limitation of this study, it was found that the protective behavior of individual peptides was different when they were used directly to coat the enamel surface as probably at that low concentration, none of the peptide could provide any protective advantage in comparison to control parotid saliva coating.

On the other hand, DR9 and DR9-DR9 both were able to provide significant protection against demineralization of the pre-coated PS pellicle enamel sections in comparison to control PS coating, simulating the environmental conditions similar to that of *in vivo* formed acquired enamel pellicle. For instance, some studies have demonstrated that in the presence of other proteins the adsorption behavior of histatins changes (Yin *et al.* 2006). Cheaib and Lussi (2011), in other study has shown that when mixed together, casein and mucin displayed enhancement in the erosion-inhibition properties of the AEP.

3.5 Conclusion

This is the first study, through which we try to address the functional effect of those newly constructed peptides in such multi-component protein film environment. However, this study represents an initial step in the direction of getting deep insights into the possible role of these peptides in enamel protection. The basic knowledge gained from this study may provide a fundamental platform in future for the development of bioinspired peptides from histatin and statherin for therapeutic use against dental erosion and dental caries.

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Chapter 4

4 Modulation of AEP by incorporation of constructed peptides

4.1 Introduction

Previous work discovered that mature AEP proteome consists of around 130 different proteins, ranging in size from >250 kDa–<5 kDa, of which about 50% are small molecular weight peptides (Siqueira *et al.* 2007, 2009) most of them without identified biological function yet. Based on the possible role of these proteins in AEP development, they have been classified into 3 major groups; Ca binding proteins, PO_4 binding proteins and protein interacting with other salivary proteins. AEP proteins have also been classified according to their putative biological functions; inflammatory responses, immune defense, antimicrobial activity & re-mineralization capacity (Siqueira *et al.* 2007).

The biomolecular events in and outside the living systems, that leads to the formation of protein–protein and other biomolecule complexes involves ionic, hydrogen bonding, hydrophobic and other non-covalent weak interactions, leading to conformational changes in the proteins, resulting in the up or down regulation of their functions (Cserhati and Szogyi 1995). Due to charge in the protein molecules because of phosphorylation, as well as the presence of acidic and basic amino acid side chains, these salivary proteins interact to a different extent with mucosal as well as enamel surfaces, apart from interacting among themselves in the oral cavity (Yin *et al.* 2006). All these protein interactions modify (enhance or diminish) their functional properties as well as impact their stability in the oral cavity.

Cheaib *et al.* (2015) studied the impact of pellicle modification on early colonizers and demonstrated that casein, mucin and a casein-mucin mixture bring about pellicle modification, resulting in the significant modulation in adhesion behavior of early colonizers on the enamel pellicle.

In an earlier study, Cheaib and Lussi (2011), demonstrated that pellicle modulation by casein and a casein-mucin mixture, augmented the enamel demineralization protection capability of acquired enamel pellicle, where a mixture of casein-mucin as pellicle modulator displayed higher demineralization protection in comparison to casein alone.

Researchers in different studies (Danielsson *et al.* 2009; Malkoski *et al.* 2001; Vacca-Smith *et al.* 1994), found that bovine α-, β- and κ-caseins (40 kDa glycoprotein-containing fraction of κ-caseins inhibited *S. mutans* adhesion to saliva-coated hydroxyapatite.

In other studies, ovalbumin has been shown to reduce the *in vitro* acidic solution induced hydroxyapatite demineralization similar to that of casein (Hemmingway *et al.* 2010, 2008).

Hannig *et al.* (2009) found that Mucin (MUC5B), present in the basal layer of the acquired enamel pellicle, is retained on enamel surface even after 2 hours of exposure to citric acid, and concluded that it is one of the contributors to the protective properties of the pellicle.

Hannig and Joiner (2006) observed that pellicle ultrastructure changes after exposure to the acidic environment. They found that after acid exposure, the global outer layer of the pellicle gets removed, while the basal layer persists.

Hannig and Hannig (2009) in another study propounded a pellicle formation model that was based on hydrophobic and ionic interactions in addition to Van der Waals forces could ultimately result in a protein layer consisting of heterotypic complexes.

In another study, Nieuw *et al.* (1987), demonstrated that a 72 hour old *in vitro* mucin pellicle from the whole saliva provided 100 % protection against erosion under citric acid challenge.

Based on the previous research from our group, two small peptides have been derived from statherin. They contain a natural N-terminal sequence of 9 amino acid residues domain as a single unit (DR9) that has the strong inhibitory property against

hydroxyapatite crystallization and DR9-DR9 as a tandem duplicate repeat from statherin so as to artificially enhance and mimic the protein evolution (Siqueira and Oppenheim 2009; Xiao *et al.* 2015). Apart from that, a small domain RR14 has been derived from histatin 3, that contain 14 amino acid residues, which has got very strong antimicrobial activity (Oppenheim *et al.* 2012). This antimicrobial histatin domain RR14 has been synthetically combined with the statherin DR9 domain to construct a histatin-statherin hybrid domain DR9-RR14 so that the antimicrobial properties of RR14 are combined with that of hydroxyapatite crystal inhibition properties of DR9 in one construct.

4.2 Material and methods

4.2.1 Cleaning and acid etching of hydroxyapatite discs

Near enamel hydroxyapatite (HA) discs (Himed, USA) were cleaned by water bath sonication for 10 minutes once in distilled water and for 10 minutes once in 100% acetonitrile. After cleaning procedure, discs were rinsed with distilled water to remove residual acetonitrile solution. Then the discs were acid etched by submerging in 32% phosphoric acid for 30 minutes (based on pilot experiments, where previously, the acid treatment kinetics was done to standardize the time of acid treatment). After 30 minutes, the acid was removed and washed under distilled water for 1 minute. The excess water from disc surface was removed by touching the tissue paper gently to the edges of the discs and discs were dried at room temperature for 1 hour before starting the experiments.

4.2.2 Saliva and peptide coating and pellicle formation on the discs

For the first set of experiments, HA discs were incubated for 2 hours at 37℃ with parotid saliva (10 ug salivary proteins/ disc/well) in a total volume of 500 µl for control or with peptide solution (20 nmol/disc/well) in a total volume of 500 µl for pellicle formation in 24 well culture plates with 4 discs/ treatment. After 2 hours of incubation and pellicle formation, the unbound peptide was removed from the discs by washing for 10 second in distilled water.

The pre-coated peptide discs were incubated for additional 2 hours at 37℃ with 500 µl parotid saliva (10 µg salivary proteins/disc/well) for salivary pellicle formation. After 2 hours of additional incubation and pellicle formation. The discs were again briefly rinsed with distilled water for 10 second to remove any unbound saliva from disc surface.

For the second set of experiments, HA discs were incubated for 2 hours at 37℃ with parotid saliva (10 µg salivary proteins/ disc/well) in a total volume of 500 µl for pellicle formation in 24 well culture plates with 4 discs/ treatment. After 2 hours of incubation and pellicle formation, the parotid saliva was removed from the discs and discs were briefly rinsed for 10 second in distilled water to remove unbound saliva from disc surfaces. Then these discs were incubated for additional 2 hours at 37℃ in peptide solutions (DR9/DR9-DR9/DR9-RR14; 20 nmol/disc/well) in a total volume of 500 µl/disc for pellicle formation. After pellicle formation, the peptide solution was removed from the discs and rinsed for 10 second in distilled water to remove unbound peptide from disc surfaces. After this step, these discs were once again incubated with (10 µg salivary proteins/ disc/well) for additional 2 hours at 37℃. After incubation, the parotid saliva was removed from the discs and discs were briefly rinsed for 10 second in distilled water to remove unbound saliva from disc surfaces.

4.3 Parotid salivary pellicle harvesting and protein estimation

Water bath sonication was used to harvest pellicle in 500 µl buffer B (80% acetonitrile, 0.1 % triflouroacetic acid and 19.9% water) following 1 minute sonication. This procedure was repeated 3 times. After pellicle harvesting, the solution was centrifuged at 4℃ at 10,000 x g for 10 minutes to remove hydroxyapatite residue. The supernatant pellicle solution was dried overnight in the speedvac. Next morning, the dried pellicle material was re-suspended in distilled water and used for protein estimation through micro-BCA assay using bovine serum albumin as protein standards.

4.4 Mass spectrometric identification of pellicle proteins

4.4.1 Trypsin digestion and desalting procedure of pellicle proteins

Ten µg equivalent of pellicle protein was dried and resuspended in 50 µl of denaturation solution (4 M urea, 10mM DTT, and 50mM NH4 HCO3, pH 7.8,) and incubated at room temperature for 1 hour. After incubation, the solution was diluted with the addition of 150 µL of 50 mM NH_4HCO_3, pH 7.8. Afterward, the pellicle proteins were digested with 5% (w/w) trypsin for 24 hours at 37℃. After digestion, the pellicle digested material was again dried and desalted using zip-tip kit (EMD Millipore, Fisher Scientific). The purified digested sample was dried overnight and submitted for nLC-MS. In MS, each treatment was run 3 times as technical replicates. This set of experiments was repeated on three independent occasions.

