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The binding of salivary proteins and its effect on the demineralization of hydroxyapatite

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THE BINDING OF SALIVARY PROTEINS AND ITS EFFECT ON THE DEMINERALIZATION OF HYDROXYAPATITE

By

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DEDICATIONS

To my late father, William G. Kells, and my mother, Marion M. Kells, for their support and encouragement throughout my life.

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ABSTRACT

Histatins, statherin and proline-rich proteins (PRPs) are important members of the human saliva proteome. They are components of the acquired enamel pellicle (AEP) which provides protection to the teeth and perform other vital functions in the oral cavity such as preventing spontaneous precipitation of calcium phosphate salts (statherin) and exhibiting fungicidal activity (histatins). The aims of the study were to (1) isolate histatins, statherin and acidic PRPs from parotid secretion, (2) develop a novel functional assay for the measurement of hydroxyapatite demineralization by modifying pre-existing methods and (3) test the binding of mixed PS proteins and purified salivary proteins (histatin 1, statherin and PRP1) to HA and the subsequent effect on demineralization.

The steps in the histatin, statherin and PRP purification process include zinc precipitation of parotid saliva, ion exchange chromatography, gel filtration chromatography and high performance liquid chromatography (HPLC). Protein purity was assessed by polyacrylamide gel electrophoresis (PAGE). The functional properties of the proteins were examined in two ways: Binding of mixed and purified proteins to ceramic hydroxyapatite (HA) powder was monitored by PAGE and HPLC, and demineralization of HA exposed to acidic conditions after incubation with mixed and purified proteins was determined in spectrophotometric assays for free calcium and phosphate.

Histatins and PRPs were successfully isolated to high levels of purity from human parotid saliva (HPS). Salivary protein binding experiments showed that the order of the three proteins studied in terms of their apparent affinity for HA is: statherin > histatin 1 > PRP1. The linearized Langmuir adsorption plots for histatin 1 and statherin fit well, showing R^2 values of 0.9880 and 0.9975, respectively. However, the adsorption isotherm for PRP1 did not exhibit linear characteristics (R^2 value of 0.61). Functional assays performed to measure the release of calcium and phosphate ions showed variable levels of protection afforded to HA by preincubation with the three proteins. On a molar basis, histatin 1 provided the most protection against demineralization. The *in vitro* data generated add to a more complete understanding of the functional characteristics of histatins, statherin and PRP1 and thereby provide insights into their potential capacity to protect enamel in the oral environment.

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List of Abbreviations

2-DE	2-Dimensional Electrophoresis
ABS	Ammonium Bicarbonate Solution
AEP	Acquired Enamel Pellicle
ATP	Adenosine triphosphate
BUGSDM	Boston University Goldman School of Dental Medicine
CHT	Ceramic Hydroxyapatite
CV	Column Volume
DEAE	Diethylaminoethanol
DI	Deionized (water)
ESRD	End Stage Renal Disease
FPLC	Fast Protein Liquid Chromatography
GCF	Gingival Crevicular Fluid
HA	Hydroxyapatite
HPLC	High Performance Liquid Chromatography
LA	Lactic Acid
LC-ESI	Liquid chromatography electrospray ionization
MALDI-TOF	Matrix-assisted laser desorption/ionization
MS	Mass Spectrometry
MWCO	Molecular Weight Cut Off
NADH	Nicotinamide adenine dinucleotide
OCPC	orthocresolphthalein complexone
PAGE	Polyacrylamide Gel Electrophoresis

PRP	Proline Rich Protein
PS	Parotid Secretion
RP-HPLC	Reversed Phase HPLC
SDS	Sodium Dodecyl Sulfate
SELDI-TOF	Surface enhanced laser desorption/ionization time-of-flight
SMSL	Submandibular / Sublingual Secretion
SN	Supernatant
SOP	Standard Operating Procedure
TFA	Trifluoroacetic Acid
UA	Uric Acid
WS	Whole Saliva

1. Introduction

1.1 Glandular Salivary Secretion

Saliva, which is 99% aqueous by volume (Helmerhorst et al., 2004), is secreted in the oral cavity by three major salivary glands and various minor salivary glands. The three major salivary glands, the parotid, the submandibular and the sublingual glands, exist in pairs. The parotid glands contribute about 50% of the total secretion when saliva flow is stimulated by mastication (Lendenmann et al., 2000). Whole saliva (WS) is primarily composed of glandular secretions. In addition it contains bacteria, leukocytes, sloughed epithelial cells and gingival crevicular fluid (GCF). GCF contributes markers to WS that are derived from the circulation. These markers make possible the diagnostic use of WS to detect HIV, periodontal disease, pancreatic cancer and acute myocardial infarction among other diseases (Thomadaki et al., 2011). Parotid and submandibular secretions are always supersaturated with respect to calcium and phosphate salts regardless of flow rate or diurnal variations. Calcium and phosphate are the major constituents of hydroxyapatite (HA) which makes up the structure of tooth enamel. This supersaturation of saliva with respect to these ions provides important protection for tooth surfaces. Except for the effects of dental caries, tooth enamel retains its integrity for many decades. Salivary supersaturation with respect to calcium and phosphate salts is responsible for this remarkable stability (Hay et al., 1979).

1.2 Functions

Being a complex fluid, saliva has a variety of functions, most of which are protective and are important for the maintenance of healthy oral tissues. Saliva facilitates the swallowing of food, the remineralization of teeth, the general lubrication of the tissues in the oral cavity and the inhibition of the growth of microorganisms. It lubricates oral mucosal tissues which facilitates speech and swallowing and attenuates mechanical, thermal and chemical irritation. Saliva serves as an ion reservoir, facilitating remineralization of tooth enamel. It acts as a buffer, neutralizing plaque pH after food intake thus reducing demineralization which occurs below pH 5.5. It acts as an antimicrobial agent, helping to control the growth of the oral microflora. Agglutinins, which include mucins and glycoproteins, in saliva aggregate bacteria facilitating their clearance from the oral cavity. A protective pellicle, known as the acquired enamel pellicle, is formed on tooth surfaces from salivary proteins. The digestion of food starts in the oral cavity with the breakdown of starch by the salivary enzyme α-amylase. Secretory Immunoglobulin A, sIgA, may participate in both adaptive immunity via its Fab regions which are involved in specific antigen recognition and in innate immunity via its glycosylated regions (Helmerhorst and Oppenheim, 2007). sIgA also aggregates *C. albicans* (yeast) cells and facilitates their removal from the oral cavity by swallowing. Other innate host defense factors exhibiting anti-bacterial and antifungal activities include lactoferrin, lysozyme, salivary peroxidase and the histatins (Helmerhorst et al., 2004). Finally, saliva facilitates the sense of taste by

acting as a solvent, bringing food constituents into intimate contact with the taste buds. The crucial role of saliva is perhaps best illustrated by the symptoms accompanying xerostomia (dry mouth syndrome), which results in a rapid deterioration of oral health and a dramatic reduction in the quality of life of the sufferer (Edgar et al., 2004).

1.3 Collection and Processing

Saliva can be collected under stimulated or unstimulated conditions. Whole saliva is collected by directing a subject to expectorate into a collection tube. Unstimulated and stimulated parotid secretion is collected via a Curby Cup securely placed over the Stensen's duct opening of the parotid glands. Stimulation is usually achieved by mastication of a parafilm bolus. The parotids are wedge shaped and are located in front of the ear and behind the ramus of the mandible. Submandibular/sublingual (SMSL) secretion is collected by means of an appliance (Truelove et al., 1967). The appliance fits over the SMSL glandular excretory ducts, also called Wharton's ducts, which are located on the floor of the mouth. Parotid secretion is usually stimulated by sweet and sour candy. Glandular secretions flow freely through tubes attached to the respective appliances. The preservation of WS is a challenge due to the complexity of its composition and high proteolytic activity. Centrifugation to separate the supernatant from the pellet and freezing of the supernatant -20° C as soon as

possible after collection is the best way to preserve intact salivary proteins. Lowering or increasing the pH or boiling also have a preservative effect on WS. Cooling on ice at 0°C only ensures protein stability for up to one hour. Compared to the drawing of blood, saliva collection is relatively easy and stress-free, making its diagnostic use highly desirable (Thomadaki et al., 2011).

1.4 Salivary Protein Separation

Early studies aimed at the separation of salivary proteins used paper electrophoresis. However, polyacrylamide gel electrophoresis (PAGE) with a stacking gel soon became the standard due to the superior resolution of proteins achieved via this method (Mandel and Ellison, 1965). Anionic PAGE, developed by Ornstein (Ornstein, 1964) and Davis (Davis, 1964), provides excellent separation of proteins by molecular size and by net negative charge. It is uniquely suited for the separation and visualization of acidic proline-rich proteins and their isoforms. Cationic PAGE on the other hand provides excellent separation of positively charged proteins such as salivary histatins (Baum et al., 1976, Oppenheim et al., 1986). Bis-Tris SDS PAGE is used to separate a broad range of proteins in their denatured state providing molecular weight determination since all proteins are rendered negative in charge by the addition of sodium dodecyl sulfate (Weber and Osborn, 1969). Larger amounts of protein can be separated by Fast Protein Liquid Chromatography (FPLC) or High

Performance Liquid Chromatography (HPLC). FPLC employs a number of different chromatographic techniques to achieve protein purification. These include size exclusion chromatography, also known as gel filtration, in which proteins are separated on the basis of their size, ion exchange chromatography, in which proteins are separated on the basis of their net charge and affinity chromatography which uses a stationary immobilized ligand that specifically binds the protein of interest. HPLC typically uses reverse phase / hydrophobic interaction columns that separate molecules on the basis of hydrophobic interactions between the sample and the stationary phase (Provost, 2012). HPLC with a GenPak FAX anion exchange column has been used to separate PRP isoforms and determine their concentrations in different glandular secretions (Hay et al., 1994).

1.5 Components of Glandular Secretions

The top five protein species in human saliva based on percentage of total protein are amylases, PRPs, cystatins, mucins and immunoglobulins. Statherin, carbonic anhydrase, histatins, lysozyme and serum albumin also have a significant presence. Serum proteins including albumin, α-, β- and γ-globulin constitute <5% of total salivary protein (Ruhl, 2012). Amylase begins the digestion of food and is the strongest antigen in saliva. Lysozyme is thought to play an important role in the defense against gram-positive oral microorganisms.

In parotid secretion, about 35% of total proteins are bound to carbohydrates (Mandel and Ellison, 1965). Salivary agglutinin is a high-molecular-weight, acidic, mucinlike glycoproteins found in parotid and submandibular secretion. Its primary function is to aggregate bacteria which facilitates its clearance out of the oral cavity by swallowing (Lamont et al., 1991). Potentially harmful microbes are subsequently destroyed in the acidic gastric environment (Ruhl, 2012). Submaxillary, previously called submandibular, secretion contains about half the total protein found in PS under unstimulated conditions. It has a much lower level of amylase, higher lysozyme activity and contains cystatins. Cystatins are cysteine containing phosphoproteins which are found in saliva, cerebro-spinal fluid, seminal fluid, serum and tears (Lamkin et al., 1991). Submandibular secretion is more difficult to collect than PS because a custom fitted device is required. With this device, submandibular and sublingual secretions are collected together, and are referred to as SMSL secretion. Most submandibular salivary proteins are anionic rather than cationic and contain a distinct group of glycoproteins. About 75% of the population demonstrates blood group substances in SMSL secretion which are represented in the glycoprotein portion. SMSL contains more glycoproteins than PS secretion. The level of lipoproteins (lipids) in WS is very low. Both PS and SMSL secretion contain a number of free amino acids (Mandel and Ellison, 1965).

Saliva contains serum-derived components resulting from passive diffusion via gingival crevices. Nitrite (NO_2^-) and uric acid (UA) build up in the

serum of patients with end stage renal disease (ESRD). Solution based colormetric detection chemistries for these analytes have been converted to a test strip format. The test strips were developed to noninvasively monitor dialysis progress using a patient's saliva. The net decreases in concentration observed for NO_2^- and UA from predialysis to postdialysis support their potential utility as markers for dialysis efficacy (Blicharz et al., 2008).

Low molecular weight salivary proteins include histatins, statherin and acidic, basic and glycosylated proline-rich proteins (PRPs). Acidic PRPs are found both in PS and SMSL and are rich in glycine, proline and glutamic acid (Kousvelari et al., 1982; Oppenheim et al., 1971). Cystatins and mucins are only present in SMSL secretion (Yao et al., 2003). Mucin and agglutinin are two of the largest proteins found in WS. Statherin and the acidic PRPs are especially important in maintaining the mineralization of tooth enamel.

1.6 Acidic PRPs

One of the unusual properties of human saliva is its high content of proline-rich proteins (PRPs). The acidic PRPs are effective inhibitors of crystal growth, but unlike statherin, do not inhibit spontaneous precipitation. There are six members of the acidic PRP family: three 150 residue proteins, PRP-1, PIF-s and PRP-2 and three 106 residue proteins, PRP-3, PIF-F and PRP-4 (Hay et al., 1988). Tryptic digestion of PRPs 1 - 4 yields a single active N-terminal peptide

from each. The N-terminal region is strongly anionic with nearly half the residues being aspartate, glutamate and phosphoserine. The N-terminal regions of the six PRPs are nearly identical. The differences occur at residues 4 and 50: PRPs 1 and 3 have Asp and Asn, PRPs 2 and 4 have Asp and Asp and PIF-s and PIF-f have Asn and Asp in those two positions, respectively (Hay et al., 1988). The mechanism behind crystal growth inhibition is thought to be adsorption of the inhibitory proteins onto the enamel surface where they block crystal growth sites and prevent mineral deposition (Hay et al., 1979). PRPs are divided into three groups: acidic, basic and glycosylated accounting, in total, for 10 - 40% of the total protein in human glandular secretion. Almost all of the phosphopeptides in WS arise from acidic PRPs. Other salivary proteins like statherin and cystatin are only a minor source of phosphoproteins (Minaguchi et al., 1988). PRPs provide a protective and reparative environment for tooth enamel and are selective mediators of bacterial adhesion to HA (Moreno and Zahradnik, 1979).

Fig. 1: The complete primary structure of PRP1 (Hay et al., 1988).

Table 1: Summary of the structural differences in the six PRPs*

	Position 4	Position 50	Total number of residues
PRP1	Asp	Asn	150
PIF-s	Asn	Asp	150
PRP2	Asp	Asp	150
PRP3	Asp	Asn	106
PIF-f	Asn	Asp	106
PRP4	Asp	Asp	106

* Hay et al., 1988

In early studies, four acidic PRPs were isolated from parotid saliva (PS) that were not detected on gel patterns obtained from WS. The proteins were purified via gel filtration chromatography (G-75 column) and ion-exchange chromatography (DEAE-Sephadex A-25 Column). The purity of the fractions was confirmed by Ornstein-Davis disc gel electrophoresis. Amino acid analysis revealed the proteins to contain: 22 – 27% proline, 20 – 22% glycine and 26 – 36% glutamate and aspartate. Sulfur containing amino acids and tyrosine were not found. The isoelectric points for the PRPs were between 4.09 and 4.71. The studies also indicated a high affinity for hydroxyapatite (Oppenheim et al., 1971).

Phosphoserine is necessary for the maximal adsorption of acidic PRPs to hydroxyapatite (HA). Acidic PRPs account for 42% of all proteins adsorbed to HA in vitro. After digesting PRPs with phosphatase, ~60% of covalently bound phosphate was removed. Experiments showed decreased binding of protein to HA after treatment with phosphatase indicating that the phosphoserine residues are necessary for optimal binding (Bennick et al., 1979). The HA binding site is located in the protein's N-terminal tryptic peptide. Trypsin digestions showed no change in the amount of valine or isoleucine (which are located in the N-terminal region) but a marked decrease in the amounts of other amino acids. These experiments showed that the C-terminal part was removed and that the N-terminal peptide remained bound to HA (Bennick et al., 1979).

PRPs are encoded by two genes in the mammalian genome, designated *PRH1* and *PRH2*. The PRH1 and PRH2 genes are closely related and differ by

only 8.7% (Azen et al., 1987). The primary products of the *PRH1* gene are the proteins Db-s, PIF-s and Pa. The primary products of the *PRH2* gene are PRP1 and PRP-2. Db-f results from cleavage of Db-s between residues 127 and 128. Cleavage of PIF-s, PRP-1 and PRP-2 between residues 106 and 107 results in PIF-f, PRP-3 and PRP-4. Total PIF-s production is lower than total PRP-1 production, indicating that the *PRH2* gene has a higher rate of transcription than the *PRH1* gene (Hay et al., 1994). Differences were found in the ability of PRP isoforms to inhibit calcium phosphate precipitation and to promote bacterial adhesion to HA. Bacteria were found to adhere much more strongly to PIF-s and PRP-1 than to the derivative peptides PIF-f and PRP-3. This is due to the presence of the pro-gln C-terminal dipeptide which is present in the 150 residue isoforms but absent in the truncated 106 residue PRPs. The relative production and post-translational cleavage of PRPs are constant in an individual's parotid and submandibular saliva samples obtained over several weeks. However, substantial inter-gland differences and variation between individuals were apparent. These variations could be significant in the resulting biological activity of the PRPs. Variations in protein degradation between subjects are thought to be due to individual differences in the degree of proteolytic activity in the parotid glands or variations in the time saliva is retained in the duct system after secretion (Azen, 1973).

The two protein pairs, PIF-s, PRP-1 and PIF-f, PRP-3 are difficult to separate by PAGE due to their identical charge. Anion exchange

chromatography using a very shallow chloride gradient was used to separate the PIF-s, PIF-f, PRP1 and PRP3 isoforms. The Asp - Asn substitution at position 4 and not the Asn – Asp substitution in position 50 is thought to account for the separation of PIF-s and PRP1 due to steric differences. Pa dimer consists of two identical 150 amino acid peptides with a disulfide bond connecting two cysteine residues at position 103. Pa dimer is heavily retarded during anion exchange chromatography because of its two negatively charged N-terminal regions. PRP 1 – 4 contain negatively charged Asp in position 4 and are also strongly retarded. Db-s, PIF-s, Pa, Db-f and PIF-f contain Asn in position 4 which results in a net charge that is less negative than PRP 1 – 4. Therefore they are the least retarded (Hay et al., 1994). Pa appears as a single band in IEF PAGE. However, the PRPs are partially cleaved at Arg 106 possibly by kallikrein in the salivary glands which results in a double-banded phenotype. One band of the pair represents intact PRP and the other band represents the larger of the cleaved fragments. Db-s and PIF-s refer to the uncleaved proteins while Db-f and PIF-f represent the larger of the cleavage products (Azen et al., 1987).

1.7 Statherin

Statherin is a 43 residue, tyrosine-rich acidic peptide with an isoelectric point of 4.2 and a molecular weight of 5380 Daltons. The complete amino acid sequence of statherin was determined by Edman degradation after digesting the

peptide separately with trypsin, chymotrypsin and *Staphylococcus aureus* protease. The resulting peptides were purified by gel filtration and the two serine residues at positions 2 and 3 were found to be phosphorylated. The supersaturation of saliva with respect to calcium salts is important for stabilizing, protecting and recalcifying enamel that has been demineralized by bacterial acids. Proteins (PRPs and statherin) in human saliva inhibit precipitation of supersaturated calcium phosphate solutions. Statherin is an unusually potent inhibitor of calcium phosphate precipitation and is regarded as a precursor of the acquired enamel pellicle (AEP) (Schlesinger and Hay, 1977). The probable mechanism for the inhibition of calcium phosphate precipitation involves the inhibitor (statherin) binding to newly formed aggregates of calcium and phosphate ions, thereby blocking their growth. At a concentration of 0.2 μM, statherin delays precipitation from a supersaturated solution for several hours. Statherin contains twelve charged amino acids, ten of which are located within the 13 N-terminal residues. All of the tyrosine, proline and glutamine are found in the C-terminal two-thirds of the peptide, where they form 75% of the residues. The highly charged N-terminal ⅓ of statherin, which includes the phosphoserine residues, plays a particularly important role in binding the peptide to HA and in the subsequent inhibition of calcium phosphate precipitation. Removal of phosphate from the phosphoserines reduced activity to less than 2% of the activity of the phosphorylated tryptic fragment (Hay et al., 1979). Statherin has a

high degree of negative charge in its N-terminus and its hydrophobic C-terminus and hydrophilic N-terminus give it striking structural asymmetry.

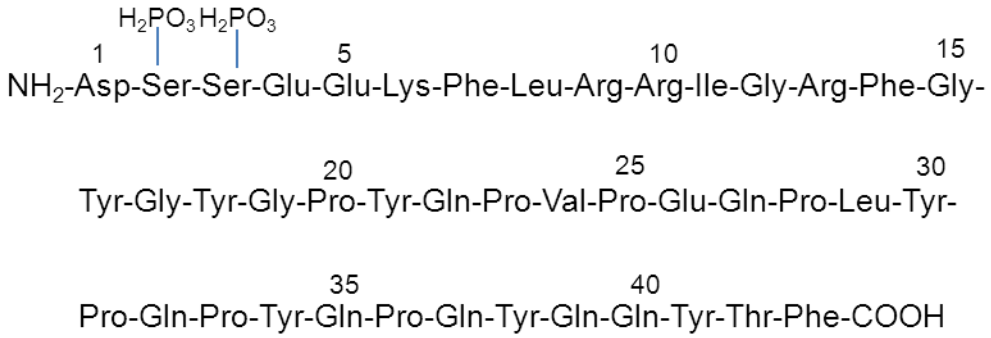

Fig. 2: The complete amino acid sequence of statherin (Schlesinger and Hay, 1977).

In a study by Tamaki and coworkers, the relationship between the concentration of salivary protein and the inhibition of calcium phosphate precipitation was found to be linear. Statherin was 14- and 66-fold more inhibitory than histatin-1 and WS respectively. Statherin was found to be the most potent salivary protein in inhibiting both primary and secondary precipitation of calcium phosphate while histatin-1 was the least inhibitory. Statherin and small PRPs adsorb to HA rapidly while large PRPs and histatins take considerably longer. Only statherin is considered to be an effective inhibitor of spontaneous precipitation at physiological concentrations (Tamaki et al., 2002).

Human statherin has been shown to inhibit primary (spontaneous) and secondary (seeded) precipitation of calcium phosphate salts. The highly negatively charged N-terminal region is important for the inhibition of both primary and secondary precipitation. The tyrosine-proline-glutamine rich C-terminal region plays a critical role in the inhibition of primary precipitation. Maximal inhibition of primary precipitation requires the entire molecule. Molecules containing multiple anionic groups, ie. phosphate, are effective inhibitors of both types of precipitation. Digestion of statherin with caboxypeptidase-A removed approximately ten residues proximal to the C-terminus and a large decrease in primary precipitation activity was observed in the truncated molecule (Schwartz et al., 1992). The biological function of statherin, concomitant with the PRPs is to inhibit potentially harmful calcium phosphate precipitation in the salivary glands and the mouth (primary precipitation) and onto tooth surfaces (secondary precipitation). Statherin levels in stimulated PS are more than sufficient to carry out its proposed function (Hay et al., 1984). However, it should be pointed out that the levels in WS are significantly diminished due to proteolytic fragmentation (Li et al., 2003, Helmerhorst et al., 2010).

1.8 Histatins

Histatins 1, 3 and 5 are members of a histidine rich protein family found in human salivary secretion. Histatins are pellicle precursor proteins that have low molecular weight, have cationic characteristics, and exhibit antibacterial and antifungal properties. They inhibit the growth of certain oral bacteria such as streptococci and fungi such as C. albicans. Histatins sequestered on the enamel surface may form a reservoir that serves diverse functional roles including the killing of yeast and bacteria cells, inhibiting enzyme function and binding to other salivary components. Of all the histatins, only histatin 1 possesses an N-terminal phosphoserine residue which greatly increases its binding affinity for HA (Fig. 3) (Yin et al., 2003).

While histatin 1's single phosphoserine residue increases it's affinity for HA compared to the non-phosphorylated histatin members, its overall affinity for HA is less than that of the PRPs or statherin. Histatin 5 has a relatively high positive charge due to the presence of seven histidine, three lysine and three arginine residues (Yin et al., 2006). The adsorption of a protein with low affinity for HA, ie. histatin 5, can be enhanced by binding to other proteins that are strongly bound to the enamel surface. Histatins kill various fungi, including *C. albicans* which is the most prevalent fungus in the oral cavity. *C. albicans* killing cannot be detected in PS, SMSL or WS. However, the fungicidal activity of PS is unmasked upon dialysis with MWCO ≥ 1000 Da tubing. Histatins were retained in samples dialyzed with 10,000 and 25,000 MWCO tubing even though their MW is <5000

Da. This is due to the fact that histatins, as well as statherin, form complexes with mucins (Iontcheva et al., 2000). Therefore, they can still be responsible for the antifungal activities in PS after dialysis.

