Characterisation of Human Salivary Proteins Inhibiting the Acid Demineralisation of Powdered Hydroxyapatite Substrates and Natural Enamel Surfaces

Nikoletta Pechlivani

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The adsorption of salivary proteins to enamel is critical for the protection against acid demineralisation but it is unclear which proteins are involved in protection or the mechanism by which they act. The aim was to investigate salivary protein adsorption and protection using powdered synthetic hydroxyapatite, powdered enamel and natural enamel as substrates and to characterise the specific salivary proteins providing protection. Human whole salivary proteins and its purified fractions were adsorbed to the various substrates and challenged with acid. Adsorbed proteins were characterised by SDS-PAGE and their protective efficacy determined by spectrophotometric assay of phosphate released into the acid during dissolution. A significantly higher degree of protection was provided by salivary proteins when adsorbed to natural enamel surfaces. It was found that the protective species in saliva reside in a small subset comprising 15% of the total protein and efficacy is dependent on conformation. The desorption of salivary proteins during an acid challenge correlated with loss of protection and it was hypothesised that the proteins desorbed during the challenge may be the protective species. Protein S100-A8 and S100 were identified as members of these putative protective proteins. Many proteins (including protein S100-A8 and S100) remained adsorbed to enamel even after 19 acid challenges but they were not protective. Size exclusion chromatography under non-denaturing conditions resulted in co-elution of two proteins identified as α-amylase and cystatin. Their adsorption behaviour was dependent on the actual substrate used. This thesis demonstrates that the use of powdered substrates may not be ideal substitutes for natural enamel surfaces when investigating salivary protein adsorption and the effect of salivary proteins on enamel demineralisation. This is an important consideration as correctly identifying the protective species and understanding the mechanism of protection will inform the development of prophylactic/therapeutic peptides for clinical use in dry mouth cases.

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Introduction

1.1 Dental enamel

The human tooth consists of three major hard tissues, enamel, dentine, cementum and a soft tissue, the pulp. Dental enamel is the outer part of the crown of the tooth and is the most mineralised biological tissue in the human body. Dental enamel is characterised by high density and is supported on dentine which is a less mineralised tissue with a lower density. Enamel is very hard but brittle so dentine functions as a supporting cushion (Jagr et al., 2014).

1.1.1 Structure and chemical composition of enamel

The enamel is primarily composed of inorganic material (at least 95% of its weight) and a very small amount of protein and other components such as water. The inorganic material is primarily hydroxyapatite (HAP) and exists in the form of long crystals which are arranged in bundles, called prisms. The crystals grow in length (c-axis growth) during the secretory phase of the enamel formation (amelogenesis) during which ameloblasts secrete enamel proteins (the three most abundant being amelogenin, ameloblastin and enamelin). The thickness of the enamel is defined by the termination of c-axis growth. At this point enamel development enters the maturation phase and the secretion of enamel proteins ends, the enamel matrix is degraded and removed, and the crystals grow in width and thickness until the tissue volume is almost totally occluded with mineral (Robinson et al., 2000, Simmer and Hu, 2001).

HAP, the main component of dental enamel, consists of calcium, phosphate and hydroxyl ions and has the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. However, small amounts of other elements, such as sodium, magnesium, potassium

as well as water and other minor ionic components such as carbonate and fluoride are also found in enamel (Weatherell, 1975).

The chemical composition of the enamel varies from the surface to the interior of the enamel. The concentrations of phosphate (17.25 to 18.25%) and calcium (36.5 to 38.5%) are high in the enamel surface and they decrease towards the interior of the enamel and so does the mineral density. Conversely, the concentration of carbonate, magnesium and sodium are low in the enamel surface and increase towards the interior of the enamel. The amount of carbonate increases from about 1% (of tissue weight) in the surface of the enamel to about 4% in the interior of the enamel. Similarly, the magnesium concentration rises from about 0.1% found in the enamel surface to 0.5% in the interior (Robinson et al., 1995). The fluoride content also varies across the different parts of enamel, with low concentration in the interior and high levels of fluoride found in the enamel surface. The high concentration of fluoride at the surface of enamel mostly arises from the period of tooth development but fluoride levels in the drinking water, diet and toothpaste have an impact on the concentration of fluoride in the enamel as it is incorporated into the surface of enamel. Fluoride, which is characterised by its small size and high electronegativity, can replace the hydroxyl ions of HAP which results in a more stabilised (less soluble) crystal structure by facilitating stronger bonds with calcium ions (Weatherell, 1975, Weatherell et al., 1975, Robinson et al., 2000). Calcium and phosphate can also be substituted. For example, phosphate can be replaced by the smaller carbonate ion and the HAP lattice dimensions are reduced creating a less stable (more soluble) mineral structure. When carbonate replaces the hydroxyl ions which are smaller, the HAP lattice becomes bigger creating a more soluble mineral by introducing a strain into the structure. Calcium can be replaced by magnesium which also results in a less stable (more soluble) crystal structure. These heteroionic substitutions cause changes in the structure and charge of the enamel and also affect its solubility. It is interesting that the term apatite is derived from the Greek word απατάω which means to deceive. The mineral was named apatite by Werner in 1786 because, due to its diversity in form and colour, it had previously been

confused with other minerals with similar appearance such as beryl and fluorite. (Weatherell, 1975, Robinson et al., 2000).

1.1.2 Enamel Pathologies

Acid erosion, dental caries and fluorosis are three main examples of oral health problems that affect the dental enamel. Acid erosion is characterised by dissolution of the enamel crystals causing softening of the enamel surface, and subsequent loss of dental hard tissue due to reduced resilience to wear. The acids responsible for acid erosion are not products of bacteria but are dietary or gastric in origin. Environmental factors such as exposure to acids by factory workers or swimming in chlorinated swimming pools have also been implicated in acid erosion, (Lussi et al., 2011, Zero, 1996). The loss of enamel will be permanent if the acid exposure persists and the softened surface of the enamel is actually lost.

Dental caries is characterised by demineralisation of the enamel as a result of acid production by plaque bacteria following the metabolism of dietary sugars which cause a decrease of pH in plaque. Factors such as saliva and fluoride can play an important role in protecting the integrity of the tooth and, therefore, demineralisation of the enamel caused by caries can be reversible (West and Joiner, 2014). Dental caries will be discussed more in section 1.3.5.

Fluorosis occurs as the result of increased levels of fluoride during amelogenesis, before tooth eruption, which can lead to enamel defects such as white specks or rough enamel surface (Carey, 2014). The mechanism involved is still under investigation but has been linked to fluoride inhibiting the removal of enamel matrix proteins prior to the enamel maturation stage which inhibits maturation stage crystal growth (Den Besten, 1999). However, fluoride has no inhibitory effect on MMP20 or KLK4; the proteases responsible for processing and degrading the developing enamel protein matrix (Tye et al., 2011). More recently, fluoride has been shown to impact directly on ameloblast (and osteoblast) cell biology leading to an

endoplasmic reticulum stress (ER stress) response that includes reduced protein synthesis and cell apoptosis (Sierant and Bartlett, 2012, Liu et al., 2015).

There are also genetic disorders of enamel collectively called amelogenesis imperfecta (AI). AI is a relatively rare inherited disorder, with an incidence of 1:700 to 1:14000 depending on the population (Crawford et al., 2007), and causes changes in the structure and appearance of the dental enamel. Hypoplasia, hypomineralisation, discolouration and sensitivity are some symptoms associated with AI but the disease can have a large impact on quality of life issues. Mutations in the amelogenin, ameloblastin and enamelin genes have been linked to AI (Crawford et al., 2007, Gadhia et al., 2012). It is interesting to note that a specific amino acid substitution in amelogenin expressed in a mouse AI model results in ER stress and ameloblast apoptosis (Brookes et al., 2014). This finding suggests that a common mechanism might underpin fluorosis and certain cases of AI.

1.2 Chemistry of de- and remineralisation

In acid erosion and dental caries the enamel dissolves due to a decrease in the pH caused by acid in the oral environment. The pH and titratable acidity of the solution around the enamel and the solubility of the enamel itself are important factors for the maintenance of the tooth integrity. The pH value at which an oral fluid which is in direct contact with the enamel is just saturated with phosphate and calcium ions with respect to enamel mineral is known as the critical pH. At pH values lower than the critical pH, the oral fluid is unsaturated and HAP starts to dissolve in order to achieve saturation of the oral fluid (Dawes, 2003).

At pH values higher than the critical pH, the oral fluid becomes supersaturated, and more phosphate and calcium ions precipitate. The value of the critical pH depends on the concentration of phosphate calcium in the

fluid surrounding the enamel mineral but a value of pH 5.5 is commonly quoted for the situation *in vivo*. The ability of a salt to dissolve in a solution is characterised by its solubility product constant Ksp which is dependent on the concentrations of the component ions in a saturated solution at equilibrium. The Ksp of HAP is very small, in other words dental enamel does not dissolve easily (Dawes, 2003).

However, below the critical pH, when acid is present, the protons react with hydroxyl ions and phosphate ions, the concentrations of hydroxyl and phosphate ions are reduced. According to Le Chatelier's principle, solid HAP dissolves to release more phosphate ions and hydroxide (and calcium ions) to restore equilibrium - in other words, the acid dissolves the HAP. In the mouth, acid dissolution of the enamel drives caries and erosion (West and Joiner, 2014).

The presence of acid also leads to the protonation of phosphate and hydroxide ions in the HAP ionic lattice. This reduces the negative charge on these ions leading to a charge imbalance which destabilises the lattice and promotes dissolution.

As described above, fluoride ions can substitute for hydroxyls in the HAP lattice and protect against acid demineralisation. The fluoride ion is much less likely to be protonated than hydroxyl and this has been suggested to explain in part the anti-caries effect of fluoride. Fluoride is known to inhibit demineralisation by adsorbing to the enamel crystal surfaces and preventing the mineral dissolution. Fluoride is also known to enhance the remineralisation by adsorbing to the enamel crystal surface and attracting calcium ions which then attract phosphate ions, resulting in the growth of a new fluoridated (less soluble) mineral. The last mechanism by which fluoride acts against caries is by inhibiting bacterial metabolism by diffusing into the bacteria in the form of hydrogen fluoride and inhibiting the function of enzymes responsible for the metabolism of carbohydrates (Featherstone, 2000).

Saliva also provides a natural protective mechanism against acid demineralisation of the enamel (properties and functions of saliva will be

discussed in more detail later in this chapter). Saliva contains calcium and phosphate ions, which together with fluoride also contribute to the reverse process of demineralisation, the remineralisation of the tooth enamel (Featherstone, 2008).

The enamel goes through many cycles of demineralisation and remineralisation after its development and eruption. Remineralisation in the presence of fluoride results in the reprecipitation of a more fluoridated mineral phase which is subsequently more resistant to acid challenges (Featherstone, 2000). Therefore, the pH and the concentrations of ions such as phosphate, calcium and fluoride (from saliva or other sources) surrounding the enamel play the most important role in the balance between the demineralisation and remineralisation processes and the subsequent fate of the tooth. In addition to this, the ability of these ions, when they are available, to interact with the enamel surface might be restricted due to the layer of organic pellicle that covers the enamel surface (Gonzalez-Cabezas, 2010). The role of this organic layer covering the enamel surface will be discussed further later in this chapter. Furthermore, advances in dental materials have introduced new methods of interfering with the demineralisation and remineralisation cycles. For instance, self-assembling peptide scaffolds were shown to be capable of increasing remineralisation and inhibiting demineralisation *in vitro* (Kirkham et al., 2007).

1.3 Saliva

Saliva is a clear, multifunctional and complex biological fluid which is secreted into the mouth by salivary glands. It is a very dilute fluid consisting of more than 99% water and its normal pH is slightly acidic (6.0-7.0). Saliva is composed of various inorganic and organic components which are responsible for a number of important functions. The presence of saliva is vital to the maintenance of oral health and its use as a diagnostic tool for oral

and systemic diseases makes saliva a very important human body fluid (Humphrey and Williamson, 2001).

Saliva as a biomarker for oral and systemic diseases

The knowledge and understanding of human saliva composition and its properties have led to the use of saliva as a biomarker for a number of oral and systemic diseases. Several compounds with diagnostic value, such as proteins, may be found in lower concentrations in saliva compared to blood samples; however, the use of advanced methods allows the detection of biomarkers of diseases even when present in low levels of saliva components.

Moreover, most of the advantages of using saliva over blood or urine for diagnostic purposes are associated with the sample collection procedure which is fast, easy, non-invasive, stress free, cost-effective and a safe method for healthcare professionals and patients. Furthermore, the fact that no specific equipment and no special training for saliva collection is required, makes saliva a useful and inexpensive tool for screening large populations (Greabu et al., 2009, Lee and Wong, 2009).

Elevated or decreased levels of various salivary components can indicate susceptibility to a disease or diagnosis of a particular disease. Differences in protein concentrations or increased numbers of oral bacteria can help the prognosis or diagnosis of oral diseases, such as dental caries and periodontal disease (Al Kawas et al., 2012). For example, Vitorino et al. (2005, 2006), in *in vivo* and *in vitro* studies of pellicle composition, found increased levels of salivary proteins such as cystatin S, statherin, PRPs, histatin 1 and IL-2 in a caries-free group compared to the caries-susceptible group which contained mainly amylase, lactoferrin and IgA (Vitorino et al., 2005, Vitorino et al., 2006).

Elevated levels of other salivary proteins were found in patients with oral squamous cell carcinoma (OSCC) compared to healthy subjects. For

instance, Hu et al. (2008), showed that many salivary proteins were present in both oral squamous cell carcinoma patients and healthy subjects; however, some proteins were differentially expressed. Five proteins, M2BP, MRP14, CD59, catalase and profilin were significantly increased in OSCC patients so they were identified as candidate biomarkers for oral squamous cell carcinoma (Hu et al., 2008). The current detection of OSCC depends on clinical examination and biopsy but potential diagnostic salivary biomarkers for OSCC may be clinically useful in the future (Yakob et al., 2014).

Various salivary proteins have been identified as potential biomarkers for the diagnosis of Sjogren's syndrome, an autoimmune disease associated with xerostomia. For instance, increased concentrations of β2-microglobulin, lactoferrin, Ig *k* light chain, polymeric Ig receptor, lysozyme C and cystatin C were found in Sjogren's syndrome patients compared to non-Sjogren's syndrome subjects with complaints of xerostomia. In addition, decreased levels of acinar proteins, proline-rich proteins, amylase and carbonic anhydrase VI were found in Sjogren's syndrome patients (Ryu et al., 2006). In the recent study by Delaleu et al., potential salivary biomarkers for Sjogren's syndrome were identified including interleukin-4 (IL-4), IL-5, and clusterin (Delaleu et al., 2015). However, further validation of the salivary biomarker signatures is needed before they can be used clinically for the detection of the disease, replacing or assisting the current salivary gland biopsy examination (Tzioufas and Kapsogeorgou, 2015).

Saliva can also be useful in the diagnosis of systemic diseases such as cancer (e.g. elevated levels of tumour marker CA125 in saliva of epithelial ovarian cancer patients), cardiovascular diseases (e.g. low levels of salivary amylase related to ruptured aortic aneurysm patients) and viral infections (e.g. decreased levels of salivary IgA linked with HIV infected patients). Saliva is also useful for detecting and measuring drugs, including alcohol and tobacco (e.g. salivary nicotine levels) (Greabu et al., 2009).

1.3.1 Saliva production

Saliva is secreted by the major salivary glands and a large number of minor salivary glands (section 1.3.2). The types of cells found in salivary glands are acinar cells, ductal system cells and myoepithelial cells (Figure 1 is adapted from Edgar et al., 2004). Different glands can produce serous secretions which are rich in water and α-amylase, mucous secretions which are glycoprotein (mucin) rich, or mixed serous and mucous secretions.

The mechanism of salivary secretion includes pathways that regulate electrolytes, fluid and protein secretion after stimulation by sympathetic and parasympathetic nerves (Humphrey and Williamson, 2001, Diaz-Arnold and Marek, 2002). Sympathetic pstimulation of β-adrenergic receptors is responsible for activating the production of the target enzyme cyclic adenosine monophosphate (cAMP) which results in protein secretion in saliva. Parasympathetic nerves activate the muscarinic M3 receptors, and the consequent activation of the target enzyme phospholipase C promotes the increase of intracellular calcium which results in the fluid secretion of saliva (Edgar et al., 2004).

Saliva is made in the serous or mucous acinar cells; this initial isotonic fluid is then driven by an osmotic gradient across the ductal system where it is converted to a hypotonic fluid by reduction in total ion concertation before finally passing into the mouth (Figure 1) (Turner and Sugiya, 2002, Carpenter, 2013).

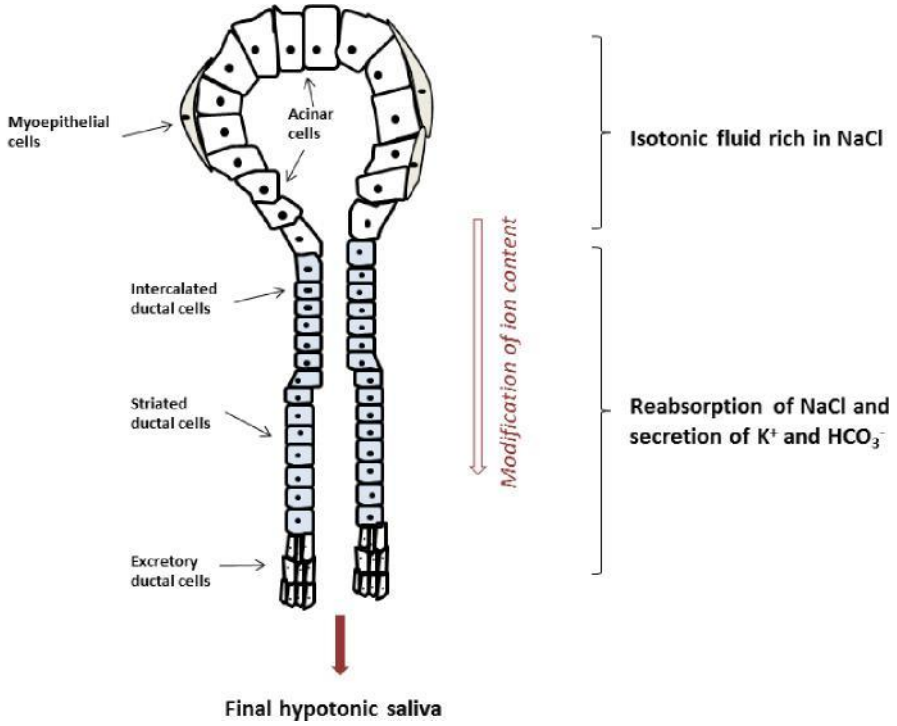


Figure 1. Salivary gland structure and function. Salivary glands are composed of acinar, myoepithelial and ductal cells. There are three types of ductal cells. Saliva is made in the acinar cells, passes through the intercalated ductal cells, enters the striated ductal cells and finally passes through the excretory ductal cells and into the mouth. The ion content of the isotonic fluid is modified when it passes through the ductal system resulting in a hypotonic secretion. Figure is adapted from Edgar et al. (2004).