4.4.2 MS Analysis

Mass spectrometric analyses were carried out with a LTQ-Velos (Thermo Scientific, San Jose, CA, USA), which allows in-line LC with the capillary-fused silica C18 column (column length 10 cm, column id 75 µm, 3 µm spherical beads, and 100 A˚pore size) linked to mass spectrometer using an ESI. All samples were dried by speedVac dryer and re-suspended in 97.5% H2 O/2.4% ACN/0.1% formic acid and then subjected to RP LC-ESI- MS. The nanoflow RP-HPLC conditions were linear 65-min gradient ranging from 5 to 55% of solvent B (97.5% ACN, 0.1% formic acid) at a flow rate of 300nL/min with a maximum pressure of 280 bars. Electrospray voltage and the temperature of the ion-transfer capillary were 1.8 kV and 250℃, respectively. The resultant MS spectra were searched against human protein databases (Swiss-Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST and Percolator algorithms in Proteome Discoverer 1.3 software (Thermo Scientific). Search results were filtered for a False Discovery Rate of 1%, employing a decoy search strategy utilizing a reverse database.

4.5 Results

4.5.1 Proteome profile of peptide-PSPL

In first set of experiments, 4 disc/group were used with the following experimental plan:

1) Parotid saliva only, (Control) (PSPL)

2) DR9 peptide coating, followed by parotid saliva, (DR9-PSPL)

3) DR9-DR9 peptide coating, followed by parotid saliva, (DR9-DR9-PSPL), and

4) DR9-RR14 peptide coating, followed by parotid saliva, (DR9-RR14-PSPL).

Only proteins common in at least two independent experiments out of three independent repeat experiments done on separate days were considered for the further analysis. A total of 139 proteins were present in all the treatments taken together. The highest number of proteins were found in group DR9-RR14-PSPL, followed by DR9-DR9-PSPL group, which was closely followed by PSPL (control) group. Lowest number of proteins were found in group DR9-PSPL. Of the 47 proteins, 28 (59.6%) were exclusive to DR9-RR14-PSPL. Out of 34 proteins, 16 (47.1%) were exclusive to PSPL (control). For DR9-DR9-PSPL, of the 39 proteins, 20 (51.3%) were specific to this group only. Similarly, out of 19 proteins, 9 (47.4%) were specific to DR9-PSPL. Out of 139 total protein, 7 proteins (5%) were common across all groups. (Table 4-1; Figure 4-1)

Table 4-1: Common proteins among all the groups of peptide coated disc pellicle.

Accession Number	Protein Name
P02814	Submaxillary gland androgen-regulated protein 3B/ Proline-rich peptide P-B/Proline-rich protein 3
P23280	Carbonic anhydrase 6/Carbonic anhydrase VI/ Carbonic dehydratase VI/ CA-VI
P02812	Basic salivary proline-rich protein 2/Salivary proline-rich protein/Con1 glycoprotein
P01833	Polymeric immunoglobulin receptor (PIgR)/ Hepatocellular carcinoma-associated protein (TB6)
G3CIG0	MUC19 variant 12
Q9UKN1	Mucin-12
Q12955	Ankyrin-3

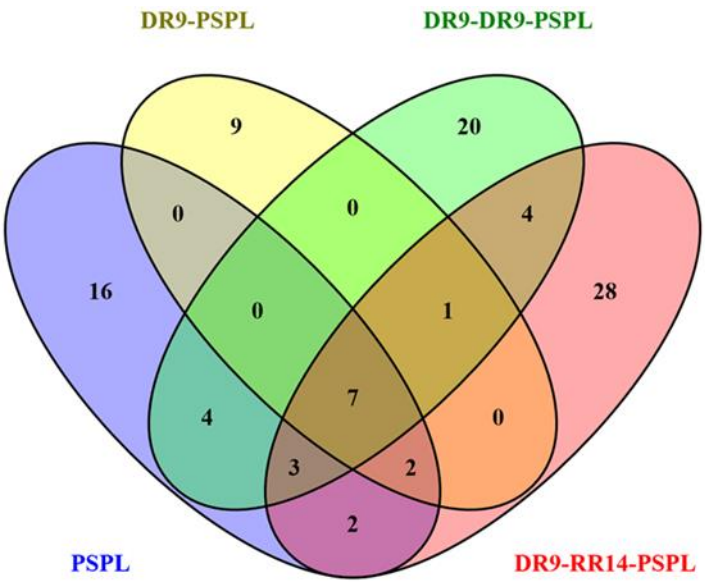

Figure 4-1: Comparison of peptide modulated AEP proteome profile on peptide coated discs.

4.5.2 Most abundant proteins from peptide-PSPL

Based on the ion abundance score count, the 10 most abundant proteins were selected from each group of peptide-PSPL modulation experiments. (Table 4-2, 4-3, 4-4, & 4-5). In PSPL (control), the relative ion abundance score ranged from a highest of 925.17 for submaxillary gland androgen-regulated protein 3B to a lowest of 21.50 for Ig alpha-2 chain region protein.

Table 4-2: Most abundant proteins based on relative ion abundance score from control group.

Accession Number	Protein Name	Ion Score
P02814	Submaxillary gland androgen-regulated protein 3B	925.17
P02808	Statherin	260.59
P23280	Carbonic anhydrase 6	215.62
Q6NSB3	Alpha-amylase (Fragment	175.35
Q8WX17	Mucin-16	77.81
P01833	Polymeric immunoglobulin receptor	72.27
P02812	Basic salivary proline-rich protein 2	67.13
Q7Z5P9	MUC19 variant 12	39.51
Q96Q06	Perilipin-4	30.04

P01877	Ig alpha-2 chain C region	21.50

In DR9-PSPL group, the relative ion abundance score ranged from a highest of 895.56 for submaxillary gland androgen-regulated protein 3B to a lowest of 13.67 for Ankyrin-3 protein.

Table 4-3: Most abundant proteins based on relative ion abundance score from DR9-PSPL group.

Accession Number	Protein Name	Ion Score
P02814	Submaxillary gland androgen-regulated protein 3B	895.56
P23280	Carbonic anhydrase 6	288.18
P02812	Basic salivary proline-rich protein 2	85.23
P01833	Polymeric immunoglobulin receptor	61.98
C0JYZ2	Titin	23.88
E9PAV3	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	22.60
Q7Z5P9	MUC19 variant 12	19.21
B4DSK7	cDNA FLJ50196, highly similar to Peroxisome proliferator-activated receptor-binding protein	16.99
Q9UKN1	Mucin-12	13.97
Q12955	Ankyrin-3	13.67

In DR9-DR9-PSPL group, the relative ion abundance score ranged from a highest of 766.95 for submaxillary gland androgen-regulated protein 3B to a lowest of 42.29 for statherin.

Table 4-4: Most abundant proteins based on relative ion abundance score from DR9-DR9-PSPL group.

Accession Number	Protein Name	Ion Score
P02814	Submaxillary gland androgen-regulated protein 3B	766.95
P23280	Carbonic anhydrase 6	389.1
Q6NSB3	Alpha-amylase	352.09
Q8WXI7	Mucin-16	94.32
P01877	Ig alpha-2 chain C region	76.47
P01833	Polymeric immunoglobulin receptor	72.38
P02812	Basic salivary proline-rich protein 2	68.36
H6VRF8	Keratin 1	66.82
P22079	Lactoperoxidase	45.26
P02808	Statherin	42.29

For DR9-RR14-PSPL group, the relative ion abundance score ranged from a highest of 359.14 for carbonic anhydrase 6 to a lowest of 26.25 for Ankyrin-3.

Table 4-5: Most abundant proteins based on relative ion abundance score from DR9-RR14-PSPL group.

Accession Number	Protein Name	Ion Score
P23280	Carbonic anhydrase 6	359.14
P02814	Submaxillary gland androgen-regulated protein 3B	278.92
Q6NSB3	Alpha-amylase	122.6
P01833	Polymeric immunoglobulin receptor	47.57
Q7Z5P9	MUC19 variant 12	44.2
Q96DA0	Zymogen granule protein 16 homolog B	39.58
P22079	Lactoperoxidase	33.7
P35527	Keratin, type I cytoskeletal 9	33.65
H6VRF8	Keratin 1	31.83
Q12955	Ankyrin-3	26.25

When the identified proteins (139) from all the groups (PSPL/ DR9-PSPL/ DR9-DR9-PSPL/ DR9-RR14-PSPL) were compared, it was found that there were 7 proteins, that were common across all the groups, but with differential ion abundance scores.

Table 4-6: Values of relative ion abundance score obtained in all peptide-PS groups.

Common proteins	PSPL (Control)	DR9-PSPL	DR9-DR9-PSPL	DR9-RR14-PSPL
Submaxillary gland androgen-regulated protein 3B	895.56	925.17	766.95	278.92
Carbonic anhydrase 6	288.18	215.62	389.1	359.14
Basic salivary proline-rich protein 2	85.23	67.13	68.36	24.3
Polymeric immunoglobulin receptor	61.98	36.83	72.38	47.57
MUC19 variant 12	19.21	39.51	33.29	44.2
Mucin-12	16.58	13.97	16.7	16.42
Ankyrin-3	14.56	13.67	16.31	26.25

The relative abundance data of the common proteins, across all the groups, clearly showed that the protein profile as well as protein abundance was modulated by the

peptide coatings of the disc surface when compared to control group. For example, submaxillary gland androgen-regulated protein 3B, the relative abundance score for control group was 895.56 while for DR9-PSPL group, the value was 925.17. In DR9-DR9-PSPL group the value obtained for submaxillary gland androgen-regulated protein 3B was less than that of control group (766.95). Relative low abundance was also observed with DR9-RR14-PSPL group (278.92).