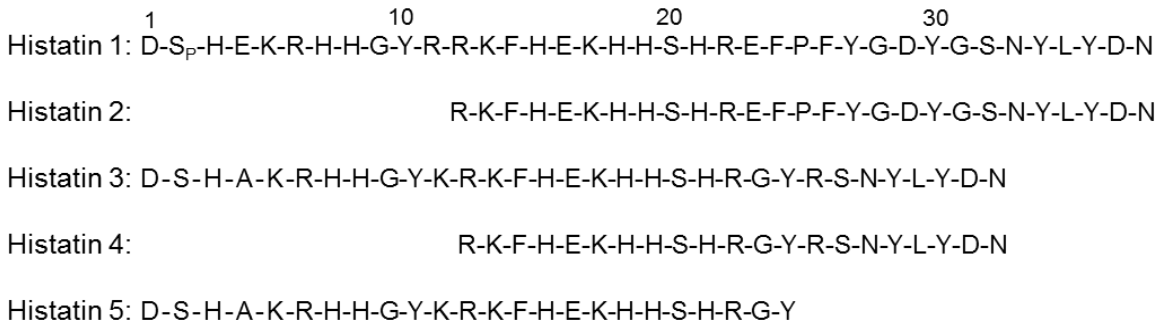

Fig. 3: Amino acid sequences of salivary histatins 1 – 5. S_p represents phosphoserine (Troxler et al., 1990).

1.9 Hydroxyapatite (HA)

HA, $Ca_5(PO_4)_3OH$, is the least soluble calcium phosphate compound found in nature and in the body. Tooth enamel is impure HA with ions other than Ca^{2+} and $(PO_4)^{3-}$ incorporated in the crystal lattice. Carbonate and fluoride are common impurities. The former makes HA more soluble and the presence of the latter results in a less soluble HA (Edgar et al., 2004). Each HA unit cell contains ten calcium ions, six phosphate groups and two hydroxyl groups, resulting in the formula: $Ca_{10}(PO_4)_6(OH)_2$. Two hydroxyl groups are stacked at z = ¼ and z = ¾ in each corner of the unit cell. Each hydroxyl group is surrounded by a triangle of

three Ca^{2+} ions. Each calcium triangle is rotated with respect to its partner triangle by 120°. Each calcium triangle is surrounded by triangles containing three phosphate $(PO_4)^{3-}$ groups. The phosphate triangles are further surrounded by a ring of six calcium ions. The phosphate triangles act as a bridge between the inner Ca^{2+} triangles and the outer Ca^{2+} ring (Kay et al., 1964). The above described arrangement of ions occurs in each corner of the unit cell parallelogram. In an analysis of the interaction of salivary proteins with HA (Hay, 1973), experiments using dental enamel powder and experiments using synthetic HA produced essentially similar results. Allowances were made due to variations in surface area. The smaller particle size and greater surface area of dental enamel powder may be due to the presence of impurities such as carbonate and fluoride in the unit cells.

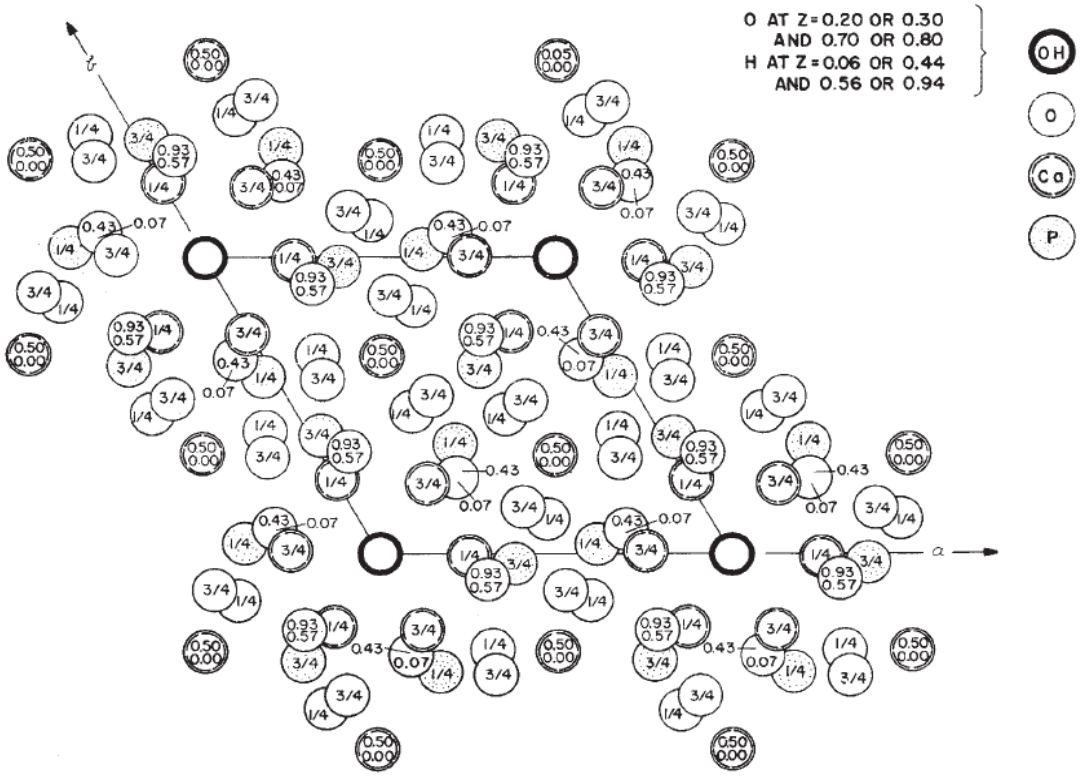

Fig. 4: HA structure projected on an x,y plane (Kay et al., 1964).

1.10 Acquired Enamel Pellicle (AEP)

The AEP is a thin layer of organic material that forms on the enamel tooth surface. It's functions include the inhibition of calcium phosphate crystallization, regulation of ion transport and a deterrent to microbial growth. Certain salivary proteins are regarded as pellicle precursors due to their affinity for HA. Amino acid analysis and Western blots of *in vivo* formed pellicle have shown the presence of many proteins including albumin, amylase, cystatins, PRPs, statherin, lysozyme and histatins. In addition to electrostatic interactions, protein

adsorption to enamel is often driven by an increase in entropy. The adsorption behavior of a single salivary protein can differ substantially from that obtained when that protein is part of a mixture of proteins (Yin et al., 2006). High-resolution gel filtration chromatography experiments have revealed that small proteins and peptides with MW<10 kDa constitute approximately 30% of total AEP material (Siqueira and Oppenheim, 2009).

The AEP covers the enamel and is not removed by tooth brushing and is only partially removed by prophylaxis. It consists of salivary proteins and lipids that form immediately when a freshly cleaned tooth surface contacts saliva. The pellicle protects the enamel from mechanical and chemical damage and delays the onset of caries and demineralization when the oral environment becomes acidic. Plaque is composed of bacteria in a polysaccharide matrix and forms a second layer between the tooth surface and the oral environment. The AEP retards the diffusion of acids formed in the dental plaque and regulates dissolution and precipitation processes. A trend towards greater plaque accumulation, expressed in terms of the number of *Streptococcus mutans* cells per unit surface area, on enamel surfaces with AEP was observed. Pellicle protection against demineralization, however, appears to be unrelated to the number of acid-producing bacteria present on tooth surfaces *in vitro* (Zahradnik et al., 1978).

1.11 Oral Bacteria and Acid Production

Glycolysis is the central ATP-producing pathway used by anaerobic bacteria such as *S. mutans* which have a dominant presence in dental plaque. From one glucose molecule the following species are generated: two molecules of pyruvate, two molecules of ATP and two molecules of NADH. In anaerobic organisms, pyruvate is either converted into lactate or ethanol and CO_2 by fermentation. NADH gives up it's electrons and is converted back into NAD^+ which is required to maintain the reactions of glycolysis (Alberts et al., 2008). Excreted lactate acquires a proton in the weakly acidic oral environment and reaches the following equilibrium (Zumdahl and Zumdahl, 2006):

$$CH_3CH(OH)COOH_{(aq)} \leftrightarrow H^+_{(aq)} + CH_3CH(OH)COO^-_{(aq)}$$

(where $CH_3CH(OH)COOH$ is lactic acid and $CH_3CH(OH)COO^-$ is lactate).

The oral environment is ideal for bacterial growth due to its humidity, temperature, large surface area for attachment and the presence of nutrients. A proper amount of saliva, with its anti-microbial agents, is necessary to maintain a balance between host defense and microbial attack. *S. mutans* and lactobacilli are commonly present in the oral cavity and are known as lactic acid producers because they convert lactose and other sugars into lactic acid. The Stephan Curve (Fig. 5) shows the change in plaque pH in response to an oral rinse with a glucose solution. The lactic acid producing bacteria in the plaque rapidly metabolize sugar to lactic acid by fermentation which accounts for the initial dip

in the curve. The rise in plaque pH is due to the diffusion of acids out of the plaque and into the saliva. The presence of acid neutralizing ammonia in saliva is due to the breakdown of urea and free amino acids.

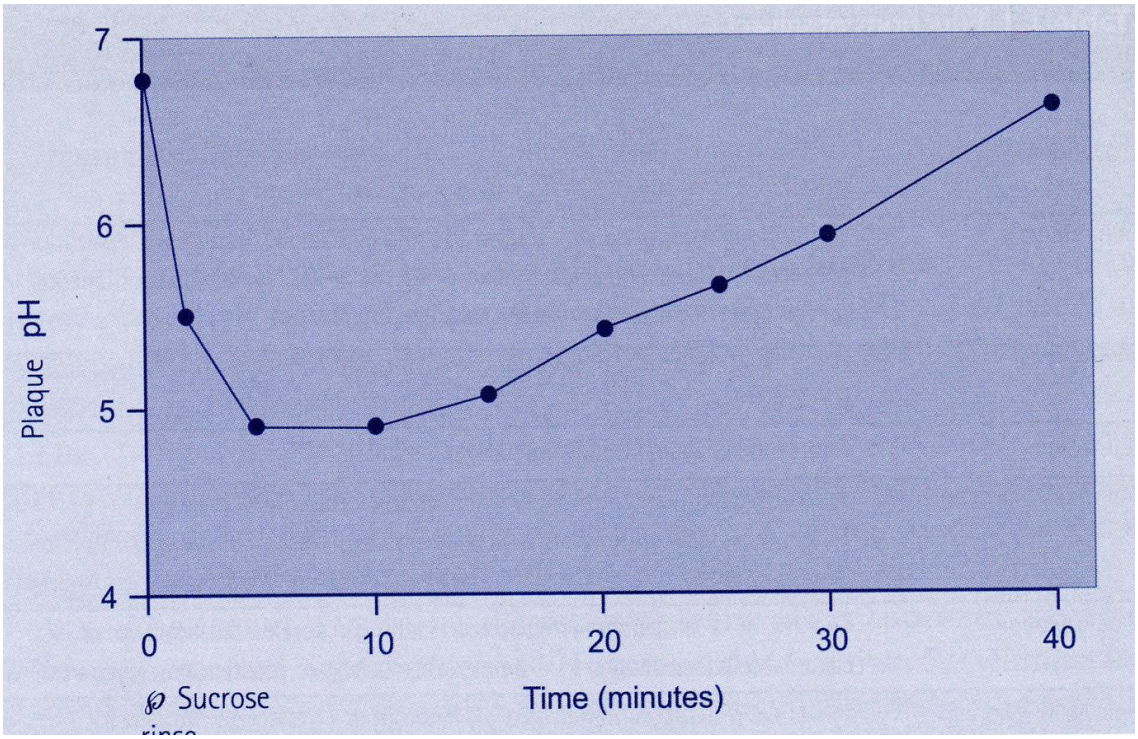

Fig. 5: Stephan Curve showing the changes in plaque pH over time following a sucrose rinse, (Edgar et al., 2004).

1.12 Proteomics

In a recent study, three research groups worked in concert to produce a catalog of the human salivary proteome with maximal coverage (Denny et al.,

2008). The objective was to improve coverage of the salivary proteome and access low-copy proteins using mass spectrometric methods. The research teams produced 11592 distinct peptide sequences that matched 2153 protein accession numbers. 57% of the proteins were shared between PS and SMSL. Well known salivary proteins such as α-amylase, cystatin, histatin, PRPs and mucins were well represented in the core proteome. The molecular weight distribution of the proteins in the core proteome is as follows: 46.6% ≤ 40 kDa, 44.6% 40 – 120 kDa and 8.7% ≥ 120 kDa. As of 2012, the proteome of human saliva has been essentially completed (Ruhl, 2012).

Human saliva is desirable for use as a diagnostic fluid due to its advantages compared to other body fluids such as blood or urine. Interest in saliva as a diagnostic fluid has increased recently along with advancements in proteomic analysis including surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), a high throughput, sensitive technique which has revealed a large number of components that are present in low concentrations and could serve as biomarkers for disease. SELDI-TOF-MS studies on WS show most peaks in the low molecular weight (MW) range indicating an abundance of peptides between 1-6 kDa. A comparison of SELDI-TOF-MS spectra reveals that a majority of WS protein originate in the parotid gland. However, high molecular weight proteins and glycoproteins such as mucins were not detected by this method (Schipper et al., 2007).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has also been a valuable tool for the cataloguing of bacterial proteomes, the identification of differences in protein expression in diseased vs. normal cells and the characterization of post-translational protein modification. Proteins found in AEP and WS were identified for the first time using MS (Yao et al., 2003). 2-D PAGE using isoelectric focusing with pH ranges of 3 – 10 and 5 – 8 in the horizontal dimension and SDS-PAGE (10%) in the vertical dimension was used to compare proteins in WS and AEP and PS and SMSL. Significant differences in the protein patterns for glandular secretions and WS were observed. The electrophoretogram for WS contains many more protein spots than in PS or SMSL. The WS spots are also distributed more evenly throughout the pH range 5-8. This indicates that WS contains a mixture of proteins both derived from different sources and having undergone significant modification upon entering the oral cavity.

2-DE allows for the separation of not only different molecules with similar masses by also different isoforms of the same molecule. MS allows for the positive identification of proteins and peptides. Calgranulin and cytokeratin family members were identified as novel AEP constituents (Yao et al., 2003). These proteins were identified by MS and are derived from non-salivary glandular sources. Calgranulin B was identified in WS and AEP. Advances in proteomics have increased the number of proteins identified and raised the level of sophistication and sensitivity of the analysis of biological materials.

Enzymatic fragmentation of proteins and peptides having MW > 5000 Da is required prior to MS analysis due to the limitations of the instrument. In an investigation of the human AEP proteome, 78 peptides originating from 29 different proteins with a MW range of 767 – 3981 Da were identified via liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) without prior proteolytic treatment (Siqueira and Oppenheim, 2009). By avoiding enzymatic fragmentation (trypsination), information on naturally occurring salivary peptides in the AEP can be obtained (Siqueira et al., 2007).

1.13 Langmuir Adsorption Equation

The Langmuir equation relates the adsorption of molecules on a solid surface to the concentration of the molecules in a medium above the surface at a constant temperature. It is used to describe the following adsorption equilibrium:

$$S + P = SP$$

where S represents empty surface binding sites, P represents protein molecules in solution and SP represents occupied binding sites (Milan et al., 2006). The Langmuir isotherm is based on three assumptions: 1) Adsorption cannot proceed beyond monolayer coverage, 2) All sites are equivalent and the surface is uniform and 3) The ability of a molecule to adsorb at a given site is independent of the occupation of neighboring sites (Atkins et al., 2002).

The Langmuir adsorption equation is used in this study to analyze the adsorption of salivary proteins to HA. The experimental adsorption data should satisfy the following equation:

$$Q = \frac{KNC}{1 + KC}$$

where Q is the moles of protein adsorbed per unit surface area (m^2) of HA, C is the equilibrium concentration (mol/L) of protein remaining in solution after equilibration, N is the maximum number of binding sites available to the protein (per unit surface area) and K is the equilibrium constant which reflects the affinity of an adsorbate for an adsorbent or, in this case, the affinity of the protein for HA. This equation can be rewritten in the form of a linear equation ($y = mx + b$):

$$\frac{C}{Q} = \frac{C}{N} + \frac{1}{NK}$$

where $\frac{1}{N}$ is the slope of the linear plot of $\frac{C}{Q}$ vs C and $\frac{1}{NK}$ is the y-axis intercept. From the slope and y-axis intercept, the values of K and N can be obtained. If the data fit reasonably well to the Langmuir model, the resulting adsorption isotherms are characterized by a steep increase followed by a leveling off which indicates saturation of the available binding sites on the surface. In the linearized adsorption plot, a correlation coefficient ≥ 0.9 and a positive y-axis intercept are required for the data to demonstrate Langmuir type adsorption (Milan et al., 2006).

2. Aims of the Study

1) To isolate histatins, statherin and acidic PRPs from parotid secretion.

2) To develop a novel functional assay for the measurement of hydroxyapatite demineralization by modifying pre-existing methods.

3) To test the binding of mixed PS proteins and purified salivary proteins (histatin 1, statherin and PRP1) to HA and the subsequent effect on demineralization.

3. Materials and Methods

All studies involving human subjects were approved by the Institutional Review Board at Boston University (protocol # H-23046, P.I. Dr. Frank G. Oppenheim).

3.1. Parotid Saliva Collection

PS was collected from healthy volunteers via a Curby Cup positioned over the orifice of the Stensen's duct in the oral cavity. The Curby Cup was securely positioned and sealed by negative pressure to avoid contamination with WS. PS flow was stimulated by use of the gustatory method employing Life Savers or similar candy. PS was collected in 50 ml conical tubes which were kept on ice.

3.2. Zinc Precipitation of PS to obtain Histatins and Statherin

The PS samples (total volume approximately 300 ml) were pooled in a beaker placed in a bucket containing crushed ice. For 80 ml of HPS, 5.5 ml of 10 mM $ZnCl_2$ and 350 µl 2M NaOH solution was added for a final $ZnCl_2$ concentration of 650 µM and a solution pH of 9.0). After raising the pH the sample solution became cloudy. After incubation on ice for one hour, the suspension was transferred to centrifuge tubes to collect the precipitate. The

tubes were balanced and centrifuged using an RC-6+ Centrifuge (Thermo Fisher, Waltham, MA, USA) and SS-34 Rotor (Sorvall / Kendro, Newtown, CT, USA) for 20 min at 20,000 RPM and 4º C. The supernatant was carefully removed from each tube using a serological pipet, pooled in a beaker, covered with aluminum foil and placed in a refrigerator for no more than one hour while preparing for dialysis (see Section 3.3.). The PS supernatant contains the proline rich proteins (PRPs) and the zinc pellet contains histatins and statherin (Flora et al., 2001).

3.3. PS Supernatant Dialysis and Lyophilization

1000 Da MWCO Dialysis Membrane Tubes (Spectrum Labs, Rancho Dominquez, CA) were used to dialyze the PS supernatant obtained after zinc precipitation. The membrane was rinsed three times before adding the supernatant, not exceeding 150 ml per membrane section. The filled membranes were sealed with tubing clips and placed in a large bucket containing 12 liters of cold, deionized (DI) water and a magnetic stirrer. The bucket was placed on a stir plate in the cold room. The bucket was observed to make certain that the stir bar was moving but not hitting the membranes. Dialysis was performed in three four hour cycles. The DI water in the bucket was changed after the first and second cycle. After dialysis, the membrane contents were transferred to lyophilization flasks, shell frozen and lyophilized. The desalted, lyophilized PS supernatant protein devoid of statherin and histatins was transferred to a labeled bottle and

stored in a -20º C. Freezer. See SOP: “Virtis FM25EL Lyophilizer, serial # 217622”, for more information on lyophilizer methodology (Appendix 6.1).

3.4. DEAE anion-exchange chromatography

A portion of the PS supernatant proteins was weighed out and dissolved in DEAE anion-exchange buffer A (50 mM TrisHCl, 50 mM NaCl, pH 8.0) to a final concentration of approximately 10 mg/ml. The solution was centrifuged for 20 min. at approximately 30,000 g using a SS-34 Rotor and the RC-6+ Centrifuge (ThermoFisher). The supernatant was removed using a pipet and the small pellet comprising insoluble minor protein components was discarded.

Aliquots of 25 ml containing 250 mg protein were applied to a DEAE Sepharose anion exchange column (40 X 2.6 cm, column volume = 186 ml) using the AKTA FPLC System (GE Healthcare, Piscataway, NJ). Before starting the actual run, buffer A was allowed to flow through the column for one hour to ensure UV baseline stability. The flow rate was 1.0 ml/min. The buffers used for anion exchange chromatography were: Buffer A: 50 mM TrisHCl, 50 mM NaCl, pH 8.0; Buffer B: 50 mM TrisHCl, 1 M NaCl, pH 8.0. The following gradient was used: 100% buffer A for 465 ml, ramp to 100% buffer B in 93 ml, 100% buffer B for 233 ml and step to 100% buffer A for 279 ml.

The first DEAE peak, which contained the void proteins, eluted between 60 and 250 ml. The fractions from several runs of the same starting solution were

combined in a flask and lyophilized. The lyophilized sample was dissolved in water, transferred to a 50 ml conical tube (labeled “VOID”) and stored in a -20º C. Freezer. The second DEAE peak, eluting between 8.2% and 35% Buffer B or 480 and 550 ml, from several runs was also combined and lyophilized. The lyophilized sample was dissolved in water, transferred to a 50 ml Conical Tube (labeled “DEAE”) and stored in a -20º freezer.

3.5. Gel filtration chromatography

Proteins in the samples labeled “Void” and “DEAE” were desalted on the G-25 Column using the Pharmacia FPLC system (Pharmacia, Uppsala, Sweden). Prior to this, the DEAE column was disconnected and sealed. The G-25 Column (100 X 2.6 cm) was connected and equilibrated with water and 25 ml sample aliquots were subsequently applied. They were run through the G-25 column using DI water as the mobile phase. The first peak, eluting between 170 - 230 ml, contained the acidic PRPs and was transferred to a flask and lyophilized. The lyophilized sample was fluffy in appearance and transferred to a 50 ml conical tube labeled “DEAE for MONO Q Column”.

3.6 Mono Q anion-exchange chromatography

Lyophilized DEAE protein desalted with the G-25 column was dissolved in Buffer A (50 mM TrisHCl, 50 mM NaCl, pH 8.0) to produce a solution with a concentration of 10 mg/ml. The solution was centrifuged for 15 min at 20,000 RPM (31,210 g) and the small pellet was discarded. The supernatant was transferred in 5 ml aliquots to 15 ml conical tubes labeled “DEAE for MONO Q”. The aliquots were run sequentially on the MONO Q HR 16/10 column (CV = 20 ml) in conjunction with an AKTA FPLC. Samples were eluted at 1.0 ml/min using the following gradient: hold at 0% buffer B for 100 ml, ramp from 0% to 14% buffer B in 100 ml, ramp from 14% to 15.5% buffer B in 800 ml, ramp from 15.5% to 22% buffer B in 180 ml and ramp from 22% to 100% buffer B in 40 ml. Buffer B consists of 50 mM TrisHCl, 1 M NaCl, pH 8.0. The resulting chromatogram showed at least eight peaks. Each peak contained one or more PRP proteins. Peaks 1 – 8 were transferred into separate lyophilization flasks and lyophilized. The lyophilized samples were dissolved separately in DI water and transferred to labeled 50 ml conical tubes and stored in a -20º C freezer. After Mono Q chromatography the samples were desalted by elution through the G-25 Sephadex Medium gel filtration column as indicated above. The first peak (containing the PRPs) was collected and lyophilized. The resulting lyophilized protein samples were light and fluffy. They were transferred to labeled tubes and stored at -20º C. See the SOP: “Pharmacia FPLC” and “FPLC AKTA PURIFIER 10” for more information (Appendix 6.2).

3.7. PAGE Technical Aspects

The proteins isolated by zinc precipitation and DEAE/gel filtration chromatography were investigated for purity using cationic and anionic PAGE. Prior to pouring a gel, the gaskets were placed in a beaker of water to soak. The glass plates were washed prior to use (short plates and 1.5 mm spacer plates). The short plates were marked at the level to which the separating gel solution should be added (1.0 cm below the comb). The glass plates were placed in the casting stand. Water was added between the glass plates and a timer was set a timer for 20 min. After 20 min. the glass plates were checked for leakage. The separating gel solution was degassed by aspiration and a 10 ml serological pipet was used to add it to the space between the glass plates. The separating gel was overlaid with a layer of butanol about 5 mm in height. The separating gel was allowed to polymerize for 45 min.

After 45 minutes the butanol was removed, the gel was rinsed with DI water and blotted dry. The stacking gel solution was added on top of the separating gel. A comb was inserted and a lamp was placed in front of the gel. Light catalyzes polymerization of the stacking gel which takes about 30 min to polymerize. While the stacking gel was polymerizing, the sample to be loaded on the gel was prepared. After 30 min had expired, the light was turned off. The comb was carefully removed and the gels were transferred to a gel box. The appropriate running buffer was added to the middle chamber of the gel box to the top of the glass plates and were checked for leakage after 10 min. If there was

any leakage, the glass plates were removed, checked and put back in the gel box, making sure they were properly sealed against the rubber gaskets. The space in the gel box outside of the glass plates was filled with running buffer to one inch above the bottom of the glass plates. The samples were loaded with a gel loading pipette. The maximum volume loaded per lane was 40 µl. The lid was placed on the gel box which was connected to a power supply. A constant voltage of 120 V was applied. Electrophoresis took approximately 1.5 hours using the BioRad Mini Protean II with a BioRad Power Supply (Bio-Rad Laboratories, Hercules, CA).

3.8. Cationic PAGE

Cationic PAGE was conducted as described previously (Baum et al., 1976; Oppenheim et al., 1986). This type of electrophoresis provides excellent separation of histatins in their native state, based on their positive charge and size.