1.3.2 Salivary glands

Salivary glands are divided into the three major and hundreds of minor glands. The major salivary glands include the parotid, submandibular and sublingual glands and the minor salivary glands include a large number of smaller glands that are located in different areas of the oral cavity such as the tongue, cheeks, palate and lips. Each gland produces a distinct and characteristic salivary secretion. For example, parotid salivary glands produce a serous watery secretion, whereas submandibular and sublingual glands secrete a more viscous saliva which contains high levels of mucins

(Schipper et al., 2007). Furthermore, the contribution made by each salivary gland to the total amount of saliva in the mouth varies. The largest contributor of stimulated saliva is the parotid gland (more than 50%), followed by the submandibular (35%) and the sublingual (8%) whereas unstimulated saliva is composed mainly of submandibular (65%) and sublingual (8%) secretions and less of parotid (20%) secretion (Humphrey and Williamson, 2001, Edgar et al., 2004). The amount of saliva produced by the minor glands in both stimulated and unstimulated saliva is relatively small (less than 10%), albeit very important for the composition of whole saliva (Sreebny, 2000). Table 1 summarises the major types of salivary glands, type of secretion and their contribution to unstimulated and stimulated whole saliva.

Table 1. The main salivary glands, their characteristic secretions, and their overall contribution to the unstimulated whole salivary volume.

Gland	Secretion	Contribution to unstimulated saliva	Contribution to stimulated saliva
Parotid	Serous	20%	50%
Submandibular	Serous and mucous	65%	35%
Sublingual	Mucous	8%	8%
Minor glands	Mucous	<10%	<10%

1.3.3 Salivary flow rate

Salivary flow rates vary amongst individuals and can be affected by different factors such as age, medication and diseases. Salivary flow rates are also characterised by circadian variation with maximum levels in the late afternoon and insignificant levels during sleep (Edgar et al., 2004). It has also been reported that salivary flow rates are affected by the circannual cycle. A study carried out in Texas showed that, during the summer, saliva

flow rates were lower probably due to dehydration (de Almeida Pdel et al., 2008). On average, between 0.5 and 1.5 L of saliva is secreted every day. The normal flow range for unstimulated whole saliva is 0.3 to 0.4 mL/min and for stimulated saliva the normal flow rate is 1 to 2 mL/min. Values below 0.1 mL/min and 0.5 mL/min, for unstimulated and stimulated whole saliva, respectively, are considered as hypofunctional. Flow rates of unstimulated saliva less than 0.1 mL/min are associated with xerostomia, the subjective feeling of dry mouth (Sreebny and Valdini, 1987, Sreebny, 2000, Humphrey and Williamson, 2001).

1.3.4 Composition and functions of saliva

Saliva is composed of 99% water, while the other 1% consists of proteins and inorganic substances. Proteins found in saliva include immunoglobulins, glycoproteins, enzymes, antimicrobial peptides and other proteins such as statherin, cystatin, histatin and proline-rich proteins. The inorganic part of saliva is composed of a variety of electrolytes, including sodium, potassium, calcium, chloride, bicarbonate and phosphate (Humphrey and Williamson, 2001, Schipper et al., 2007). Table 2 shows the amounts and concentrations of the main inorganic and organic components of unstimulated and stimulated saliva (Edgar et al., 2012) (the table is adapted from Edgar et al. 2012).

Table 2. Inorganic and organic constituents of unstimulated and stimulated whole saliva (adapted from Edgar et al. 2012)

	Unstimulated	Stimulated
Inorganic components (mmol/L)		
Phosphate	5.69	2.70
Calcium	1.32	1.47
Sodium	5.76	20.67
Potassium	19.47	13.62
Chloride	16.40	18.09
Bicarbonate	5.47	16.03
Organic components		
Total protein (mg/L)	1630	1350
Total lipids (mg/L)	12.1	13.6
Amino acids (µmol/L)	780	567
Ammonia (mmol/L)	6.86	2.57
Urea (mmol/L)	3.57	2.65

Saliva performs a number of very important functions that maintain the oral health. These can be summarised into five major categories: 1) lubrication and protection, 2) buffering action and clearance, 3) antimicrobial activity, 4) maintenance of tooth integrity, and 5) taste and digestion (Humphrey and Williamson, 2001). It is important to note that the biological function of salivary proteins can be dependent upon their conformation or the formation of homotypic and heterotypic complexes with other similar or different salivary proteins. Furthermore, most salivary proteins are multifunctional, can have overlapping functions and a single protein can have both protective and harmful properties (Levine, 1993). Figure 2 depicts the functions of saliva and the main salivary proteins associated with each function (figure is

adapted and updated from Levine, 1993; Humphrey and Williamson, 2001; Devine and Cosseau, 2008 and Carpenter, 2013). Some of the proteins are involved in more than one activity, emphasizing the multifunctional nature of salivary molecules.

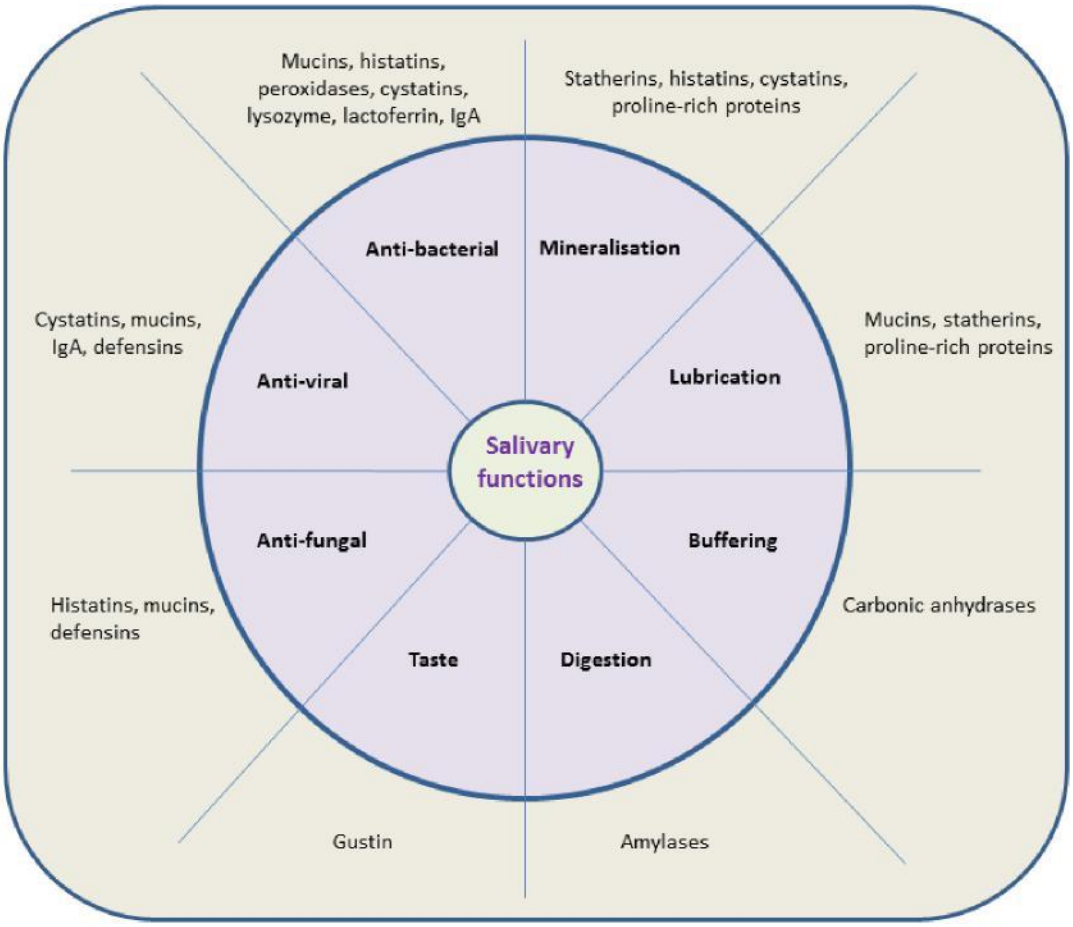


Figure 2. Functions of saliva and the main salivary proteins involved (adapted and updated from Levine, 1993; Humphrey and Williamson, 2001; Devine and Cosseau, 2008 and Carpenter, 2013).

1.3.5 Role of saliva in oral health and disease

The oral environment is constantly moistened with saliva at a neutral pH, is kept warm and frequently flooded with sources of carbon and nitrogen (dietary components) which all make for a highly favourable environment for the growth of micro-organisms. Saliva and bacteria form a layer on the enamel surface (dental plaque) and the microbiota in dental plaque is distinct in health and disease. The composition of the oral microbiota can remain stable over time (microbial homeostasis) but various factors, such as poor oral hygiene, sugar-rich diet, reduced salivary flow and smoking, can cause disruption of this stability within the microbial communities and lead to disease. The balance between health and disease being dependent on the relationship between the oral microbiota and the host environment is the basis of the ecological plaque hypothesis (Marsh, 2003).

Dental caries is a major oral health problem that affects children and adults having a significant impact on the quality of life. According to the FDI World Dental Federation report (Oral Health Worldwide) in 2012, dental caries is the most common childhood disease (worldwide, between 60 and 90% of school children have dental caries).

It has been shown that dental caries (tooth decay) is associated with acidogenic and acid tolerant bacteria such as mutans streptococci and lactobacilli. The consumption of dietary fermentable carbohydrates, which are catabolised to acids (e.g. lactic acid) creating a low pH, promote the growth of the acidogenic and acid tolerant bacteria and the acid production results in the demineralisation of the enamel (Marsh, 2003, Marsh, 2006).

High saliva flow rates have been associated with fast clearance and better buffering action, while low flow rates may suggest slow clearance, low buffering capacity and susceptibility to colonisation by microbes. The link between flow rate and buffering is obvious from Table 2, which shows that the concentration of bicarbonate is increased by about 3-fold in stimulated saliva. The evolutionary advantage here is that food in the mouth and the action of chewing triggers the salivary response so both flow rate (clearance

rate) and buffering power are increased when potentially erosive or fermentable material is present in the mouth. Various salivary proteins have the ability to adsorb to enamel surfaces, inhibit enamel dissolution and the growth of bacteria, which are important properties for protection against enamel dissolution (Lenander-Lumikari and Loimaranta, 2000). This is discussed in more detail in section 1.4.

Xerostomia

Xerostomia is the subjective feeling of dry mouth which is usually associated with decreased secretion of unstimulated saliva, while hyposalivation is the objective finding of reduced saliva flow rate (Nederfors, 2000). In this review, the term xerostomia is used to describe patients that complain of dry mouth and have salivary hypofunction too. Unstimulated saliva flow rates lower than 0.1 mL/min can be regarded as hyposalivation. The most common methods for the diagnosis of hyposalivation are the measurement of saliva flow rate at rest or under stimulation as well as questionnaires or interviews (Villa et al., 2015). The most important factors known to cause xerostomia and hyposalivation are drugs that restrict the salivary flow, a number of systemic diseases such as Sjogren's syndrome and radiation therapy in patients with neck and head cancer. The treatment of xerostomia and hyposalivation is aimed at the alleviation of symptoms by increasing the saliva flow when possible or protection of the oral mucosa by providing moisture by other means (saliva substitutes, mechanical or gustatory stimulants). There are also specific drugs that promote the increase of saliva flow, such as pilocarpine, which stimulates the secretion by salivary glands. Xerostomia patients may also benefit from the elimination of the drugs causing xerostomia, or the decrease of their dosage, or the replacement of these medicines (Sreebny and Valdini, 1987, Saleh et al., 2015).

The protein composition of unstimulated whole saliva and residual saliva (saliva remaining in the mouth after swallowing) plays an important role in regulating various functions in the mouth. Pramanik et al. (2010) showed

that xerostomia patients had reduced amounts of residual saliva on mucosal surfaces but this saliva contained a higher protein concentration compared to saliva from healthy subjects. It was also shown that some proteins, in particular, high-molecular-weight mucin, cystatin S and statherin were present in dry mouth patients despite the decreased saliva flow rate (Pramanik et al., 2010). Their results were in agreement with those of a previous study from Lee et al. (2002) in which it was found that patients with severe xerostomia had the highest protein concentrations of residual saliva compared to the other two groups of patients with milder form of xerostomia and control subjects (Lee et al., 2002). This may suggest that the biosynthesis of salivary proteins is not tightly coordinated with a reduced flow rate in severely affected xerostomia patients. In contrast, in healthy volunteers the total protein content is similar regardless of the volume being secreted (see Table 2) suggesting that biosynthetic control of salivary proteins is compromised in patients with the severe form of xerostomia.

1.4 Major salivary proteins

Salivary proteins can be divided into six major families: the proline-rich proteins (PRPs), statherins, cystatins, histatins, amylases, mucins and antimicrobial proteins. This classification is somewhat clumsy as there is cross over between the groups, e.g. histatins are antibacterial but also have other functions such as complexing dietary tannins in order to reduce the inhibitory impact of tannins on digestive enzymes (Yan and Bennick, 1995). The concentrations of salivary proteins found in human parotid, submandibular/sublingual and whole saliva are shown in Table 3 (table is adapted from Huq et al., 2007, Oppenheim et al., 2007 and Levine 2011). Some of the main functions of the major salivary proteins are described next (Oppenheim et al., 2007, Levine, 2011, Huq et al., 2007).

Table 3. Concentrations of salivary proteins in salivary gland secretions and whole saliva (adapted from Huq et al., 2007, Oppenheim et al., 2007 and Levine, 2011)

Protein	Concentration in saliva (µg/mL)		
	Whole saliva	**Parotid**	**Submandibular/ sublingual**
Amylase	380-500	650-2600	-
MG1 (MUC5B)	80-500	-	80-560
MG2 (MUC7)	10-200	-	21-230
PRPs	90-180	230-1251	270-1335
Cystatin	240-280	2-4	92-280
Histatins	2-30	30-55	13-70
Statherins	2-12	16-147	20-150
sIgA	19-439	20-230	41-56
Lactoferrin	194	12	13
Lysozyme		7	21

The salivary proteome comprises several hundred proteins and it is fair to say that the role of most of these specific components is unclear. However, some proteins are relatively well studied and will be introduced below.

Proline-rich proteins

Proline-rich proteins (PRPs) are secreted by parotid and submandibular glands and constitute 70% of the total human parotid saliva secretion. They are highly polymorphic, contain a large amount of proline and can be divided into acidic, basic and glycosylated sub groups. The acidic PRPs are encoded by two gene loci, *PRH1* and *PRH2*, which contain several alleles

while basic PRPs are encoded by genes in four separate loci comprising *PRB1, PRB2, PRB3,* and *PRB4*, which also contain various alleles resulting in a variety of PRP polymorphisms (Oppenheim et al., 2007). The acidic and basic PRPs have molecular mass of 10-40 kDa and the glycosylated form a mass of 60-70 kDa (Lamkin and Oppenheim, 1993, Carpenter, 2013, Fabian et al., 2012). The main activities of the acidic PRPs include calcium binding, HAP binding, inhibition of enamel crystal growth and a role in formation of the acquired enamel pellicle and lubrication (Bennick, 1982, de Almeida Pdel et al., 2008). The ability of PRPs to bind free calcium is important because it modulates the activity of calcium. The binding of calcium to PRPs may prevent calcium precipitation and the potential formation of calculus or salivary stones but it may also inhibit the remineralisation process. It has also been reported that the larger PRPs assist the attachment of bacteria and the smaller proteins appear to decrease the initial microbial adherence. The reason for this is because both large and small PRPs bind to the same positions on HAP but the large PRPs also have dominant binding sites for bacteria (Lamkin and Oppenheim, 1993).

Statherins

Statherins are 43-residue acidic peptides, rich in tyrosine and proline, and phosphorylated at Ser-2 and Ser-3. Statherins have a molecular mass of 6 kDa and are found in human parotid and submandibular/sublingual saliva, have a high binding affinity for HAP and comprise major components of the acquired enamel pellicle (Schlesinger and Hay, 1977, Raj et al., 1992). Furthermore, when statherins are adsorbed to HAP, they also promote the attachment of microorganisms (e.g. *Actinomyces viscosus*). One of the main activities of these proteins is associated with the inhibition of crystal growth of calcium and phosphate salts and the primary precipitation of calcium phosphate salts from supersaturated saliva (Schlesinger and Hay, 1977, Lamkin and Oppenheim, 1993). In other words, statherins prevent the formation of calculus by inhibiting the precipitation or crystallisation of

supersaturated calcium phosphate. Statherins also can reduce the adhesion of *Streptococcus mutans* on HAP surfaces (Shimotoyodome et al., 2006).

Cystatins

The human cystatin gene family contains 14 genes and the major cystatins present in saliva are cystatin-A, cystatin-B, cystatin-C, cystatin-D, cystatin-S, cystatin-SA and cystatin-SN. Cystatins have molecular mass of about 13 kDa and are secreted from the human submandibular and sublingual glands, as well as from the parotid gland albeit in very low concentrations (Lupi et al., 2003, Fabian et al., 2012). Cystatins are a family of cysteine protease inhibitors that prevent the function of bacterial proteases and like other salivary proteins, they appear to have several other functions in the oral environment (Gorr, 2009). Phosphorylated and non-phosphorylated cystatins bind to HAP, like statherins, but with a lower affinity than the latter, and together they play an important role in the mineral balance of the tooth by inhibiting the crystal growth of calcium phosphate salts (Johnsson et al., 1991, Lamkin and Oppenheim, 1993). Salivary cystatins have antimicrobial properties (e.g. inhibiting the growth of *Porphyromonas gingivalis*) and an association has also been found between decreased levels of cystatins and inflammatory periodontal disease (Baron et al., 1999b, Gorr, 2009, Gorr and Abdolhosseini, 2011).