For carbonic anhydrase 6 protein, the relative ion abundance score for control group was 288.18, whereas for DR9 group it was lowest and less than control (215.62; 74.8%), but both in DR9-DR9-PSPL and DR9-RR14-PSPL groups, it was highest (DR9-DR9) and more than control group (389.1; 135%) and (359.14; 124.6%), respectively.

In case of basic salivary proline-rich protein 2, the relative ion abundance scores for all the groups were less than the control group. It was 85.23 in control group, whereas for DR9-PSPL and DR9-DR9-PSPL groups, it was almost similar but less than control group (67.13; 78.8%), (68.36; 80.2%), respectively. The relative ion score was lowest and less than control group in case of DR9-RR14-PSPL group (24.3; 28.5%).

For polymeric immunoglobulin receptor (PIgR), the relative ion abundance score was 61.98 in control group. For DR9-DR9-PSPL group, it was highest and more than that of control (72.38; 116.8%), while for DR9-PSPL (lowest, 36.83; 59.4%) and DR9-RR14-PSPL (47.57; 76.8%) groups, it was lower than that of control group relative ion abundance score, respectively.

In case of mucin-19 variant 12 protein, the relative ion abundance score for control group was 19.21. For all the peptide coated groups, the relative ion abundance score was more than the control with DR9-RR14-PSPL group having the highest score (44.2; 230%) in comparison to the control group, followed by DR9-PSPL group (39.51; 205.7%) and DR9-DR9-PSPL group (33.29; 173.3%) in comparison to control group, respectively.

For mucin-12, the relative ion abundance score for control group was 16.58. For DR9-PSPL group, it was less than the control group (13.97; 84.3%). In case of DR9-DR9-PSPL group, the relative ion abundance score was higher than control group (16.7;

100.7%), while DR9-RR14-PSPL group ion abundance score was lower than the control group (16.42; 99 %), respectively.

Table 4-7: Proteome profile of control (PSPL) group.

Accession Number	Protein Name	Ion Score
Q96Q06	Perilipin-4	30.04
P01877	Ig alpha-2 chain C region	21.50
Q5HYC2	Uncharacterized protein KIAA2026	20.84
Q9Y6V0	Protein piccolo	20.64
P35527	Keratin, type I cytoskeletal 9	19.25
P08493	Matrix Gla protein	18.21
Q9UKN1	Mucin-12	16.58
Q12955	Ankyrin-3	14.56
Q9NR09	Baculoviral IAP repeat-containing protein 6	14.42
Q07954	Prolow-density lipoprotein receptor-related protein 1	13.15
O75592	E3 ubiquitin-protein ligase MYCBP2	11.74
Q8WWA1	Transmembrane protein 40	11.31
Q09666	Neuroblast differentiation-associated protein	10.98
Q15751	Probable E3 ubiquitin-protein ligase HERC1	10.19
Q15772	Striated muscle preferentially expressed protein kinase	9.94
Q75MN6	Putative uncharacterized protein MLL3	9.84
Q6W4X9	Mucin-6	9.70
A1YBP1	Breast and ovarian cancer susceptibility protein 2 truncated variant	9.61
L0R8C7	Alternative protein PRIC285	7.60
Q86XX4	Extracellular matrix protein FRAS1	7.14
Q9P0K0	Putative zinc finger protein	7.06
A0A087WU78	Nance-Horan syndrome protein	7.02
Q5CZC0	Fibrous sheath-interacting protein 2	6.63
O75443	Alpha-tectorin	6.39
Q59FP8	Neogenin	6.33
Q5D862	Filaggrin-2	5.71

Table 4-8: Proteome profile of DR9-DR9-PSPL group.

Accession Number	Protein Name	Ion Score
P02808	Statherin	42.29
Q9Y5I3	Protocadherin alpha-1	38.74
P35527	Keratin, type I cytoskeletal 9	38
G3CIG0	MUC19 variant 12	33.29
Q5T4S7	E3 ubiquitin-protein ligase UBR4	29.78
A0A0C4DGN4	Zymogen granule protein 16 homolog B	24.82
B7ZA42	cDNA, FLJ79056	20.49
Q96Q06	Perilipin-4	19.21
Q9UKN1	Mucin-12	16.7
Q14676	Mediator of DNA damage checkpoint protein 1	16.41
Q12955	Ankyrin-3	16.31
Q9Y6V0	Protein piccolo	14.35
E9PAV3	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	14.13
Q86VD1	MORC family CW-type zinc finger protein 1	13.43
H0YEF4	Palmitoyltransferase	13.17
I0B0K5	Truncated profilaggrin	13.05
Q5D862	Filaggrin-2	12.86
Q5QP82	DDB1- and CUL4-associated factor 10	12.4
A8K119	cDNA FLJ76742, highly similar to Homo sapiens deleted in liver cancer 1 (DLC1), transcript variant 2	10.35
Q9UF83	Uncharacterized protein DKFZp434B061	10.08
A6NKB5	Pecanex-like protein 2	9.67
F8W6X9	Protein sidekick-1	9.22
Q8WXG9	G-protein coupled receptor 98	7.19
Q02388	Collagen alpha-1(VII) chain	6.93
H0Y3R4	SET domain-containing protein 5	6.8
B4DF20	cDNA FLJ58172, highly similar to Zinc finger protein 238	6.54
L8E7G9	Alternative protein ZNF74	6.44
E5RJ29	PH and SEC7 domain-containing protein 3	6.35
Q9NR48	Histone-lysine N-methyltransferase ASH1L	6.32
Q96L91	E1A-binding protein p400	5.71

Table 4-9: Proteome profile of DR9-PSPL group.

Accession Number	Protein Name	Ion Score
Q12955	Ankyrin-3	13.67
Q14333	Facioscapulohumeral muscular dystrophy	9.95
Q15772	Striated muscle preferentially expressed protein kinase	9.48
Q10571	Probable tumor suppressor protein MN1	8.60
Q9H158	Protocadherin alpha-C1	8.45
B7Z7S7	cDNA FLJ60964, weakly similar to Homo sapiens dentin sialophosphoprotein, mRNA	7.35
B3KUS4	cDNA FLJ40508 fis, clone TESTI2045850, highly similar to AP-3 complex subunit beta-2	7.14
Q6W4X9	Mucin-6	6.79
E7EWQ5	Microtubule-associated serine/threonine-protein kinase 4	6.41
Q9C0F0	Putative Polycomb group protein ASXL3	6.39

Table 4-10a: Proteome profile of DR9-RR14-PSPL group.

Accession Number	Protein Name	Ion Score
Q12955	Ankyrin-3	26.25
P02812	Basic salivary proline-rich protein 2	24.3
F8VV32	Lysozyme	22.08
P51587	Breast cancer type 2 susceptibility protein	18.95
Q9UGM3	Deleted in malignant brain tumors 1 protein	18.02
Q9UKN1	Mucin-12	16.42
Q96Q06	Perilipin-4	15.86
B3KWI5	cDNA FLJ43124 fis, clone CTONG3004072	15.43
P13611	Versican core protein	13.7
P35908	Keratin, type II cytoskeletal 2 epidermal	13.69
Q5VST9	Obscurin	11.66
Q17R98	Zinc finger protein 827	10.99
Q86YZ3	Hornerin	9.88
O75592	E3 ubiquitin-protein ligase MYCBP2	9.59
E9PAV3	Nascent polypeptide-associated complex subunit α	9.27
Q6W4X9	Mucin-6	9.27
Q13535	Serine/threonine-protein kinase ATR	9.23
O95714	E3 ubiquitin-protein ligase HERC2	8.97
Q9H195	Mucin-3B	8.93
Q15751	Probable E3 ubiquitin-protein ligase HERC1	8.6

Table 4-10b: Proteome profile of DR9-RR14-PSPL group.

Accession Number	Protein Name	Ion Score
Q9ULT8	E3 ubiquitin-protein ligase HECTD1	7.86
P13645	Keratin, type I cytoskeletal 10	7.79
Q9UQ35	Serine/arginine repetitive matrix protein 2	7.7
A0A024QZW7	Nucleoporin 153kDa, isoform CRA_a	7.36
A0A087WW06	Tetratricopeptide repeat protein 28	7.01
O15265	Ataxin-7	6.83
G3V3H7	A-kinase anchor protein 6	6.82
A6NM28	Zinc finger protein 92 homolog	6.72
Q49AJ0	Protein FAM135B	6.59
Q9UF83	Uncharacterized protein DKFZp434B061	6.57
H7BXI1	Extended synaptotagmin-2	6.49
B3KPG0	clone NT2RI2007096, highly similar to Pleckstrin homology domain-containing family G member 1	6.01
P49792	E3 SUMO-protein ligase RanBP2	5.53
P08F94	Fibrocystin	5.47
P11137	Microtubule-associated protein 2	5.39
Q15772	Striated muscle preferentially expressed kinase	4.91
Q96SZ4	Zinc finger & SCAN domain-containing protein 10	4.64
Q6PRX3	Transducin-like enhancer of split 3 splice variant 1	4.51
Q12955	Ankyrin-3	26.25

The proteome profiles of all 4 groups were clearly different from each other for majority of proteins. For instance, keratin, type I cytoskeletal 9 protein and alpha-amylase were common in control, DR9-DR9-PSPL and DR9-RR14-PSPL groups but completely absent from DR9-PSPL group. Most of the proteins were present in only two groups out of four. For example, mucin-12 and ankyrin-3 were present only in DR9-PSPL and DR9-RR14-PSPL groups and absent from control and DR9-DR9-PSPL groups. Similarly, Ig alpha-2 chain C region and statherin were present only in control and DR9-DR9-PSPL groups and absent from DR9-PSPL and DR9-RR14-PSPL groups. Likewise, zymogen granule protein 16 homolog B and keratin 1 were present in DR9-DR9-PSPL and DR9-RR14-PSPL groups, but absent from control and DR9-PSPL groups.