The concentration of the ingredients in the separating gel solution, pH=2.74, are as follows: 14.6% acrylamide, 0.78% bis-acrylamide, 0.125 M KOH, 8.0 mM TEMED, 1.64 mM ammonium persulfate, 13 µM riboflavin 5-phosphate, 4.4 M acetic acid.

The concentration of the ingredients in the stacking gel solution, pH=5.9, are as follows: 2.5% acrylamide ,0.625% bis-acrylamide, 60 mM KOH, 8.3 mM

TEMED, 19.4 µM riboflavin 5-phosphate and 0.584 M sucrose. The cationic PAGE sample buffer (1X) contains 1.17 M sucrose and 1.22 mM methyl green. The upper (anode) running buffer (1X) contains 0.37 M glycine. The pH is adjusted to 4.0 with glacial acetic acid. The lower cathode running buffer (1X) contains 0.72 M glacial acetic acid. The pH was adjusted to 4.3 with 45% KOH.

3.9. Ornstein / Davis Polyacrylamide Gel Electrophoresis (Anionic PAGE)

The method for anionic PAGE is based on two articles (Ornstein, 1964 and Davis, 1964) and is summarized in a doctoral dissertation (Oppenheim, 1973). Anionic PAGE electrophoresis provides excellent separation of proteins by molecular weight and by negative charge. It is uniquely suited for the separation and visualization of acidic proline-rich proteins.

The concentrations of the ingredients in the separating gel solution, pH = 8.9, are as follows: 375 mM Tris Base, 1.917 mM TEMED, 7.3% acrylamide, 0.195% bis-acrylamide, 3.067 mM ammonium persulfate. The concentrations of the ingredients in the stacking gel solution, pH = 6.7, are as follows: 83 mM Tris Base 2.56 mM TEMED, 3.33% acrylamide, 0.833% bis-acrylamide, 8.86 µM riboflavin, 779 mM sucrose.The concentrations of the ingredients in the sample buffer are: 60 mM Tris base, 7.7 mM TEMED, 0.56 M sucrose, 12 mM bromphenol blue. The concentrations of the ingredients in the running buffer, pH

= 8.3, are as follows (10X solution): 0.25 M Tris Base, 1.92 M glycine. The solution was diluted 1:10 with DI water before use.

3.10. Hydroxyapatite (HA) binding assay

Aliquots of 1.0 mg HA powder (Bio-Rad Macro-Prep Ceramic HA Type I 40 µm) were weighed into fifteen 1.5 ml centrifuge tubes. To the HA samples 1.0 ml of histatin 1, statherin or PRP1 solutions were added, the concentrations are indicated in Table 1. Each concentration indicated was added to the HA powder in triplicate. Samples were incubated in an orbital hybridization incubator mixer for one hour. The samples were removed from the incubator and centrifuged at 21,130 x g for 1 min (Eppendorf Centrifuge model 5424). A 0.75 ml aliquot of the supernatant was removed without disturbing the pellet at the bottom of the tube and transferred to another tube. From this aliquot, 75 ul was subjected to RP-HPLC (see Section 3.11).

3.11 Reversed-phase high performance liquid chromatography (RP-HPLC)

RP-HPLC analysis was performed using a HPLC Model 715 (Gilson, Middleton, WI) with a C-18 column (TSK-GEL 5 µm, ODS-120T, 4.6 mm X 250 mm, TOSOHaas, Montgomeryville, PA). Samples in the Histatin 1, Statherin and PRP1 experiments were eluted using a linear gradient from 0 to 55% buffer B

(80% acetonitrile, 0.1% trifluoroacetic acid) over a 74 min. time interval at a flow rate of 1.0 ml/min. Buffer A was acidified DI water (0.1% trifluoroacetic acid). The eluate was monitored at 219 nm. This was done to determine C, the amount of protein remaining in the supernatant after incubation with HA.

3.12 HA demineralization assay

The pellet obtained after the protein adsorption assay (Section 3.10) was washed two times with water. To each pellet 1 ml of lactic acid (10 mM) was added, the samples were incubated for 30 min. in the rotating incubator at room temperature and centrifuged. After centrifugation, the supernatant was removed and diluted for the Calcium and Phosphate Assays, 1:10 and 1:40 respectively.

Table 2: Summary of Histatin 1 binding and demineralization experiments.

First incubation, 1 hr. @ 37º C with HA	Second Incubation, 30 min. @ room temp.
DI water	10 mM Lactic Acid
20 µM histatin 1 solution	10 mM Lactic Acid
50 µM histatin 1 solution	10 mM Lactic Acid
100 µM histatin 1 solution	10 mM Lactic Acid
200 µM histatin 1 solution	10 mM Lactic Acid
DI water	DI water

Table 3: Summary of Statherin binding and demineralization experiments.

First incubation, 1 hr. @ 37º C with HA	Second Incubation, 30 min. @ room temp.
50 mM NH_4HCO_3	10 mM Lactic Acid
25 µM statherin solution	10 mM Lactic Acid
50 µM statherin solution	10 mM Lactic Acid
100 µM statherin solution	10 mM Lactic Acid
50 mM NH_4HCO_3	50 mM NH_4HCO_3
25 µM statherin solution – no HA	
50 µM statherin solution – no HA	
100 µM statherin solution – no HA	

Table 4: Summary of PRP1 binding and demineralization experiments.

First incubation, 1 hr. @ 37º C with HA	Second Incubation, 30 min. @ room temp.
DI water	10 mM Lactic Acid
25 µM PRP1 solution	10 mM Lactic Acid
50 µM PRP1 solution	10 mM Lactic Acid
100 µM PRP1 solution	10 mM Lactic Acid
DI water	DI water
25 µM PRP1 solution – no HA	
50 µM PRP1 solution – no HA	
100 µM PRP1 solution – no HA	

3.13. Assay to measure free calcium (Ca^{2+})

Free calcium in the lactic acid treated HA samples was measured using orthocresolphthalein complexone (OCPC) (Connerty and Briggs, 1966). Two 25 µl aliquots of each standard and diluted supernatant were transferred to a 96 well plate followed by the addition of 250 µl OCPC Reagent. The OCPC Reagent consisted of: 4.5% (v/v) aminoethanol , 1.4 % (v/v) ethanol , 29 mM Boric Acid,

0.25 mM KOH, 43 mM Acetic Acid, 5 mM 8-Quinolinol and 62 mM o-cresolphthalein complexone. The Ca standards contained 0, 0.24, 0.5, 0.7, 1.0 or 1.2 mM Ca respectively using a $CaCO_3$ solution.

The OCPC Reagent absorption maximum is approximately 350 nm. After complexation with Ca^{2+} the absorbance maximum shifts to 560 nm. The absorbance was measured at 560 nm against a reagent blank and the Ca standards. The absorbance was measured using the Genios plate reader (Tecan, Research Triangle Park, NC, USA). Before measuring the absorbance, the 96 well plate was covered with sealing tape and incubated at 37º C for 30 minutes. All glassware was rinsed with DI water and 0.5 N HCl to remove any traces of calcium and baked in the oven until completely dry. Pyrex glass-stoppered volumetric flasks were used.

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3.14. Assay to measure free phosphate $(PO4)^{3-}$

Free phosphate in the lactic acid treated HA samples was measured using an established method for the determination of inorganic free phosphate (Work, 1981). Two 86 µl aliquots of each standard and diluted supernatant were transferred to a 96 well plate followed by 200 µl reaction solution. The phosphate reaction solution was 0.86 N sulfuric acid, 81 mM ascorbic acid and 2.9 mM ammonium molybdate. The phosphate standards contained 0.03, 0.08, 0.17,

0.25 and 0.3 mM phosphate respectively and were prepared using a Na_2HPO_4 stock solution.

The phosphate reaction solution's absorption maximum is approximately 250 nm. After complexation with phosphate the absorbance maximum shifts to 825 nm. The absorbance was measured at 825 nm against a reagent blank and the phosphate standards. The absorbance was measured using the Genios plate reader (Tecan, Research Triangle Park, NC, USA). Before measuring the absorbance, the 96 well plate was covered with sealing tape and incubated at 37º C for 30 minutes.

4. RESULTS

4.1 Histatin and Statherin Isolation

The first goal was to isolate histatins and statherin from human parotid secretion (PS). PS from several donors was incubated with zinc chloride under basic conditions to precipitate histatins and statherin. The precipitate was subsequently dissolved in 2% TFA, and aliquots were subjected to reversed phase HPLC. This yielded four major peaks representing, in order of elution, histatin 5, 3, 1, and statherin (Flora et al., 2001). The individual peaks were collected and dried. Cationic PAGE was used to assess the purity of the histatins (Fig. 6A). As a reference, whole PS protein was analyzed on the same gel (lane 8). The darkest bands in the PS sample corresponded to histatins 1, 3 and 5. The isolated histatins were relatively pure but contained some impurities which reflect other, minor histatins. The histatin 1 preparation likely contained traces of histatin 2, the histatin 3 preparation likely contained traces of histatin 4, and the histatin 5 preparation traces of histatin 6. The histatin 3 sample (lane 5) appeared to be of the highest purity. The histatins shown in Fig. 6A were further purified by isocratic elution using RP-HPLC. The histatin 1 shown in Fig. 6B, lane 1, representing the phosphorylated histatin family member, was used in the binding and functional assays described in sections 3.10 and 3.12.

The purity of the statherin preparation was evaluated by anionic PAGE (results not shown). The statherin preparation that was used for in the binding

and functional assays described in sections 3.10 and 3.12 was isolated by Dr. Traboulsi, and the purity of this preparation has been reported earlier (Traboulsi et al., 2010).

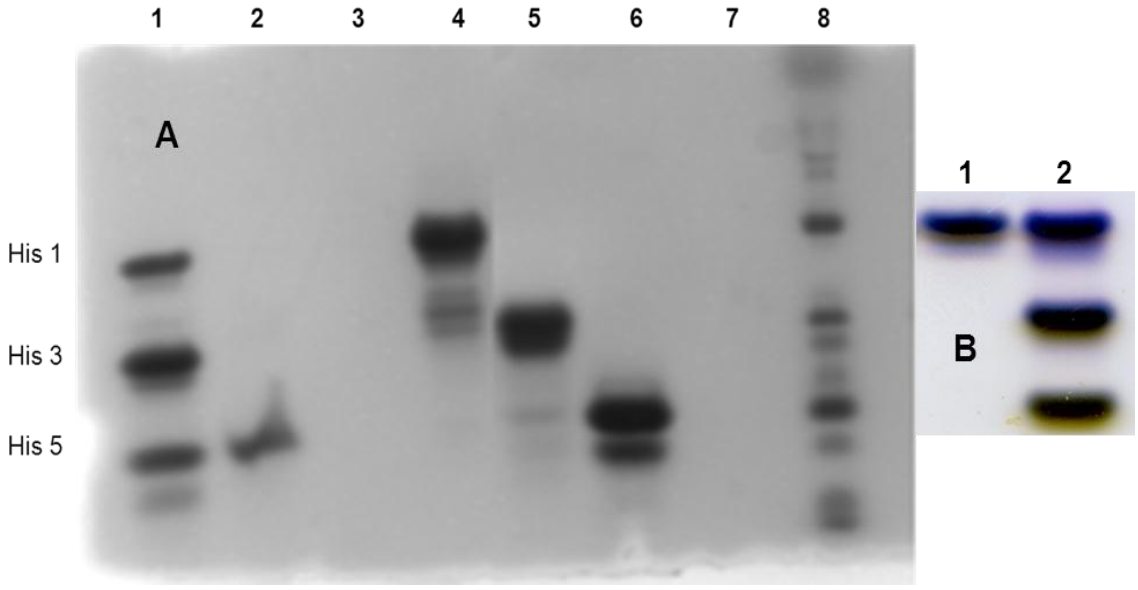

Figure 6A: Cationic PAGE of proteins in zinc-precipitated pellet from PS. Lane 1: Histatins 1, 3 and 5 (mixed standards), 19 µg total; Lane 2: Histatin 5 (synthetic), 3 µg; Lane 3: empty; Lane 4: Histatin 1, 20 µg; Lane 5: Histatin 3, 20 µg, Lane 6; Histatin 5, 20 µg; Lane 7: empty; Lane 8: HPS, 200 µg.

Figure 6B: Cationic PAGE of histatins purified from zinc-precipitated PS pellet by RP-HPLC (isocratic elution). Lane 1: Histatin 1, 20 µg, Lane 2: Histatin 1, 3 and 5 (standards), 20 µg. The gels were stained with Brilliant Blue.

4.2 Acidic PRP Isolation

The PRPs were purified from the desalted and lyophilized PS supernatant devoid of histatins and statherin. DEAE anion exchange chromatography was used to separate the cationic proteins appearing in the void volume eluting between 75 and 275 ml from the anionic PRPs, eluting between 480 and 550 ml. Figure 7 shows the sample separation between the two peaks with the beginning of PRP elution and the increase in conductivity caused by an increase in the percentage of buffer B occurring simultaneously. After DEAE chromatography, the fraction containing the PRPs was desalted by G-25 gel filtration chromatography. The elution pattern, presented in Fig. 8 showed two major peaks, one eluting between 160 – 230 ml and one eluting between 350 and 450 ml. The fractions from the first peak were collected and lyophilized. The mixture of PRPs in the lyophilized material were then dissolved in buffer A (50 mM Tris HCl, 50 mM NaCl) and applied to a Mono Q Column (Fig. 9). The Proteins were eluted using a shallow gradient (14% to 15.5% buffer B in 40 CV). Each peak in the Mono Q chromatogram represents a different PRP isoform. The level of purity of the proteins in each of the Mono Q Peaks was evaluated by anionic PAGE (Fig. 10). The position of the individual PRPs is indicated in the margin of the gel and is known from previous studies conducted in our lab as well as published studies. The PRP 1, PRP 2 and Pa Dimer were relatively pure. However, the other PRP isoforms appeared to contain various amounts of impurities and may require further purification. The PRP1 preparation (lane 5)

was used in the binding and functional assays described in sections 3.10 and 3.12.

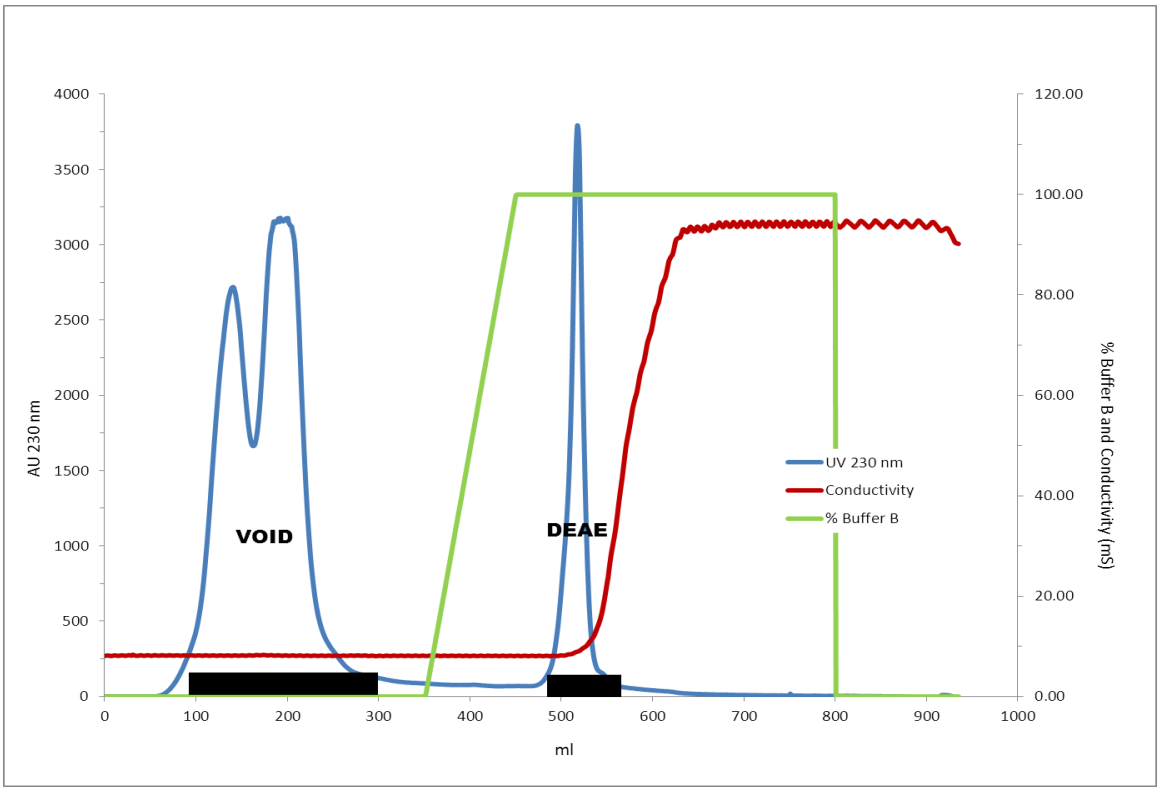

Figure 7: Anion Exchange Chromatography using a 40 cm DEAE Sepharose Column to separate the PRP Proteins (DEAE Peak) from the Void proteins. The black rectangles correspond to the fractions that were collected and pooled. The amount loaded was 250 mg.

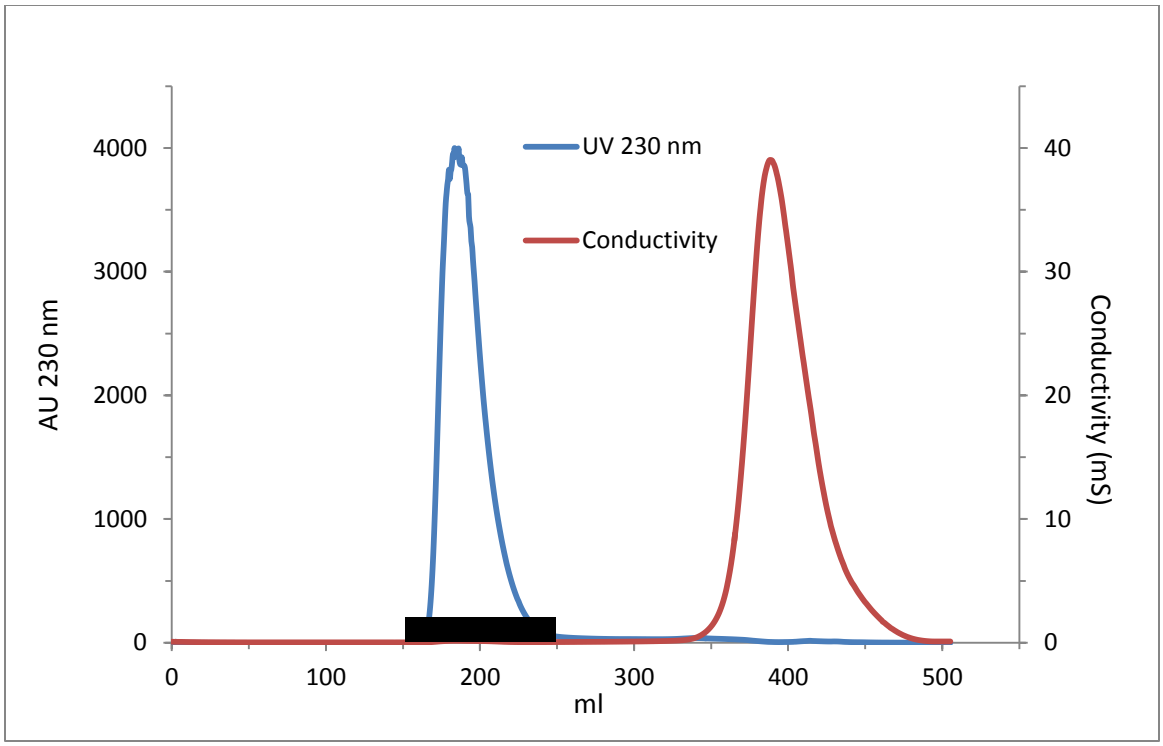

Figure 8: Gel Filtration (Size Exclusion) Chromatography of DEAE Peak using a 100 cm Column containing Sephadex G25 Medium. The black rectangle indicates the fractions containing PRP proteins that were pooled and collected. Mixed PRP solution (pooled from multiple DEAE anion exchange runs) was loaded in 25 ml aliquots.

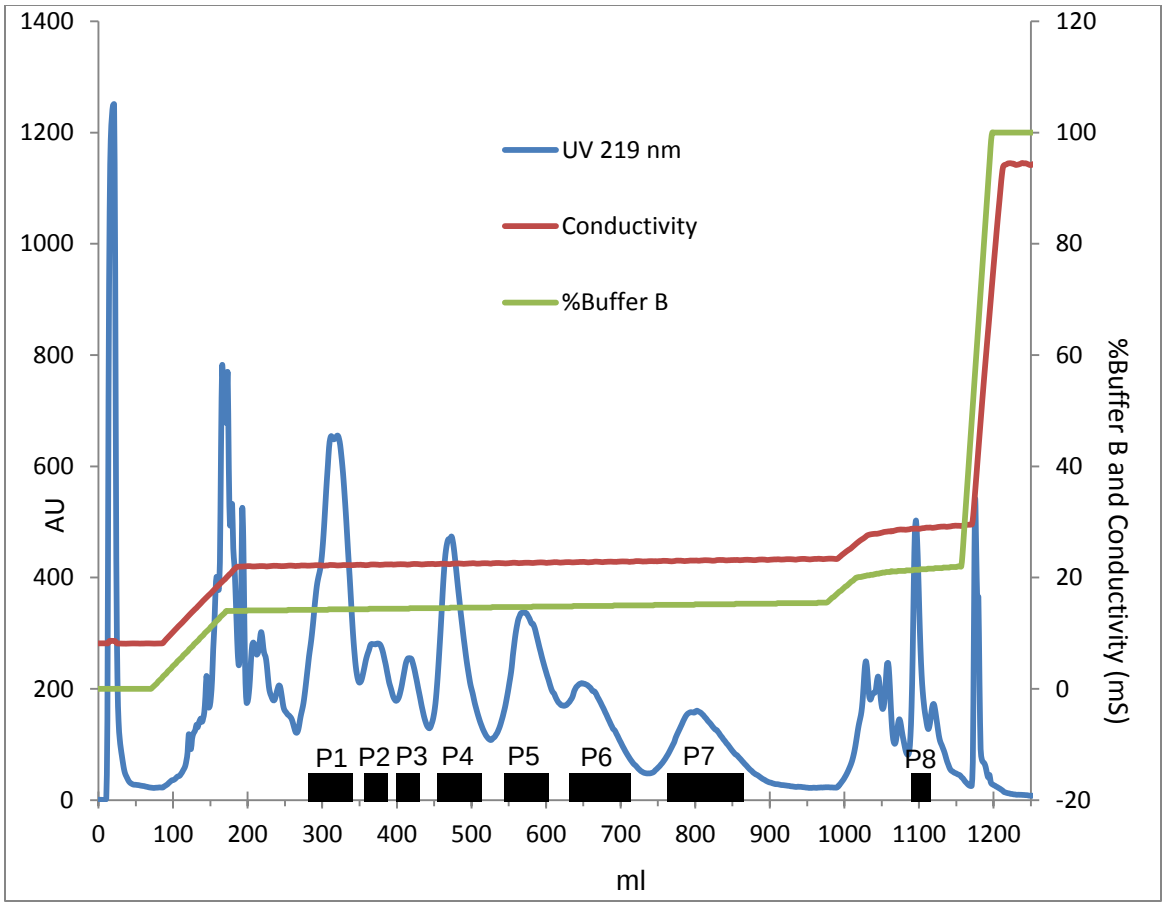

Figure 9: Anion Exchange Chromatography using a Mono Q HR 16 Column to separate individual PRP Proteins. The black rectangles correspond to the fractions that were pooled from each peak (P1 – P8). The amount of protein applied to the column was 30 mg.

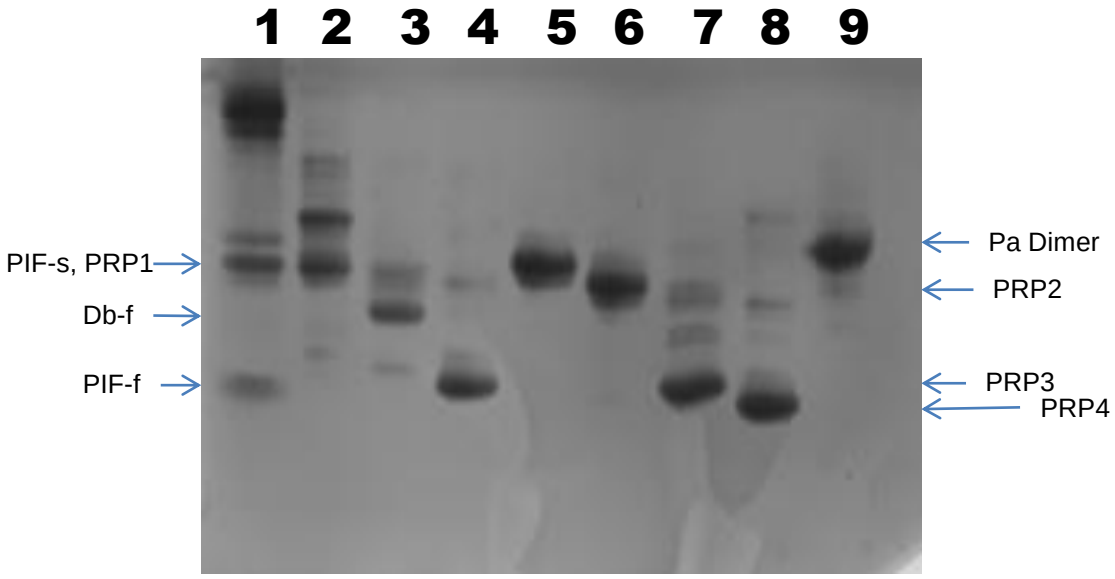

Figure 10: Anionic PAGE of Mono Q peaks 1-8. The individual peaks obtained from anion exchange chromatography (Fig. 9) were subjected to anionic PAGE. Lane 1: PS 200 µg; Lane 2: Peak 1 (PIF-s) 20 µg; Lane 3: Peak 2 (Db-f) 20 µg, Lane 4: Peak 3 (PIF-f) 20 µg; Lane 5: Peak 4 (PRP1) 20 µg; Lane 6: Peak 5 (PRP2) 20 µg; Lane 7: Peak 6 (PRP3) 20 µg; Lane 8: Peak 7 (PRP4) 20 µg; Lane 9: Peak 8 (Pa Dimer) 20 µg. The gel was stained with Amido Black. The bands are identified based on previous anionic PAGE experiments carried out in the lab of Dr. Frank Oppenheim at BUGSDM (Helmerhorst and Oppenheim, 2007) and elsewhere (Zakhary et al., 2007).