Histatins

Histatins are low molecular weight proteins (3 to 6 kDa), rich in histidine and are secreted by both parotid and submandibular/sublingual glands. Two human genes are responsible for the production of histatin 1 and histatin 3 and proteolysis of these two peptides generates more fragments. For example, the cleavage of histatin 3 generates at least 24 different peptides. The three major histatins are 1, 3 and 5. Histatin 1 is a neutral protein and phosphorylated at residue 2, but histatins 3 and 5 are basic and non-phosphorylated proteins (Castagnola et al., 2004). Histatins were shown to

prevent calcium and phosphate precipitation from supersaturated saliva and inhibit crystal growth (Oppenheim et al., 1986, Oppenheim et al., 1988). There is a dichotomy between the positive effect of the inhibition of calcium and phosphate precipitation and the negative effect of the inhibition of remineralisation by the histatins again here. However, the two mechanisms may be in balance resulting in a protective effect against acid dissolution of the enamel. Histatins, like statherins and PRPs, are characterised by strong affinity for HAP and have been detected in the human acquired enamel pellicle (Vitorino et al., 2005). Histatins were also shown in early reports to have antimicrobial activity by inhibiting the growth and viability of *Candida albicans* (Pollock et al., 1984). Furthermore, it was shown that the *Streptococcus mutans* adherence to HAP *in vitro* is prevented by histatin 1 (Shimotoyodome et al., 2006). Histatin 5 plays a role in the inhibition of the growth and haemagglutination of *P. gingivalis* by inhibiting the activity of the bacterial proteolytic enzymes (Gusman et al., 2001, Devine and Cosseau, 2008). More recent studies described histatins as human saliva components which have wound healing function (Oudhoff et al., 2008, Oudhoff et al., 2009). From an evolutionary perspective, it is interesting that animals are known to lick their wounds and humans also react in a similar manner when injured. For example, the first thing that someone does is to put their finger into their mouth when it has been injured by a cut. Histatins have also been shown to have a protective effect against influenza A virus, a human virulent pathogen that causes severe illness or death (White et al., 2009).

Amylase

α-Amylase is secreted by the human parotid salivary gland and it is the most abundant protein in human saliva. Salivary amylases are divided into two families of isoenzymes. Family A consists of isoenzymes 1, 3 and 5 which are N-glycosylated with molecular weight of 61-63 kDa, and family B, consists of non-glycosylated isoenzymes 2 and 4 with molecular weight of 56-59 kDa (Keller et al., 1971, Yao et al., 2003). Besides its main role in digestion of starch to glucose and maltose, α-amylase has also been shown

to interact with bacteria. For example it binds with great affinity to different species of oral streptococci (Fabian et al., 2012). In a recent study, alpha-amylase was identified as an *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) binding protein. Alpha-amylase was found to bind to *A. actinomycetemcomitans* (a bacterium strongly associated with an aggressive form of periodontitis) with high affinity and inhibit bacterial adhesion and further biofilm formation (Baik et al., 2013). Moreover, amylase is a component of the acquired enamel pellicle which suggests that amylase might assist the attachment of bacteria to the enamel surface and in this way may also contribute to plaque development (Scannapieco et al., 1993). What is more, the presence of an enzyme that breaks down starch into simple sugars that are immediately available for bacterial metabolism, intuitively would have a less than desirable effect on oral health. α-Amylase helps the adhesion of bacteria to enamel surface but also inhibits the adhesion of other bacteria and the biofilm formation. This is a slight conundrum but it may be suggested that α-amylase plays an important role in controlling which species bind to enamel and as such is an important player in plaque development.

Mucins

Human salivary mucins are highly glycosylated proteins secreted by submandibular and sublingual glands. There are two types of mucins, a high-molecular-weight protein, MG1 or MUC5B with a molecular mass higher than 1000kDa, and a lower-molecular-weight mucin, called MG2 or MUC7 of approximately 180-200kDa. Mucins adsorb to the enamel surface and take part in the formation of the acquired enamel pellicle, as well as providing lubrication to hard and soft tissues of the oral cavity (Tabak, 1990, Levine et al., 1987, Fabian et al., 2012). Many oral bacteria can produce enzymes to remove sugars from glycan side chains of mucins in order to use them for their growth. Therefore, these highly glycosylated proteins act as a major microbial nutrient source (Derrien et al., 2010). In addition, mucins have antimicrobial activity which might be linked with the formation of heterotypic

complexes with other salivary proteins. Formation of complexes between MG1 and amylase, histatins, statherins and proline-rich proteins have been found but their biological function has not been reported (Iontcheva et al., 1997). MG2 along with amylase, glycosylated proline-rich protein, sIgA, lactoferrin and lysozyme are proteins that have been identified as complexes in salivary micelles (Soares et al., 2004). Antiviral properties of MUC5B and antifungal activity of a peptide derived from MUC7 have also been reported (White et al., 2009, Lis et al., 2010).

Immunologic and non-immunologic antibacterial proteins

There are a number of immunologic and other non-immunologic proteins present in human saliva, exhibiting various important biological functions, which are not all relevant to this study. Immunoglobulins (with IgA as the most dominant), and enzymes such as lysozyme, lactoferrin, peroxidase, as well as, antimicrobial peptides (such as defensins) are some examples of salivary proteins with important antimicrobial activity. Although these proteins might exist in low concentrations in human saliva, they play an important role in the maintenance of the oral health through different mechanisms. Immunoglobulin A can act as an antibody against bacterial antigens, promote bacterial aggregation and neutralise viruses, bacterial and enzyme toxins while the antibacterial properties of lysozyme are based on its ability to hydrolyse the cellular wall of Gram positive bacteria and promote bacterial aggregation. The iron binding protein lactoferrin can kill or inhibit the growth of various microorganisms by depriving them of the iron necessary for their growth and survival. Salivary peroxidases exhibit antiviral, antifungal and bacteriostatic activities through the oxidation of salivary thiocyanate ion into hypothiocyanite by hydrogen peroxide (Humphrey and Williamson, 2001, de Almeida Pdel et al., 2008, Farnaud et al., 2010, Singh Mamta, 2013).

1.5 Acquired enamel pellicle

The inorganic part of dental enamel consists primarily of HAP. It has been shown that salivary proteins are rapidly adsorbed onto tooth enamel surfaces, and this organic film has been referred to as the acquired enamel pellicle (Figure 3). Positively charged amino groups of proteins can bind to negatively charged phosphate ions at the enamel surface and negatively charged carboxyl groups of proteins can bind to positively charged calcium sites at the enamel surface (Gorbunoff and Timasheff, 1984). In early studies, it was shown that protein adsorption to enamel is selective. When whole saliva was incubated with HAP or enamel powder, the range of proteins adsorbed to the two powders was not identical. Moreover, protein adsorption was not a general property; only specific proteins were found to be adsorbed to HAP or enamel powder while other proteins remained unbound in the surrounding solution (Hay, 1967). Similarity between *in vivo* and *in vitro* formed acquired enamel pellicles has also been reported, suggesting that bacteria do not have any part in the pellicle formation (Mayhall, 1970).

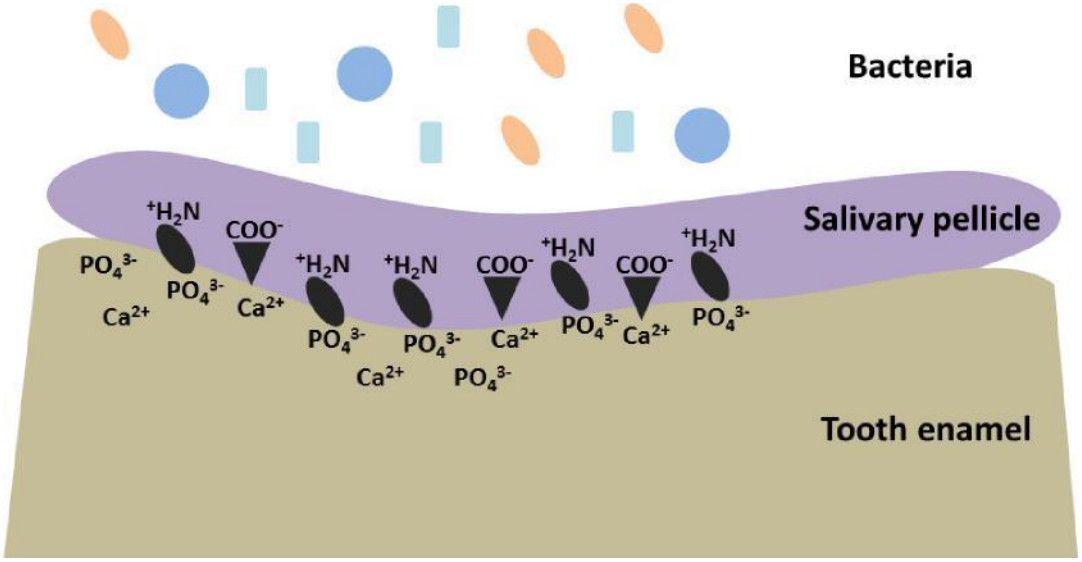


Figure 3. Salivary proteins are adsorbed onto tooth enamel surface and form the acquired enamel pellicle. Proteins are adsorbed via electrostatic interactions involving calcium and phosphate groups on the enamel surface.

Protein composition of acquired enamel pellicle

With regard to amino acid composition, the acquired enamel pellicle has been shown to be different from whole saliva, submandibular/sublingual and parotid saliva (Al-Hashimi and Levine, 1989, Lendenmann et al., 2000). Furthermore, differences have been reported in amino acid composition between the *in vitro* and *in vivo* formed pellicles especially regarding the proline content, due to the decreased amount of acidic proline-rich proteins in the *in vivo* pellicles (Yao et al., 2001). What is more, due to post-sampling modifications, the binding affinity of salivary proteins on enamel surfaces *in vitro* might differ from the affinity of the same proteins when they are in the oral environment due to the presence of other important saliva components and to interactions with other salivary proteins. It is important to note that

degradation occurs during the processing of saliva, and several proteins are lost through centrifugation.

Comparison of the amino acid composition of 2 hour pellicles formed *in vivo* on different teeth has been carried out and it has been shown that there is no difference in amino acid composition among pellicles derived from different areas in the mouth (Sonju and Rolla, 1973). However, a serious weakness with this conclusion was that the pellicles compared in this study were all removed from the buccal surfaces of the upper molars, upper incisors and lower anterior teeth. The finding of this study would have been more useful if the investigators had included comparisons with pellicles obtained from other tooth surfaces such as lingual or palatal.

An attempt by Rykke and co-workers was made to investigate the variation of amino acid composition in *in vivo* salivary pellicles among individuals and also within individuals over a two year period of time. Though the amino acid composition of whole saliva varied between individuals in the study, no major differences were found in the amino acid profiles of pellicles generated by the participants (Rykke et al., 1990). However, these results were based upon salivary pellicles collected from a small number of individuals (3 subjects took part in the study).

In regard to the actual protein composition, salivary pellicles formed *in vivo* in different regions of the mouth were characterised by variation in proteins adsorbed to enamel, due to the different types of saliva dominating in each particular area (Carlen et al., 1998). In this thorough study, the compared pellicles were removed from buccal and palatal surfaces of premolars and front teeth of the upper jaw as well as buccal and lingual surfaces of premolars and front teeth of the lower jaw.

Furthermore, differences have been reported between the protein composition of human acquired enamel pellicle and whole saliva (Yao et al., 2003). This is not surprising, considering the fact that protein adsorption onto the enamel surface is a selective process and not all proteins in whole saliva have the ability to bind to HAP and contribute to the formation of the protective layer of the acquired enamel pellicle (Hay, 1967).

Salivary pellicles formed *in vitro* by proteins from parotid saliva and those from submandibular saliva were found to have some similarities, although submandibular-derived pellicles, as might be predicted from the compositional data shown in Table 3, were still differentiated by having higher cystatin levels and lower levels of α-amylase (Jensen et al., 1992).

Some of the proteins that have been identified as significant components of the acquired enamel pellicle included secretory IgA, proline-rich protein (PRP), cystatin SA, α-amylase, high-molecular weight mucin MG1, histatin, statherin and lysozyme (Bennick, 1982, Al-Hashimi and Levine, 1989, Jensen et al., 1992, Lamkin et al., 1996, Lendenmann et al., 2000). However, with the advent of more sensitive proteomic analysis, a much larger range of proteins comprising the acquired pellicle are being found than the impression given here.

Identification of proteins of the human acquired enamel pellicle using advanced proteomic analysis

Recently, researchers have shown an increased interest in identifying the protein components in human acquired enamel pellicle and achieving a better understanding of the role of these proteins in the maintenance of oral health. Developments in the field of proteomics have allowed researchers to achieve the identification of a larger number of proteins found in acquired enamel pellicle (Table 4). In the study conducted by Yao et al., lactoferrin, albumin, amylase, PRPs, lysozyme, cystatin SN, statherins and peroxidase were identified as *in vitro* pellicle proteins by combining gel electrophoresis and matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry techniques (Yao et al., 2001).

A different approach to pellicle protein analysis identified MG1, histatin 1, albumin, amylase, statherin and IgA as major components of the *in vivo* human acquired pellicle, but also demonstrated that lysozyme, PRP, MG2, carbonic anhydrase, lactoferrin and peroxidase were absent from *in vivo* formed pellicles (Li et al., 2003). The method for protein identification used in this study was based upon the production of monoclonal antibodies after

immunization of mice with pellicle components, followed by immunologic assays. However, some proteins were not identified as constituents of the acquired pellicle by this method and this could be for a number of reasons such as because they were absent or found in low concentration, were proteolytically fragmented, had reduced ability to induce immune response or they formed complexes with other proteins.

In 2003, Yao et al. described the results of the use of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) for analysis of *in vivo* enamel pellicle and whole saliva. Some components of the human acquired enamel pellicle have been identified in this study including statherin, histatin 1, histatin 3, lysozyme, calgranulin B, cytokeratins (different from those in skin and hair usually considered to be laboratory contaminants) and phosphodiesterase (Yao et al., 2003).

With shotgun proteomics becoming more popular, researchers have identified a large number of proteins comprising the human acquired enamel pellicle. Shotgun proteomics refers to protein identification by the use of a combination of high performance liquid chromatography and mass spectrometry. The great advantages of this method are that it decreases the amount of time each sample must be handled and that it minimizes the quantity of material needed for proteomic analysis of the acquired enamel pellicle. Using shotgun proteomics a total of 130 proteins/peptides have been identified as components of *in vivo* formed human acquired enamel pellicle (Siqueira et al., 2007). In this study the acquired enamel pellicle was obtained from three healthy subjects and it was formed naturally for a 2 hour time period.

As an overview, some of the main proteins that have been found in 2h *in vivo* and *in vitro* acquired enamel pellicles, and have been reported in the literature more than once, are presented in Table 4. Most of the proteins have been found in both *in vivo* and *in vitro* formed pellicles, but some others have been found only in *in vivo* pellicles. Taken at face value, the data in Table 4 suggest that there are differences between *in vitro* and *in vivo* formed pellicles, presumably due to the influence of additional factors

associated with the oral environment. However, the proteomic analysis carried out in the various studies was not complete in that not all proteins were identified (the complete identification of all proteins would be technically challenging and perhaps not possible). For example, calgranulin B has been positively identified in *in vivo* pellicles but to date not in *in vitro* pellicles; this may simply reflect the fact that a calgranulin B spot on a 2-D gel was not chosen for further characterisation – and not necessarily indicate it was absent. The situation is further complicated by the fact that processing saliva for *in vitro* studies modifies the composition. For example, prior to use in *in vitro* studies, saliva is invariably centrifuged to remove cellular and dietary debris. This procedure may also remove mucins (Nieuw Amerongen et al., 1987) and any other proteins associated with the mucins which will clearly impact on the composition of the *in vitro* pellicle produced.

Table 4. Proteins found in human acquired enamel pellicle

Proteins	*In vivo*	*In vitro*	References
amylase	✓	✓	(Carlen et al., 1998, Yao et al., 2001, Jensen et al., 1992, Al-Hashimi and Levine, 1989, Lamkin et al., 1996, Li et al., 2003, Siqueira et al., 2007, Lee et al., 2013)
statherin	✓	✓	(Jensen et al., 1992, Lamkin et al., 1996, Carlen et al., 1998, Yao et al., 2001, Li et al., 2003, Yao et al., 2003)
cystatins	✓	✓	(Al-Hashimi and Levine, 1989, Jensen et al., 1992, Lamkin et al., 1996, Yao et al., 2001, Siqueira et al., 2007, Lee et al., 2013)
histatins	✓	✓	(Jensen et al., 1992, Lamkin et al., 1996, Li et al., 2003, Yao et al., 2003, Lee et al., 2013)
lysozyme	✓	✓	(Yao et al., 2001, Yao et al., 2003, Siqueira et al., 2007, Lee et al., 2013)
PRPs	✓	✓	(Jensen et al., 1992, Lamkin et al., 1996, Yao et al., 2001, Siqueira et al., 2007, Lee et al., 2013)
IgA	✓	✓	(Al-Hashimi and Levine, 1989, Carlen et al., 1998, Li et al., 2003, Siqueira et al., 2007)
albumin	✓	✓	(Yao et al., 2001, Li et al., 2003, Yao et al., 2003, Siqueira et al., 2007)
lactoferrin	✓	✓	(Yao et al., 2001, Siqueira et al., 2007, Lee et al., 2013)
mucin MG1	✓		(Li et al., 2003, Siqueira et al., 2007, Lee et al., 2013, Al-Hashimi and Levine, 1989)
S100 calcium binding protein	✓		(Siqueira et al., 2007, Lee et al., 2013)
calgranulin B	✓		(Yao et al., 2003, Siqueira et al., 2007, Lee et al., 2013)
keratins	✓		(Siqueira et al., 2007, Lee et al., 2013)

Apart from the identification of a large number of pellicle proteins, Siqueira et al. have provided a useful separation of these proteins into groups according to their chemical properties and biological function as shown in Table 5. With regard to their role in the acquired enamel pellicle formation, 17.5% of the identified proteins have been characterised by their ability to bind calcium ions, 15.4% by their ability to bind phosphate ions and 28.2% by their tendency to interact with other proteins. Categories of the identified proteins in relation to their biological function are high affinity for enamel surface/remineralisation (15.5%), inflammatory response (12.5%), immune response (11.3%), antimicrobial activity (8.3%) and buffer capacity and lubrication (<2%). In addition to the above categories, proteins can also be grouped according to their origin. Identified proteins have been derived from exocrine salivary secretions (14.4%), cells (67.8%) or serum (17.8%) (Siqueira et al., 2007).