4.6 Peptide-PSPL proteome profile classification

Based on molecular functions, cellular components, biological functions, and protein class, the 139 pellicle proteins from Peptide-PSPL were classified as follow:

4.6.1 Protein classification based on molecular functions

By molecular functions, the proteins were grouped under binding, catalytic activity, structural molecule activity, transporter activity and others (Figure 4-2). Of the total 139 pellicle proteins, 37.5% of the proteins were involved molecular binding activities with either other or with different ion species like calcium and phosphate. 35% of the proteins were involved in different kind of catalytic activities. Only 5 % proteins were involved in the transporter activity including ion transport. Around 7.5% proteins were either unknown proteins or proteins without known functions.

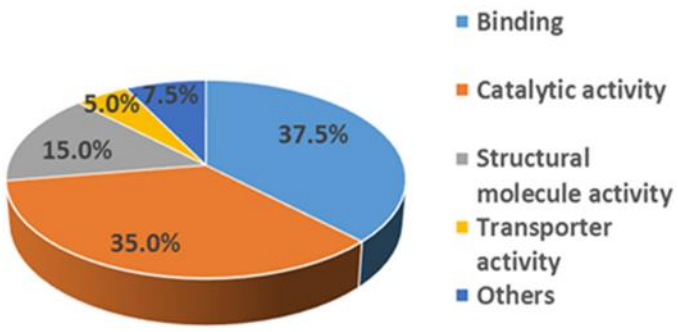

Figure 4-2: Protein classification based on molecular functions in peptide-PSPL group.

4.6.2 Protein classification based on cellular components

By cellular components, the proteins were grouped into cell part, extracellular region, macro molecular complex, organelles, and others (Figure 4-3). When the proteins were grouped based on the cellular components, out of 139 total pellicle proteins, 38.2% of the proteins were either part of the cellular components or associated with cellular components. Extracellular region proteins constituted 14.7% of the pellicle proteins. Around 6% of the proteins were part of the macromolecular complex with other proteins. 26.5% of the proteins were either part of the organelles or associated with the organelles. About 14.7% of the proteins were either unknown proteins or proteins without known functions.

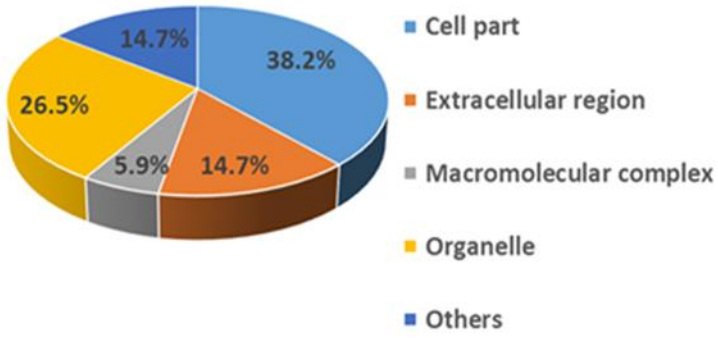

Figure 4-3: Protein classification based on cellular components in peptide-PSPL group.

4.6.3 Protein classification based on biological processes

Based on biological processes, the proteins were grouped under biogenesis, cellular process, immune system process, metabolic process, and others (Figure 4-4). When proteins were grouped according to biological processes, 31% of the total pellicle proteins were involved in various cellular processes, while 21.4% proteins were involved in metabolic processes. Immune system processes, comprise 3.6% of the pellicle proteins. Around 37% of the proteins were either unknown proteins or proteins with unknown functions, whereas 8.3% proteins were involved in biogenesis related activities.

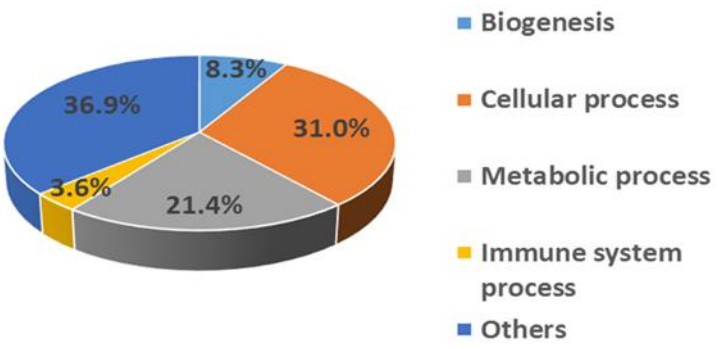

Figure 4-4: Protein classification based on biological processes in peptide-PSPL group.

4.6.4 Protein classification based on protein classes

Based on protein classes, the proteins were classified as calcium binding proteins, cell adhesion molecule, cytoskeletal proteins, immunity proteins, nucleic acid binding proteins, and others unknown or uncharacterized proteins (Figure 4-5). When the proteins

were grouped according to different classes, 1.6% of the proteins were calcium binding proteins, around 10% of the proteins were those associated with or involved in cell adhesion processes. Another ~10% proteins were either associated with cytoskeleton or part of the cytoskeletal system. About 5 % of the proteins were either part of the immune system or associated with immunity. About ~10% of the pellicle proteins were either nucleic acid associated or nucleic acid binding proteins. Around 6.6% of the proteins were structural proteins. ~57% of the proteins were either unknown proteins or proteins with unknow functions.

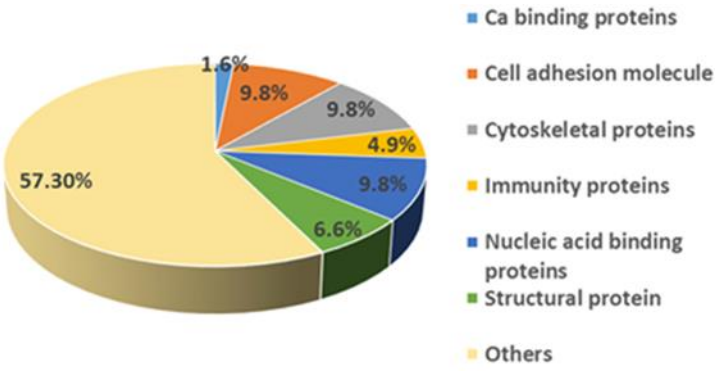

Figure 4-5: Protein classification based on protein classes in peptide-PSPL group.

4.7 Proteome profile of PS-Peptide-PSPL

In the second set of experiments, 4 disc/group were used with the following experimental plan:

1) Parotid saliva Control, (PSPL),

2) Parotid saliva coating, followed by DR9 peptide coating, followed by parotid saliva, (PS-DR9-PSPL),

3) Parotid saliva coating, followed by DR9-DR9 peptide coating, followed by parotid saliva, (PS-DR9-DR9-PSPL), and

4) Parotid saliva coating, followed by DR9-RR14 peptide coating, followed by parotid saliva, (PS-DR9-RR14-PSPL).

In the second set of pellicle modulation experiments, only those proteins were considered for the further analysis which were common in at least two independent experiments out of three independent repeat experiments done on separate days. There were in all 65 proteins from all the groups taken together. The highest number of proteins were present in PS-DR9-PSPL group, closely followed by PS-DR9-RR14-PSPL group, followed by PS-DR9-DR9-PSPL group.

Of the 12 proteins, 2 (16.7%) were exclusive to the PSPL (control) group. For PS-DR9-PSPL group, there were 20 proteins of which, 8 (40%) were exclusive to the PS-DR9-PSPL group. For PS-DR9-DR9-PSPL group, there were 15 proteins in all of which only 4 (26.7%) were exclusive to the group. In case of PS-DR9-RR14-PSPL group, there were in all 18 proteins of which 5 (27.8%) were exclusive to the group. Overall, a total of 7 proteins (~10.8%) were found to be shared among all groups (Table 4-11; Figure 4-6).

Table 4-11: Common proteins among all the groups of parotid saliva coated disc pellicle.