4.3. Binding and Demineralization Experiments with PS and HA

Different amounts of HA (1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg) were incubated for 15 min with lyophilized parotid saliva protein solutions (2.0 mg/ml). After various time intervals, the suspensions were centrifuged and 100 ul aliquots of the supernatant were loaded on the gel to assess PS protein binding to HA. Anionic PAGE (Fig. 11) clearly showed that as the amount of HA that was incubated with PS increased, (lanes 4 – 9) less and less protein was detectable in the supernatant and no PRPs were visible after incubation with >6.0 mg HA. Based on this result, it is reasonable to assume that all of the binding sites in 1.0 mg HA are occupied after incubation with 1.0 ml PS solution (2.0 mg/ml).

In the next experiment 1.0 mg of HA was incubated with PS protein solutions (1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mg/ml). After the supernatant was removed, the HA was incubated with 1.0 mM lactic acid. The released calcium was determined using the OCPC assay (see section 3.13), and the mM calcium released was established by comparison to a standard curve. The results presented in Fig. 12 indicate that approximately the same amount of calcium was released from HA regardless of preincubation with PS protein. Therefore, calcium release from HA by lactic acid could not be prevented by preincubation of HA with PS protein.

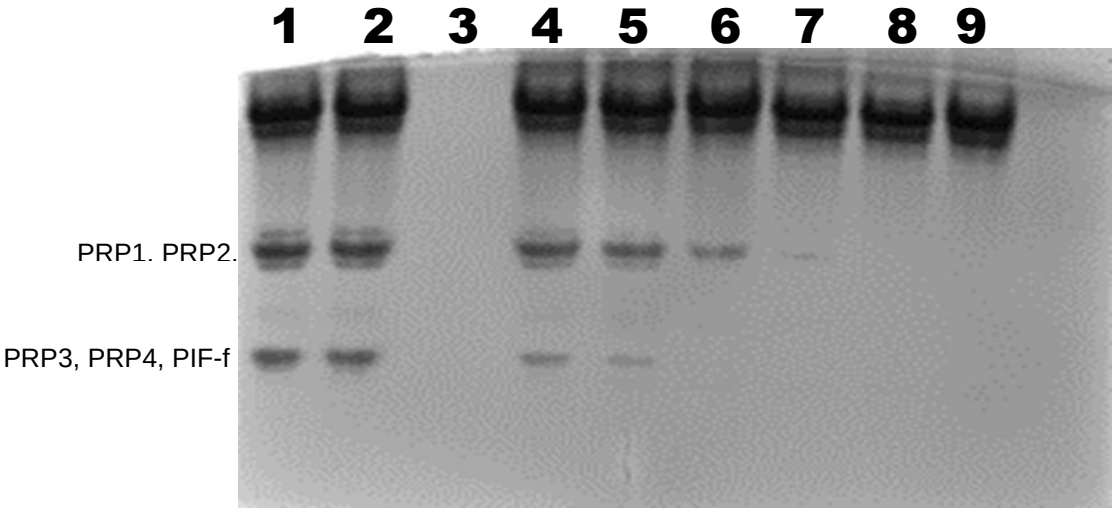

Figure 11: Anionic PAGE of PS before and after incubation with different amounts of HA. Lanes 1 and 2: PS protein, 200 µg/lane. Lanes 4-9: PS protein solution (2 mg/ml) was incubated with increasing amounts of HA (1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg respectively), 100 ul/lane. The supernatants were applied to lanes 4 – 9. The gel was stained with Amido Black.

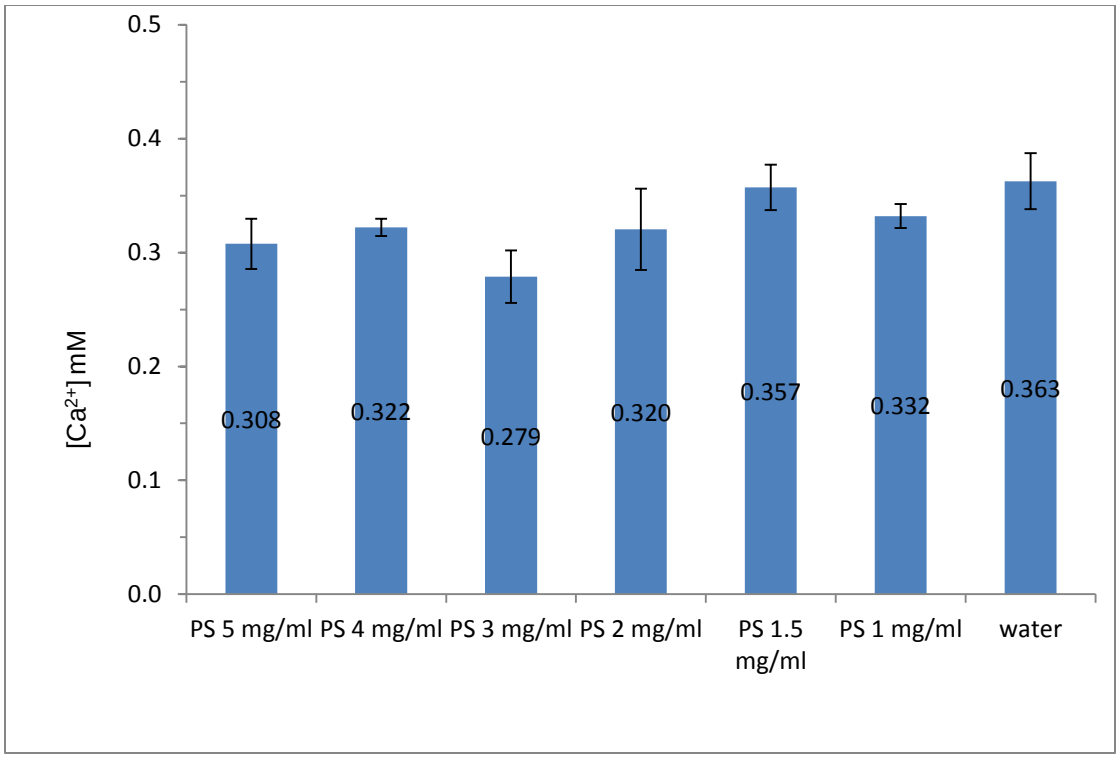

Figure 12: Calcium release assay. An aliquot of 1.0 mg HA was incubated with the indicated concentrations of PS for one hour before being treated with 1.0 mM lactic acid (pH 3.5). The supernatants were subsequently assayed for free calcium.

4.4 Binding Experiments with Pure Proteins and HA

4.4.1. Histatin 1 binding to HA

To study the binding of histatin 1, HA was incubated with isocratically purified histatin 1 solutions or with water (Table 2) and the supernatants were analyzed by Bis-Tris Gels (Fig. 13) and by HPLC (Fig. 14). The gels and HPLC results both show positive binding of His 1 to HA. In Fig. 13, the bands in Gel #1, lanes 2 – 5 (stock solutions) are noticeably more prominent than the bands in Gel #1 lanes 6 – 9 and in Gel #2 lanes 2 – 5 and 6 – 9 which correspond to the histatin 1 solutions after incubation with HA. In Fig. 14, the peaks in Chromatogram B, which correspond to the histatin 1 solutions after incubation with HA are 40 – 50% lower than the peaks in chromatogram A which correspond to the histatin 1 solutions not incubated with HA. Both of these results indicated that histatin 1 bound to HA. Fig. 15 is a graphical representation of the results obtained in the HPLC experiment (Fig. 14). After incubation with HA, the 2, 4, 8 and 16 nmol histatin 1 solutions were depleted of protein by 76%, 67%, 53% and 39% respectively with respect to the stock solutions.

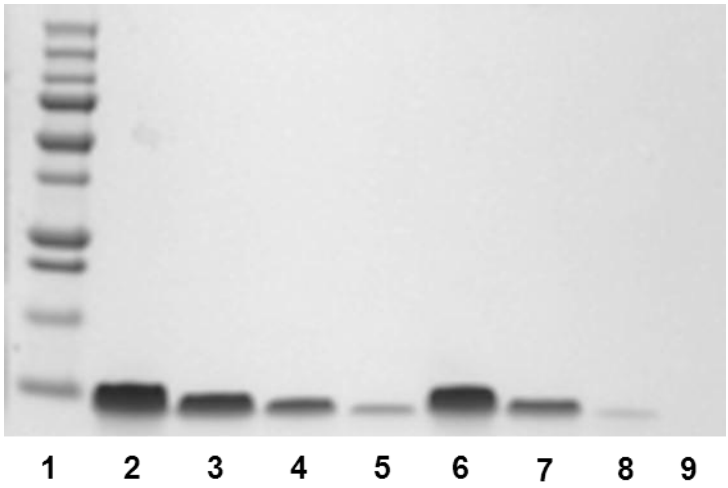

Figure 13: 12% Bis-Tris PAGE of histatin 1 solutions before and after incubation with HA. Lane 1: molecular weight standards; lanes 2 – 5: histatin 1 stock solutions (200, 100, 50 and 20 µM respectively); lanes 6 – 9: histatin 1 stock solutions (200, 100, 50 and 20 µM respectively) after incubation with HA.

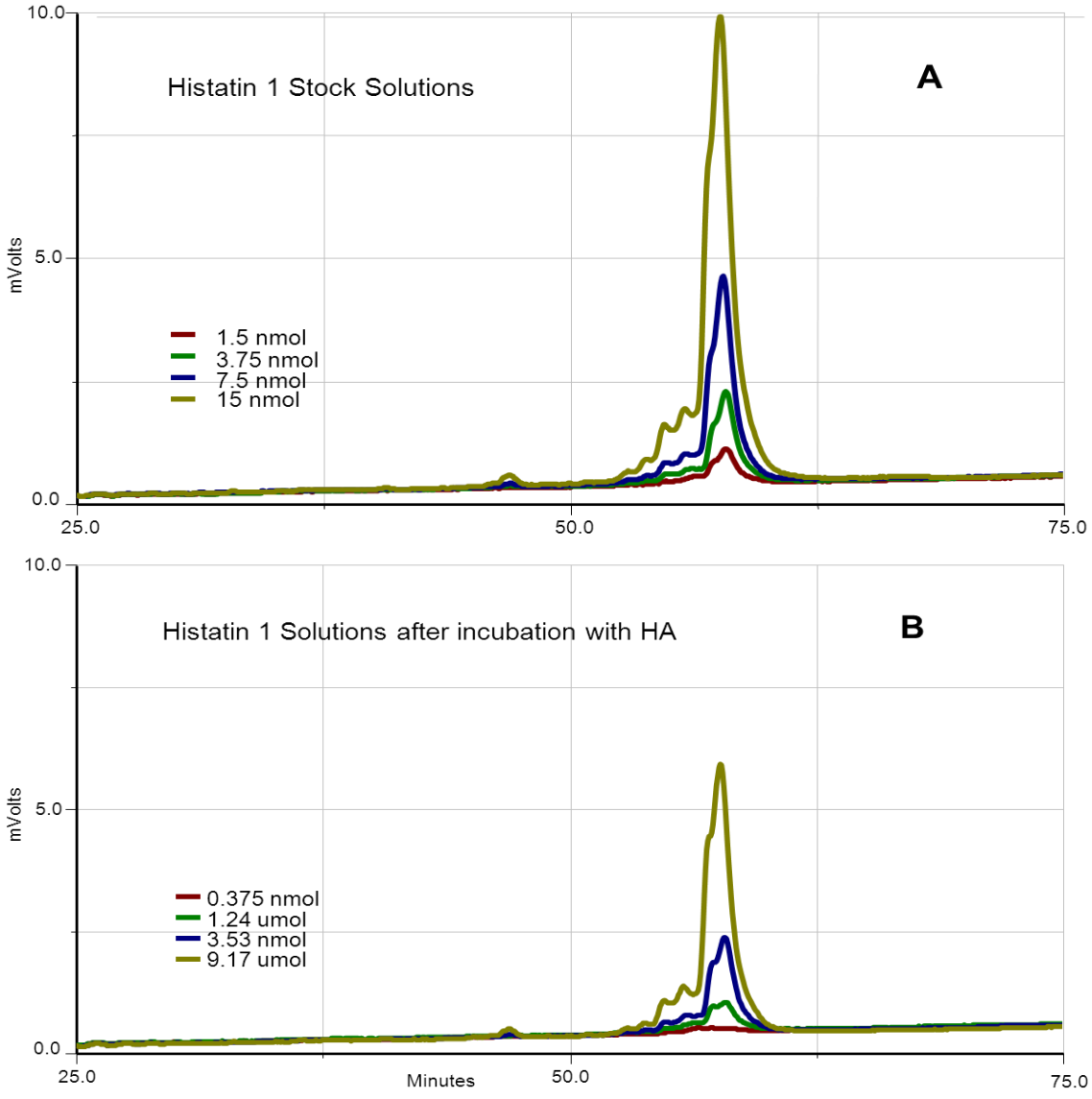

Figure 14: RP-HPLC Chromatograms (UV 219 nm) of histatin 1 incubated without and with hydroxyapatite. 1 ml aliquots of four different histatin 1 solutions (200, 100, 50 and 25 µM) were incubated without or with 1 mg HA. After 1 hr incubation at 37º C, samples were centrifuged and 75 µl aliquots of the supernatant, containing the indicated amounts of histatin 1, were analyzed by RP-HPLC. A) Histatin 1 stock solutions, B) Histatin 1 stock solutions after incubation with HA.

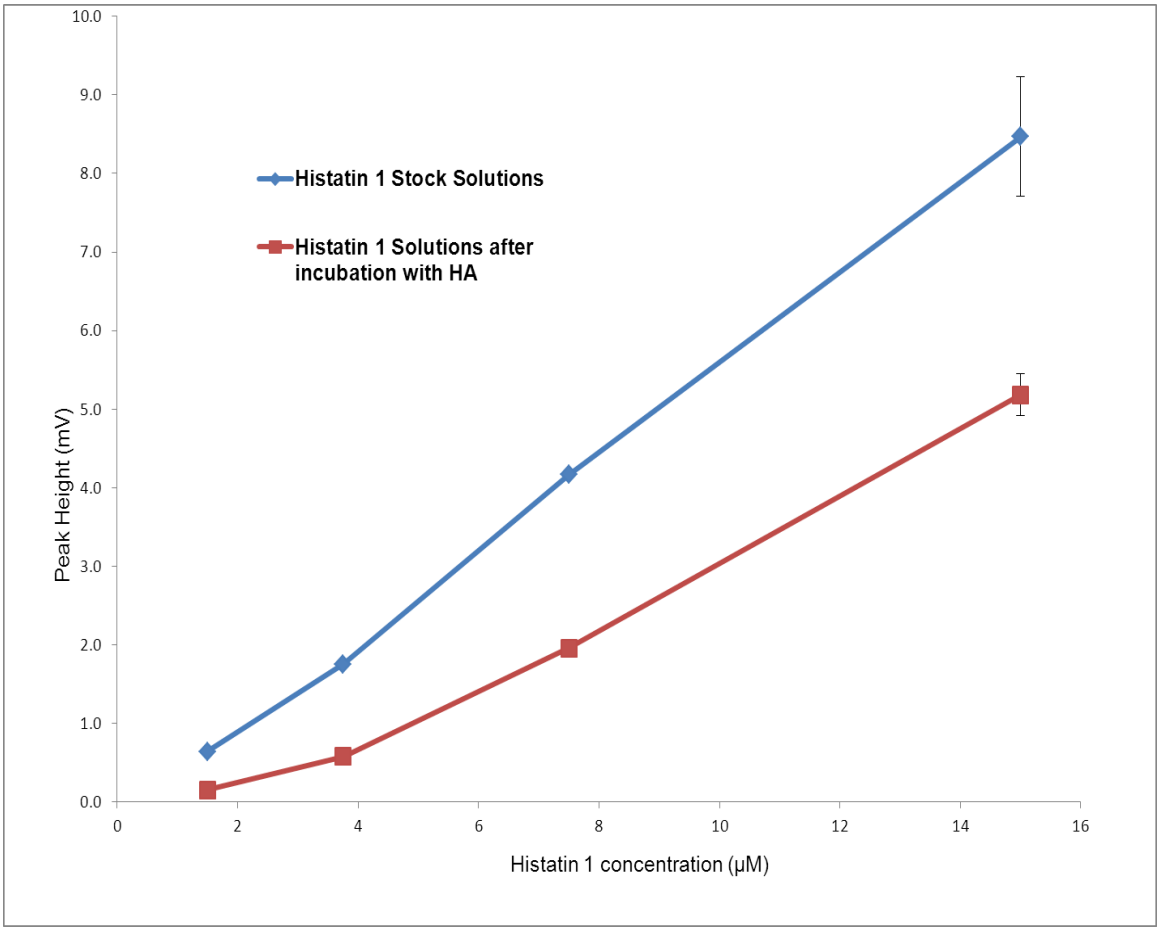

Figure 15: Peak heights of His 1 in HPLC experiment presented in Fig. 14. The blue line corresponds to the peak heights in Figure 14A and the red line corresponds to the peak heights in Figure 14B.

4.4.2. Statherin binding to HA

To study the protective effects of statherin, HA was incubated with pure statherin solutions or with ammonium bicarbonate solution (ABS) before being treated with 10 µM lactic acid (Table 3). The supernatants obtained after the first incubation were analyzed by RP-HPLC to assess binding of statherin to HA. In this experiment, a control was included to assess the stability of statherin during the 1h incubation at 37º C. Chromatogram A (Fig. 16A) shows the statherin peaks of the stock solutions which had been stored at -20º C (control solutions). Chromatogram B (Fig. 16B) shows the stock solutions incubated for 1h at 37ºC without HA and chromatogram C (Fig. 16C) shows the corresponding peaks from the stock solutions after incubation with HA. The results indicated that incubation alone did not cause degradation of statherin (Figure 16B). The presence of HA substantially removed statherin from the solution (Figure 16C). Fig. 17 is a graphical representation of the statherin peak heights obtained in the HPLC experiment. After incubation with HA, the 2, 4 and 8 nmol statherin solutions were depleted of protein by 96%, 77% and 49% respectively with respect to the stock solutions.

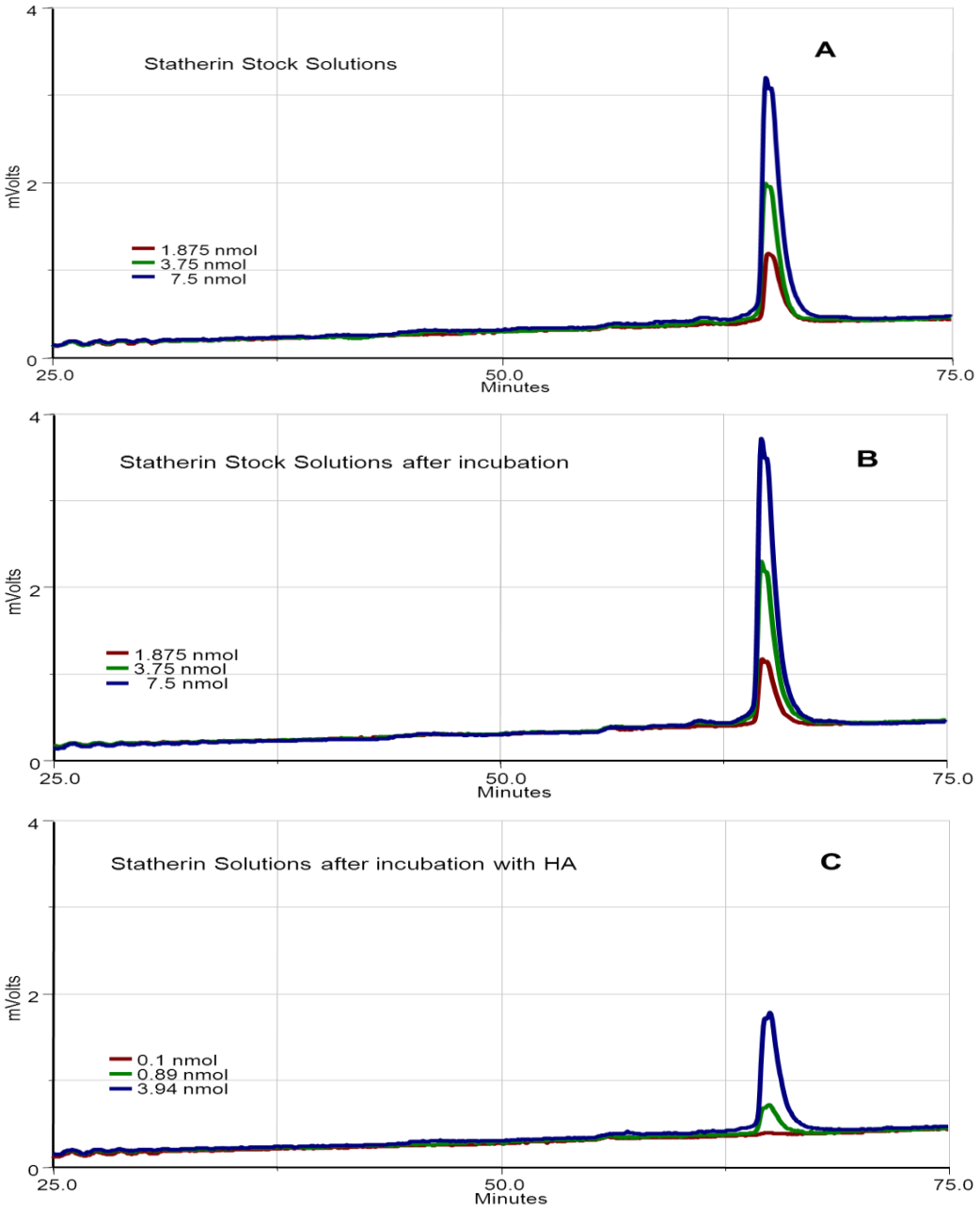

Figure 16: RP-HPLC Chromatograms (UV 219 nm) of statherin incubated without and with hydroxyapatite. 1 ml aliquots of three different statherin solutions (100, 50 and 25 µM) in 50 mM NH_4HCO_3 were incubated without or with 1 mg hydroxyapatite. After 1 hr incubation at 37º C, samples were centrifuged and 75 µl aliquots of the supernatant, containing the indicated amounts of statherin, were analyzed by RP-HPLC. A) Statherin stock solutions (untreated, control), B) Statherin stock solutions incubated without HA, C) Statherin stock solutions after incubation with HA.