Table 5. Categorisation of identified acquired enamel pellicle proteins in regard to their (a) chemical properties and (b) biological function (adapted from Siqueira et al., 2007)

(a) Chemical properties	%	(b) Biological function	%
Protein/protein interaction	28.2	Antimicrobial function	8.3
Calcium binding	17.5	Immune response	11.3
Phosphate binding	15.4	Remineralisation	15.5
Unknown	38.9	Inflammatory process	12.5
		Buffer capacity	<1
		Lubrication	<1
		Unknown function	51.2

Some pellicle proteins from Table 4, such as histatins, statherins and S100 calcium binding protein are characterised by their ability to bind calcium and phosphate ions. These proteins are also considered precursors of the

human enamel pellicle due to their ability to bind directly to enamel which consists mainly of the calcium phosphate salt HAP (Siqueira et al., 2007). Mucin MG1 is an example of proteins that show interactions with other proteins, since it is known that mucin MG1 forms complexes with amylase, proline-rich proteins, statherin and histatins (Iontcheva et al., 1997). Other proteins discovered in enamel pellicle, including lysozyme, lactoferrin, cystatins and calgranulin B, are involved in immune response or possess antimicrobial properties (Siqueira et al., 2007).

In vivo human acquired enamel pellicle on deciduous teeth has also been analysed resulting in the identification of 76 proteins and 38 naturally occurring peptides from various proteins such as histatin and statherin (Zimmerman et al., 2013). Most of the common salivary proteins which are found in permanent enamel pellicle, including all proteins in Table 4, have also been found in deciduous acquired enamel pellicle. However, there are still a considerable number of differences, in terms of protein composition, between the *in vivo* pellicles formed on deciduous and permanent enamel. This can be explained again by considering the fact that the enamel surface between permanent and deciduous teeth is different and, therefore, protein adsorption may differ as well (Zimmerman et al., 2013). However, a major factor here is that the protein spectrum of whole saliva from adults is significantly different to that of children (Sivakumar et al., 2009). Other factors that should be taken into account in these findings are differences in the diet and salivary flow between young children and adults.

Recently, investigators have examined how the protein composition of acquired enamel pellicle alters at four specific time-points within the two hours of formation *in vivo*. It has been reported that the enamel pellicle at initial stages of formation (5-10 minutes) is rich in proteins with an affinity for calcium and phosphate ions and the final stage pellicle (60-120 minutes) is more abundant in proteins known to interact with other proteins (Lee et al., 2013).

The human acquired enamel pellicle exhibits major functions in the oral cavity, such as lubrication and antimicrobial activity. Acquired enamel pellicle is also

known for playing an important role in the regulation of mineral homeostasis and the protection against acid demineralisation, which will be discussed in more detail in the next section (Lendenmann et al., 2000, Siqueira et al., 2012a).

1.6 Effect of the acquired pellicle on erosive demineralisation of enamel

Dental erosion is described as the loss of dental hard tissue as a result of chemical etching and dissolution by acids that are not derived from bacteria (Imfeld, 1996). There are several factors, such as environmental factors, medication, diet and lifestyle, that contribute to the development of dental erosion. Tooth erosion is a progressive lifetime process and it is possible that all factors act together or at some point in a person's life (Zero, 1996). Dietary acids such as lactic, malic, phosphoric and citric acids, found in low pH drinks are major components that cause tooth erosion. It has been demonstrated that demineralisation of enamel *in vitro* by lactic, malic or citric acid is reduced when the pH of the acid is increased, the acid concentration (titratability) is decreased or a small concentration of calcium is added to the solution which increases the ion solubility product for HAP (Hughes et al., 2000).

Saliva is comprised of 2290 proteins, 130 of which have been identified in acquired enamel pellicle (Siqueira and Dawes, 2011). Such an impressive range of proteins in the mouth means that there is also a large network of protein interactions with various functions, including protection of the enamel against acid demineralisation. In early studies *in vitro*, it has been demonstrated that salivary pellicles are able to protect enamel against acid-induced demineralisation (Zahradnik et al., 1976). It should be noted that saliva is not the only source of proteins that can adsorb to enamel surfaces and by some definition comprise the pellicle. Protective proteins include not only salivary proteins, but also dietary proteins and compounds, food polymers, synthetic proteins and protein complexes that will also be

discussed next (Vukosavljevic et al., 2014). In the next section the protective effect of the pellicle is considered in more detail.

1.6.1 Protection against acid-induced demineralisation by whole saliva and the importance of the maturation period of pellicle formation

Featherstone et al. (1993) used an *in vitro* enamel demineralisation model to confirm that salivary pellicle (from whole saliva) reduced the rate and severity of enamel demineralisation and this effect was dependent on the time of development of the pellicle. Pellicle formed over 7 days provided better protection than pellicle formed over 18 hours (Featherstone et al., 1993). However, Hannig and Balz (1999) investigated the role of 24-hour or 7-day *in vivo* formed salivary pellicles on citric acid-induced erosion of bovine enamel. Their results from scanning (SEM) and transmission electron microscopy (TEM), as well as measurement of surface microhardness (SMH) did not reveal distinct differences between the behaviour of 24-hour and the 7-day pellicles with respect to 0.1% and 1% citric acid challenge over 30, 60 and 300 sec (Hannig and Balz, 1999). In 2003, Hannig et al. assessed the protective effect of *in vivo* salivary pellicles formed over periods of 2 to 24 hours against citric acid induced demineralisation of bovine enamel. Measurement of the calcium release did not show any significant differences among the 2-, 6-, 12- and 24-hour pellicles but TEM analysis showed that the 2-hour pellicle layer was partially lost after one minute citric acid challenge (Hannig et al., 2003).

One criticism of these studies is the extremely long incubation periods used to develop these pellicles. Under normal *in vivo* conditions, it is questionable whether pellicles ever age to this degree given the chemical and mechanical factors at work in the mouth that serve to continually remove the pellicle layer (e.g. acid foods/beverages, shear forces from mastication and tooth brushing etc). In other words, pellicle is a continual state of turnover.

Short-term salivary pellicles were also assessed by Hannig et al. (2004), and it was shown that pellicles formed in situ for 3, 60 or 120 min did not differ in terms of their protective properties against enamel demineralisation. Their results revealed that even 3 min salivary pellicles can decrease significantly the citric acid induced demineralisation of the enamel (Hannig et al., 2004).

The results of another study (Nekrashevych and Stosser, 2003) also suggested that the salivary pellicle protects the enamel against acid erosion. In this study, human whole salivary pellicle was deposited on bovine enamel blocks for 24 hours and the blocks were exposed to 0.1% or 1% w/v citric acid for 1, 5 or 10 min. Measurement of calcium released was not significantly different from enamel surfaces with and without pellicle. However, the loss of surface microhardness and the roughness of the eroded surfaces were decreased in the presence of *in vitro* pellicles, and scanning electron microscopy revealed that the salivary pellicle protected the enamel surface from serious destruction.

In another study by Hannig and Balz (2001), 24-hour *in vivo* pellicles were formed and similar methods were used to investigate the protective effect and the ultrastructure of salivary pellicles formed near to the ductal orifices of the parotid and sublingual/submandibular glands. Their findings indicated that 24-hour pellicles formed near the ductal orifices of the parotid and sublingual/submandibular glands did not differ significantly in terms of the protection offered to the enamel surface against acid demineralisation (Hannig and Balz, 2001). The use of toothpaste was avoided in these studies in order to minimize the chance of the pellicle being partially removed. However, using toothpaste when cleaning the enamel specimens during their exposure to the oral cavity would have been a better simulation of the effect of daily tooth brushing.

Hall et al. (1999) used both *in vitro* and *in vivo* experiments to evaluate the capacity of saliva to inhibit the mineral loss from the enamel surface after exposure of human enamel specimens to acid. It was found that there is a higher level of protection against acid demineralisation *in situ* compared with the *in vitro* environment (Hall et al., 1999). Possible explanations for this

finding are the composition and amount of saliva in the oral environment being different compared to *in vitro* environment, the constant bathing of the enamel by fresh saliva and the presence of other organic components on the enamel in an *in situ* environment, or the protein degradation in *in vitro* environment.

It has also been demonstrated that the thickness of the acquired salivary pellicle affects the degree of protection against acid demineralisation. In a previous study, Amaechi et al.(1999), it was shown that the thickness of acquired salivary pellicle formed on bovine enamel slabs after 1 hour of intraoral exposure was different among individuals and within the dental arches. Therefore, there is variation of the thickness of the pellicle not only with time but also due to location in the mouth and among individuals. It was also shown that the degree of dissolution can be dependent on this variation within the arches but more importantly it was demonstrated that the 1-hour pellicle did protect the teeth from dental erosion (Amaechi et al., 1999). Taken together, it can be suggested that although increasing the maturation period of pellicle formation may result in gradual increase of protection, short term salivary pellicles still cause a significant inhibition of demineralisation.

Another study *in vitro* also showed individual variations in the protection from whole saliva against erosion. Human enamel specimens were exposed to whole saliva for 2 hours followed by 10 min challenge with 0.3% citric acid, pH 3.2, and the tissue loss was measured by contact profilometry. Their results showed that the saliva of all fourteen subjects except one reduced the enamel erosion but the degree of protection varied between the subjects (Wetton et al., 2007). One criticism of this study is that the observed protective effect cannot unequivocally be attributed to salivary proteins as the whole saliva used was not centrifuged and therefore cellular debris and bacterial proteins were still present in saliva samples and may have contributed to the observed effect. The standardisation of the collection and processing of saliva is important especially when a large number of subjects is examined and compared.

Bruvo et al., investigated the effect of *in vitro* formed parotid and submandibular salivary pellicles from Scandinavians and non-Scandinavians in an effort to examine whether individuals with different ethnic background offer different degree of protection against acid demineralisation. Bovine enamel blocks were exposed to pellicles derived from parotid or submandibular saliva for 12 hours and then challenged with 1% tartaric acid solution at pH 2.3. Measurement of the surface microhardness showed that saliva from Scandinavians offered less protection compared to saliva from non-Scandinavians and the protein analysis found a slightly more intense staining of the SN-isoform of S-type cystatin in saliva from non-Scandinavians (Bruvo et al., 2009).

In summary, results from several studies suggest that regardless of the maturation time, pellicles offer a degree of protection against acid demineralisation of the enamel. Consistent findings showed that salivary proteins are rapidly adsorbed to enamel surface to form the pellicle layer which can reduce the enamel dissolution (Hannig et al., 2004).

1.6.2 Protection against acid-induced demineralisation by non salivary proteins

Several proteins have been investigated individually for their potential protective effect against acid demineralisation. For example, Arends et al (1986) investigated the effect of albumin on lactic acid-induced demineralisation of bovine enamel *in vitro*. Various concentrations of albumin were tested on salivary pellicle-free enamel blocks and the lesion depth was measured after 3, 9 and 21 days of demineralisation. The results of this study showed that albumin inhibited the acid dissolution of the enamel, however, it is not possible to make a direct comparison between the protective effect of albumin and that of salivary pellicle since the study was carried out only on salivary pellicle-free enamel surfaces (Arends et al., 1986).

Many studies have examined various substances that reduce HAP dissolution aiming for their potential use in food products. It is important that researchers find a way to modify soft drinks focusing on the reduction of the erosive potential but without significantly changing the taste. Most studies have focused on the addition of calcium and phosphate supplements, and although this is effective, it affects the flavour and taste of the products (Grenby, 1996). Barbour et al., investigated the potential inhibitory effect of ordinary food ingredients and additives on the rate of HAP dissolution. Their results indicated that polyphosphate and a mixture of polyphosphate and xanthan at a concentration 0.02% (w/v) decreased the HAP dissolution rate in a 0.3% citric acid solution, pH 3.2, more efficiently than 10mM calcium. However, as Barbour et al. reported, it is critical that the protective effect of these agents is tested in combination with salivary pellicles in order for these findings to be validated (Barbour et al., 2005).

In 2008, the effect of casein, a phosphoprotein found in bovine milk, in reducing acid erosion was also investigated by Barbour et al. (2008). They found that the addition of casein to a citric acid solution of a range of pH values similar to those typical for soft drinks resulted in the reduction of HAP dissolution, and that the addition of a low concentration of calcium further decreased the dissolution. Moreover, this study revealed that the inhibitory effect of casein was exhibited in the presence of salivary pellicle. However, the study was carried out *in vitro* using HAP discs and the effect of casein could be different on natural enamel surfaces (Barbour et al., 2008).

In another *in vitro* study, the effect of the addition of a protein mixture (casein and mucin) and casein and mucin individually to the enamel pellicle was investigated. In contrast to the results of the study carried out using HAP discs (Barbour et al., 2008), the results of this study showed that the combination of casein and salivary pellicle did not decrease the HAP dissolution. The same results were observed for the combination of mucin and salivary pellicle, although the combination of the mixture of casein and mucin and salivary pellicle better maintained microhardness (Cheaib and Lussi, 2011).

1.6.3 Protection against acid-induced demineralisation by specific salivary proteins

The previous section described the protective effects of whole salivary proteins when adsorbed to HAP surfaces. Here, the focus is on specific salivary proteins. Nieuw Amerongen et al. (1987) investigated the effect of salivary pellicles and salivary mucins on the citric acid-induced demineralisation of enamel *in vitro*. Their results indicated that salivary mucins play a significant part in the protection of enamel via pellicle formation (Nieuw Amerongen et al., 1987). However, their experimental method involved removing mucins from whole saliva by centrifugation and measuring the effect this had on the protective effects of the remaining salivary components. They also used mucins directly following their crude "purification" from saliva by a centrifugational technique. One criticism of this approach is that mucins bind other salivary proteins and removal of the mucin by centrifugation may have removed other salivary components during centrifugation so the protective effects seen cannot be unequivocally ascribed to just the mucins.

In a microradiographic study Kielbassa et al. (2005) used bovine enamel specimens which were challenged with a demineralising solution of pH 5.0 for 10 days while different concentrations of "salivary" proteins were added to it. However the "salivary" proteins used were bacteriological mucin, bovine milk casein, an unspecified IgG, human serum albumin and, most strangely, as it is not a protein at all, free proline. Nevertheless, their results suggested that mucin reduced the enamel dissolution *in vitro* and they suggested mucin as an added ingredient for saliva substitutes or mouthwashes for patients with dry mouth. They also reported that proline and casein exhibited an inhibitory effect against acid demineralisation of the enamel but only at higher concentrations (Kielbassa et al., 2005). In contrast to mucin and casein, albumin and IgG were found to have no protective effect.

Siqueira et al. (2010) aimed to address the effect of another salivary protein, for which researchers have shown an increased interest in recent years, histatin. In this study, intact histatins 1, 3 and 5 were found in the human *in vivo* acquired enamel pellicle. Interestingly, it was also found that phosphorylated histatins offered a higher degree of protection against acid demineralisation of the enamel. Measurement of phosphate and calcium loss after 12 days of acetic acid-induced demineralisation of enamel revealed that native histatin 1 (which is phosphorylated at residue 2) and synthetic histatin 3 (phosphorylated at residue 2) were more effective in inhibiting the enamel dissolution compared to unphosphorylated histatins (Siqueira et al., 2010). However, it should be noted that isolated histatins were used for the formation of the enamel pellicles in this study and the effect of these proteins in the presence of other salivary proteins or peptides found in a natural pellicle was not examined. Siqueira and Oppenheim (2009) identified 78 natural pellicle peptides with small molecular weights which may play a significant part in the function of the acquired enamel pellicle (Siqueira and Oppenheim, 2009).

Most recently, it has been reported that statherin-like peptides protected enamel against acid-induced demineralisation *in vitro*. Blocks of HAP pellets were pre-treated with 0.1M acetic acid, pH 4.0, for 5 days, then exposed to statherin-like peptides for 24 hours and finally exposed to acid for a further 5 days. It was shown that statherin-like peptides containing 15 N-terminal residues or more were able to reduce the HAP dissolution rate by about 50% while statherin-like peptides composed of only 10 or 5 N-terminal residues had no effect (Shah et al., 2011).

Though obvious, it should be stressed that it is difficult to relate *in vitro* findings to the clinical relevance of the various salivary proteins in terms of protecting against demineralisation. The design of studies to test the clinically relevance of salivary components in humans is challenging but *in vitro* studies will remain an important tool for elucidating the functional roles of salivary proteins.

1.7 Summary

Acid demineralisation of human enamel is a process that occurs every day in every dentate person's life and, under certain circumstances, it may affect the oral cavity and have negative effects on oral health. This indicates a need to understand the various relationships that exist among the proteins, microorganisms and inorganic components associated with the enamel surface.

Although a number of studies have examined the effect of the acquired enamel pellicle or other proteins on the acid-induced demineralisation of the enamel, the specific proteins that provide significant protection have not been identified and the mechanism by which they protect is not clear.

Bovine enamel specimens or human enamel surfaces, as well as powdered substrates such as HAP or human enamel powder, have been used to investigate the effect of saliva on acid demineralisation of dental enamel *in vitro* and *in vivo*. However, one question that needs to be asked is how similar the results obtained from the use of different enamel substrates are, and whether they are clinically relevant.

1.8 Aims of the study

Salivary proteins are known to provide protection against acid demineralisation of the human enamel, however, it is not clear which specific proteins are involved.

The aims of this study are:

- To investigate the protective effect of whole saliva and purified salivary proteins against acid demineralisation of the enamel using synthetic HAP powder, human enamel powder and natural enamel surfaces as model substrates.
- To investigate the adsorption of salivary proteins onto the model enamel surfaces.
- To identify, purify and further characterise the protective salivary proteins.

The long-term translational targets of this study are:

- To inform on the design of protective peptides for therapeutic use (e.g. mouthwashes and artificial saliva substitutes used by patients with dry mouth).
- To screen for salivary polymorphisms in the general population that are poorly protective allowing for early prophylactic intervention.