Accession Number	Protein Name
P02814	Submaxillary gland androgen-regulated protein 3B/ Proline-rich peptide P-B/Proline-rich protein 3
P23280	Carbonic anhydrase 6/Carbonic anhydrase VI/ Carbonic dehydratase VI/ CA-VI
P35908	Keratin, type II cytoskeletal 2 epidermal protein
P35527	Keratin, type I cytoskeletal 9
Q0KKI6	Immunoglobulin light chain
P13645	Keratin, type I cytoskeletal 10
Q9UGM3	Deleted in malignant brain tumors 1 protein

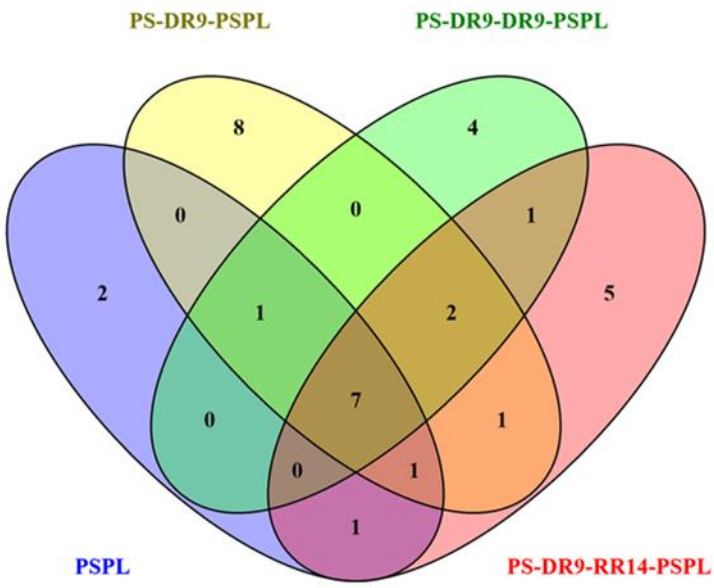

Figure 4-6: Comparison of peptide modulated AEP proteome profile on parotid saliva coated discs.

4.7 .1 Most abundant proteins from PS-peptide-PSPL

Based on the relative ion abundance score count, the 10 most abundant proteins were selected from each group of PS-peptide-PSPL modulation experiments. (Table 4-12, 4-13, 4-14, & 4-15). In control, the relative ion abundance score ranged from a highest of 111.16 for keratin 1 to a lowest of 14.05 for mucin 16 protein.

Table 4-12: Most abundant proteins from control group.

Accession Number	Protein Name	Ion Score
H6VRF8	Keratin 1	111.16
P02814	Submaxillary gland androgen-regulated protein 3B	81.54
P23280	Carbonic anhydrase 6	79.2
P35908	Keratin, type II cytoskeletal 2 epidermal	50.71
P35527	Keratin, type I cytoskeletal 9	48.36
Q0KKI6	Immunoglobulin light chain	23.78
P01833	Polymeric immunoglobulin receptor	23.15
G3CIG0	MUC19 variant 12	20.55
P13645	Keratin, type I cytoskeletal 10	18.07
B5ME49	Mucin-16	14.05

In PS-DR9-PSPL group, the ion abundance score ranged from a highest of 238.65 for carbonic anhydrase to a lowest of 41.32 for C1 segment protein (Fragment).

Table 4-13: Most abundant proteins from PS-DR9-PSPL group.

Accession Number	Protein Name	Ion Score
P23280	Carbonic anhydrase 6	238.65
H6VRF8	Keratin 1	184.2
Q0KKI6	Immunoglobulin light chain	118.2
P35908	Keratin, type II cytoskeletal 2 epidermal	95.41
P35527	Keratin, type I cytoskeletal 9	82.33
A0A075B6K9	Ig lambda-2 chain C regions	69.20
P01877	Ig alpha-2 chain C region	59.26
P13645	Keratin, type I cytoskeletal 10	58.33
P02814	Submaxillary gland androgen-regulated protein 3B	48.17
A0M8Q9	C1 segment protein	47.32

In PS-DR9-DR9-PSPL group, the relative ion abundance score ranged from a highest of 277.97 for carbonic anhydrase to a lowest of 38.94 for basic salivary proline-rich protein 2.

Table 4-14: Most abundant proteins from PS-DR9-DR9-PSPL group.

Accession Number	Protein description	Ion Score
P23280	Carbonic anhydrase 6	277.97
H6VRF8	Keratin 1	275.64
P35908	Keratin, type II cytoskeletal 2 epidermal	176.01
P13645	Keratin, type I cytoskeletal 10	160.70
Q0KKI6	Immunoglobulin light chain	124.99
Q6MZV6	Putative uncharacterized protein DKFZp686L19235	90.14
P35527	Keratin, type I cytoskeletal 9	69.69
P02814	Submaxillary gland androgen-regulated protein 3B	58.00
P12273	Prolactin-inducible protein	40.35
P02812	Basic salivary proline-rich protein 2	38.94

In PS-DR9-RR14-PSPL group, the relative ion abundance score ranged from a highest of 414.26 for carbonic anhydrase to a lowest of 46.89 for matrix Gla protein

Table 4-15: Most abundant proteins from PS-DR9-RR14-PSPL group.

Accession Number	Protein description	Ion score
P23280	Carbonic anhydrase 6	414.26
B7ZMD7	Alpha-amylase	390.09
Q0KKI6	Immunoglobulin light chain	148.32
P02814	Submaxillary gland androgen-regulated protein 3B	94.58
P35908	Keratin, type II cytoskeletal 2 epidermal	74.33
P01833	Polymeric immunoglobulin receptor	70.35
P13645	Keratin, type I cytoskeletal 10	68.69
B4E1M1	cDNA FLJ60391, highly similar to Lactoperoxidase	65.27
A0A0C4DGN4	Zymogen granule protein 16 homolog B	52.99
P08493	Matrix Gla protein	46.89

When the identified proteins from all the groups (PS/PS-DR9-PSPL/PS-DR9-DR9-PSPL/PS-DR9-RR14-PSPL) were compared, it was found that there were 7 proteins, that were common across all the groups, but with differential relative ion abundance scores.

Table 4-16: Values of ion abundance score obtained in all PS-peptide-PSPL groups.

Common Proteins	PSPL (Control)	PS-DR9-PSPL	PS-DR9-DR9- PSPL	PS-DR9-RR14- PSPL
Submaxillary gland androgen-regulated protein 3B	81.54	48.17	58.00	94.58
Carbonic anhydrase 6	79.2	238.7	277.97	414.26
Keratin, type II cytoskeletal 2 epidermal protein	51.71	95.41	176.01	74.33
Keratin, type I cytoskeletal 9	48.4	82.33	69.69	46.61
Immunoblobulin light chain	23.8	118.2	124.99	148.32
Keratin, type I cytoskeletal 10	18.1	58.33	160.7	68.69
Deleted in malignant brain tumors 1 protein	6.72	25.74	28.48	35.44

The relative ionic abundance data of the common proteins across all the groups, clearly showed that the protein profile as well as abundance was modulated by the peptide coatings on the PS pellicle coated surface, in comparison to control group. For example, in case of submaxillary gland androgen-regulated protein 3B, the relative ion abundance score for control was 81.54. For both, PS-DR9-PSPL and PS-DR9-DR9-PSPL groups, it was lowest and less than that of control group (48.17; 59.1%) and (58.0; 71.13%),

respectively. The score was highest for PS-DR9-RR14-PSPL coated disc (94.58; 116%), compared to control.

For carbonic anhydrase 6 protein, the ion abundance score for control was 79.2, whereas for PS-DR9-PSPL, PS-DR9-DR9-PSPL groups it was higher and more than control (238.7; 301.4%), (277.97; 351%) and highest for PS-DR9-RR14-PSPL (414.26; 523.1%) in comparison to control, respectively.

In case of keratin, type II cytoskeletal 2 epidermal protein, the ion abundance score for all the treatments was more than the control. It was 51.71 in control, whereas for PS-DR9-PSPL, PS-DR9-RR14-PSPL it was higher than control (95.41; 184.5 %) and (74.33; 143.7%) and highest for PS-DR9-DR9-PSPL group (176.01; 340.4%), respectively compared to control.

For keratin, type I cytoskeletal 9 protein, the ion abundance score was 48.4 in control. For PS-DR9-RR14-PSPL group, it was lowest and less than that of control (46.61; 96.3%). Ion scores for PS-DR9-PSPL (highest) was followed by PS-DR9-DR9-PSPL, (82.33; 170.1%) and (69.69; 144 %) respectively, were higher than that of control.

In case of immunoglobulin light chain protein, the ion abundance score for control was 23.8. For all the peptide coated groups, the ion score was more than the control with DR9-RR14-PSPL group having the highest ion score (148.32; 623.2%), followed by PS-DR9-DR9-PSPL group (124.99; 525.2%) and PS-DR9-PSPL group (118.2; 496.6%) of the control, respectively.

For keratin type I cytoskeletal 10 protein, the ion abundance score for control was 18.1. In comparison to PS control, the ion abundance score was highest for PS-DR9-DR9-PSPL group (160.7; 887.9%) followed by PS-DR9-RR14-PSPL (68.69; 379.5%), and followed by PS-DR9-PSPL (58.33; 322.3 %), respectively, and was more than the control.

In case of deleted in malignant brain tumors 1 protein, the control had an ion abundance count of 6.72. Compared to control, ion abundance count was higher in all the groups

with PS-DR9-RR14-PSPL group being the highest (35.44; 527.4%), followed by PS-DR9-DR9-PSPL (28.48; 423.8%) and lowest PS-DR9-PSPL group (25.74; 383%), respectively.

Table 4-17: Proteome profile of control group.

Accession Number	Protein description	Ion Score
Q59ED1	Cubilin variant	10.31
Q9UGM3	Deleted in malignant brain tumors 1 protein	6.72

Table 4-18: Proteome profile of PS-DR9-PSPL group.