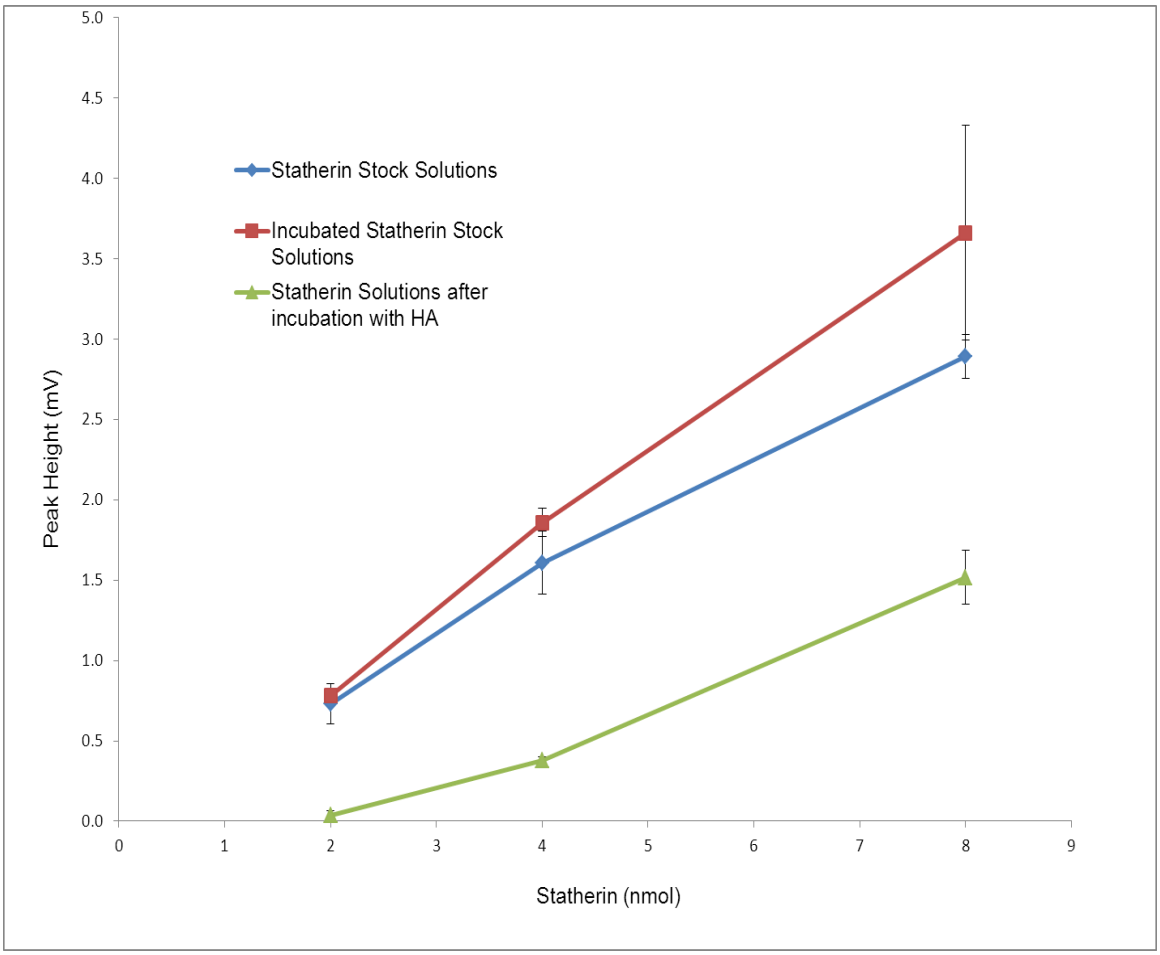

Figure 17: A chart showing the statherin peak heights (results of the HPLC experiment in Fig. 16). The blue line corresponds to the peaks in Fig. 16A and the green line corresponds to the peaks in Fig. 16C. The red line corresponds to statherin stock solutions incubated without HA (Fig. 16B).

4.4.3. PRP1 Binding to HA

To study the protective effects of PRP1, HA was incubated with different concentrations of PRP1 solutions (Table 4). After protein binding, the suspension was centrifuged and the amount of protein in the supernatant was measured by RP-HPLC (Fig. 18). Chromatogram A (Fig. 18A) shows the PRP1 peaks of the stock solutions which were stored at -20º C (control solutions). Chromatogram B (Fig. 18B) shows the PRP1 peaks from the stock solutions incubated at 37º C without HA and Chromatogram C (Fig. 18C) shows the PRP1 peaks from the stock solutions after incubation with HA. Fig. 19 is a graphical representation of the peak height results obtained in the RP-HPLC experiment. After incubation with HA, the 2, 4 and 8 nmol PRP1 solutions were depleted of protein by 20%, 7% and 6% respectively with respect to the stock solutions. This contrasts with the corresponding values obtained for statherin which were 96%, 77% and 49%.

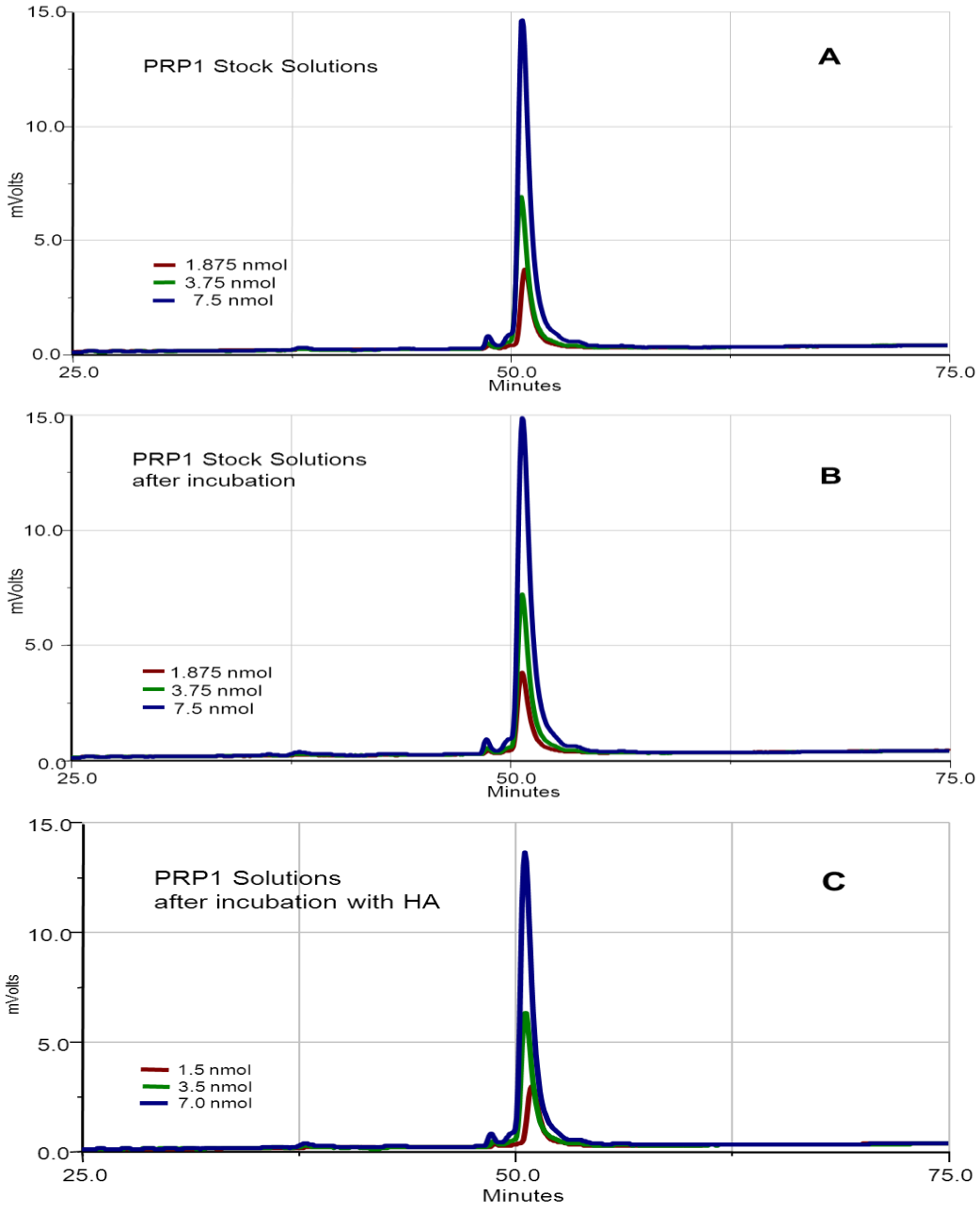

Figure 18: RP-HPLC Chromatograms (UV 219 nm) of PRP1 incubated without and with hydroxyapatite. 1 ml aliquots of three different PRP1 solutions (100, 50 and 25 µM) in water were incubated without or with 1 mg HA. After 1 hr incubation at 37º C, samples were centrifuged and 75 µl aliquots of the supernatant, containing the indicated amounts of PRP1, were analyzed by RP-HPLC. A) PRP1 stock solutions (untreated, control), B) PRP1 stock solutions incubated without HA, C) PRP1 stock solutions after incubation with HA.

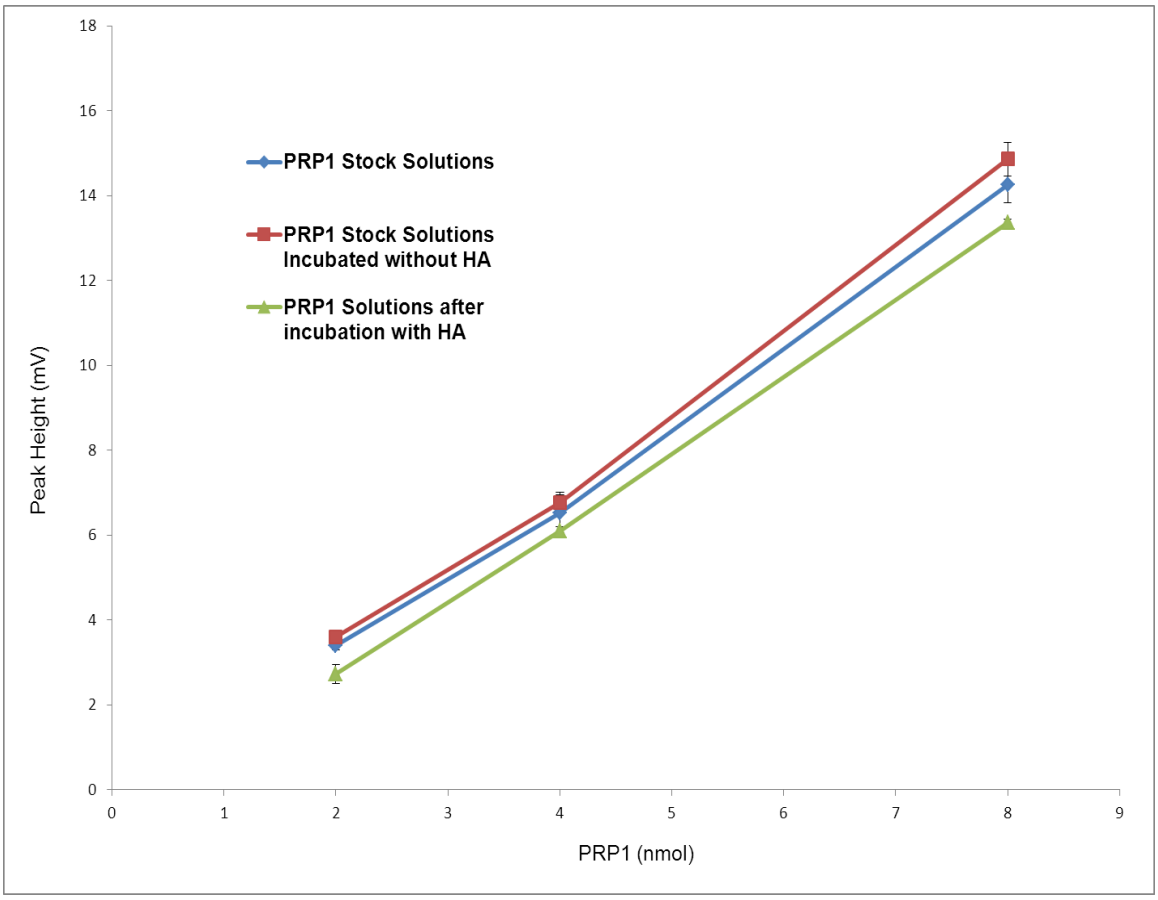

Figure 19: A chart showing the PRP1 binding results of the HPLC experiment in Fig. 18. The blue line corresponds to the peaks in Fig. 18A (control), the red line corresponds to the peaks in Fig. 18B and the green line corresponds to the peaks in Fig. 18C.

4.5 Analysis of the Histatin 1, statherin and PRP1 binding in Langmuir plots

The Langmuir adsorption equation was used in this study to analyze the adsorption of pure histatin 1, statherin and PRP1 to HA. The average peak heights from the HPLC binding experiments are given in Table 5. The total amount of protein in the stock solutions, S, was reflected by the peak heights of the stock solutions incubated without HA. The amount of protein remaining in solution after incubation with HA, C, was reflected by the peak heights of the stock solutions after incubation with HA. Finally, Q, the amount of protein adsorbed per unit surface area of HA, is determined by subtracting C from S. The values for S, C and Q are given in Table 6. C and Q values were subsequently used to obtain the Langmuir linear plots and adsorption isotherms for histatin 1, statherin and PRP1. The HPLC chromatograms for histatin 1, statherin and PRP1 are shown in Figures 14, 16 and 18 respectively. As a control, aliquots of the statherin and PRP1 stock solutions were analyzed by HPLC without incubation. The difference between the incubated and unincubated stock solutions was not significant.

The histatin 1 and statherin adsorption isotherms (Figs. 20B and 21B) were characterized by a steep increase followed by a leveling off which indicates saturation of the available binding sites on the surface. Based on the characteristics in the linear plot, the values for N and K_a were determined (Table 7). The values of N, the number of binding sites and K_a, the binding affinity

constant, were not calculated for PRP1 because the R^2 value obtained from the plot of the linearized data was too low (0.78). Indeed, PRP1 did not show typical adsorption characteristics (Figure 22). The two variants of the constant K are K_d, the dissociation constant with units of molarity (mol/L) and K_a, the association constant which is defined as $K_a = 1/K_d$. K_a has units of L/mol. A greater value of K_a indicates greater affinity of the ligand (protein) for the substrate (HA). According to the data presented in Table 7, the difference between histatin 1 and statherin in terms of the number of binding sites (N) is not significant. However, the K_a of statherin is an order of magnitude higher than that of histatin 1. This indicates that statherin has a much greater affinity for HA than histatin 1.

Table 5: HPLC Peak heights (mV) from Histatin 1, Statherin and PRP1 Binding Experiments. A linear relationship between the average peak heights and protein concentration is assumed.

Protein Solution	nmol present in 75 µl	Avg. peak height (mV)*	SD	Avg. peak height (mV), after incubation with HA	SD
Histatin 1	1.5	0.64	0.050	0.16	0.015
	3.75	1.76	0.049	0.58	0.075
	7.5	4.17	0.026	1.96	0.047
	15	8.47	0.761	5.18	0.265
Statherin	1.875	0.73	0.123	0.04	0.029
	3.75	1.61	0.194	0.38	0.024
	7.5	2.89	0.137	1.52	0.168
PRP1	1.875	3.40	0.102	2.72	0.231
	3.75	6.52	0.481	6.10	0.096
	7.5	14.27	0.433	13.36	0.090

*****Peak heights less background

Table 6: The variables C and Q for the Langmuir plots determined from the HPLC peak heights (see Table 5).

Protein Solution	Total conc. (µM)	nmol present in 1 ml	Conc. in supernatant (C) after incubation with HA (µM)	nmol protein bound to 1 mg HA*	Protein bound per unit surface area (Q, µmol/m^2)
Histatin 1	20	20	5.0	15.0	0.375
	50	50	16.4	33.5	0.838
	100	100	47.0	53.0	1.325
	200	200	122.3	77.7	1.943
Statherin	25	25	1.37	23.63	0.591
	50	50	11.8	38.2	0.955
	100	100	52.6	47.4	1.185
PRP1	25	25	20	5	0.125
	50	50	46.8	3.2	0.80
	100	100	93.6	6.4	0.160

* 1 mg HA has a surface area of 0.04 m^2

Table 7: N and K values determined from the linear Langmuir plots

	Number of Binding Sites (N) µmol/m^2	Binding Affinity Constant (K_a) l/µmol	Correlation Coefficient (R^2)
Histatin 1	2.40	0.0323	0.99 (n=4)
Statherin	1.23	0.425	0.99 (n=3)
PRP1	*	*	0.61 (n=3)

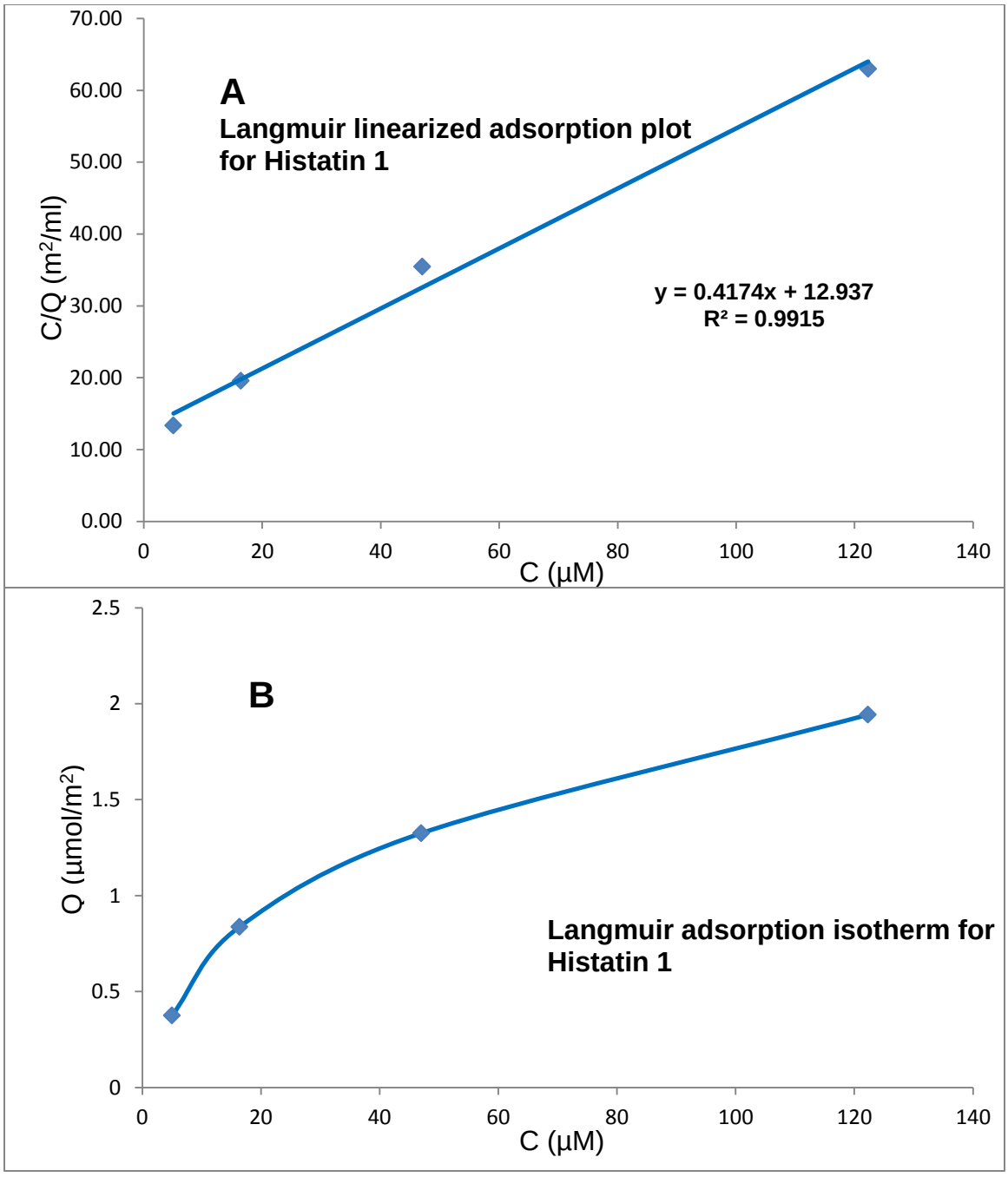

Fig. 20: Langmuir Plots of Histatin 1 adsorption onto HA. A is the linear plot and B is the calculated adsorption isotherm (see Section 1.13).

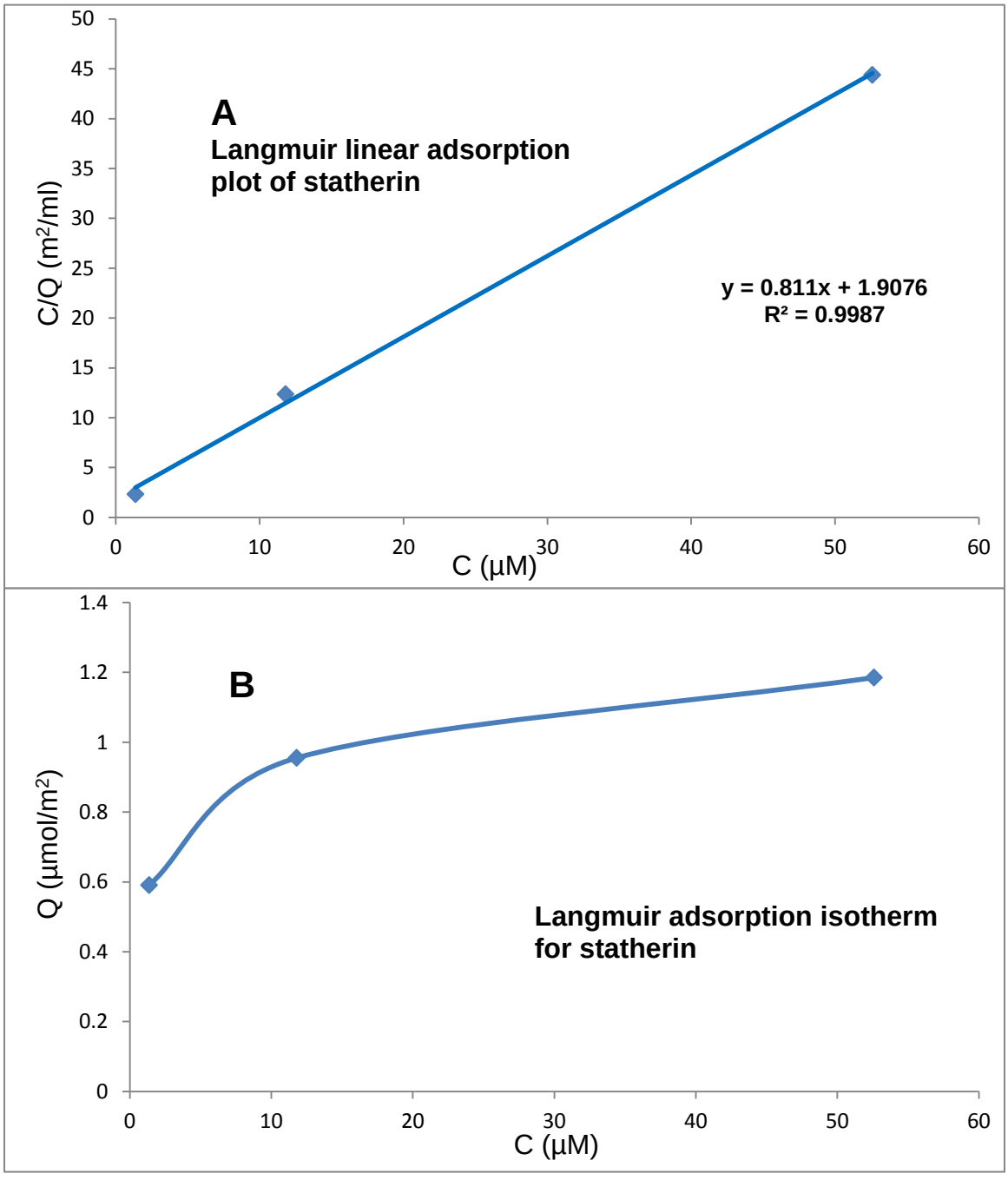

Fig. 21: Langmuir Plots of statherin adsorption onto HA. A is the linear plot and B is the calculated adsorption isotherm (see Section 1.13).

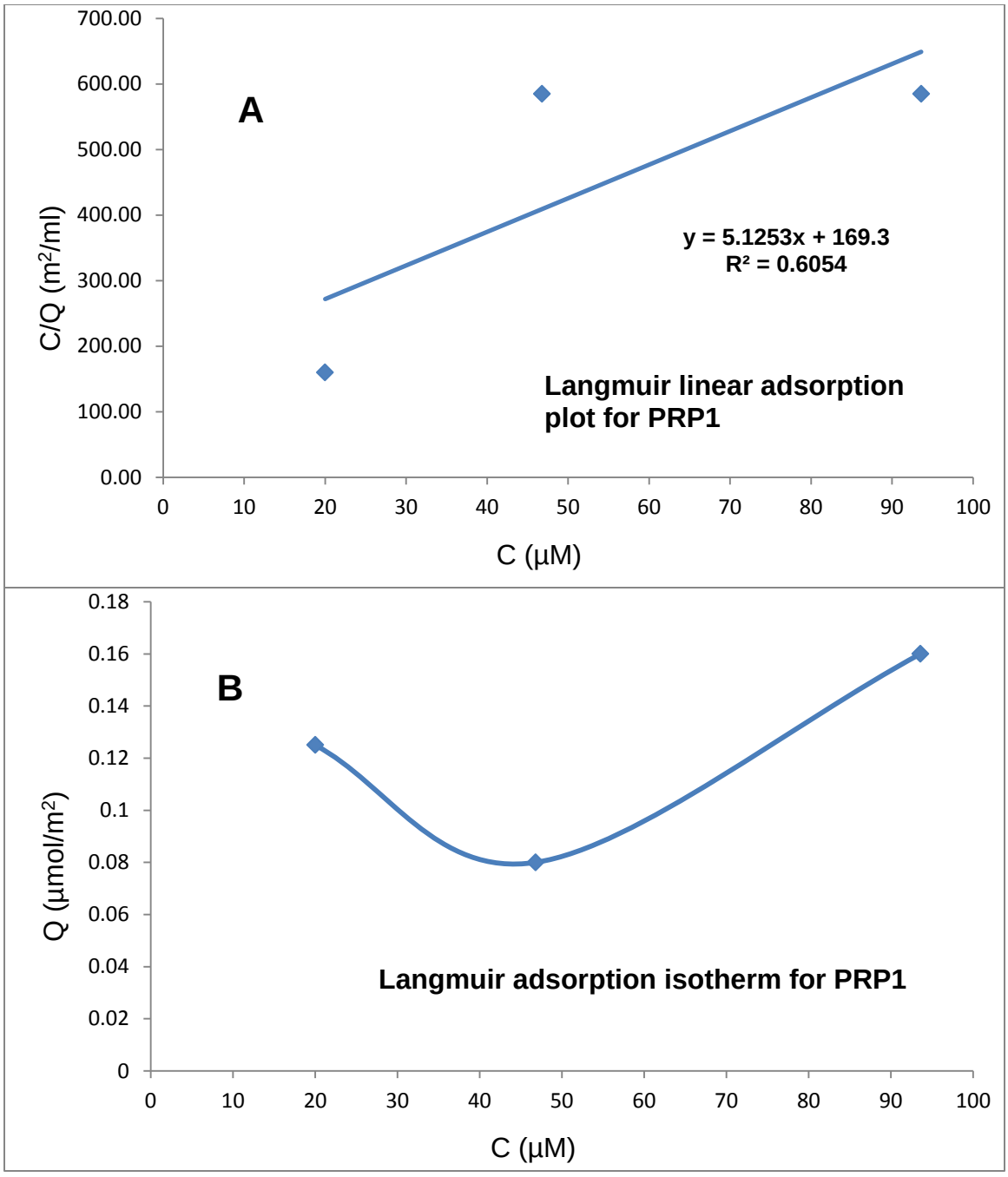

Fig. 22: Langmuir Plots of PRP1 adsorption onto HA. A is the linear plot and B is the calculated adsorption isotherm (see Section 1.13).