Chapter 2
Materials and methods

2.1 Collection of whole saliva and processing

Ethical approval from the Dental Research Ethics Committee (DREC No: 090212/SB/80) was obtained in order to carry out the experiments using saliva from healthy volunteers. A signed consent form was obtained from the volunteers after they read the participant information sheet.

Paraffin wax (Parafilm)-stimulated whole saliva from smokers and non-smokers (n=7) (3 males and 4 females, aged 26-50) was collected between 9 and 11 am on the day of an experiment. Subjects were asked to refrain from eating, drinking or smoking for 1 hour prior to the collection. The saliva was immediately clarified by centrifugation at 20800g for 10 minutes to remove bacteria, cells and other debris, and was immediately used.

2.2 Effect of salivary proteins on acid demineralisation of enamel

2.2.1 Adsorption of saliva to synthetic hydroxyapatite (HAP) powder or human enamel powder and acid demineralisation

2.2.1.1 Binding ratio of proteins to HAP powder

In order to decide the ratio of whole saliva to HAP powder sufficient to saturate the binding capacity of the HAP powder, the following method was carried out. Various amounts (5mg, 10mg, 20mg, 30mg, 50mg and 75mg) of HAP powder (ICN Biomedicals, Inc, Germany) were prepared, weighed and added to six microcentrifuge tubes (Eppendorf, Hamburg, Germany) that contained 1mL of clarified whole saliva. The mixture in each tube was incubated for 1hour, at 37°C with shaking. After 1 hour incubation, the samples were centrifuged for 10 minutes at 20800g in an Eppendorf Centrifuge 5417R (Eppendorf, Hamburg, Germany). The supernatant from each tube was carefully removed and the pellet was washed twice with

50mM Tris pH 7.4 and was then resuspended in 500µl of phosphate buffer (100mM, pH 7.4). Phosphate buffer was used in order to desorb the proteins from the HAP (Gorbunoff and Timasheff, 1984). The quantity of bound proteins was determined by UV absorbance at 280nm using a plate reader (Thermo Scientific Varioskan Flash). In each well of a 96 well microplate (Greiner Bio-One Ltd, Stonehouse, Great Britain), 150µl of sample was added and all samples were analysed in duplicate. Increasing the amount of HAP added to saliva did not seem to cause an increase in the proteins binding to HAP (Figure 4a). The absorbance of whole saliva was approximately 0.9 units and the absorbance of the adsorbed proteins remained similar, approximately 0.2 units, for all of the amounts of HAP tested (the values for the adsorbed proteins are twice relative to whole saliva since 500µl of phosphate was used to desorb proteins for 1mL of whole saliva incubated with HAP).

The bound proteins in each case were also analysed by SDS-PAGE (Figure 4b). The supernatants were diluted 3:1 into 4x SDS loading buffer ((0.625M Tris-HCl, pH 6.8, 40% (v/v) glycerol, 2% (w/v) SDS and 0.00125M bromophenol blue)) and briefly heated (2 min, 90°C) in a heat block. A sample of whole saliva was also loaded for comparison. Proteins were resolved using gels comprising 12% separating gel and 4% stacking gel (detailed SDS-PAGE protein analysis in section 2.2.1.5). The salivary proteins that bound to HAP when whole saliva was incubated with 5mg of HAP were the same to those bound when saliva was incubated with larger amounts of HAP.

The ratio of 1mL whole saliva to 5mg of HAP powder was chosen for the experiments in this study as this was found to saturate the protein binding capacity of the HAP (Figure 4).

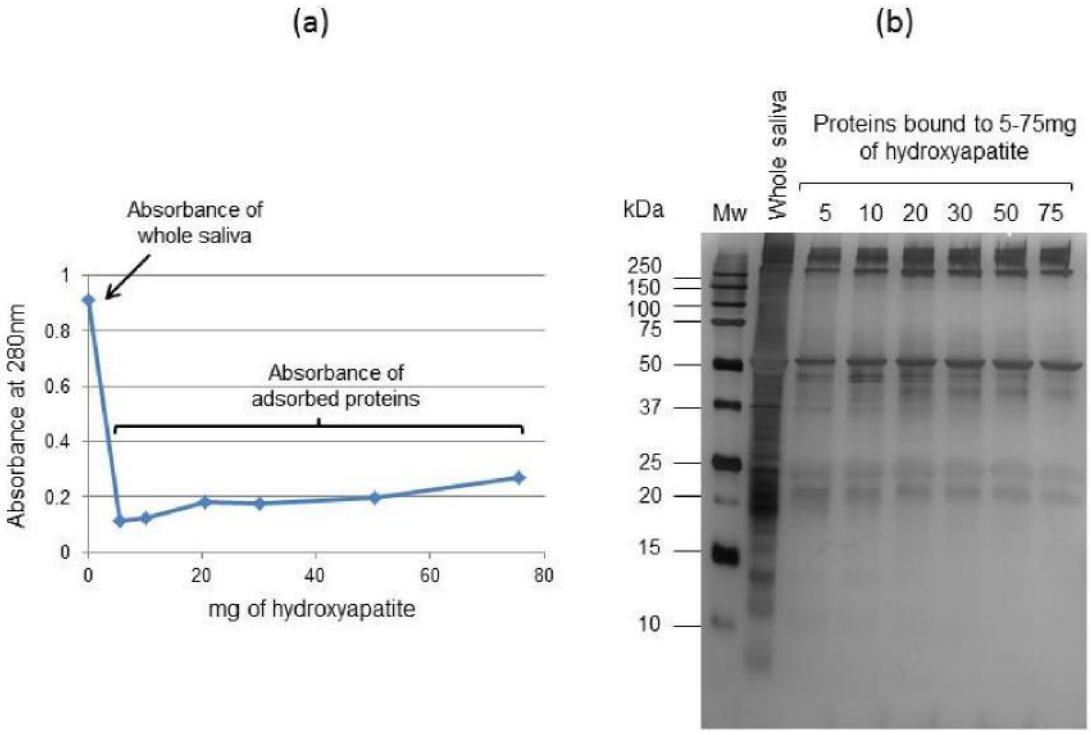


Figure 4. Binding ratio of proteins to HAP. (a) Quantity of salivary proteins bound to different amounts of HAP powder. (b) SDS-PAGE analysis of pure whole saliva (first lane) and whole salivary proteins bound to 5mg, 10mg, 20mg, 30mg, 50mg and 75mg of HAP.

2.2.1.2 Acid demineralisation of synthetic HAP and enamel powder with and without adsorbed salivary proteins

Following clarification, freshly collected whole saliva was added to hydroxyapatite (ICN Biomedicals, Inc, Germany) or human enamel powder prepared in house (Brookes et al., 2003) in a ratio of 1mL saliva : 5mg powder (n=8). As a negative control, 50mM Tris, pH 7.4 was added to HAP or enamel powder (in the same ratio) instead of saliva. After being thoroughly mixed by shaking at 37°C for 1 hour, the mixture was centrifuged for 10 minutes at 20800g and the supernatant which contained the unbound proteins was removed. The pellet, which contained the adsorbed proteins,

was washed twice with 50mM Tris, pH 7.4 since, unlike phosphate, Tris has not been reported to have the capacity to desorb proteins bound to HAP.

The HAP was challenged by resuspending the pellet in 10mM acetic acid, pH 3.30, or 100mM citric acid, pH 3.50 (1mL acid/5mg HAP or enamel powder), and vortexing for 30 seconds. These conditions were chosen as they are commonly used in dental research with citric acid modelling citrus fruit drinks (Hughes et al., 2000, Nekrashevych and Stosser, 2003, Wetton et al., 2007, Penniston et al., 2008). Additionally, pH values ranging from 3.30 to 5.50 for acetic acid and pH values ranging from 2.79 to 5.50 for citric acid, as well as a range of different concentrations were tested for both acids. After 30-sec vortexing, the sample was centrifuged at high speed (20800g) for 20 seconds and the supernatant was removed exactly 1 min and 25 sec after the initiation of the acid challenge.

2.2.1.3 Determination of phosphate

The level of the acid demineralisation was determined by measuring the phosphate released into the acid solution during acid attack. Phosphate was measured using a modified version of the spectrophotometric phosphomolybdate assay (Chen et al., 1956) as published previously (Brookes et al., 2003). A 96 well microplate (Greiner Bio-One Ltd, Stonehouse, Great Britain) was used and 100µl of sample and 100µl of a reagent A were added to each well. Reagent A consisted of four volumes of 1.5 mol/L sulphuric acid containing 0.625% (w/v) ammonium molybdate solution and one volume of 10% (w/v) ascorbic acid. Standard phosphate solutions containing 1000, 500, 250, 125 and 62.5 µg/mL were used to develop a standard curve and distilled water was used as a blank. Samples were diluted with distilled water (1:40) and analysed in duplicate. After 2 hours incubation of the 96 well plate at 37°C, the absorbance of the samples was measured at 820 nm using the plate reader (Thermo Scientific Varioskan Flash). The standard curve was used to determine the phosphate concentration of the samples.

2.2.1.4 Determination of calcium

A method described by Attin et al. was used to measure the calcium concentrations in each acid solution after acid attack (Attin et al., 2005). Standard calcium solutions with final concentrations of 400, 200, 100, 50 and 25 µM were used for generating a standard curve, and distilled water was used as a blank for calibration of zero absorbance. The samples and standards were diluted with distilled water (1:10) and analysed in duplicate. One hundred microlitres of samples were added to the wells of a 96 well microplate (Greiner Bio-One Ltd, Stonehouse, Great Britain) followed by 100µl of reagent R (100 mmol/L imidazole buffer (pH6.5) and 0.12 mmol/L Arsenazo III). Absorbance was read at 650 nm in the microplate reader (Thermo Scientific Varioskan Flash) at room temperature.

2.2.1.5 Protein analysis by SDS-PAGE

After acid exposure, the adsorbed proteins were extracted (desorbed) by resuspending each pellet in 0.4 mL of phosphate buffer (100mM, pH 7.4) by vortexing. The samples were centrifuged for 10 minutes at 20800g and the supernatants containing the desorbed proteins were removed. Protein samples were diluted 3:1 into 4x SDS loading buffer ((0.625M Tris-HCl, pH 6.8, 40% (v/v) glycerol, 2% (w/v) SDS and 0.00125M bromophenol blue)) and briefly heated (2 min, 90°C) in a heat block. The resolving gel consisted of 15% and in some cases 12% total concentration of acrylamide and bisacrylamide monomer (30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide stock solution (37.5:1)) in gel buffer ((1.5M Tris-HCl, 0.4% (w/v) SDS, pH 8.8)) with a 4% stacking gel in gel buffer ((0.5M Tris-HCl, 0.4% (w/v) SDS, pH 6.8)). 10-20µl aliquots of protein samples were loaded in each sample well onto the SDS-PAGE gels and also 5µl of protein standards were loaded to allow molecular weight estimation. Gel electrophoresis was carried out for 60 minutes at a constant 200V.

2.2.1.6 Gel staining

Various stain protocols for protein visualisation were used and so various protein molecular markers were loaded onto SDS-PAGE gels depending on the purpose. Coomassie blue, silver staining and fluorescent dyes were the most frequently methods used.

For silver staining the gels, the Pierce Silver Stain Kit (Thermo Scientific, Rockford, USA) was used according to the manufacturer's instructions. Instant Blue (Expedeon, Cambridge, UK) and Quick Coomassie (Generon, Maidenhead, UK) were mainly used as Coomassie based gel stains, following the one step protocol suggested by the manufacturers. Oriole fluorescent gel stain (Bio-Rad, Hertfordshire, UK) was also used for visualising proteins separated by SDS-PAGE.

Precision plus protein prestained standards in dual colour (Bio-Rad, Hertfordshire, UK) were commonly used for silver staining and Coomassie blue staining. Unstained broad range SDS-PAGE molecular weight standards (Bio-Rad, Hertfordshire, UK) were used for Oriole fluorescent protein staining.

Some posttranslational phosphorylation of proteins was detected by fluorescence using the Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen, Eugene, USA) which was used for selectively staining phosphoproteins in the SDS polyacrylamide gels.

The Pierce Glycoprotein Staining Kit (Thermo Scientific, Rockford, USA) was chosen for staining and detecting glycosylated proteins in SDS polyacrylamide gels. The protocol is based on the Periodic acid-Schiff (PAS) staining method and provides detection of the glycoprotein sugar groups.

2.2.1.7 Statistical analysis

Data were analysed using Excel (Microsoft) to calculate the means and standard deviations for saliva treated and control samples. The two groups

were then compared using an unpaired t-Test. P values less than 0.05 were considered statistically significant.

2.2.2 Adsorption of saliva to natural enamel surfaces and acid demineralisation

2.2.2.1 Preparation of natural enamel surfaces

The use of sound permanent human teeth for the study was approved by the Dental Research Ethics Committee of the University of Leeds (DREC No: 070213/NP/92). Human permanent molars and premolars were obtained from the Skeletal Tissues Research Bank of the School of Dentistry at the University of Leeds and stored in 70% ethanol. Prior to use, the teeth were thoroughly cleaned using pumice powder and a toothbrush, rinsed in distilled water and then cut in half mesio-distally using a precision cutting machine (Accutom-5, Struers, Denmark). The root and all cut surfaces were nail varnished leaving the natural enamel surface exposed. The tooth halves were rinsed with distilled water, air dried overnight and then stored in 20% ethanol until use.

2.2.2.2 Acid challenges against natural enamel surfaces

In these experiments, a tooth half (prepared as described above) was immersed in 10mM acetic acid (pH 3.3) for 30 seconds, after which it was transferred to another vial containing 10mM acetic acid (pH 3.3). This was repeated so that the tooth was immersed sequentially in 5 vials in order to establish a baseline for the mineral loss. After each acid challenge the tooth was rinsed with distilled water and the excess water removed with a paper towel. The mineral loss was determined by measuring the phosphate dissolved into acid as described in section 2.2.1.3. The tooth was then incubated with whole saliva or purified salivary proteins for 10 minutes at 37°C with shaking. After rinsing with distilled water, the tooth was similarly subjected to a series of fifteen sequential acid challenges, and mineral loss was measured after each challenge (Figure 5).

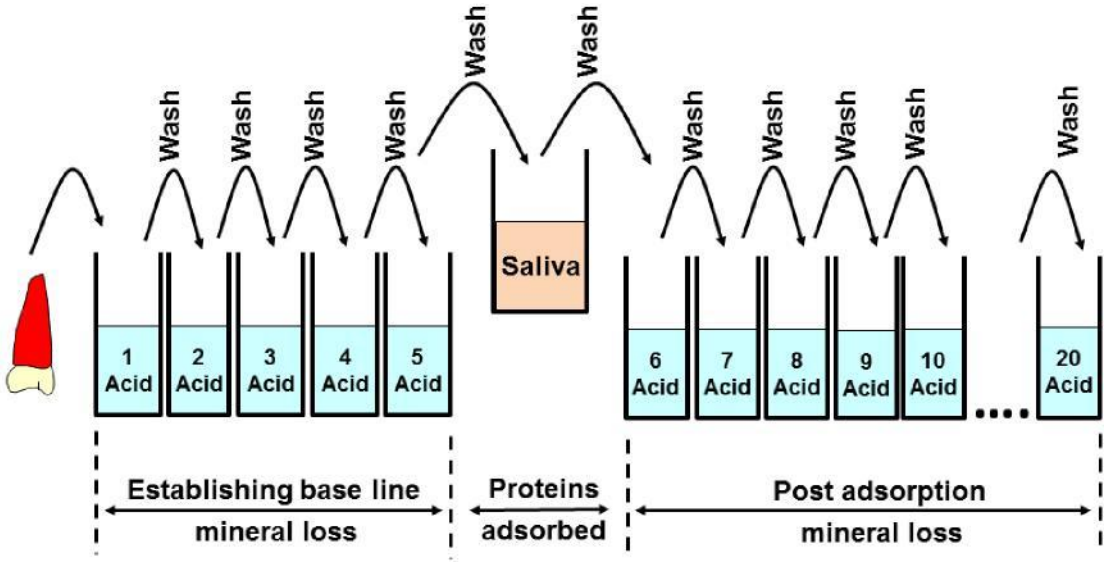


Figure 5. Acid challenges against natural enamel surfaces.

2.2.2.3 Determination of phosphate

The phosphate concentration was determined as described previously in section 2.2.1.3 except samples were not diluted. Standard phosphate solutions containing 20, 10, 5, 2.5 and 1.25 µg/mL phosphate were used to develop a standard curve.

2.2.2.4 Statistical analysis

Data were analysed using Excel (Microsoft) to calculate the means and standard deviations for the phosphate dissolved in each vial. Phosphate dissolved in the last acid challenge (vial 5), before saliva treatment, was compared with the phosphate present in the following vials (vials 6-20). The data were analysed using a paired t-Test. P values less than 0.05 were considered statistically significant.

2.2.2.5 Protein analysis by SDS-PAGE

From vials post absorption of salivary proteins (vials 6-20 containing 1 mL of acidic solution), 200µl was used for the determination of phosphate and 800µl was lyophilised for protein analysis by SDS-PAGE. After lyophilisation of the proteins released into acid, each protein sample was resuspended in 15µl of 1x SDS loading buffer and briefly heated (2 min, 90°C) in a heat block.

The proteins that had remained adsorbed to the enamel surface after all 20 acid challenges were desorbed from the enamel surface by agitating the tooth half in 150µl of phosphate buffer (100mM, pH 7.4). The desorbed protein samples were diluted 3:1 into 4x SDS loading buffer and briefly heated as described before.

All protein samples were loaded and run in 15% acrylamide gels with 5µl of protein standards loaded for molecular weight estimation.

2.2.2.6 Gel staining

Silver staining and Coomassie blue staining methods were used as described in section 2.2.1.6.

2.3 Identification of proteins by Western Blotting

After SDS-PAGE, the resolved proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) for 60 minutes at 70 Volts in transfer buffer ((25mM Tris, 192mM glycine, 20% (v/v) methanol)) cooled by ice packs.

The membranes were then blocked with 3% (w/v) blocking agent (non-fat dry milk) (Bio-Rad, Hertfordshire, UK) in TBS-T ((1.37M NaCl, 0.027M KCl, 0.25M Tris/Tris-HCl, pH 7.4 and 0.05% (v/v) Tween 20)) for 1 hour or overnight.