Accession Number	Protein description	Ion Score
P01833	Polymeric immunoglobulin receptor	44.71
P22079	Lactoperoxidase	43.66
A0A0C4DGN4	Zymogen granule protein 16 homolog B	40.92
P61626	Lysozyme C	40.05
A2KBC1	Anti-(ED-B) scFV	38.52
Q9UGM3	Deleted in malignant brain tumors 1 protein SV=2	25.74
P09228	Cystatin-SA	17.85
P01034	Cystatin-C	16.02
P12273	Prolactin-inducible protein	14.07
E9PKG6	Nucleobindin-2	8.96

Table 4-19: Proteome profile of PS-DR9-DR9-PSPL group.

Accession Number	Protein description	Ion Score
A0A087X2C0	Ig mu chain C region	28.98
Q9UGM3	Deleted in malignant brain tumors 1 protein	28.48
A0A0C4DGN4	Zymogen granule protein 16 homolog B	26.01
C9JEV0	Zinc-alpha-2-glycoprotein	14.22
A8K739	cDNA FLJ77339	10.32

Table 4-20: Proteome profile of PS-DR9-RR14-PSPL group.

Accession Number	Protein description	Ion Score
P35527	Keratin, type I cytoskeletal 9	46.61
A8K739	cDNA FLJ77339	36.07
Q9UGM3	Deleted in malignant brain tumors 1 protein	35.44
P01034	Cystatin-C	26.40
P12273	Prolactin-inducible protein	21.22
B4DI70	cDNAhighly similar to Galectin-3-binding protein	16.13

P10909	Clusterin	13.62
G3CIG0	MUC19 variant 12	6.32

The proteome profiles of all the 4 groups (control and 3 PS-peptide-PS pellicle), were clearly different from each other. For instance, alpha-amylase (Fragment) was absent in control, PS-DR9-PSPL, and PS-DR9-DR9-PSPL and present in PS-DR9-RR14-PSPL group only. Mucin-16 protein was present in control only and was absent in all other groups. Lysozyme C was present only in PS-DR9-PSPL and absent from all other groups, including control. Cystatin-SA was present only in PS-DR9-PSPL and from all other groups whereas cystatin-C was present in PS-DR9-PSPL and PS-DR9-RR14-PSPL groups and absent in control as well as PS-DR9-DR9-PSPL. On the contrary, prolactin-inducible protein and zymogen granule protein 16 homolog B were absent in control and present in all the peptide groups. Keratin I was absent in PS-DR9-RR14-PSPL but present in all other groups. Similarly, MUC 19 variant 12 protein was present in control and PS-DR9-RR14-PSPL and absent from PS-DR9-PSPL and PS-DR9-DR9-PSPL groups.

There were some proteins like keratin type II cytoskeletal 2 epidermal, keratin type I cytoskeletal 10, keratin type I cytoskeletal 9, submaxillary gland androgen-regulated protein 3B, carbonic anhydrase 6, immunoglobulin light chain (Fragment) and deleted in malignant brain tumors 1 protein, that were present in all the groups, irrespective of presence or absence of peptides on the PS pellicle surface.

4.8 PS-Peptide-PSPL proteome profile classification

Based on molecular functions, cellular components, biological functions and protein class, the 65 pellicle proteins from PS-peptide-PSPL were classified as follow:

4.8.1 Protein classification based on molecular functions

By molecular functions, the proteins were grouped under binding, catalytic activity, structural molecule activity, transporter activity and others (Figure 4-7).

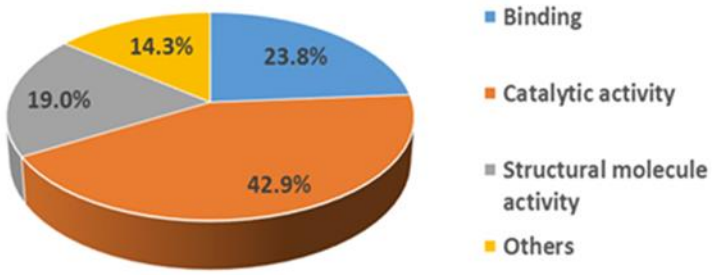

Figure 4-7: Protein classification based on molecular functions in PS-peptide-PSPL group.

Of the total 65 pellicle proteins, 23.8% of the proteins were involved molecular binding activities with either other proteins or with different ion species like Ca and Phosphate. About 43% of the proteins were involved in different kind of catalytic activities. 19% of the proteins had structural molecule activity. Around 14% proteins were either unknown proteins or proteins without known functions.

4.8.2 Protein classification based on cellular components

By cellular components, the proteins were grouped into cell part, extracellular region, macro molecular complex, organelles, and others. (Figure 4-8).

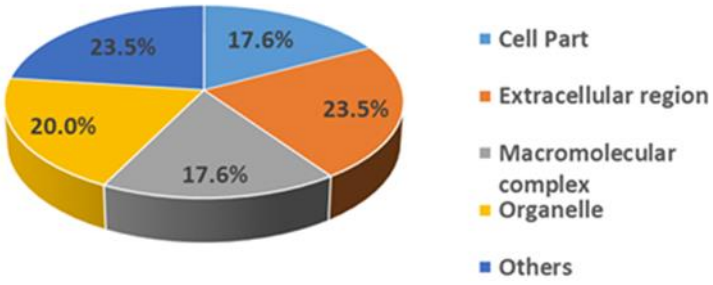

Figure 4-8: Protein classification based on cellular components in PS-peptide-PSPL group.

When the proteins were grouped based on the cellular components, out of 65 total pellicle proteins, 17.6% of the proteins were either part of the cellular components or associated with cellular components. Extracellular region proteins constituted 23.5% of the pellicle proteins. About 17.6% of the proteins were part of macromolecular complex with other proteins. 20 % of the proteins were either part of organelle system or associated with organelles. Around 23.5% proteins were either unknown proteins or proteins without known functions.

4.8.3 Protein classification based on biological processes

Based on biological processes, the proteins were grouped under biogenesis, cellular process, immune system process, and metabolic process.

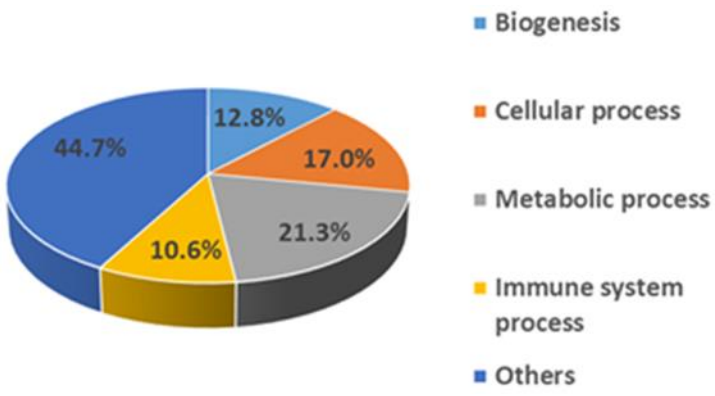

Figure 4-9: Protein classification based on biological processes in PS-peptide-PSPL group.

When proteins were grouped according to biological processes, 12 % of the proteins were involved in the biogenesis related processed and 17% of the total pellicle proteins were involved in various cellular processes, while 21.3% proteins were involved in metabolic processes. Immune system processes, comprise 10.6% of the pellicle proteins. Around 45% of the proteins were either unknown proteins or proteins with unknown functions.

4.8.4 Protein classification based on protein classes

Based on protein class, the proteins were classified calcium binding proteins, cell adhesion molecule, cytoskeletal proteins, immunity proteins, and others.

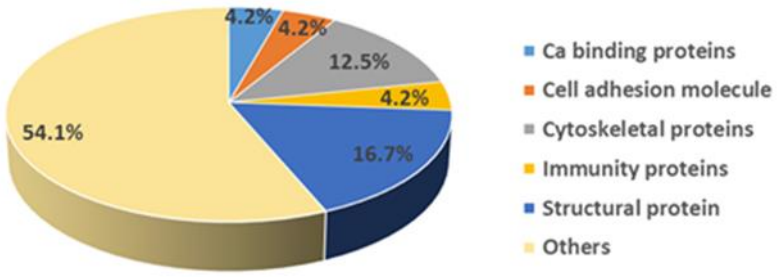

Figure 4-10: Protein classification based on protein classes in PS-peptide-PSPL group.

When the proteins were grouped according to different classes, 4.2% of the proteins were calcium binding proteins, around 4.2% of the proteins were those associated with or involved in cell adhesion processes. Another 12.5% proteins were either associated with cytoskeleton or part of the cytoskeletal system. About 4.2 % of the proteins were either part of the immune system or associated with immunity. About Around 17% of the proteins were structural proteins. ~54% of the proteins were either unknown proteins or proteins with unknow functions.