4.6. Demineralization assay results

4.6.1. Histatin 1 protection against demineralization

After the histatin 1 solutions were incubated with HA, the supernatant was removed. The HA was rinsed with water and incubated with 10 mM lactic acid solution (Table 2). The supernatants were removed and tested for free calcium and phosphate by colorimetric assays. The standard curves for the calcium and phosphate assays are given in Figure 23. The results of the calcium and phosphate release assays from histatin 1-coated HA are given in Fig. 24. Both assays indicate a trend towards greater protection against calcium or phosphate release with increasing histatin 1 concentration. However, the trend is somewhat understated and is more apparent in the calcium assay.

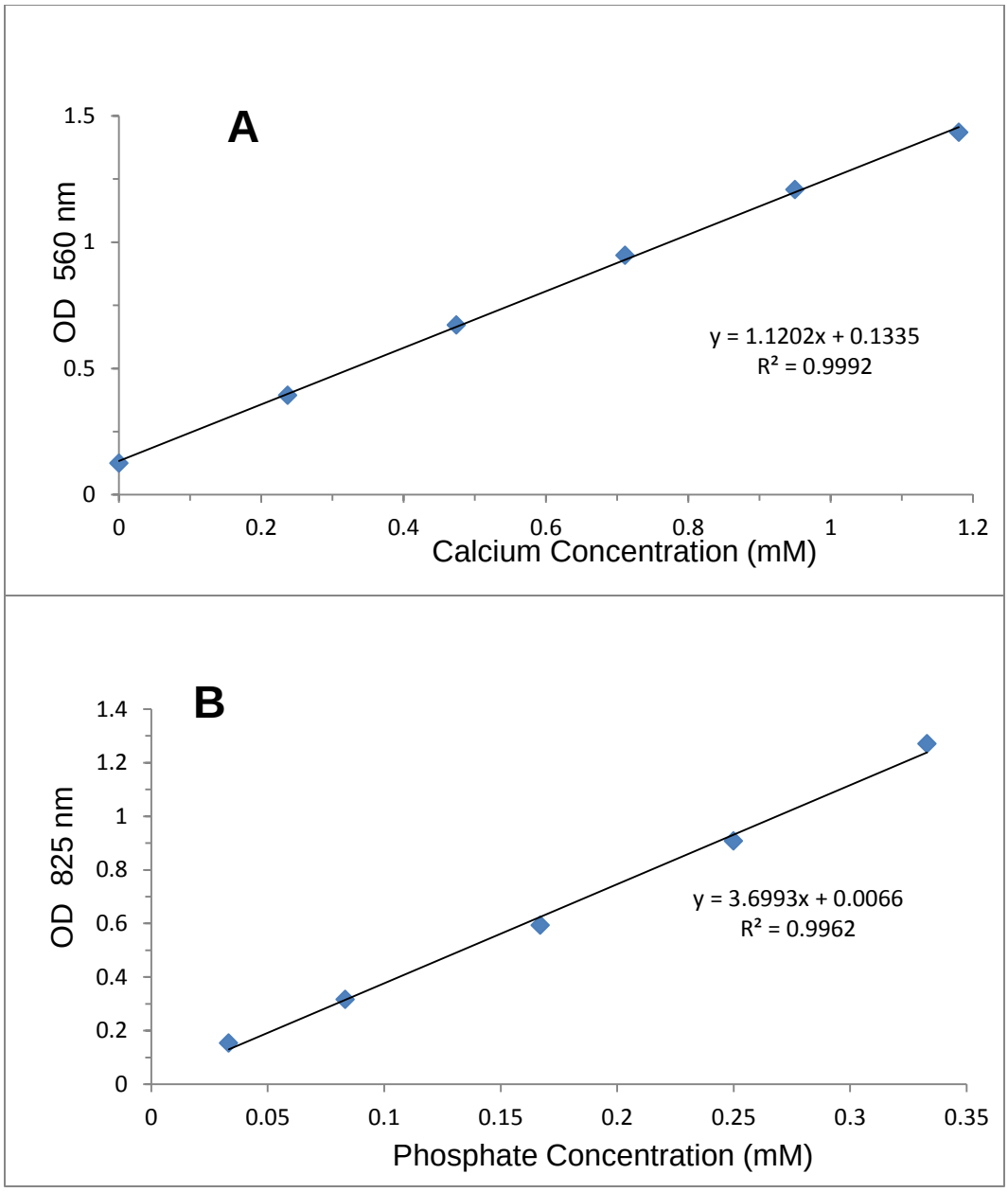

Figure 23: Representative standard curves for the detection of calcium and phosphate ions in solution. A) Calcium standards from dissolved $CaCO_3$, and B) Phosphate standards from dissolved Na_2HPO_4. Indicated are the concentrations of the free Ca^{++} and $(PO_4)^{3-}$ ions.

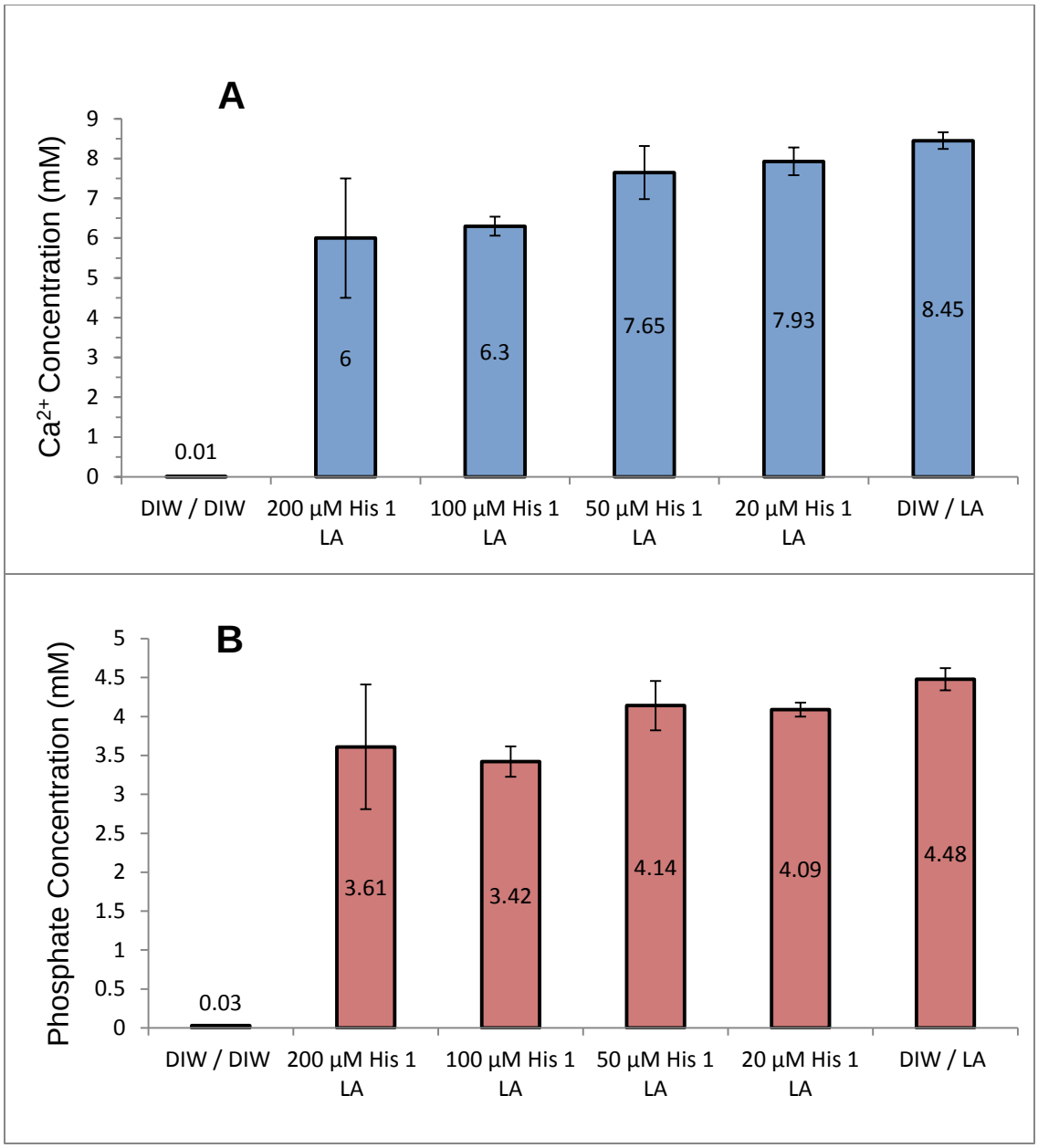

Figure 24: Assays for calcium and phosphate released from HA after incubation with histatin 1: A) Calcium Release Assay, B) Phosphate Release Assay. HA, incubated with water or with different concentrations of His1, was treated with 10 mM Lactic Acid. HA = Hydroxyapatite, DIW = Deionized Water, LA = 10 mM Lactic Acid. Calcium and phosphate released were determined by comparison to the standard curves shown in Figure 23.

4.6.2. Statherin protection against demineralization

As with histatin 1, colorimetric assays were performed on the supernatants from the second incubation (Table 3) to determine the amount of calcium and phosphate released from statherin coated HA after incubation with lactic acid (Fig. 25). Both assays indicate a trend towards greater protection against calcium or phosphate release with increasing statherin concentration. However, it is unlikely that the differences are statistically significant.

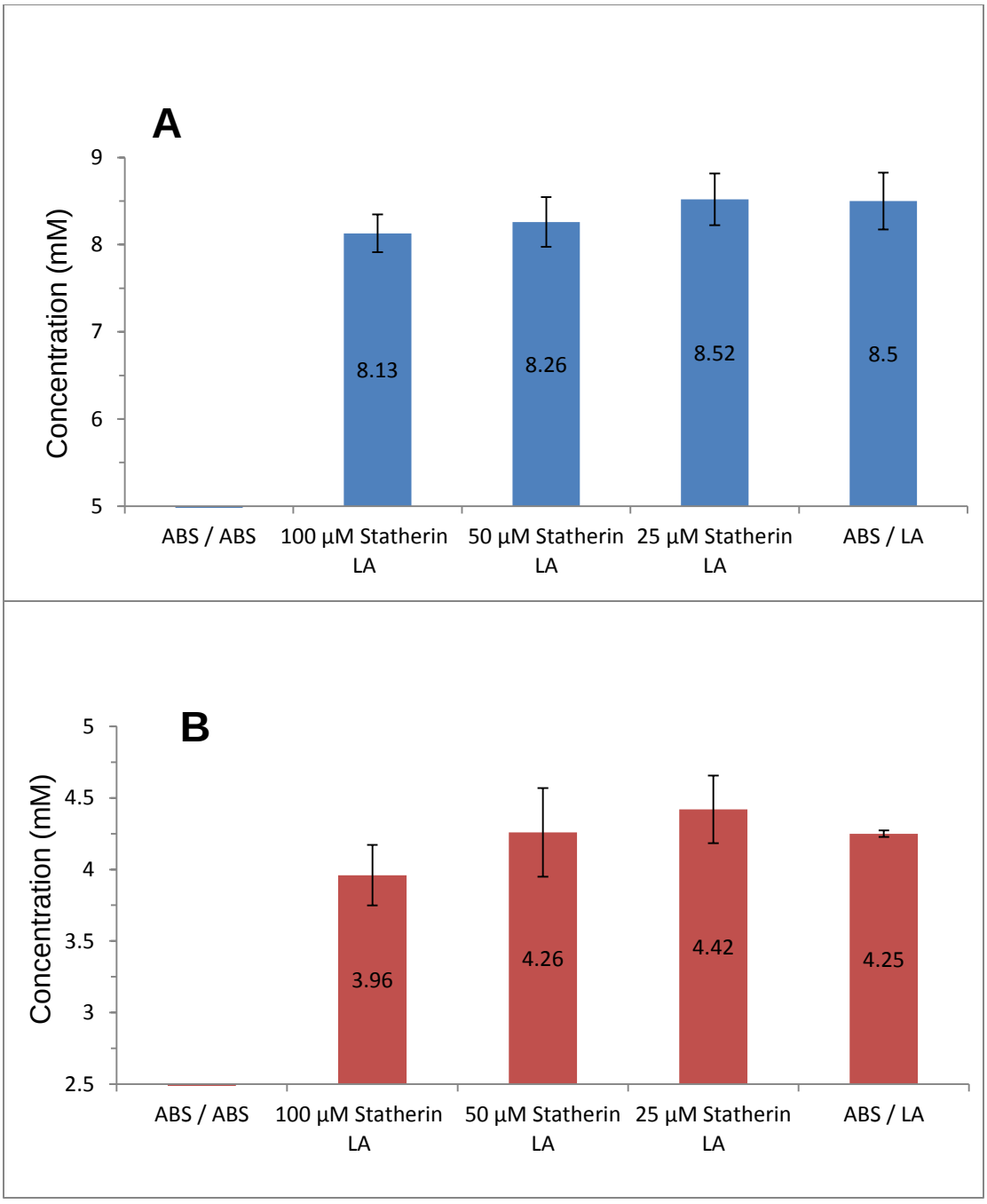

Figure 25: Assays for calcium and phosphate released from HA after incubation with statherin solutions or ammonium bicarbonate solutions: A) Calcium Release Assay, B) Phosphate Release Assay. Concentrations were determined by reference to standard curves similar to those shown in Figure 23.

4.6.3. PRP 1 protection against demineralization

For PRP1, colorimetric assays were performed on the supernatants from the second incubation (Table 4) to determine the amount of calcium and phosphate released from HA after incubation with lactic acid (Fig. 26). The trend towards some protection against calcium and phosphate release with increasing protein concentration that was observed with histatin 1 and statherin was not seen with PRP1. The level of calcium and phosphate released was about the same for all the samples that were treated with lactic acid.

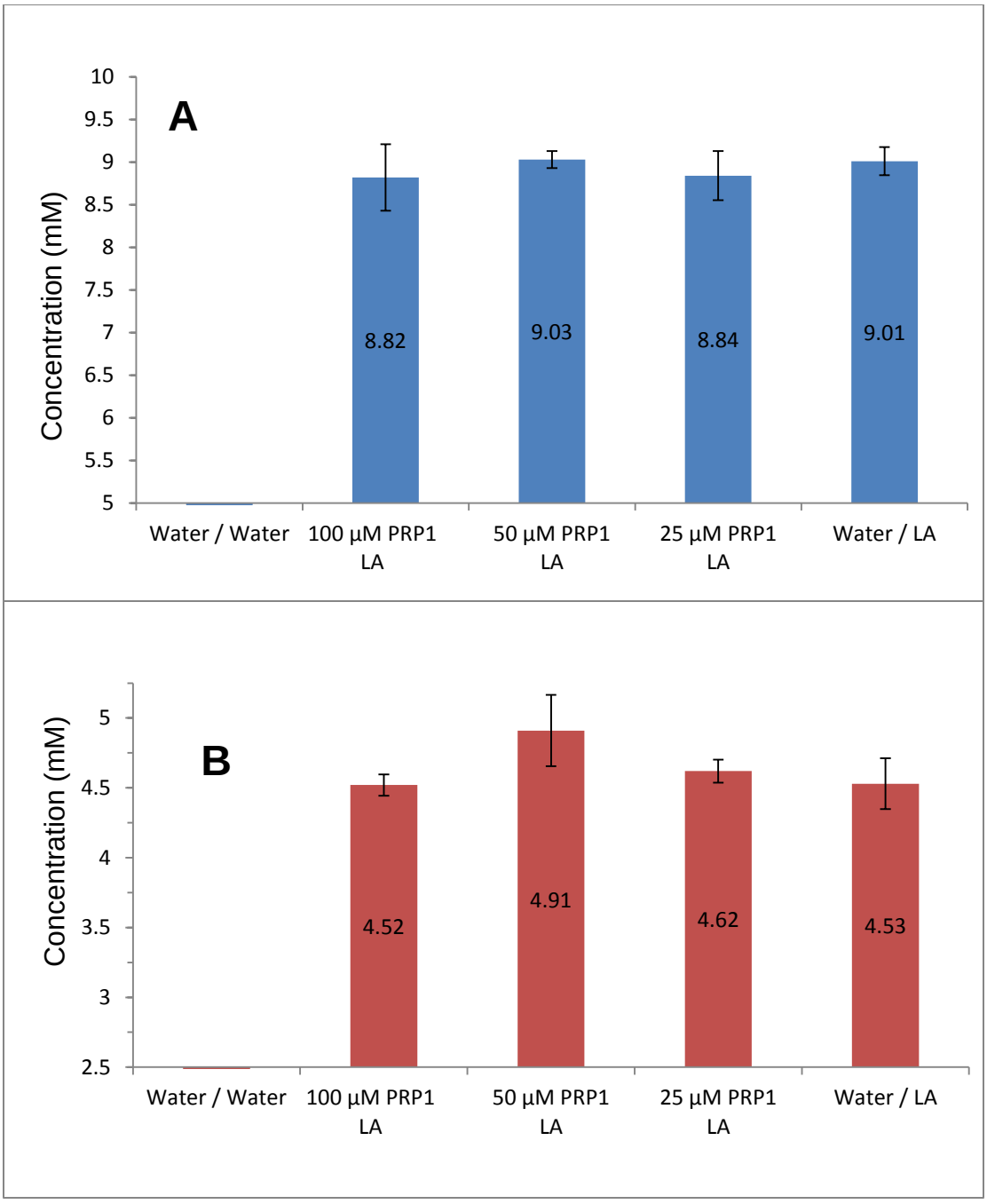

Figure 26: Assays for calcium and phosphate released from HA. HA incubated with water or different PRP1 solutions was treated with 10 mM Lactic Acid (LA). A) Calcium Release Assay, B) Phosphate Release Assay. Concentrations were determined by reference to standard curves similar to those shown in Figure 23.

4.6.4. The effect of extended incubation times on PRP1 binding and protection of HA.

The final experiment attempted to determine the effect of longer incubation times of the PRP1 solution with HA on the amount of protein bound to HA after incubation. The supernatants collected after four incubation times were analyzed by HPLC (Fig. 27). Fig. 28 is a histogram of the average peak heights from Fig. 27 and the two other sample series (not shown). There was a 12% depletion of PRP1 after the first hour of incubation, 16% depletion after 8 hours and 15% after 24 hrs, indicating the maximum amount adsorbed was reached within 1 h incubation. The PRP1 depleted from the supernatants is assumed to have bound to HA. In addition, calcium and phosphate assays were performed on the supernatants obtained after HA incubation (Fig. 29). For this experiment, samples incubated for 24 hours were used. The calcium and phosphate release data confirm that the protection achieved after pre-incubation with PRP1 is minimal.

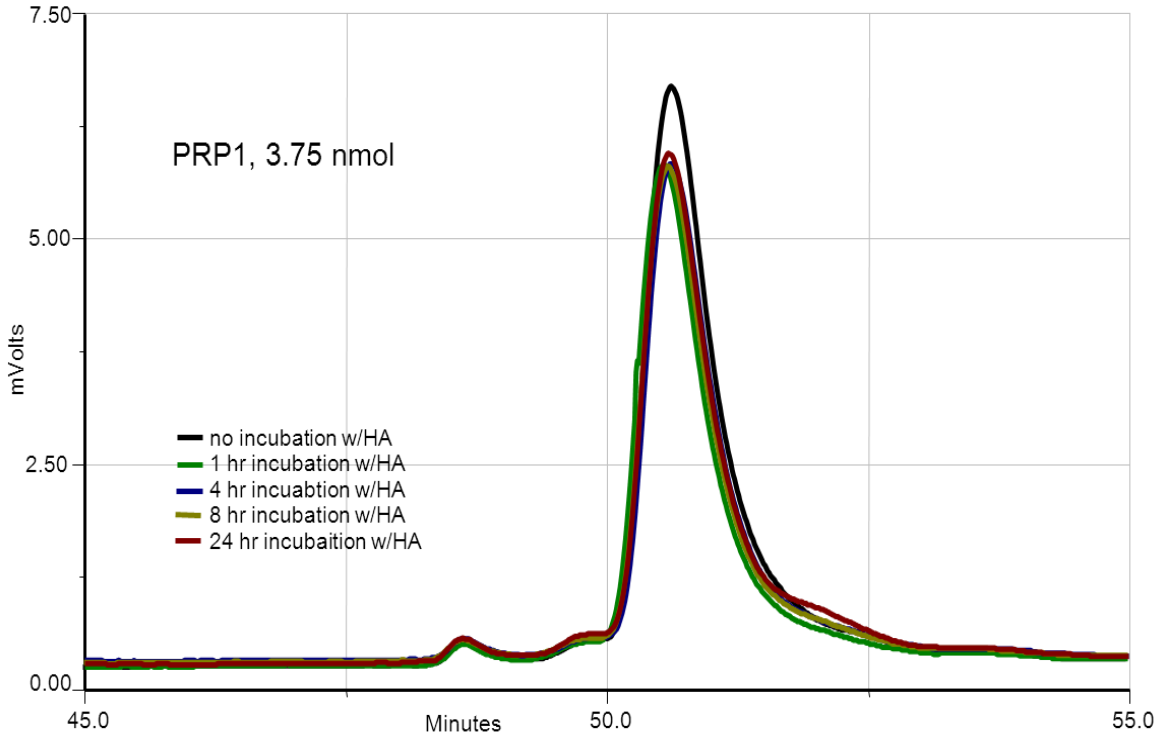

Figure 27: The binding of PRP1 to HA after different incubation times. The amount of PRP1 after incubations of 1, 4, 8 and 24 hours in the supernatant was assessed by quantitative RP-HPLC using a C-18 column. Protein quantitation was derived from the average peak heights from triplicate experiments.

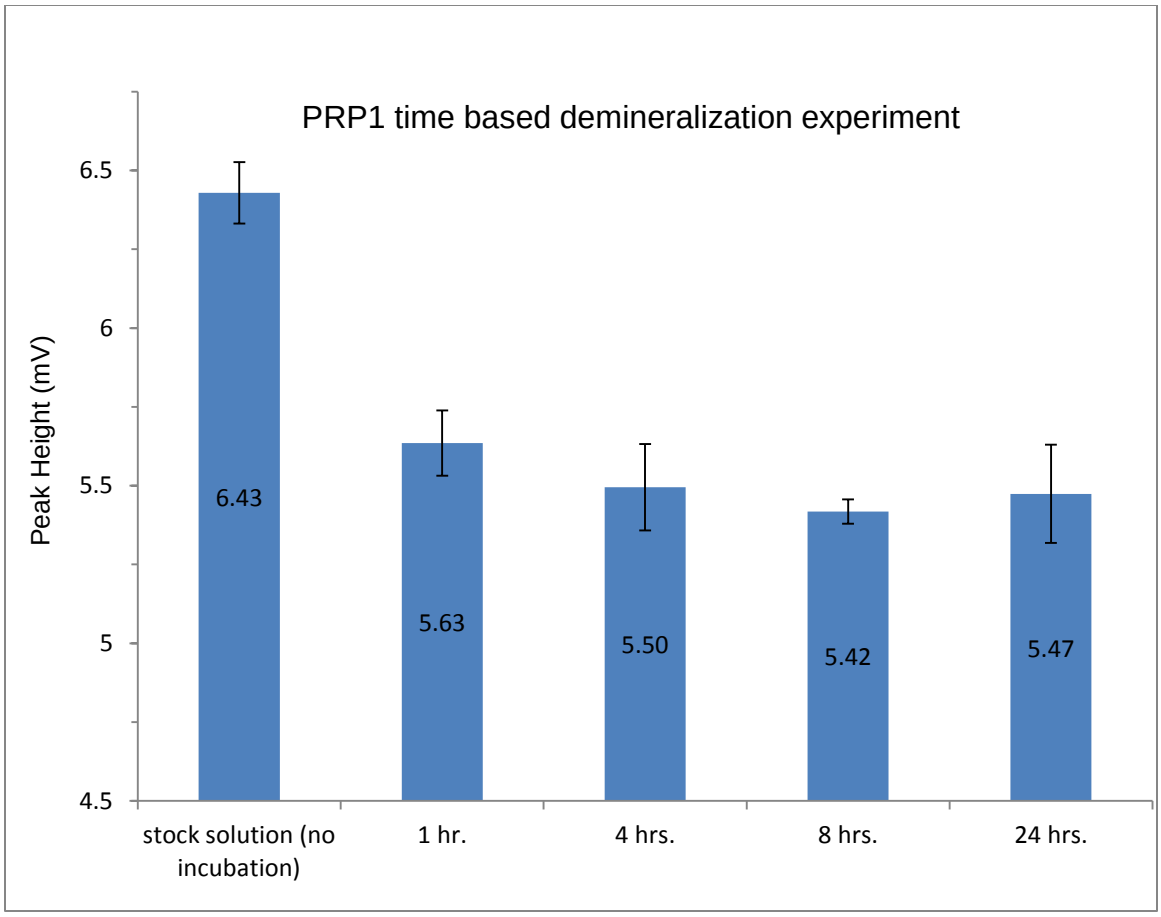

Figure 28: PRP1 peak heights after various incubation times with HA. The results of an HPLC experiment to determine whether the amount of PRP1 bound to HA increases as the incubation time with HA increases. The number of hours on the x-axis indicates the incubation time of the PRP1 stock solution with HA.