Membranes were washed with TBS-T twice, for 10 minutes, while shaking, and then were incubated with the appropriate primary antibodies (diluted in TBS-T) for 2 hours at room temperature. Primary antibodies used for this study were anti-cystatin SN mouse polyclonal antibody produced in rabbit (diluted 1:500) (abcam, Cambridge, UK) and rabbit anti-human α-amylase antibody (diluted 1:3000) (Sigma-Aldrich, Dorset, UK). After incubation with primary antibodies, the membranes were washed twice, for 10 minutes with TBS-T with gentle agitation.

The membranes were then incubated with appropriate secondary antibodies (diluted 1:4000 in TBS-T) for 1 hour at room temperature while shaking. Secondary antibodies used were anti-rabbit IgG peroxidase and anti-mouse IgG peroxidase antibody produced in rabbit (Sigma-Aldrich, Dorset, UK).

Finally, after washing the membranes with TBS-T five times for 5 minutes, DAB (3,3'-Diaminobenzidine tetrahydrochloride) with metal enhancer tablet set (Sigma-Aldrich, Dorset, UK) was used as precipitating substrate for the localization of peroxidase activity. After the development of the blot, an image of it was taken using a ChemiDoc MP Imaging System (Bio-Rad, Hertfordshire, UK).

2.4 Purification of salivary proteins

Different purification methods were used in order to obtain fractions of salivary proteins that could be tested individually for their ability to protect against acid demineralisation. Size exclusion chromatography and HAP chromatography were the main techniques used for this study (Table 6).

2.4.1 Protein purification by size exclusion chromatography

Size exclusion chromatography is a method by which the molecules are separated according to their size. Proteins transport through a column, the stationary phase of which consists of spherical porous particles, according to their molecular size using an aqueous solution as the mobile phase. Large proteins are expected to elute from the column first while smaller proteins can enter into pores and come off the column later (Figure 6). Cross-linked dextran, agarose and polyacrylamide gels are some examples of materials used to pack columns.

Ultraviolet (UV) detectors at wavelengths of 270, 275 and 280 nm are commonly used for detection of the proteins but fluorescent detectors or a combination of multiple detectors can also be used. The correct size of the pores and the appropriate conditions of the mobile phase (buffer) are important steps for the development of the size exclusion chromatography method. Generally, the use of buffers with salt concentration (ionic strength) at 50-200mM decreases non-specific interactions between the protein and the column. The pH of the buffers commonly used is between 6 and 7.2 but ideally it should be close to the isoelectric point of the protein. Furthermore, the flow rate of the buffer and the sample loading are two more factors important for the development of the method. As a general rule, the appropriate sample size for loading is 1 to 5 % of the column volume and the reduction of flow rate usually results in an improvement of the resolution (Hong et al., 2012).

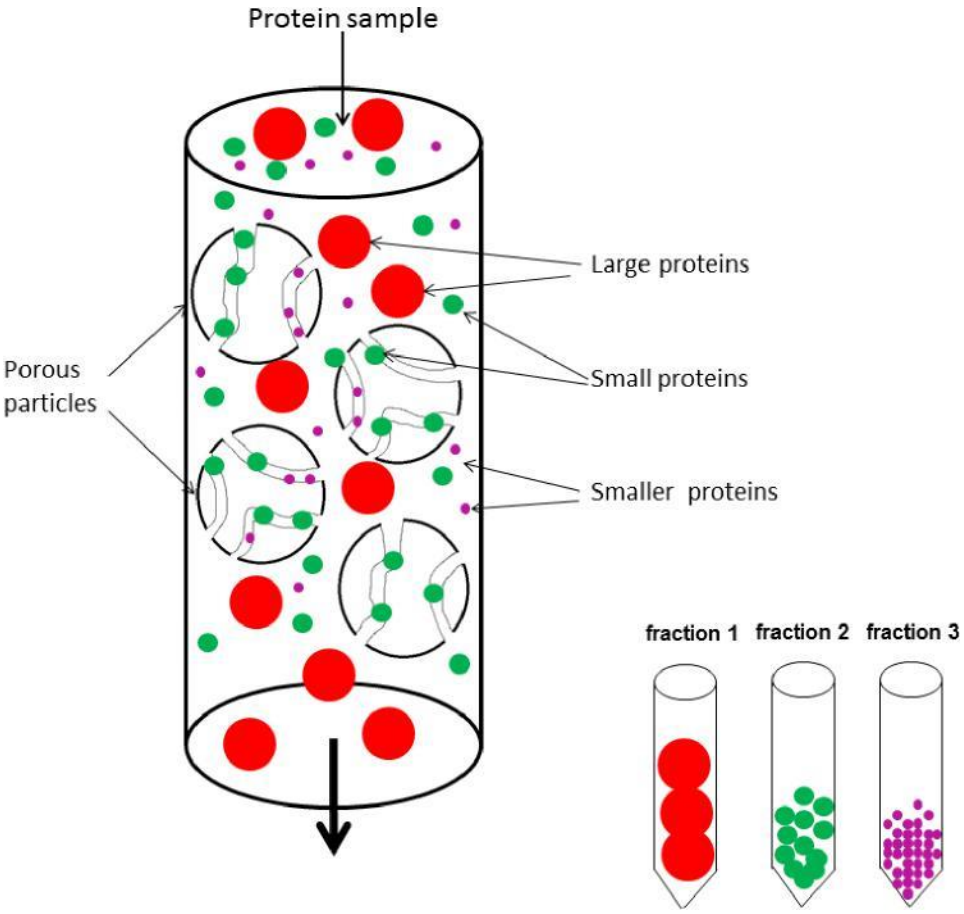


Figure 6. An illustration of the theory behind size exclusion chromatography. A mixture of proteins is loaded onto a chromatography column which is packed with porous particles. Large proteins cannot penetrate into the pores and they are eluted first while smaller proteins enter into the pores and elute last.

2.4.1.1 Protein purification under non-denaturing conditions using a HiLoad 16/600 Superdex 75 pg column

Purification of salivary proteins by size exclusion chromatography under non-denaturing conditions was conducted following the method described by Baron et al. with some modifications (Baron et al., 1999a). Paraffin wax-stimulated whole saliva was collected between 9 and 11 am as described before and protease inhibitors (Roche Diagnostics, Mannheim, Germany) were added (one tablet per 10mL saliva). For this experiment, 20mL of saliva was collected and centrifuged for 30 minutes at 8000g, at 4°C. The supernatant was centrifuged again for 30 minutes and then frozen at -80°C before lyophilisation. The lyophilised sample was resuspended in 2mL of buffer A (25mM Tris-HCl, pH 7.0, 50mM NaCl) and centrifuged for 10 minutes at 20800g, at 4°C. Due to the high viscosity of the sample, centrifugation was repeated and the supernatant was filtered through a filter with 0.45 μm pore size. The resulting supernatant was loaded onto a HiLoad 16/600 Superdex 75 pg (GE Healthcare Life Sciences, Buckinghamshire, UK) that had been pre-equilibrated in buffer A and protein fractions were eluted at a flow rate of 1mL/min. Proteins eluting from the column were detected spectrophotometrically at 280nm and the fractions (5mL each) were automatically collected. A 30μl aliquot from each fraction was lyophilised for SDS-PAGE analysis and the rest was lyophilised for storage and further use.

The fractions were combined according to their approximate size (as low, mid and high molecular weight groups) and resuspended in a volatile buffer of 0.15M ammonium hydrogen carbonate. In order to remove the Tris and NaCl and concentrate the samples they were then applied onto a 26/10 HiPrep desalting column (GE Healthcare Life Sciences, Buckinghamshire, UK) that had been pre-equilibrated in 0.15M ammonium hydrogen carbonate and the column was run at a flow rate of 5mL/min. The desalted proteins were collected and, after lyophilisation, they were resuspended in 20mL (initial volume of whole saliva) of 50mM Tris pH 7.4 in order to be used in acid demineralisation assays and to be tested for their ability to protect against an acid challenge.

2.4.1.2 Protein purification under denaturing conditions using a HiLoad 16/600 Superdex 75 pg column

SDS-PAGE analysis of the fractions purified following the method described above showed that proteins of greatly varying molecular size were co-eluted from the column in the same fraction. Possible explanations for this were that low molecular weight proteins interacted with larger proteins and passed through the column as complexes or that large proteins were interacting with the column matrix and were thus being retarded and were therefore anomalously eluting later with the smaller proteins. In order to help prevent non-specific protein interaction with the column, the concentration of NaCl in the chromatography buffer was increased from 50mM to 150mM. To dissociate any protein complexes, 4-6M urea (a denaturing agent) was also added. Whole saliva was loaded onto the HiLoad 16/600 Superdex 75 pg (GE Healthcare Life Sciences, Buckinghamshire, UK) as described before but the column was pre-equilibrated in a modified buffer A (25mM Tris-HCl, pH 7.0, 150mM NaCl, 6M urea). The size of the eluted fractions was also decreased to 3ml in order to achieve better protein separation. Immediately after collection, fraction aliquots of 30µl were analysed by SDS-PAGE and all protein fractions were then lyophilised, resuspended in 0.15M ammonium hydrogen carbonate and desalted ready for further use as described in section 2.2.1.2 and 2.2.2.2.

The same protocol was also followed using 0.1% (v/v) Tween 20 instead of urea as a mild detergent, and the buffer A was then 25mM Tris-HCl, pH 7.0, 150mM NaCl, 0.1% Tween 20.

2.4.2 Protein purification by HAP chromatography

Another method used for purification of salivary proteins was HAP chromatography. This method is based on non-specific interactions between positively and negatively charged ions on a HAP column with positively and negatively charged protein groups. Positively charged protein amino groups bind to the negatively charged phosphates of the HAP column, while negatively charged protein carboxyl groups are repelled from the negatively

charged sites of the column and bind to the positively charged calcium ions on the column (Figure 7). Phosphate buffer is commonly used for the elution of acidic proteins from the HAP column as phosphate binds more strongly to calcium sites of the column. For the elution of basic proteins, Ca^{2+} and Mg^{2+} ions can be used as these interact with the negatively charged phosphate ions of the column (Cummings et al., 2009).

A TricornTM Empty High Performance Column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was packed according to the column instructions (Instructions 28-4094-88 AC). The packing medium consisted of DNA Grade Bio-Gel HTP Hydroxyapatite powder (Bio-Rad Laboratories, Hemel Hempstead, UK) or Macro-Prep Ceramic Hydroxyapatite TYPE II 40µm (Bio-Rad Laboratories, Hemel Hempstead, UK) and buffer A (50mM Tris, pH 7.4).

The whole saliva sample was collected and processed as described in section 2.1 and was then loaded onto the packed HAP column that had been pre-equilibrated with buffer A (50mM Tris, pH 7.4) and was run at a flow rate of 1mL/min using the buffer A. The first UV peak contained the flow through; the proteins that did not bind to the HAP column. A phosphate gradient (0-500mM phosphate buffer, 50mM Tris, pH 7.4) was then used to elute proteins according to their binding affinity to a HAP column and their desorbability by phosphate buffer. Buffer B (50mM Tris, 500mM phosphate buffer, pH 7.4) was introduced to the system and its concentration was gradually increased in order to generate the 0-500mM phosphate gradient. Proteins were detected at 280nm and the fractions (3.5mL each) were automatically collected. Immediately after collection, fraction aliquots of 30µl were analysed by SDS-PAGE and all protein fractions were then lyophilised, resuspended in 0.15M ammonium hydrogen carbonate and desalted as described in the last paragraph of section 2.4.1.1.

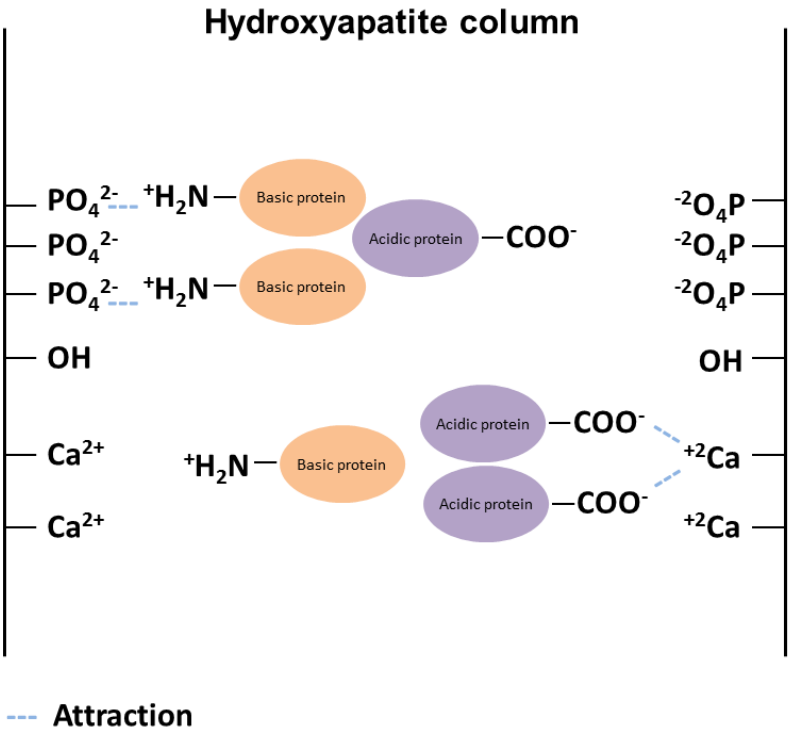


Figure 7. An illustration of the adsorption of proteins to HAP column (HAP chromatography). Basic proteins bind to phosphate sites and are repelled from the positively charged calcium sites of the column. Acidic proteins are attracted and bind to calcium sites of the column and are repelled from the negatively charged phosphate sites.

Table 6. Types of column chromatography used in this study.

Type	Column	Conditions	Buffer A	Buffer B
Size exclusion	HiLoad 16/600 Superdex 75pg	Non-denaturing	25mM Tris-HCl, pH 7.0, 50mM NaCl	
	HiLoad 16/600 Superdex 75pg	Denaturing	25mM Tris-HCl, pH 7.0, 150mM NaCl, 6M urea	
	26/10 HiPrep desalting	Non-denaturing	0.15M ammonium hydrogen carbonate	
HAP (ion exchange)	Tricorn Empty High Performance	Non-denaturing	50mM Tris, pH 7.4	50mM Tris, 500mM phosphate buffer, pH 7.4

2.4.3 Protein purification by isoelectric focusing

Isoelectric focusing (IEF) is an electrophoretic technique used to separate proteins according to their isoelectric point (the pH at which the proteins have zero net charge). A pH gradient is created, and with an applied electric field the proteins migrate towards the positive anode or the negative cathode depending on their charge. When a protein migrates to a point in the pH gradient which corresponds to its isoelectric point, the net charge on the protein becomes zero and the protein stops migrating. Proteins are thus separated according to their isoelectric points and can be harvested.

The protective salivary protein fractions that had been purified by HAP chromatography were combined and further fractionated using the MicroRotofor cell (Bio-Rad Laboratories, Inc., Hertfordshire, UK). The device was operated at 10°C to prevent heat denaturation of the proteins. Ion exchange membranes (cathode and anode membranes) were equilibrated in

electrolyte solution overnight. The sample preparation involved resuspending the lyophilised protein sample in 12% (v/v) glycerol solution which contained 2% (w/v) concentration of ampholytes (pH 3-10 range). The protein sample was then loaded and run under constant power conditions at 1W, while the voltage was increased over time. The run was complete when the voltage stabilised and the protein fractions were collected 15-20 minutes later.

2.5 Identification of salivary proteins by mass spectrometry

2.5.1 Preparation of the protein samples

Proteins that gained interest throughout this study were prepared for mass spectrometry analysis which was carried out at the Cambridge Centre for Proteomics.

Firstly, the fractions that were purified by size exclusion chromatography under non-denaturing conditions (section 2.4.1.1) containing two proteins of 14 and 60 kDa, were pooled and desalted. The protein sample was then diluted 3:1 into 4x SDS loading buffer and briefly heated (2 min, 90°C) before it was loaded and run in a 12% acrylamide gel, at 200V. After SDSPAGE, the gel was silver stained using the Pierce Silver Stain for Mass Spectrometry kit (Thermo Scientific, Rockford, USA). The protein gel bands at 14 and 60 kDa were cut in sterile conditions and stored in two different Eppendorf tubes with 50µl distilled water. A small area of stained gel with no bands was also cut to be used as a negative control.

Secondly, the salivary proteins released during each acid challenge and those that remained adsorbed to the enamel were also prepared for mass spectrometry analysis. In order to retrieve an adequate amount of these proteins for identification by mass spectrometry, the acid challenges on natural enamel surfaces was carried out as described in section 2.2.2.2 but using five times the amount of whole saliva (5mL) and five tooth halves.

From each acid vial 300µl was used for determination of phosphate dissolved. The contents of the acid vials from the 7^{th},8^{th} and 9^{th} acid challenges were pooled and lyophilised and the protein sample was resuspended in 40µl of 1xSDS loading buffer and briefly boiled. The proteins that remained adsorbed to the enamel were desorbed by adding 750µl of 100mM phosphate buffer, pH 7.4, in total for the five tooth halves. The protein sample was then diluted 3:1 into 4x SDS loading buffer and briefly boiled. Both samples (i.e. proteins released into acid and proteins that had remained adsorbed to the enamel) were then loaded and run in a 12% acrylamide gel at 200V. The gel was silver stained using the Pierce Silver Stain for Mass Spectrometry kit (Thermo Scientific, Rockford, USA). The strong, well separated gel bands were cut and stored individually in microcentrifuge tubes with 50µl distilled water. A small stained gel piece was also cut as a negative control.

2.5.2 Mass spectrometry analysis

The gel bands were further analysed in the Cambridge Centre for Proteomics by Dr Mike Deery. The samples were processed as described below.

The gel bands were excised and transferred into a 96-well PCR plate. The gel bands were cut into $1mm^2$ pieces, destained, reduced (DTT) and alkylated (iodoacetamide) and subjected to enzymatic digestion with trypsin overnight at 37°C. After digestion, the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis.

All LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC system (Thermo Fisher Scientific Inc, Waltham, MA, USA) and a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Separation of peptides was performed by reverse-phase chromatography at a flow rate of 300nL/min and a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2µm particle size, 100A pore size, 75µm i.d. x 50cm length). Peptides were

loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5µm particle size, 100A pore size, 300µm i.d. x 5mm length) from the Ultimate 3000 autosampler with 0.1% formic acid for 3 minutes at a flow rate of 10 µL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water and 0.1% formic acid and solvent B was 80% acetonitrile, 20% water and 0.1% formic acid. The linear gradient employed was 2 to 40% of solvent B in 30 minutes.