4.9 Discussion

In the current study, the peptide pellicle was allowed to accumulate on the surface of hydroxyapatite discs, and then the saliva pellicle was accumulated on top of peptide pellicle. We found that this resulted in the different profile of majority of salivary proteins that constituted the pellicle in all the peptide groups in comparison to control where no peptide was used. Only ~4% of the proteins, out of a total of 139 proteins were found to be common across all the treatments. Another interesting aspect observed was that 45-60% of the proteins were specific to each treatment, according to the peptide used in the treatment. This further indicates that peptide coating of the disc surface was responsible for the modulation as well as specificity of the proteins to each group. This can be explained by the fact that all the three peptides (DR9/DR9-DR9/DR9-RR14) carried different net charges at pH 7 and had different pI values. Peptide having different net charges interact differently to the same pool of parotid saliva proteins. Due to the presence of seven histidine, three arginine and three lysine residues, histatin 5 has a got very high positive charge (Yin *et al.* 2006). The adsorption of such proteins, with low

affinity, to hydroxyapatite, can be augmented through their interaction with other strongly bound proteins to the dental enamel. Besides electrostatic interactions, an increase in entropy drives the protein adsorption to dental enamel surface. Moreover, histatins, statherin, and acidic proline-rich proteins (aPRPs), comprise the basal layer of the enamel pellicle. These proteins modulate the ionic concentration of calcium and phosphate in the oral cavity and are thought to exert control over the dental enamel erosion. (Siqueira *et al.* 2007).

In the process of enamel erosion, the proteomic composition of the pellicle play an important role. High-molecular-weight glycoproteins, salivary mucins (MUC5B) are one of the main pellicle components, and constitute 7 to 26 % of total salivary proteins in the pellicle (Slomiany *et al.* 1996). In our study, we also found that mucins in different isoforms was present in PS pellicle under different peptide treatments.

Perinpanayagam, *et al.* (1995), did the characterization of low molecular peptides from parotid saliva. They concluded that these peptides are the result of post translation proteolysis of the parotid salivary antimicrobial proteins. They also estimated that these small peptides are present in nanomole (nM) concentration in the parotid saliva. Whether these levels are sufficient to exert any biological effect is open to speculation. Therefore, the rationale for using 20 nmol concentration in our experiments was based on the nanomole concentration of these peptides in the parotid saliva. We wanted to test if these levels of salivary peptides are sufficient to exert any biological effect in terms of demineralization protection as well as pellicle modulation. What we found that these peptides (DR9/DR9-DR9/DR9-RR14) at 20 nmol concentration could exert their biological effect in terms of the modulation of pellicle composition.

Since the MUC5B, a high-molecular-weight mucin, has around 6 x affinity to hydroxyapatite, compared to MUC7, a low-molecular weight mucin, the MUC5B adsorbs to the tooth surface and contributes to the formation of the acquired enamel pellicle by quickly adsorbing to enamel surface. (Tabak 1990). In this study, we found that different isoforms of mucin were part of the pellicle, the binding of which was selectively modulated by the peptides in case of PS-peptide-PSPL where mucin -16 and 19 isoforms

were present in control but absent in both PS-DR9-PS and PS-DR9-DR9-PS treatment and only mucin-19 was detected in PS-DR9-RR14-PS treatment, whereas in peptide-PSPL the mucin isoforms were present in all the treatments to different extent based on their ionic abundance scores. This clearly showed that the peptides were modulating the proteomic composition of the pellicle in a very selective manner depending upon whether they were present directly on the surface of hydroxyapatite or on the surface of PS pellicle.

Low-molecular-weight salivary proteins, histatins, are synthesized and secreted in the oral cavity by the parotid and other minor salivary glands. (Siqueira *et al.* 2008; Oppenheim *et al.* 1986).

Three main types of histatins (1, 3, and 5), have been detected as the components enamel pellicle. (Siqueira *et al.* 2010). Being multifunctional proteins in nature, besides the fungicidal activity, (Xu *et al.* 1991) these proteins display significant protective activity against demineralization challenges as well (Siqueira *et al.* 2010). Histatin and statherin phosphoproteins have been shown to display acid demineralization protection against acid challenges (Siqueira *et al.* 2010; Li *et al.* 2004). Phosphorylated proteins have been speculated to display more affinity to hydroxyapatite, therefore, phosphorylated histatin 1 and statherin provide higher protection in comparison to non-phosphorylated residues. (Siqueira *et al.* 2012, Richardson *et al.* 1993).

Apart from the phosphoproteins, another high molecular weight acidic, hydrophilic protein albumin, has been implicated to play a role in demineralization protection. (Hemingway *et al.* 2010).

Salivary protein statherin is most potent inhibitor of both primary and secondary calcium phosphate precipitation, and histatin-1 displayed the least inhibition. Tamaki *et al.* (2002), found that only statherin was able to effectively inhibit calcium phosphate spontaneous precipitation at physiological concentrations.

Yin *et al.* (2003) found that only histatin 1 contained N-terminal phosphorylated serine, among all the histatins, which greatly increased its hydroxyapatite binding affinity.

Due to the technical limitations imposed by the instrument, for MS analysis, the protease digestion of the proteins and peptides bigger than 5 kDa becomes a necessity. Siqueira and Oppenheim (2009), in LC-ESI MS/MS mediated salivary proteomic profiling of the human pellicle proteome, identified 78 natural peptides (with a MW range of 767 – 3981 Da) that originated from 29 different proteins without any prior proteolytic treatment. Siqueira and Oppenheim (2009), by avoiding trypsinization, demonstrated that naturally occurring salivary peptides in the enamel pellicle can be identified.

Yao *et al.* (2000) proposed that statherin, histatins and proline-rich proteins (PRPs) are thought to initiate pellicle formation, which in turn, through transglutaminase enzyme follow cross-linking via covalent bonds between c-amide groups of a specific glutamine and an e-amino group of a specific lysine of adsorbed salivary proteins, leading to their complex formation with each other. (Bradway *et al.* 1992; Yao *et al.* 1999; Hannig *et al.* 2008).

Yao *et al.* (2000) observed that under *in vitro* conditions, transglutaminase catalyzed acidic PRP-1 and statherin cross-link. They also observed that under *in vitro* conditions the PRPs is involved in cross-linking with histatins salivary proteins as well.

Lendenmann *et al.* (2000) observed that IgA is the component of enamel pellicle and has been observed in the saliva as well (Lee *et al.* 2007).

IgA is implicated to promote some specific bacterial clearance, which was also observed in the gut (Mantis *et al.* 2011), besides neutralizing certain viruses in the oral cavity (McNabb and Tomasi, 1981). These protective immunological properties of the pellicle are very important for overall health protection, and maintenance of the normal functioning of oral cavity.

Salivary proteins statherin, agglutinins, PRPs, histatins and cystatins can help in aggregation of bacterial cells, thereby reducing their adherence and colonization on the enamel pellicle surface. This can significantly control and modulate the quality of biofilms in the oral cavity (Humphrey and Williamson, 2001). In this study, we also found that polymeric immunoglobulin receptor protein, Ig alpha-2 chain C region,

immunoproteins, and statherin, were part of Peptide-PSPL proteome, thus confirming earlier observations by Lendenmann *et al.* (2000).

In addition to immunoglobulin light chain, Ig alpha-2 C region proteins, PRP, polymeric immunoglobulin receptor protein, Ig lambda-2 chain C regions and cystatins were part of PS-Peptide-PSPL proteome, supporting the earlier observations of Lendenmann *et al.* (2000). Presence of these proteins in the pellicle further strengthen and support the important role of overall oral health protection, normal functioning of the oral cavity, in addition to the modulation of colonization and biofilm formation in the oral cavity, that is played by pellicle (Mantis *et al.* 2011; McNabb and Tomasi, 1981; Humphrey and Williamson, 2001).

Different enzymological, electrophoretic, immunological and mass spectrometer methods have been employed to detect amylase in the enamel pellicle. Amylase has been shown to present both *in vitro* enamel pellicles (Rølla *et al.* 1983; Siquiera *et al.* 2012) as well as *in situ* pellicles as a major component of pellicle (Carlen *et al.* 1998; Siquiera *et al.* 2007; Siqueira and Oppenheim 2009; Deimling *et al.* 2004). In this study, we also found that alpha-amylase was one of the major component of pellicle in both types of pellicle (peptide-PSPL and PS-Peptide-PSPL), thus supporting previous studies. Apart from alpha-amylase, carbonic anhydrase 6, lactoperoxidase were found to be present in the peptide-PSPL.

Among acquired enamel pellicle proteins, lysozyme is one of the main enzymatic protein (Siqueira *et al.* 2007; Li *et al.* 2004). Lysozyme has been found in an active form both in the *in situ* enamel pellicles (Li *et al.* 2004), as well as in the *in vitro* experimental pellicles (Hennig et al 2005; Siqueira *et al.* 2009). In this study, the lysozyme was identified in the pellicle modulated by DR9-RR14 peptide coated disc only. In case of PS-DR9-PSPL, the lysozyme was detected, thereby supporting previous studies in relation to presence of lysozyme in the *in vitro* pellicle. It is speculated, that presence of other two peptide coating on the HA surface (DR9 and DR9-DR9), could have modified the surface electrochemistry of HA surface, that might have led to nonadherence and hence integration of lysozyme into *in vitro* pellicle. In case of PS-DR9 coating, the

presence of multiprotein PS coating on HA surface might have changed the surface electrochemistry in a way, that preferentially favored lysozyme over the DR9 in the pellicle.

Tellefson and Germaine, (1986) and Cimasoni *et al.* (1987) found that lysozyme specifically binds to certain bacteria and may help them not only to aggregate but also in bacterial adherence to the enamel pellicle. On the other hand, in a contrasting study, Roger *et al.* (1994) showed that lysozyme inhibited the adsorption of *Streptococcus* to saliva-coated hydroxyapatite.