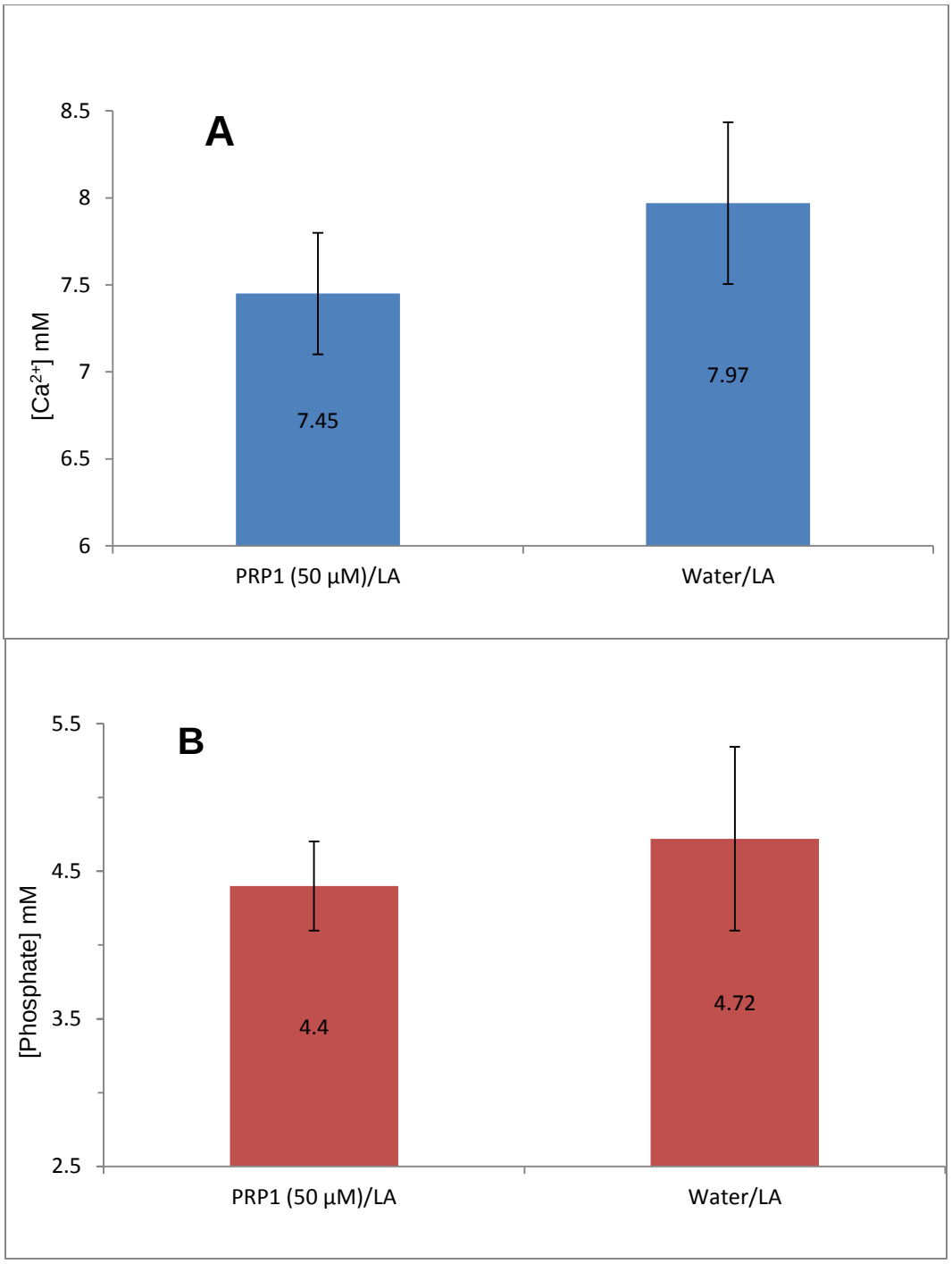

Figure 29: Assays for calcium and phosphate release. HA, incubated with 50 µM PRP1 solution or water, was treated with 10 mM lactic acid (LA) for 30 min. A) Calcium Release Assay, B) Phosphate Release Assay.

5. DISCUSSION

The goal of the present investigation was to isolate and purify salivary proteins and to evaluate the effect of selected proteins on tooth enamel protection in functional assays. Histatins were successfully isolated from parotid saliva (PS) by zinc precipitation and RP-HPLC. The histatin 1 sample, which contained traces of histatin 2, was used in subsequent demineralization experiments. The acidic PRPs were purified from desalted and lyophilized PS supernatant obtained after zinc precipitation to remove histatins and statherin. They were purified via column chromatography in three steps: low resolution anion exchange, gel filtration and high resolution anion exchange using a Mono Q Column. The separation of the individual acidic PRP isoforms was achieved in the final step. The peak containing PRP1 (Peak 4 in Fig. 9 and Lane 5 in Fig. 10) had good resolution and a high level of purity. This isoform was chosen for use in the subsequent binding and demineralization experiments. The statherin that was used in these experiments had been previously purified and isolated in our laboratory (Helmerhorst et al., 2011).

In the past, various chromatographic approaches have been used to isolate histatins, statherin and acidic PRPs. In a method described by Oppenheim and coworkers (Oppenheim et al., 1986), histatins were isolated in three steps: 1) fractionation of PS protein on a DEAE-Sephadex A-25 ion exchange chromatography column equilibrated with 50 mM Tris-HCl and 25 mM

NaCl, pH 8.0 using a linear NaCl gradient (0.025 – 1.5 M), 2) desalting of the histatin-rich fractions on a Bio-Gel P-2 column and chromatography on a CM Bio-Gel A column equilibrated with 25 mM sodium acetate and 25 mM NaCl, pH 5.0 using a linear NaCl gradient (25 – 800 mM), 3) final purification by gel filtration on a Bio-Gel P-6 column equilibrated and developed with 50 mM ammonium bicarbonate, pH 8.0. CM Bio-Gel A is a cation exchange resin which is necessary to separate histatins and statherin which elute from the DEAE-Sephadex A-25 Column together. The eluate was monitored at 230 nm which is the wavelength of absorbance for a peptide bond. A method for the preparation of highly purified statherin was reported (Schlesinger and Hay, 1977). This method includes fractionation of PS on a DEAE Sephadex ion exchange column, gel filtration using Bio-Gel P-6 and anion exchange chromatography using Bio-Rad DEAE-agarose with a NaCl gradient. Flora and coworkers in 2001 first discovered the precipitation of histatins with zinc under alkaline conditions. The method was further optimized (Helmerhorst et al., 2011) and is now the first step in the isolation of histatins and statherin in our and other laboratories.

A procedure for PRP isolation from human parotid secretion was described first by Oppenheim and coworkers (1971). The method that resulted in the purification of the four major salivary acidic proteins (PRP 1 – 4) included the following steps: 1) isolation of the protein components of interest by ammonium sulfate fractionation, 2) gel filtration on a Sephadex G-75 Column, 3) low resolution ion exchange chromatography on DEAE-Sephadex A-25 using a steep

NaCl gradient, 4) final purification on DEAE-Sephadex A-25 using a shallow NaCl gradient and 5) Disc gel electrophoresis to monitor the purity of the proteins (PRP 1 – 4). Another article (Bennick, 1975) described a modified approach: 1) Gel filtration on a Sephadex G-50 Column, 2) purification by cation-exchange chromatography on CM-cellulose CM-32, 3) further purification by anion exchange chromatography on DEAE-Sephadex A-25 and 4) desalting on a Sephadex G-25 Column. Bennick used this procedure to purify a single salivary phosphoprotein, Protein A, which corresponded to PRP-3 that had been isolated by Oppenheim and coworkers in 1971. Its primary structure is identical to the first 106 residues of acidic PRP-1. In recent years, we have optimized PRP isolation in our laboratory. The current approach minimizes the use of column chromatography in order to reduce protein loss, particularly in ion exchange chromatography. The starting material used is PS protein that had been depleted of histatins and statherin by the zinc precipitation procedure. The initial desalting is accomplished via dialysis. The cation exchange chromatography step is unnecessary because histatins (and statherin) were already removed. Using two chromatography steps, low resolution (DEAE Sepharose) and high resolution (Mono Q) anion exchange chromatography, the major acidic PRP isoforms are purified.

After the proteins were obtained in adequate quantities at a sufficient level of purity, functional assays could be performed. Four experiments were carried out to study the binding of salivary protein (histatin 1, statherin and acidic PRP1)

to hydroxyapatite (HA). In a qualitative experiment, the binding of mixed proteins from parotid saliva to HA was investigated by anionic PAGE (Fig. 11). The data showed that as the HA amount increased PRPs were depleted from the PS. Subsequent binding experiments were carried out with purified proteins. HPLC was used to quantitatively assess the binding of histatin 1, statherin and PRP1 to HA. The results of these experiments show that statherin exhibits the highest affinity for HA followed by histatin 1 and PRP1 (Table 8). In partial agreement with these findings, statherin has been shown to be a much stronger inhibitor of seeded calcium phosphate precipitation than either histatin 1 or PRP1 (Tamaki, et al., 2002).

Table 8: Percentage of protein bound to HA after incubation with the indicated pure salivary protein (from Figs. 14, 16 & 18).

	2 nmol protein	4 nmol protein	8 nmol protein
Histatin 1	76%	67%	53%
Statherin	96%	77%	49%
PRP1	20%	7%	6%

It is logical to assume that statherin, which has two N-terminal phosphoserine residues, has greater affinity for HA than histatin 1 which has only one phosphoserine. The negatively charged phosphoserines bind via favorable electrostatic interaction to the Ca^{2+} ions in the HA crystal lattice. The extra

phosphoserine is largely responsible for statherin's lower PI (4.2) compared to that of histatin 1 (7.0). In a comparative study (Hay, 1973), statherin, followed by histatin 1, were shown to exhibit the highest affinity for HA among all salivary proteins. Hay's finding was the result of examinations of the proteins adsorbed to 5, 20 and 50 mg of HA from 10 ml samples of PS. With 5 mg of HA, the only component to be significantly adsorbed was statherin, indicating its high affinity. With 20 mg of HA, adsorption of both statherin and histatin 1 was observed. PRPs only adsorbed at the 50 mg level, indicating their lower affinity for HA. It is noted in the study that statherin and histatin (which were referred to at the time as Components I and II) each contain about 25% acidic amino acid residues whereas PRPs contain about 36%. However, a major cause of the reduced affinity for HA by PRPs is that a significant percentage of its acidic residues (Asp and Glu) are present in the amidated form (Asn and Gln), as determined by free amine titration after hydrolysis (Oppenheim, 1973). Amidation increases the overall IEP of the protein, making its binding to HA less favorable.

In this study, functional assays were performed to measure the release of calcium ion and phosphate. In the three experiments carried out with histatin 1, statherin and PRP1, the protection provided to HA by protein binding was variable (Table 9). The trend towards protection against demineralization was most pronounced for histatin 1 where a 29% decrease in free calcium between the supernatant from unprotected HA and the supernatant from HA pre-incubated with 200 µM protein solution was measured (Fig 24A). The differences in free

calcium levels for statherin and PRP1 were within the margin of error in both experiments and therefore are not significant. The results for free calcium and free phosphate after pre-incubation with intermediate protein concentrations did not always follow the expected trend, where greater protein concentration leads to greater protection.

Table 9: Comparison of the results of the calcium and phosphate assays.

	Free Calcium (% decrease)	Free Phosphate (% decrease)
Histatin 1	29	19.4
Statherin	4.4	6.8
PRP1	2.1	0.2

* The values indicate the percent difference between the supernatant from unprotected HA after incubation with lactic acid and the supernatant from HA pre-incubated with the most concentrated protein solution in each experiment and subsequent incubation with lactic acid.

Several factors could explain the demineralization results. One possibility is that the protein to HA ratio was insufficient to adequately protect the HA from dissolution. However, the data presented in Table 8 suggest that a sufficient amount of protein was present, when the protein concentration increased, the percentage of protein bound to HA decreased. It appears that the proteins adsorb to HA with insufficient affinity (coverage) to protect against demineralization following acid exposure. Another possible explanation for the

lack of protection of HA by protein pre-incubation is that the protein could have been removed during the washing step. The interaction of the proteins with HA is reversible, and washing with water, a polar solvent, may have broken the electrostatic bonds between the proteins and HA, especially when coupled with the mechanical shearing that occurred when the solution was vortexed.

To understand the interaction of the negatively charged salivary proteins with hydroxyapatite, it is important to consider the physical properties of the HA with respect to protein binding in some more detail. Hay (Hay, 1973) compared the interaction of human PS proteins with synthetic HA and dental enamel powder. Essentially no differences in binding affinity were observed. It should be noted that greater amounts of dental enamel powder were required to obtain comparable surface areas since synthetic HA powder has a smaller particle size. Carbonate constitutes up to 2.25% of the dry weight of dental enamel and its presence causes HA crystals to be smaller and more spherical therefore approximating ceramic hydroxyapatite particles. In general, the surface area of HA powder is much greater than that of the available tooth enamel surface in the oral cavity.

The HA used in this study was Macro-Prep Ceramic Hydroxyapatite (CHT) Type I (40 µm particle size, surface area ~ 40 m^2/g) from Bio-Rad. HA has been used since the 1950s as a medium in affinity chromatography for the separation of biomolecules. Mineral HA is mined from quarries and until recently its use has

been limited by the fragile nature of its crystalline hexagonal rods. CHT is a spherical, macroporous form of mineral HA that has been sintered at high temperatures to modify it from a crystalline to a ceramic form (Fig. 30). In this process, the HA morphology changes from hexagonal rods (Fig. 31) to larger, spherical particles. CHT is extremely stable, both chemically and physically and has been used in column chromatography to separate proteins. Type I CHT exhibits a higher binding capacity for proteins, in particular acidic proteins whereas type II CHT is better suited for the separation of nucleic acids (Bio-Rad, 1996). Hay's findings (Hay, 1973) showed no essential difference between synthetic HA and dental enamel powder with respect to protein binding. The chemical composition of ceramic hydroxyapatite (CHT) is virtually identical to synthetic HA. The principal observations made in this study could apply to the *in vivo* adsorption process of salivary proteins to tooth enamel.

Salivary proteins which exhibit a high affinity for HA are known as "pellicle precursor proteins." Significant modifications and proteolytic processing of proteins including statherin and histatin often occurs before or after their incorporation into the pellicle structure and the life span of full-length histatins in the *in vivo* whole saliva environment is very short (Helmerhorst et al., 2006). In spite of their relatively weak affinity for HA, PRPs are larger in size and are present at higher concentrations than histatin 1 and statherin so therefore play a critical role in pellicle formation. It should not be assumed that proteins with a high affinity for HA in vitro play a significant role in the in the *in vivo* AEP. The

salivary proteins which are the focus of this study, like individual musicians in an orchestra, interact with other members and their concerted effort may well enhance the protection of tooth enamel.

Overall, the first aim of this study, to isolate histatins, statherin and acidic PRPs from parotid secretion, was successfully achieved. The second aim, to establish a spectrophotometric assay for Ca^{2+} and Phosphate release from hydroxyapatite in our laboratory based on pre-existing methods, was realized. The third aim was to test the binding of PS proteins to HA and examine the subsequent effect on demineralization. N and K values for statherin and histatin 1 were unequivocally established. The demineralization protection by salivary proteins tested was not very dramatic but was clearly discernable. Future experiments could mimic the *in vivo* conditions under which tooth demineralization occurs using weakly acidic buffers and prolonged incubation times.

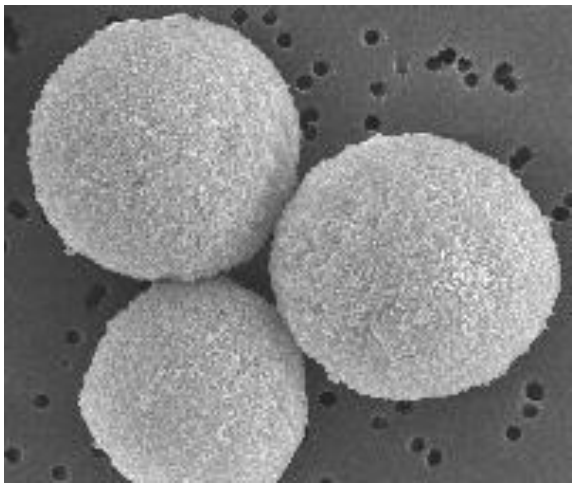

Fig. 30: Ceramic Hydroxyapatite (CHT) Beads – 40 µm diameter (Pentax. Pentax New Ceramics Site).

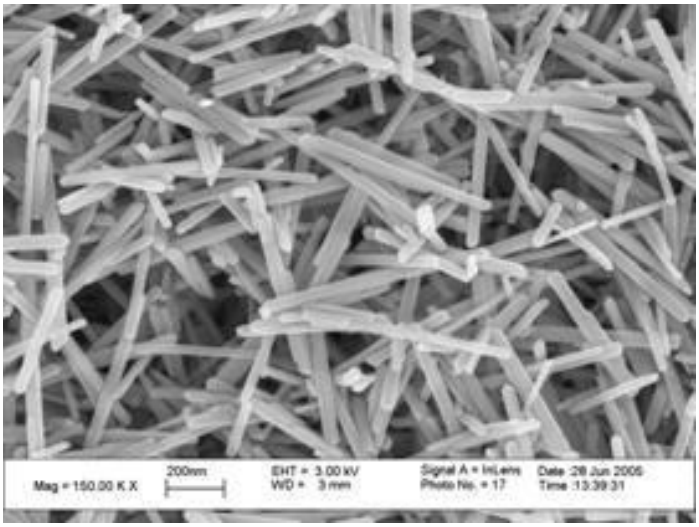

Fig. 31: HA Crystals in dental enamel (White - Color me for Life).

6. APPENDIX

6.1 SOP: Virtis FM25EL Lyophilizer

Written by: Hank Kells | Issue Date: Jan. 19, 2010

Verified by: Dr. Eva J. Helmerhorst | Document nr:

Title: Virtis FM25EL Lyophilizer, serial # 217622

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1. PURPOSE/SCOPE

1.1 To describe the method of using the Virtis FM25 EL Lyophillizer

2. GENERAL

The Virtis Lyophilizer is used to dry aqueous samples. Samples containing acid or organic solvents are not to be put on the lyophilizer.

3. PROCEDURE

3.1) The maximum fill volume of a lyo flask is half of its total volume, ie. the max. fill volume for a 600 ml flask is 300 ml. Do not overfill the flask.
3.2) Always use a clean lyo flask and insert a new filter paper disk and a clean O- Ring into the flask cover every time.
3.3) Shell freeze the flask in the Shell Bath on the Lyophilizer.
- 3.3.1) The Bath should be filled with methanol or acetone until the solvent level is about one inch above the rollers. Make sure the drain hose is plugged before adding solvent to the bath!!! Make sure the bath is turned on (go to main menu) and allow the bath to cool to at least -70º C before proceeding.
- 3.3.2) Insert a polypropylene plug into the flask top. Place a Stainless Steel Bracket Chain above the Bath.
- 3.3.3) Lay the flask in the bath at an angle and make sure the chain goes into the groove in the polypropylene plug.
- 3.3.4) Adjust the chain length so that the fluid in the flask is close to the top but does not come into contact with the flask top.

3.4) After the sample is frozen solid, remove the flask from the Shell Bath and attach it to one of the ports on the manifold using a stainless steel adapter. Rotate the white selector knob 180º to put the flask under vacuum.
3.5) Most samples require at least 24 hours to fully dry. Samples in which the solution is thicker will take longer to dry.
3.6) If the flask feels cold, the sample is not dry yet. The sample is probably dry when the flask is at room temp. and the vacuum reading on the lyophilizer reaches its ultimate low (<20 millitorr).

3.7) Release the vacuum in the flask by turning the white selector knob 180º. Release the vacuum slowly to reduce turbulence inside the flask.

4. Maintenance

4.1) Sign the user's list and enter the number of flasks and the total volume being lyophilized.

4.2) To shut down the Lyophilizer:

- 4.2.1) Release the system vacuum by opening one of the valves on the manifold (turn the white selector knob 180º). Immediately press "Auto" on the control panel, this turns off the compressors and the vacuum pump.
- 4.2.2) Shut down the lyophilizer if it is not going to be used over a long weekend or intersession.
- 4.2.3) To restart: Make sure all the valves on the manifold are closed and press "Auto". Wait for about 30 min. (until the vacuum is <100 millitorr) before adding samples.

4.3) Follow the maintenance log. The vacuum pump oil must be changed monthly.

4.4) Changing the vacuum pump oil:

- 4.4.1) Shut down the lyophilizer (see 4.2).
- 4.4.2) Get an 8 mm Allen Wrench from the drawer opposite the lyophilizer.
- 4.4.3) Open one of the fill ports on the front of the vacuum pump.
- 4.4.4) Attach a length of clear plastic tubing to the drain port on the vacuum pump and place the other end of the tubing into a container.
- 4.4.5) Open the drain valve and drain all of the oil out of the vacuum pump. Elevate the motor end of the pump to ensure all of the oil has been drained.
- 4.4.6) Close the drain valve.
- 4.4.7) Add fresh oil to the vacuum pump with a funnel. Use "Elite Z" Synthetic Hydrocarbon Oil from Eastern Scientific. Add oil until the level is ¾ of the way to the top of the sight glass.
- 4.4.8) replace the plug in the fill port using the 8 mm Allen Wrench.
- 4.4.9) dispose of the used oil as hazardous waste.

4.5 Defrosting the condenser:

- 4.5.1) Shut down the lyophilizer (see 4.2)
- 4.5.2) If a fast defrost is desired, press "DEFR" (Quick Defrost). This melts the outside of the ice cylinder in about one hour. The ice can then be pulled out of the condenser by hand. If a fast defrost is not necessary, the ice in the condenser will melt overnight.
- 4.5.3) Open the condenser door and place a plastic bucket or pan on the floor in front of the lyophilizer. Place a 500 ml Graduated Cylinder under the condenser drain port. Insert the drain hose and connector into the drain port. Place the other end of the drain hose into the bucket.
- 4.5.4) After the ice has melted, remove any remaining ice from the condenser and wipethe condenser chamber dry with paper towels. Wait a few minutes before removing the drain hose to make sure all the water has drained out.

4.5.5) Replace the condenser gasket, if necessary, and close the condenser door.
4.5.6) Restart the lyophilizer (see 4.2 c)

4.6) Alarms

4.6.1) Alarm conditions include: Power Outage, Condenser over Temperature, Vacuum Exceeded and Vacuum Pump Maintenance. The most common one is for Vacuum Pump Maintenance.

4.6.2) The Alarm set point for Vacuum Pump Maintenance is set for 1000 hours. This means that the alarm will sound after the system has been running for 1000 hours to prompt the user to change the oil in the vacuum pump. The vacuum pump oil should be changed on a monthly basis so this alarm is superfluous.

4.6.3) To silence the alarm press MENU on the control panel.

4.6.4) Access the alarm screen by pressing MENU, scroll to “Alarms” and press MENU again. Any alarm currently active will be indicated within the alarm screen by a solid box in front of the active alarm. To clear an alarm use the arrow buttons to scroll to the desired alarm and press the MENU button.

4.6.5) For more information, see the Operator’s Manual.

5. Manual Stoppering Assembly

5.1) Release the vacuum in the lyophilizer by turning one of the white selector knobs in a quickseal valve 180º. Turn off the lyophilizer by pressing “AUTO”. Wait a few minutes and turn on the refrigeration by pressing “COND”. This will keep the condenser cold for faster vacuum pulldown. Remove the Stainless Steel Drum Manifold from the top of the lyophilizer (pull it off vertically) and put it aside.

5.2) Pull the black manifold connector gasket up so that it extends about ½” above the top of the lyophilizer (to ensure vacuum seal) and place the manifold adapter plate on it. The hole in the center of the adapter plate should line up with the hole in the top of the lyophilizer.