The LC eluent was sprayed into the mass spectrometer by means of an Easy-spray source (Thermo Fisher Scientific Inc.). All *m/z* values of eluting ions were measured in an Orbitrap mass analyser, set at a resolution of 70000. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD) in the quadrupole mass analyser and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17500. Peptide ions with charge states of 2+ and above were selected for fragmentation.

Post-run, the data were processed using Protein Discoverer (version 1.4., ThermoFisher). Briefly, all MS/MS data were converted to mgf files and these files were then submitted to the Mascot search algorithm (Matrix Science, London, UK) and searched against the Uniprot human database using a fixed modification of carbamidomethyl (C) and variable modifications of oxidation (M) and deamidation (NQ). Incorporated into this database were common contaminant protein sequences which were searched to reduce the number of false-positive identifications. The peptide mass tolerance was set to 5ppm, the fragment ion mass tolerance to 0.1Da and the maximum number of missed cleavages to 2. Peptide identifications were accepted if they could be established at greater than 95.0% probability.

Chapter 3
Results

3.1 Protective effect of adsorbed salivary proteins against acid demineralisation of synthetic hydroxyapatite powder

Synthetic hydroxyapatite (HAP) powder was initially selected as a model substrate because it is commonly used in dental research (Hay, 1967, Barbour et al., 2005, Shah et al., 2011). Acetic acid was selected as it is often used as demineralising solution in studies investigating the effect of saliva on enamel dissolution (Siqueira et al., 2010, Shah et al., 2011), and citric acid as a real world example. Different concentrations of acids and a range of pH values were tested but the study was then carried out using specific conditions for each acid.

3.1.1 Protective effect of adsorbed whole salivary proteins

The protective effect of whole saliva against acid demineralisation was first investigated. Salivary proteins from a volunteer were adsorbed onto synthetic HAP and then the HAP was exposed to acid. The phosphate released was determined as a measure of demineralisation. Controls comprised synthetic HAP without adsorbed salivary protein.

3.1.1.1 Exposure to 100mM citric acid, pH 2.79 and pH 3.50

Citric acid at 100mM was chosen as this is typical of the concentration in orange juice but the pH value of this solution was 0.7 pH units lower than in orange juice at about 2.79. Figure 8 shows the salivary proteins bound to synthetic HAP before acid exposure and the proteins that remained adsorbed after 1 minute and 25 seconds exposure to citric acid, pH 2.79 and pH 3.50. Most salivary proteins survived the citric acid attack at pH 2.79 but some proteins such as the 25 kDa protein and a protein of about 23 kDa

were lost or decreased in concentration after the acid attack (Figure 8a) and salivary proteins did not reduce acid demineralisation compared to the control (no adsorbed salivary proteins) (Figure 9). However, after a citric acid challenge at pH 3.50, all proteins remained adsorbed to synthetic HAP. Moreover, salivary proteins significantly reduced the mineral loss by about 20%, ($p<0.01$), compared to the control (Figure 10). It was hypothesised that the proteins remaining bound to synthetic HAP during the acid attack were protective.

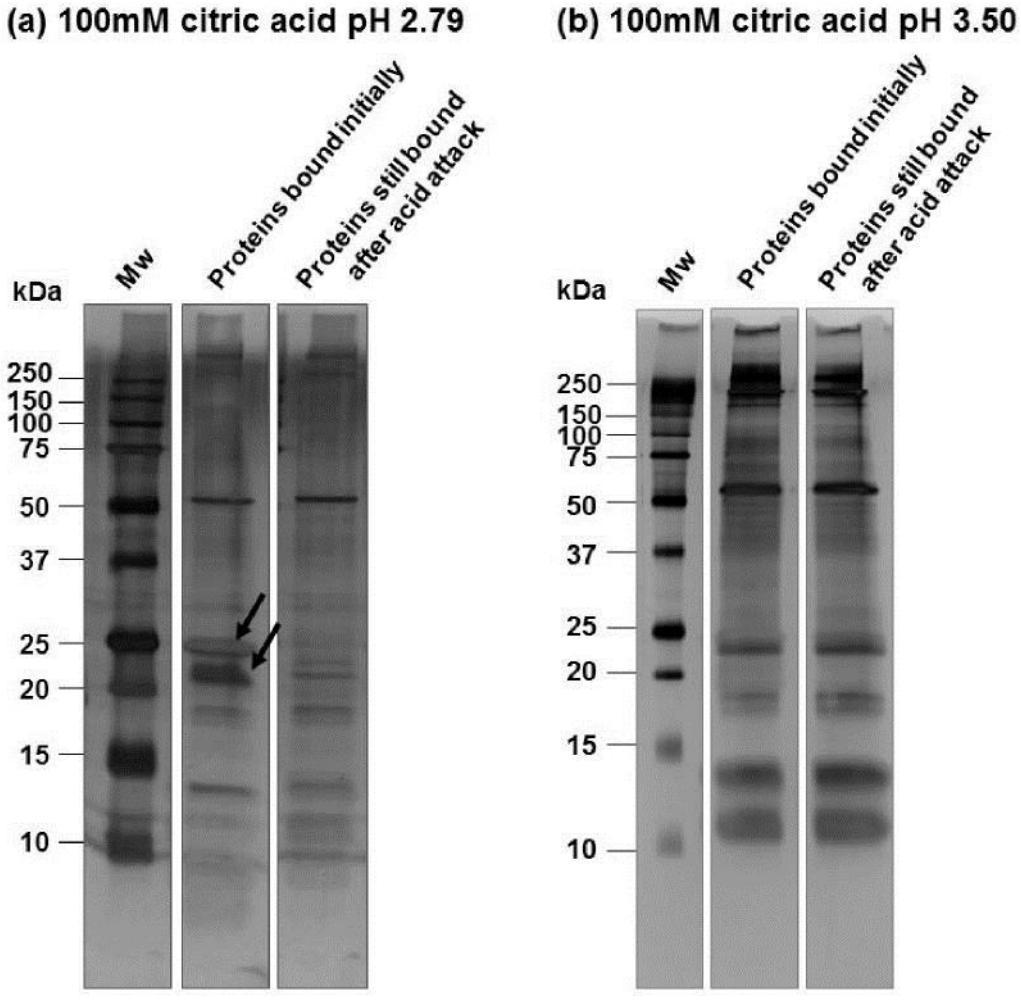


Figure 8. SDS-PAGE analysis of salivary proteins adsorbed to synthetic HAP before and after exposure to 100mM citric acid, (a) pH 2.79 and (b) pH 3.50. Note that proteins at around 23 and 25 kDa did not survive attack by 100mM citric acid at pH 2.79 (arrowed).

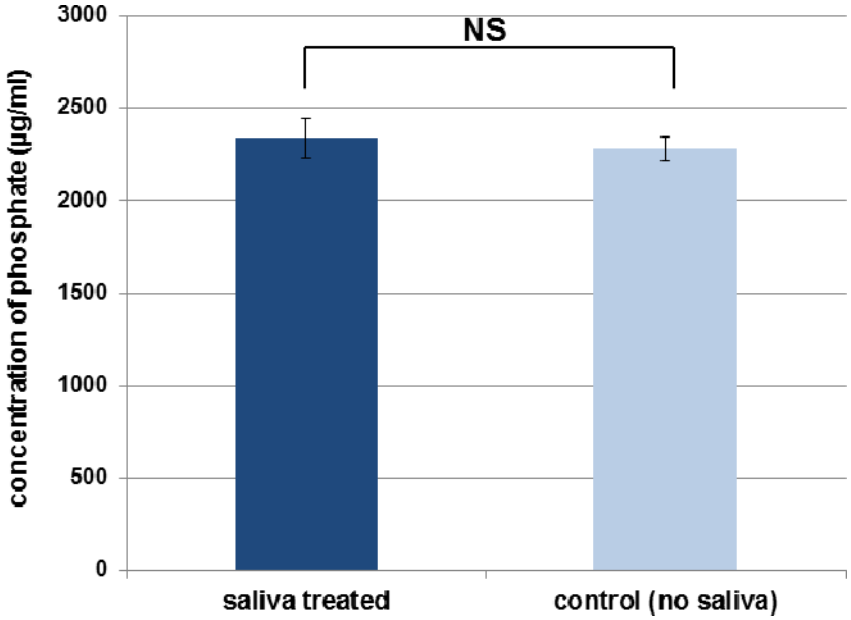


Figure 9. Release of phosphate after 100mM citric acid pH 2.79 challenge to synthetic HAP. Mean of 3 repeats (± SD) with n=8 for each repeat. Adsorbed salivary proteins had no significant protective effect.

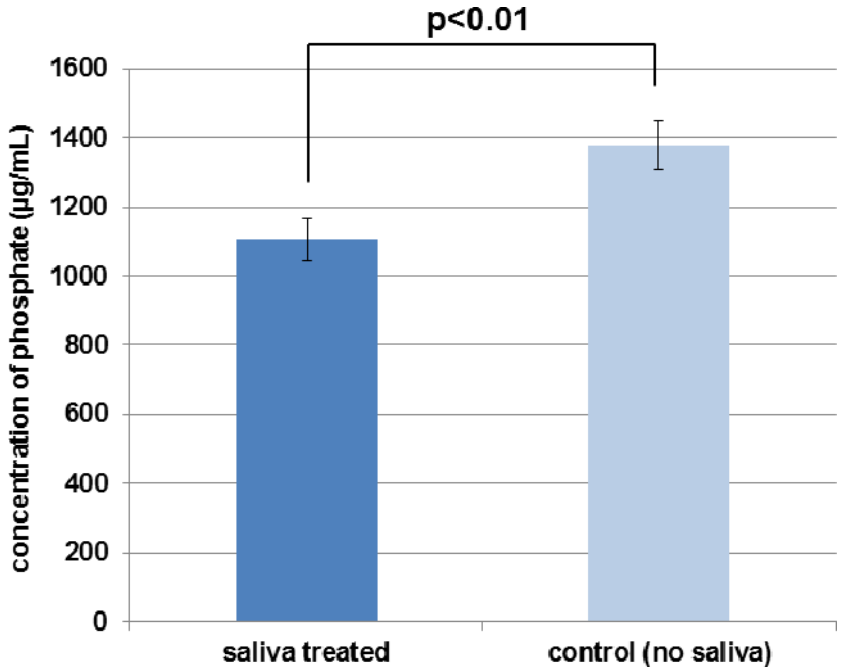


Figure 10. Release of phosphate after 100mM citric acid pH 3.50 challenge to synthetic HAP. Mean of 3 repeats (± SD) with n=8 for each repeat. Adsorbed proteins had a significant protective effect.

3.1.1.2 Exposure to 10mM acetic acid pH 3.30

The protective effect of salivary proteins against 10mM acetic acid, pH 3.30 was then tested. All salivary proteins survived the acid attack and remained adsorbed to the synthetic HAP (Figure 11). Adsorbed salivary proteins significantly reduced the mineral loss by 30%, ($p<0.0001$) compared with the control (Figure 12). It was hypothesised that the proteins remaining adsorbed during the acid attack were protective.

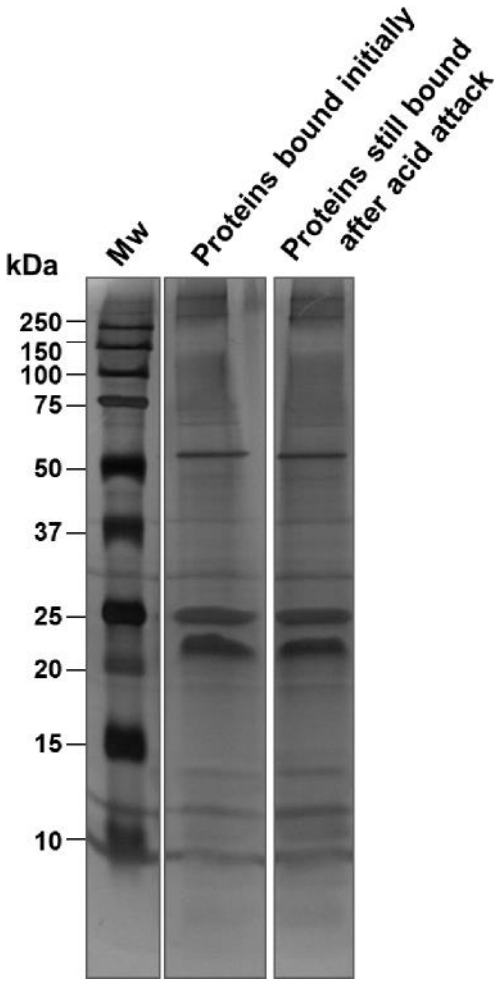


Figure 11. SDS-PAGE analysis of salivary proteins adsorbed to synthetic HAP before and after exposure to 10mM acetic acid pH 3.30. Note that proteins survived acid attack by 10mM acetic acid pH 3.30.

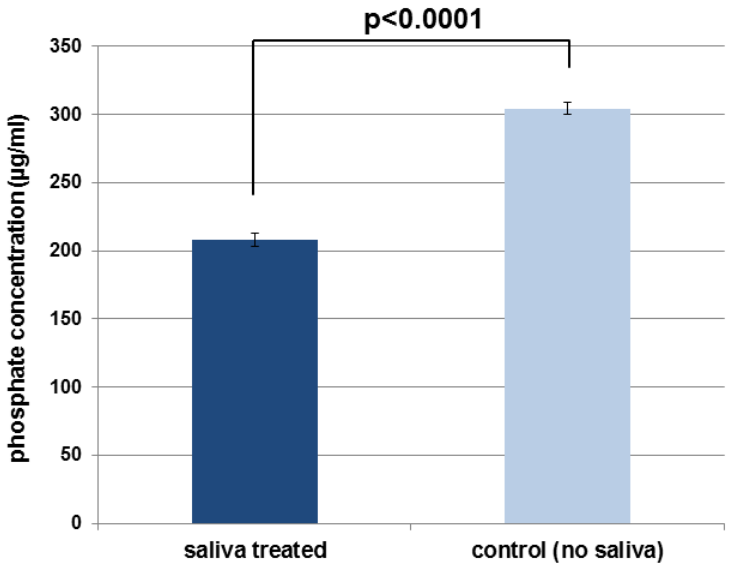


Figure 12. Release of phosphate after 10mM acetic acid pH 3.30 challenge to synthetic HAP. Mean of 3 repeats (± SD) with n=8 for each repeat. Adsorbed proteins had a significant protective effect.

3.1.2 Protective effect of adsorbed purified salivary proteins

In order to test the protective effect of different salivary protein groups or specific proteins, size exclusion chromatography was chosen as a protein purification method. Purified salivary protein fractions were then tested for their protective properties against acid demineralisation.

3.1.2.1 Purification of proteins under non-denaturing conditions

Size exclusion chromatography under non-denaturing conditions was carried out first and as a result various protein fractions, derived from whole saliva, were collected. The size exclusion chromatogram in Figure 13 shows several peaks which correspond to salivary proteins that were eluted under

non-denaturing conditions. The peaks represent the adsorption recorded at a wavelength of 280nm as the column eluent flows through a UV detector. Each peak represents different protein components and the area under each peak is proportional to the concentration of the particular component. Collected fractions exhibiting UV absorbance above baseline (A2-C3) were analysed by SDS-PAGE. Large proteins were expected to be eluted off the column first followed by the smaller proteins in order of their sizes. However, it is clear from the SDS-PAGE in Figure 14 that the proteins were not separated according to their size. A protein with a molecular weight of around 60 kDa appeared to be co-eluting with a smaller protein with a molecular weight of around 14 kDa (lanes A12-B11 in Figure 14). Possible explanations for this are that the 60 and 14 kDa proteins exist as a complex under non-denaturing size exclusion chromatography conditions but during the denaturing conditions of SDS-PAGE, the proteins migrate on the gel as monomers; alternatively the 60 and 14 kDa proteins interact with the column matrix resulting in their slow elution.

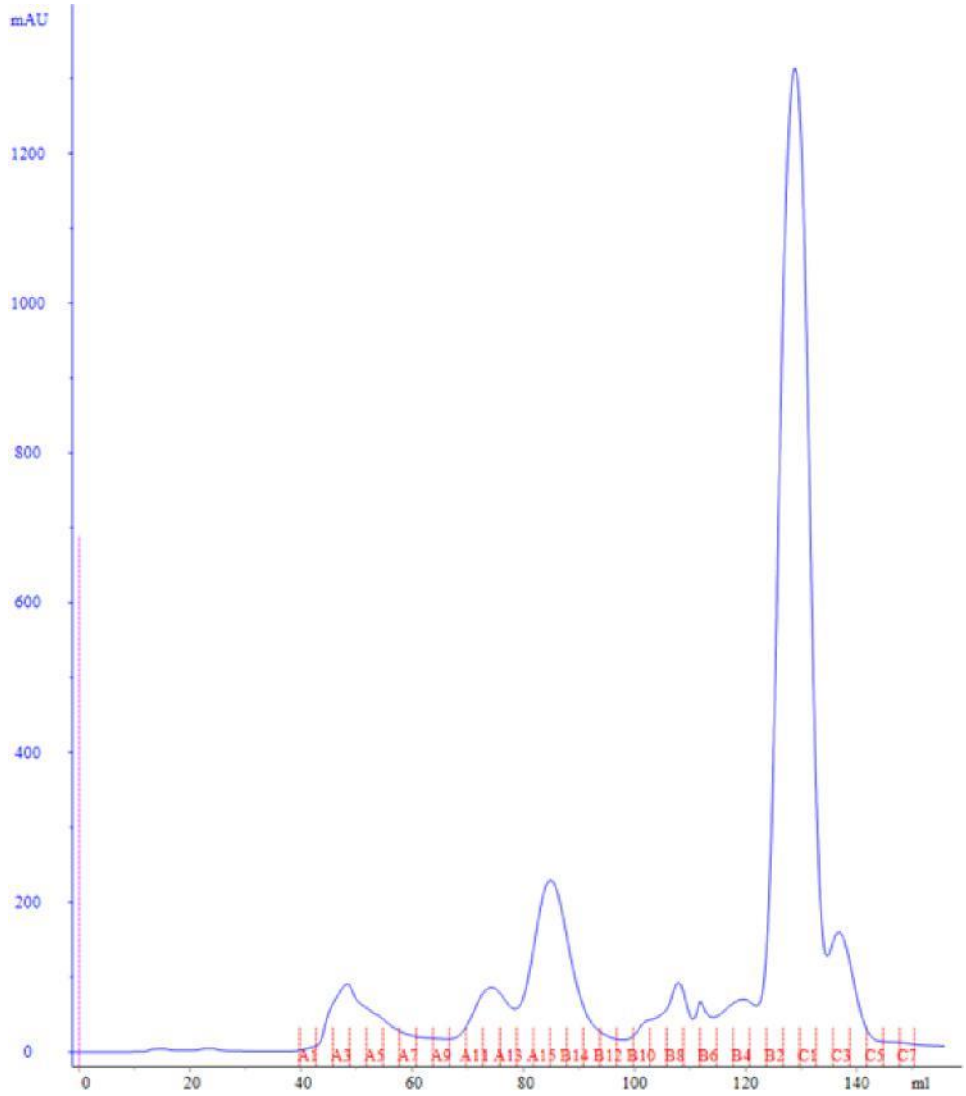


Figure 13. Size exclusion chromatogram of whole saliva on a Superdex 75 prep grade column with 25mM Tris-HCl, pH 7.0, 150mM NaCl buffer as eluent at a flow rate of 1mL/min. Several chromatographic peaks are evident indicating the separation and elution of different proteins from the column.