It has been demonstrated, that peroxidase adsorb irreversibly and retain the enzymatically active conformation, to human enamel, when saliva was incubated with enamel powder (Tenovuo *et al.* 1977; Hennig *et al.* 2005). Pruitt and Adamson (1977), showed that 75% of activity of the immobilized enzyme in the pellicle was retained. In this study, we also identified the peroxidase, thus confirming the previous observations regarding presence of peroxidase in the *in vitro* pellicle. We identified lactoperoxidase in DR9-RR14 PSPL and PS-DR9-RR14-PSPL. This can very well be explained by the selective interaction of DR9-RR14 with peroxidase both in presence and absence of salivary pellicle on the disc surface.

Leinonen *et al.* (1999), employed immunostaining antibody techniques, to detect carbonic anhydrase VI (CA VI) in *in situ* formed enamel pellicles. In another study, Li *et al.* (2004) detected CA II in the *in situ* enamel pellicle, by employing monoclonal antibodies. In this study, we also found, that CA was present in the *in vitro* pellicle.

PRPs are one of the major constituent of enamel pellicle and they represent around one-third of the salivary proteins (Bennick, 1987; Hay *et al.* 1988; Gibbons and Hay 1989). These proteins adsorb on to the enamel surface and incorporated as an integral part of the enamel pellicle due to their negatively charged amino acid. (Lamkin *et al.* 1996). In this study, we also found that PRPs were present in PS saliva control and all the peptide coated discs (DR9, DR9-DR9, DR9-RR14).

Through general consensus, it is accepted that, enzymes adsorb into the pellicle in a selective fashion similar to other salivary proteins, since only a fraction of enzymes present in saliva has been identified in the enamel pellicle (Yao *et al.* 2003; Hay, 1967).

Pruitt *et al.* (1969), proposed the idea that salivary proteins could compete for adsorption and inhibit certain enzymes from immobilization in the pellicle. From a clinical view point, the main objective would be to modulate the pellicle composition, which plays a crucial role in the attachment of bacteria, for the biofilm establishment, that will be harmless, non-cariogenic and ecologically stable. Basically, this forms the basis of our hypothesis that salivary peptides can modulate the pellicle composition. We found in this study that when peptides were used for coating the disc surface, they did selectively modulate and inhibit adsorption of certain enzymes (alpha-amylase), which was absent from the peptide treated pellicle (DR9 peptide coating) in comparison to the control.

On the other hand, carbonic anhydrase was one such enzyme, the adsorption of which was not affected by presence or absence of the peptides, but the extent of its presence in the pellicle was differential according to different ionic abundance count across the treatments in both the Peptide-PSPL as well as PS-peptide-PSPL.

In case of peptide coating of disc surface, it is speculated that there was possible uniform charge due to homo-molecular layer of peptide, and hence there was direct interaction between the peptide layer (different surface electrochemistry for each peptide) and the PSPL proteins, resulting in increasing trend of ion abundance. On the contrary in case of PS-Peptide-PSPL, the ion abundance trend was irregular. This can be possibly attributed to the influence and competition of the disc surface PSPL on the adsorption of specific peptide, possibly due to surface charge interactions of PSPL proteins and peptide with 2nd layer of PSPL proteins. This in turn influenced the further differential binding of second layer of PSPL on the peptide layer.

The adsorption behavior of a single salivary protein can be significantly different than one that can be observed if that protein is present in a mixture of different proteins (Yin *et al.* 2006). In this study, we observed that there was indeed difference in the behavior of salivary peptides in absence and presence of mixture of different salivary proteins.

4.10 Conclusion

Overall, this study provided direct evidence that salivary peptides at nanomole concentration are capable of exerting and involved in the modulation of the pellicle proteome composition as well as protein abundance based on their ion count, possibly through the modification in the protein-protein interactions. This modulation both in proteome profile and abundance of proteins is influenced, in parts due to presence and absence of PSPL proteins on the hydroxyapatite surface as was observed in terms of lower number of pellicle proteins recovered from PS-peptide-PSPL in comparison to peptide-PSPL. This is initial step in the direction of further understanding the actual molecular mechanism of how the protein-protein interactions bring about the pellicle proteome modulation to pave the way for dental caries and other oral healthcare management in the future.

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Chapter 5

5 Summary and conclusion

1. Under the current experimental conditions, the direct coating of HA surface with peptide constructs (DR9, DR9-DR9, DR9-RR14) at low concentrations of the peptides (20 nmol), did not provide any advantage or significant demineralization protection against calcium and phosphate losses from enamel surface compared to control.

2. When the PS pre-coated HA disc were coated with peptide constructs (DR9, DR9-DR9, DR9-RR14), DR9 and DR9-DR9 peptides exhibited similar and significant level of calcium and phosphate demineralization protection, compared to control.

3. At lower concentration, DR9-DR9, did not provide any advantage over DR9, over demineralization protection against calcium and phosphate losses from enamel surface.

4. At lower concentration, the peptide DR9-RR14 did not provide any protection against calcium and phosphate demineralization.

5. In the peptide mediated pellicle proteome modulation experiments, out of 139 proteins identified in all the groups from peptide coated discs, (PS/ DR9-PSPL/ DR9-DR9-PSPL/ DR9-RR14-PSPL), interestingly, 45-60% of the proteins were specific to each group according to the peptide used. Only 7 proteins (~4%), were common across all the groups, but with differential ion abundance scores.

6. The proteome profiles of all 4 groups were clearly different from each other for majority of proteins, since most of the proteins were present in only two groups out of four.

From the current study, it can be concluded that DR9 (single & tandem repeat construct) provided significant enamel demineralization protection, Additionally, all the tested peptide constructs (DR9, DR9-DR9, DR9-RR14) modulated the enamel pellicle proteome composition. Overall, this study, provided direct evidence, that salivary

peptides at nanomolar concentration are capable of exerting and involved in the modulation of the pellicle biological functions.

Appendix A

Abbreviations

µg	Microgram
µl	Microliter
µm	Micrometer
2-D PAGE	2-dimensional poly acrylamide gel electrophoresis
3-D	Three dimensional
AEP	Acquired enamel pellicle
AFM	Atomic force microscopy
aPRPs	Acidic proline rich proteins
BSA	Bovine serum albumin
Buffer B	80% acetonitrile, 0.1 % triflouroacetic acid and 19.9% water
℃	Degree Celsius
CA	Carbonic anhydrase
Ca and PO_4	Calcium and phosphate
$CaCl_2$	Calcium chloride
PSPL	Parotid saliva pellicle
DR9-PSPL	Parotid saliva pellicle on top of DR9 coating
DR9-DR9-PSPL	Parotid saliva pellicle on top of DR9-DR9 coating
DR9-RR14-PSPL	Parotid saliva pellicle on top of DR9-RR14 coating
PS-DR9-PSPL	Parotid saliva pellicle on top of PS-DR9 coating
PS-DR9-DR9-PSPL	Parotid saliva pellicle on top of PS-DR9-DR9 coating
PS-DR9-RR14-PSPL	Parotid saliva pellicle on top of PS-DR9-RR14 coating
DTT	Dithiothreotol
HA	Hydroxyapatite
HPLC	High performance liquid chromatography
kDa	Kilo Dalton
LC-ESI-MS/MS	Liquid chromatography-electron spray ionization-tandem mass spectrometry
MALDI-TOF-MS	Matrix assisted lased desorption ionization-time of flight-mass spectrometry
micro-BCA	micro-BiCinconinic Acid protein estimation assay
mM	Millimolar
NaH_2PO_4	Ammonium dihydrogen phosphate
NH_4 HCO_3	Ammonium bicarbonate
nLC-ESI-MS	Nano liquid chromatography-electron spray ionization- mass spectrometry
RP-HPLC	Reverse phase high performance liquid hromatography
nM	Nano mole
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
pI	Isoelectric point
PRPs	Proline-rich proteins
PS	Parotid saliva
SEM	Scanning electron microscopy
TEM	Tunneling electron microscopy

Curriculum Vitae

Name: Rajesh Gupta

Post-secondary Education and Degrees:
Dr.YS Parmar University of Horticulture & Forestry, Solan, HP, India
1990-1994 B.Sc. Horticulture (Major Plant Biotechnology)
GB Pant University of Agriculture & Technology, Pantnagar, UA, India
1994-1996 M.Sc. Agriculture (Molecular Biology & Biotechnology)
The University of Western Ontario, London, Ontario, Canada
2010-2012 M.Sc. Biology (Cell Biology)

Honours and Awards:
DBT All India Merit Fellowship 1994-1996
CSIR-UGC NET Fellowship 1996-2002
TATA International Fellow Endowment Award 2010-11
Deans Award 2013-2016
WGRS 2010-2016

Related Work Experience
Graduate Teaching Assistant, The University of Western Ontario, 2010-2015
Graduate Research Assistant, The University of Western Ontario 2013-2017

Publications:
Possmayer, M., Gupta, R.K., Szyszka-Mroz, B., Maxwell, D.P., Lachance, M.A., Huner, N.P.A., and Smith, D.R. (2016). Resolving the Phylogenetic Relationship between Chlamydomonas sp. UWO 241 And Chlamydomonas raudensis SAG 49.72 (Chlorophyceae) With Nuclear and Plastid DNA sequences. J. Phycol. DOI: 10.11 11/jpy.12383

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