5.3) Place two aluminum racks on the bottom shelf of the stoppering assembly to serve as spacers. All of the vials to be stoppered should be placed on the top shelf. No more than 40 (the number of spaces in one Whatman Vial File) 2 ml vials should be lyophilized per run.

5.4) The sample containing vials should be placed in a Whatman Vial File and left in the -80º C. freezer overnight. Freeze the vials without stoppers.

5.5) Pre-cool the stoppering assembly and the stoppers by placing them in the cold room for at least one hour before loading. Sample melting is greatly reduced when the vials are loaded at 4º C rather than room temp.

5.6) When ready, quickly bring the Whatman Vial File from the -80º freezer into the cold room. Remove the frozen vials from the Vial File, partially insert a rubber stopper into the top of each vial and place each vial on the top shelf of the stoppering assembly. The stoppers should be partially inserted so that water vapor can escape from the sample vial. The vials should be evenly distributed on the top shelf of the stoppering assembly.

5.7) When all of the vials are loaded gently and carefully pick up the stoppering assembly and take it to the lyophilizer (ask someone to hold the doors for you). Place the stoppering assembly on the adapter plate so that the three feet of the stoppering assembly are each in one of the three depressions on the adapter plate. Carefully place the acrylic manifold over the stoppering assembly and make sure the gasket on the bottom of the manifold is seated in the groove in the adapter plate. Insert the stoppering crossbar and plug into the hole in the top of the acrylic manifold making sure that the grooves mesh with the T at the top of the stoppering assembly.

5.8) As soon as possible turn on the “VAC” switch to start the vacuum pump. The sooner the vacuum is started, the less chance of sample melting. Make sure that the vacuum level begins to decrease (check the display on the front panel of the lyophilizer) and that there are no obvious vacuum leaks. Check again in 15-20 minutes to make sure that the vacuum level has dropped below 100 milltorr.

5.9) The samples should be dry after 24 hours. Connect one end of a length of Tygon Tubing to the nitrogen tank and the other end to the 3/8” port on the T connector. Take one Quickseal Valve from the Stainless Steel Manifold and use it to plug one end of the T connector. Attach the other end of the T connector to one of the ports on the acrylic manifold.

5.10) Nitrogen Backfill: 1) Turn off “VAC” switch, 2) Open the valve on the nitrogen tank and set the regulator at 10 psi. (you should hear the gas flowing), 3) Open the Quickseal Valve on the Acrylic Manifold by turning the white selector knob 180º, 4) After one minute, close the quickseal valve and stop the backfill. Ideally the lyophilizer should remain under a slight vacuum but it is ok to proceed even if the vacuum is completely released.

5.11) Stoppering: Turn the crossbar clockwise until the top plate of the stoppering assembly makes contact with the stoppers. Then turn the crossbar slowly until the stoppers are fully seated in the vials. Do not overtighten or the vials can be crushed.

5.12) After the vials are stoppered, turn the crossbar counterclockwise to raise the top shelf. It may be necessary to separate the top two shelves manually.

Remove the stoppered vials from the shelf. Put them into a Wheaton Vial File and bring them to the lab.

5.13) Obtain the Aluminum Seals and the Seal Crimping Tool. Place an aluminum seal over the stopper and use the crimping tool to attach it to the vial. Crimp-seal all of the vials and place them in a Wheaton Vial File. Label the Vial File. Place the Vial File in the -20 Freezer and/or give to Eva for long-term storage.

Consumable items (order from Fisher Scientific):

2 ml Thin Wall Serum Vial, Part # 06-406-37, (144/pk)
13 mm Split Rubber Stopper, Part # 06-447E, (1000/pk)
13 mm Aluminum Seals, Part # 06-406-14A, (1000/pk)

6.2 SOP: PRP Isolation by Anion-exchange Chromatography

Written by: Hank Kells — Issue Date: Jan. 22, 2010

Verified by: Dr. Eva J. Helmerhorst — Document nr:

Title: PRP Isolation by Anion-exchange Chromatography

1. PURPOSE/SCOPE

1.2) To describe the procedure for the isolation of acidic Proline-rich Proteins (aPRPs) from human parotid secretion.

2. GENERAL

The acidic PRP family contains various isoforms including PIF-slow, Db-slow and Pa (encoded by the *PRH1* gene) and PRP1 and PRP2 (encoded by the *PRH2* gene). Individuals can be homozygous or heterozygous for *PRH1* and *PRH2*. *PRH1/PRH1* can yield 3x3=9 possible phenotypes, *PRH1/PRH2* can yield 3x2=6 phenotypes, and *PRH2/PRH2* can yield 2x2=4 phenotypes. Thus in total there are 9+6+4=19 different PRP phenotypes. Therefore, for isolating the complete set of PRPs it is important to

include several parotid saliva donors (Allele frequencies have been described in the literature, Hay et al., *J. Dent. Res.* 73(11): 1717-1726, 1994).
PRP1 and PRP2 undergo partial post-translational degradation within the salivary gland, yielding PRP3 and PRP4, respectively, and PIF-slow and Db-slow are partially degraded into PIF-fast and Db-fast. Pa is the only PRP isoform that contains a cysteine residue and forms a dimer (Oppenheim et al., *Ann N Y Acad Sci*. 098:22-50, 2007). All PRP isoforms can be separated by sequential anion-exchange chromatography (DEAE followed by MONO Q) and visualized by Ornstein-Davis PAGE and staining with Amido Black. Once purity of the isolated PRP isoforms is confirmed, proteins are lyophilized and stored for further studies.

3. APPLICABLE DOCUMENTS

3.1) SOP – "Large scale isolation and purification of individual histatins and statherin from parotid secretions using zinc chloride precipitation".
3.2) SOP – "Virtis FM25EL Lyophilizer"
3.3) SOP – "Pharmacia FPLC"
3.4) SOP – "AKTA FPLC"
3.5) SOP – "Ornstein Davis Gel"

4. PROCEDURE

4.1) Parotid Saliva (PS) is delivered to the Lab (about 300 ml per week).
- 4.1.1) PS is subjected to zinc precipitation to deplete the secretion from histatins and statherins, as described in SOP "large scale isolation and purification of histatins and statherin from parotid secretions using zinc chloride precipitation".
- 4.1.2) Obtain the supernatant by centrifugation for 20 minutes at 20,000 RPM (27,000 x g)in the Sorvall centrifuge (SS-34 rotor).

4.2) Dialyze SN using MWCO=1000d membrane (Spectrum Labs, Rancho Dominquez, CA), for 3X4hrs.
4.3) Transfer SN to lyo flasks and lyophilize (see 3.2).
4.4) Transfer dry sample to the square bottle in Freezer #4 labeled "Zn-precipitated HPS, contains no histatins".
4.5) Weigh out 500 mg (or 1000 mg) of Zn precipitated HPS from the square bottle.
- 4.5.1) dissolve in 50 ml (or 100 ml) Buffer A.
- 4.5.2) Centrifuge 20 min. at 20,000 rpm. Use the SS34 Rotor on the Sorvall Centrifuge
- 4.5.3) Remove SN by pipet and discard the pellet.
- 4.5.4) Split SN into equal (25ml) aliquots. Transfer aliquots to 50ml Falcon Tubes with labels (250mg HPS for DEAE).

4.6) Run one aliquot at a time on DEAE Column (old FPLC).
- 4.6.1) Use Buffer A as the mobile phase.
- 4.6.2) Use the "sephvm" method. Manually change the name of the run to include the next number in the sequence.
- 4.6.3) Set fraction collector to 25 min. The flow rate = 1 ml/min.
- 4.6.4) Let Buffer A flow through the column (manual) until all traces are stable

before starting. The 214nm UV trace should be about 0.1 AU.

4.7) DEAE VOID Peak

4.7.1) Combine the eluent from the tubes containing the DEAE Void Peak in a 600 ml lyo flask and lyophilize.

4.7.2) Dissolve the dry sample in water and transfer to a 50ml Falcon Tube in Freezer #4 labeled “VOID”. Keep Void 1 and Void 2 Peaks separate.

4.7.3) When the VOID Tube is full, run the sample (≤50 ml) through the G-25 Column to desalt (old FPLC).

4.7.4) To switch from DEAE to G-25 Column:

- Before changing from DEAE to G-25, let buffer A flow through the DEAE Column until the conductivity drops below 10 mS.
- Switch inlet and outlet lines.
- Place both buffer lines (A&B) into the water container and fill container with water.
- Do pump wash three times to flush lines (turn injection valve to waste and start wash). After three washes turn injection valve to load and inject sample.
- Change method to G25. The next run number in the sequence appears when the run is started.
- Change fraction size to 5 min. The flow rate is 5 ml/min (25 ml fraction size).

4.7.5) Transfer the first peak (from G-25) containing the void proteins to a lyo flask and lyophilize. The second peak contains the salt.

4.7.6) Transfer the lyophilized Void sample to the box labeled “DEAE Void Peaks” in Freezer #4. When the Tube is full, give it to Eva.

4.8) DEAE Peak (contains the acidic, proline rich proteins).

4.8.1) Transfer DEAE Peak to a lyo flask and lyophilize.

4.8.2) Dissolve dry sample in water and transfer to 50ml Falcon Tube in Freezer #4 labeled “DEAE”. This tube must be kept frozen. Dissolved proteins degrade quickly.

4.8.3) When the tube is full, run the sample through the G-25 Column (see instructions above).

4.8.4) Transfer the first peak from the G-25 column (contains the acidic PRP Proteins) to a lyo flask and lyophilize.

4.8.5) Transfer the dry sample to the square bottle labeled “DEAE for MONO Q Column” in Freezer #4.

4.9) AKTA FPLC

4.9.1) Weigh out 50 mg dry “DEAE for MONO Q Column” protein and dissolve in 5 ml Buffer A.

4.9.2) Filter the solution using a syringe filter (wet the filter with Buffer A first) and inject on the MONO Q Column.

4.9.3) Refer to the FPLC AKTA SOP.

4.9.4) Transfer Peaks 1-8, X,Y,Z, etc. to different lyo flasks and lyophilize.

4.9.5) Dissolve the salty samples in water (separately) and transfer to the corresponding Falcon Tubes in Freezer #4.

4.9.6) When a tube is full (or nearly full), run the sample through the G-25 Column to remove the salt and lyophilize.

4.9.7) When the sample is light and fluffy, transfer it to the corresponding tube

on the rack labeled “For Davis Gel” in Freezer #4.

4.10) Davis Gel

4.10.1) Follow SOP “Ornstein David Gel” to confirm the purity and identity of the proteins in the different MONO Q Peaks.

4.11) Final Product

4.11.1) Once the samples are confirmed to be pure, check their concentration using the Cary 50 UV-Vis at 215nm. Add 10µl of sample to 990µl of water in a quartz cuvette. Obtain the OD. The actual concentration of the protein in solution is 5X the OD (mg/ml). The concentration should be at least 1.0 mg/ml. If the concentration is <1.0mg/ml, lyophilize the sample and make it more concentrated.

4.11.2) Label 2ml Autosampler vials. The label should indicate the protein, the amount of protein and the date. Aliquot the samples into the vials. Do not exceed 1.0 ml per vial.

4.11.3) For each vial insert one cotton plug into a black cap and tighten the cap on the vial. Make sure that the liquid sample does not come into contact with the cotton plug.

4.11.4) Place the vials in a plastic box and put the box in the -80º C freezer for 16 h (O/N).

4.11.5) Prepare a 600ml Lyo Flask. Separate the vials into groups of 8-10 vials and place a rubber band around each group of vials. Quickly transfer the frozen vials to the flask and put it on the lyophilizer.

4.11.6) The samples should remain on the lyophilizer for at least 24 hours. After the samples are dry, remove the cotton plugs and insert Teflon discs into each cap (red side up). Give the vials to Eva for storage in the locked -20º C freezer.

5. Equipment

5.1) Virtis FM25EL Lyophilizer, serial # 217622.
5.2) Pharmacia FPLC with DEAE and G-25 Columns.
5.3) AKTA FPLC with MONO Q Column
5.4) Sorvall RC-6+ Centrifuge and SS-34 Rotor

6. Buffers for DEAE and MONO Q Columns

6.1) Stock Solutions

6.1.1) 1M TrisHCl pH=8 (2 liters)

1) Dissolve 242.28 g Tris Base (MW=121.14 g/mol) in 800ml ultrapure water in a 1000ml beaker with a magnetic stirrer.
2) Transfer the solution to a 2 liter graduated cylinder.
3) Use the pH meter to determine the pH of the solution.
4) Fill the cylinder to the 1800 ml mark with water and allow the pH to stabilize.
5) add 1:1 diluted HCl dropwise until the pH stabilizes at 8.0. than add water tol 2 liter line.
6) Transfer the solution to a labeled 2 liter container

6.1.2) 2M NaCl (2 liters)

1) Dissolve 233.76 g NaCl (MW=58.44 g/mol) in 1 liter of ultrapure water in a 1000 ml beaker with a magnetic stirrer.
2) Transfer the solution to a 2 liter graduated cylinder.
3) Fill the cylinder to the 2 liter line with water.
4) Transfer the solution to a labeled 2 liter container.

6.2) Buffers

6.2.1) Buffer A (50mM Tris/HCl and 50 mM NaCl)

1) Add 1800 ml ultrapure water to a 2 liter graduated cylinder.
2) Add 100 ml of 1M TrisHCl pH=8 stock solution and 50 ml of 2M NaCl stock solution to the cylinder.
3) Fill to the 2 liter line with ultrapure water and filter.
4) Transfer solution to a properly labeled bottle.

6.2.2) Buffer B (50mM Tris/HCl and 1M NaCl)

1) Add 800 ml ultrapure water to a 2 liter graduated cylinder.
2) Add 100 ml 1M TrisHCl and 1 liter 2M NaCl to the cylinder.
3) Fill to the 2 liter line with ultrapure water and filter.
4) Transfer solution to a properly labeled bottle

7. "Buffers" for Gel Filtration Columns

The objective here is to desalt saliva samples. For this application, pure water is used as the running "buffer" in gel filtration chromatography.

7. REFERENCES

7.1 List of Journal Abbreviations

Adv Dent Res	Advances in Dental Research
Am J Clin Pathol	The American Journal of Clinical Pathology
Am J Hum Genet	American Journal of Human Genetics
Ann NY Acad Sci	Annals of the New York Academy of Sciences
Arch Biochem Biophys	Archives of Biochemistry and Biophysics
Arch Oral Biol	Archives of Oral Biology
Biochem Genet	Biochemical Genetics
Biochem. J.	The Biochemical Journal
Calcif Tissue Int	Calcified Tissue International
Clin Chem	Clinical Chemistry
Eur J Oral Sci	European Journal of Oral Sciences
Expert Rev Proteomics	Expert Review of Proteomics
Infect Immun	Infection and Immunity
Inorg Perspect Biol Med	Inorganic Perspectives in Biology and Medicine
J Biol Chem	Journal of Biological Chemistry
J Chromatogr B Analyt Technol Biomed Life Sci.	Journal of chromatography. B, Analytical technologies in the biomedical and life sciences
J Dent Res	Journal of Dental Research
J Proteome Res	Journal of Proteome Research
Oral Microbiol Immunol	Oral Microbiology and Immunology
Protein Expr Purif	Protein Expression and Purification

7.2 References

Alberts B, Johnson A, Lewis J et al. (2008). Molecular Biology of the Cell - 5th Edition pg. 89-90. New York, NY: Garland Science.

Atkins P, dePaula J (2002). Physical Chemistry – 7th Edition pg. 989-990. New York, NY: WH Freeman & Co.

Azen EA (1973). Properties of salivary basic proteins showing polymorphism. Biochem Genet 9:69-86.

Azen EA, Kim H-S, Goodman P et al. (1987). Alleles at the PRH1 locus coding for the human salivary-acidic proline-rich proteins Pa, Db and PIF. Am J Hum Genet 41:1035-1047.

Baum BJ, Bird JL, Millar D et al. (1976). Studies on histidine-rich polypeptides from human parotid saliva. Arch Biochem Biophys 177:427-436.

Bennick A (1975). Chemical and physical characteristics of a phosphoprotein from human parotid saliva. Biochem J 145, 557-567.

Bennick A, Cannon M, Madapallimattam G (1979). The nature of the hydroxyapatite-binding site in salivary acidic proline-rich proteins. Biochem J 183, 115-126.

Bio-Rad (1996) Macro-Prep Chromatography Supports - Ceramic Hydroxyapatite. Bulletin 1971, Hercules, CA.

Blicharz TM, Rissin DM, Bowden M et al. (2008). Use of colorimetric test strips for monitoring the effect of hemodialysis on salivary nitrite and uric acid in patients with end-stage renal disease: a proof of principle. Clin Chem 54:1473-80.

Connerty HV, Briggs AR (1966). Determination of serum calcium by means of ortho-cresolphthalein complexone. Am J Clin Pathol 45 290-296.

Davis BJ (1964). Disc Electrophoresis - II: method and application to human serum proteins. Ann NY Acad Sci 121:404-27.

Denny P, Hagen FK, Hardt M et al. (2008). The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. J Proteome Res 7:1994-2006.

Edgar M, Dawes C, O'Mullane D. (2004), Saliva and Oral Health - 3rd Edition. London: British Dental Association.

Flora B, Gusman H, Helmerhorst EJ et al. (2001). A new method for the isolation of histatins 1, 3 and 5 from parotid secretion using zinc precipitation. Protein Expr Purif 23:198-206.

Hay DI (1973). The interaction of human parotid salivary proteins with hydroxyapatite. Arch Oral Biol 18:1517-1529.

Hay DI, Moreno EC, Schlesinger DH (1979). Phosphoprotein-inhibitors of calcium phosphate precipitation from salivary secretions. Inorg Perspect Biol Med 2:271-285.

Hay DI, Smith DJ, Schluckebier SK et al. (1984). Basic biological sciences relationship between concentration of human salivary statherin and Inhibition of calcium phosphate precipitation in stimulated human parotid saliva. J Dent Res 63:857-863.

Hay DI, Bennick A, Schlesinger DH et al. (1988). The primary structures of six human salivary acidic proline-rich-proteins (PRP-1, PRP-2, PRP-3, PRP-4, PIF-s and PIF-f). Biochem J 255:15-21.

Hay DI, Ahern JM, Schluckebier SK et al. (1994). Human salivary acidic proline-rich protein polymorphisms and biosynthesis studied by high-performance liquid chromatography. J Dent Res 73:1717-1726.

Helmerhorst EJ, Flora B, Troxler RF et al. (2004). Dialysis unmasks the fungicidal properties of glandular salivary secretions. Infect Immun 72:2703-9.

Helmerhorst EJ, Alagi AS, Siqueira WL et al. (2006). Oral fluid proteolytic effects on histatin 5 structure and function. Arch Oral Biol 51:1061-70.

Helmerhorst EJ, Oppenheim FG (2007). Saliva: a dynamic proteome. J Dent Res 86:680-693.

Helmerhorst EJ, Traboulsi G, Salih E et al. (2010). Mass spectrometric identification of key proteolytic cleavage sites in statherin affecting mineral homeostasis and bacterial binding domains. J Proteome Res 9:5413-21.

Iontcheva I, Oppenheim FG, Offner GD et al. (2000). Molecular mapping of statherin- and histatin-binding domains in human salivary mucin MG1 (MUC5B) by the yeast two-hybrid system. J Dent Res 79:732-739.

Kay MI, Young RA, Posner AS (1964). Crystal structure of hydroxyapatite. Nature 204:1050 - 1052.

Kousvelari EE, Oppenheim FG, Cutler LS (1982). Ultrastructural Localization of Salivary Acidic Proline-rich Proteins from Macaca fascicularis. J Histochem Cytochem 30:274-278.

Lamkin MS, Jensen JL, Setayesh MR et al. (1991). Salivary cystatin SA-III, a potential precursor of the acquired enamel pellicle, is phosphorylated at both its amino- and carboxyl-terminal regions. Arch Biochem Biophys 288:664-70.

Lamont RJ, Demuth DR, Davis CA et al. (1991). Salivary-agglutinin-mediated adherence of *streptococcus mutans* to early plaque bacteria. Infect Immun 59:3446-3450.

Lendenmann U, Grogan J, Oppenheim FG (2000). Saliva and dental pellicle – a review. Adv Dent Res 14:22-8.

Li J, Helmerhorst EJ, Corley RB et al. (2003). Characterization of the immunologic responses to human in vivo acquired enamel pellicle as a novel means to investigate its composition. Oral Microbiol Immunol 18:183-91.

Mandel ID, Ellison SA (1965). Organic components of human parotid and submaxillary saliva. Ann NY Acad Sci 131:802-11.

Milan AM, Sugars RV, Embery G et al. (2006). Adsorption and interactions of dentine phosphoprotein with HA and collagen. Eur J Oral Sci 114:223-231.

Minaguchi K, Madapallimattam G, Bennick A (1988). The presence and origin of phosphopeptides in human saliva. Biochem J 250:171-177.

Moreno EC, Zahradnik RT (1979). Demineralization and Remineralization of Dental Enamel. J Dent Res 58(Spec Issue B):896-903.

Oppenheim FG, Hay DI, Franzblau C (1971). Proline-rich proteins from human parotid saliva: isolation and partial characterization. Biochem J 10:4233-4238.

Oppenheim FG. (1973) Protein Components of Saliva (doctoral dissertation). Boston University, Boston.

Oppenheim FG, Yang Y-C, Diamond RD et al. (1986). The primary structure and functional characterization of the neutral histidine-rich polypeptide from human parotid secretion. J Biol Chem 261:1177-1182.

Ornstein L (1964). Disc electrophoresis I: background and theory. Ann NY Acad Sci 121:321-49.

Ruhl S (2012). The scientific exploration of saliva in the post-proteomic era: from database back to basic function. Expert Rev Proteomics 9:85-96.

Schipper R, Loof A, de Groot J et al. (2007). SELDI-TOF-MS of saliva: methodology and pre-treatment effects. J Chromatogr B Analyt Technol Biomed Life Sci. 847:45-53.

Schlesinger DH, Hay DI (1977). Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva. J Biol Chem 252:1689-1695.

Schwartz SS, Hay DI, Schluckebier SK et al. (1992). Inhibition of calcium phosphate precipitation by human salivary statherin: structure-activity relationships. Calcif Tissue Int 50:511-517.

Siqueira WL, Zhang W, Helmerhorst EJ et al. (2007). Identification of protein components in in vivo human acquired enamel pellicle using LC-ESI-MS/MS. J Proteome Res 6:2152-60.

Siqueira WL, Oppenheim FG (2009). Small molecular weight proteins/peptides present in the in vivo formed human acquired enamel pellicle. Arch Oral Bio l 54:437-444.

Tamaki N, Tada T, Morita M et al. (2002). Comparison of inhibitory activity on calcium phosphate precipitation by acidic proline-rich proteins, statherin and histatin-1. Calcif Tissue Int 71:59-62.

Thomadaki K, Helmerhorst EJ, Tian N et al. (2011). Whole saliva proteolysis and its impact on salivary diagnosis. J Dent Res 90:1325-1330.

Traboulsi G (2011). Proteolytic Cleavage Sites in Statherin (masters dissertation). Boston University, Boston.

Troxler RF, Offner GD, Xu T et al. (1990) Structural relationship between human salivary histatins. J Dent Res 69:2-6.

Truelove EL, Bixler D, Merritt AD (1967). Simplified method for collection of pure submandibular saliva in large volumes. J Dent Res 46(6):1400-3.

Weber K, Osborn M (1969). The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J Biol Chem 244:4406-4412.

Work A (1981). Laboratory Techniques in Biochem and Molecular Biology. New York, NY: Elsevier Science.

Yao Y, Berg EA, Costello CE et al. (2003). Identification of Protein Components in Human Acquired Enamel Pellicle and Whole Saliva Using Novel Proteomics Approaches. J Biol Chem 278:5300-5308.

Yin A, Margolis HC, Grogan J et al. (2003). Physical parameters of hydroxyapatite adsorption and effect on candidacidal activity of histatins. Arch Oral Biol 48:361-368.

Yin A, Margolis HC, Yao Y et al. (2006). Multi-component adsorption model for pellicle formation: The influence of salivary proteins and non-salivary phospho proteins on the binding of histatin 5 onto hydroxyapatite. Arch Oral Biol 51:102-110.

Zahradnik RT, Propas D, Moreno EC (1978). Effect of salivary formation time on in vitro attachment and demineralization by Streptococcus mutans. J Dent Res 57:1036-1042.

Zakhary GM, Clark RM, Bidichandani SI et al. (2007). Acidic proline-rich protein Db and caries in young children. J Dent Res 86:1176-1180.

Zumdahl SS, Zumdahl SA (2006). Chemistry – 6th Edition pg. 675. Boston: Houghton Mifflin.

Webpages:

Provost J, MSUM Biotech - Chromatography, Theories, FPLC and beyond. [Cited: August 29, 2012.] http://webmnstate.edu/biotech/chrom_fplc.pdf.

Pentax. Pentax New Ceramics Site. [Online] [Cited: Nov. 18, 2012.] http://www.bone.pentax.jp/cht_e.php.

White - Color me for Life. [Online] [Cited: Nov. 18, 2012.] http://colorme4life.wordpress.com/life-colors/white/.

8. VITA

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