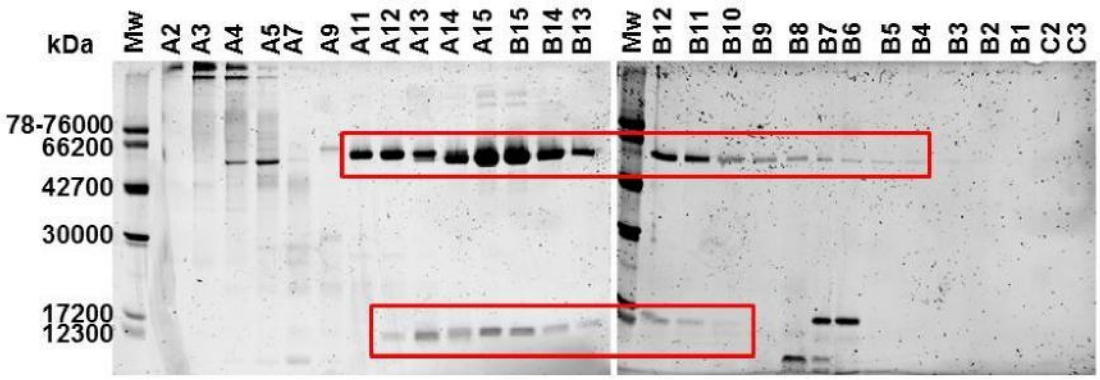


Figure 14. SDS-PAGE of salivary protein fractions purified by size exclusion chromatography under non-denaturing conditions. The 60 and 14 kDa proteins (boxed) elute in the same fractions suggesting they exist as a complex during non-denaturing size exclusion chromatography but under the denaturing conditions experienced during SDS-PAGE, they migrate on the gel in their monomeric form. Alternatively, the 60 and 14 kDa proteins interact with the column matrix resulting in their slow elution from the column.

3.1.2.2 Purification of proteins under denaturing conditions

As described above, non-denaturing size exclusion chromatography suggested that the 60 and 14 kDa proteins either existed as a complex (the complex being broken into its component monomers during SDS PAGE) or interaction between the 60 and 14 kDa proteins with the column occurred, which delayed the elution of both proteins from the column.

To investigate this, the size exclusion chromatography was re-run in the presence of a mild detergent, 0.1% v/v Tween 20, in an attempt to reduce non-specific interactions of the proteins with the column. This detergent was chosen because it is commonly used to prevent non-specific interactions between antibodies and proteins in Western blotting, enzyme linked immunosorbent assays (ELISA) and immunohistochemistry without denaturing the antibodies employed in those techniques. The chromatogram, Figure 15, shows quite defined peaks, representing

separated compounds of whole saliva passed through the column. However, SDS-PAGE analysis of each protein fraction (Figure 16) proved that proteins of different sizes were eluted as a mixture and a separation based on molecular size was not achieved. Therefore, Tween 20 was deemed not suitable for use in size exclusion chromatography for salivary protein purification.

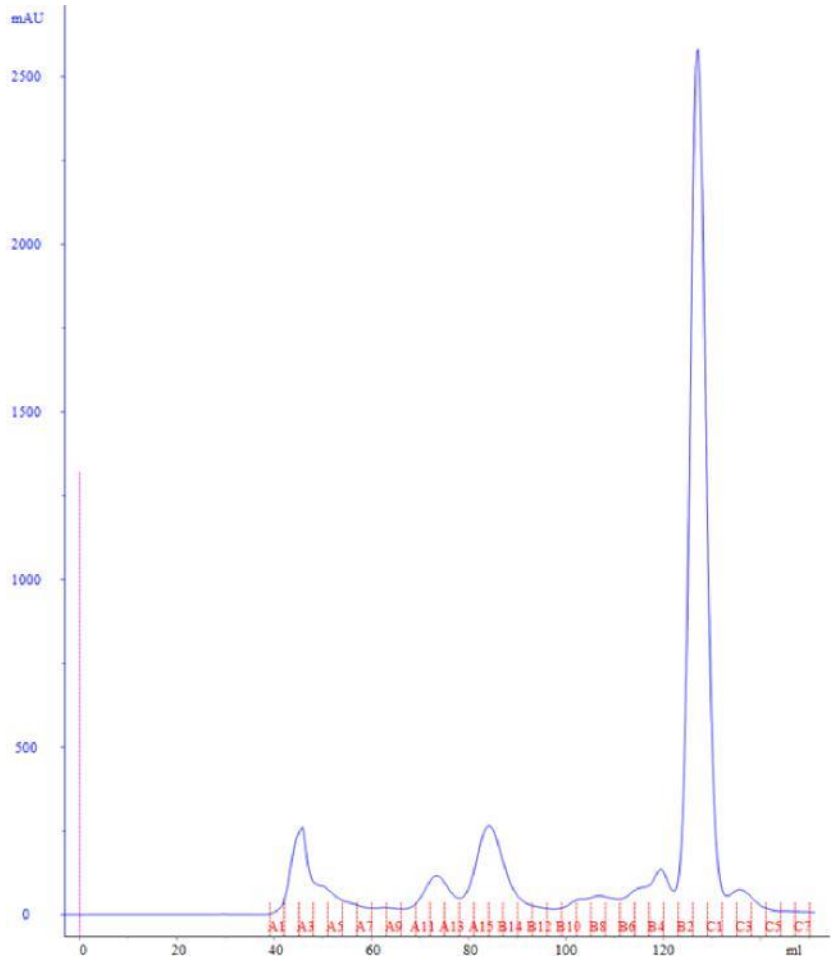


Figure 15. Size exclusion chromatography of whole saliva on a Superdex 75 prep grade column with 25mM Tris-HCl, pH 7.0, 150mM NaCl, 0.1% Tween 20 buffer as eluent at a flow rate of 1mL/min. Several peaks which represent different compounds of whole saliva eluting from the column are observed.

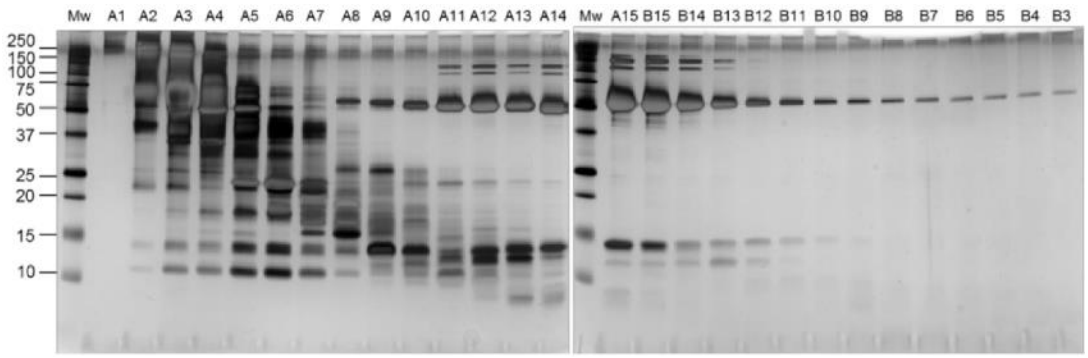


Figure 16. SDS-PAGE of salivary protein fractions purified by size exclusion chromatography under mild denaturing conditions (use of 0.1% Tween 20). Each fraction contained proteins of different sizes and protein separation according to size was not achieved.

For that reason, size exclusion chromatography was re-run in the presence of 6M urea, a strong denaturing agent, which would dissociate any protein complexes passing through the column into their monomeric forms; i.e. proteins would elute according to their true monomeric size. Urea, as a chaotropic agent, would also disrupt any hydrogen bonds between proteins and the column matrix that might result in protein-matrix interactions (causing unexpected elution characteristics). In addition, the column was calibrated by running protein standards of known molecular weight in order to better interpret the results. Knowing the elution time (volume) of the standard proteins enables the estimation of the molecular weight of unknown proteins eluted from the same column under the same conditions. Figure 17 shows the elution profile of the standard proteins.

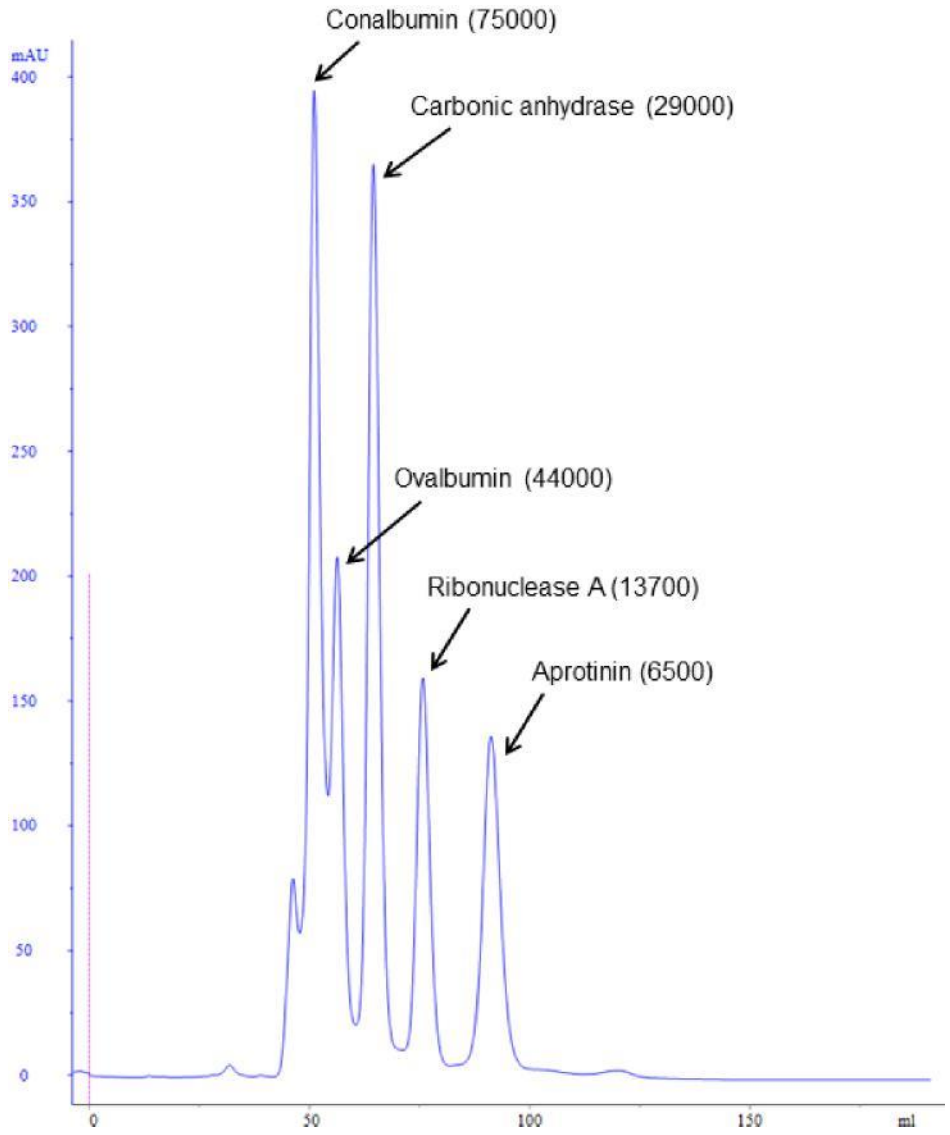


Figure 17. Size exclusion chromatography of standard proteins on a Superdex 75 prep grade column with 25mM Tris-HCl, pH 7.0, 150mM NaCl, 6M urea buffer as eluent at a flow rate of 1mL/min. Conalbumin (75 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) were the standard proteins contained in the low molecular weight calibration kit used for the molecular weight determination by size exclusion chromatography.

The chromatogram in Figure 18 shows a series of peaks representing the whole salivary proteins eluting from the calibrated column. Clear differences in the chromatographic peak profile are observed between this run under

denaturing conditions and the one under non-denaturing conditions shown in Figure 13. The dotted lines mark the elution volumes of the standard proteins shown in Figure 18 and serve as calibration markers to estimate the molecular weight of the peaks generated from the whole saliva sample loaded on the column.

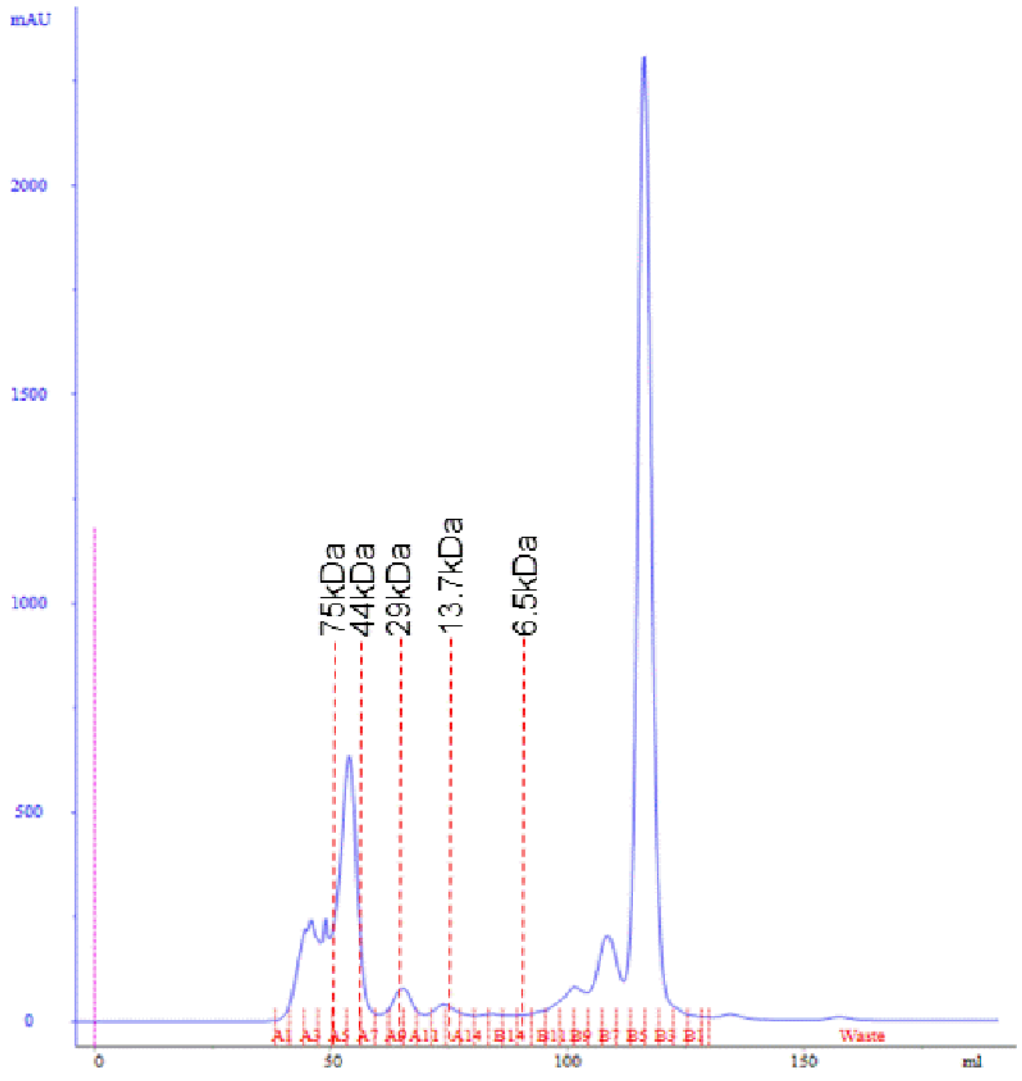


Figure 18. Size exclusion chromatogram of whole saliva on a Superdex 75 prep grade column with 25mM Tris-HCl, pH 7.0, 150mM NaCl, 6M urea buffer as eluent at a flow rate of 1mL/min. The dotted lines mark the elution volumes of the standard proteins and serve as calibration markers to estimate the molecular weight of the peaks generated from the whole saliva sample loaded onto the column.

The purified whole salivary protein fractions shown in Figure 18 were analysed on SDS- PAGE, (Figure 19). This gel effectively represents a “2 dimensional separation” of the whole salivary proteins (the first dimension being separation by size exclusion chromatography and the second dimension being separation by SDS PAGE). Going from left to right, the lanes A1-B3 represent each chromatographic fraction from Figure 18 with higher molecular protein being present in the early eluting fractions (A1-A6). The molecular calibration data shown in Figure 18 have been transposed onto Figure 19 so the molecular weight of each fraction as determined by size exclusion chromatography can be compared to the molecular weight as determined by SDS PAGE. The proteins in the peak eluting in fractions 5 and 6 in Figure 18 were eluting at a molecular weight midway between the 75 and 44 kDa standards; likewise the major bands in these fractions were migrating between the 42.7 and 66.2 kDa standards on SDS PAGE (Figure 19). The 14 kDa protein eluted in fractions A9-A12 corresponding to a molecular weight between 29 and 13.7 kDa during size exclusion chromatography (Figure 18). However, the SDS PAGE showed the protein in fractions A9-A12 migrating at a far more defined molecular weight similar to the 12.3 kDa SDS PAGE standard.

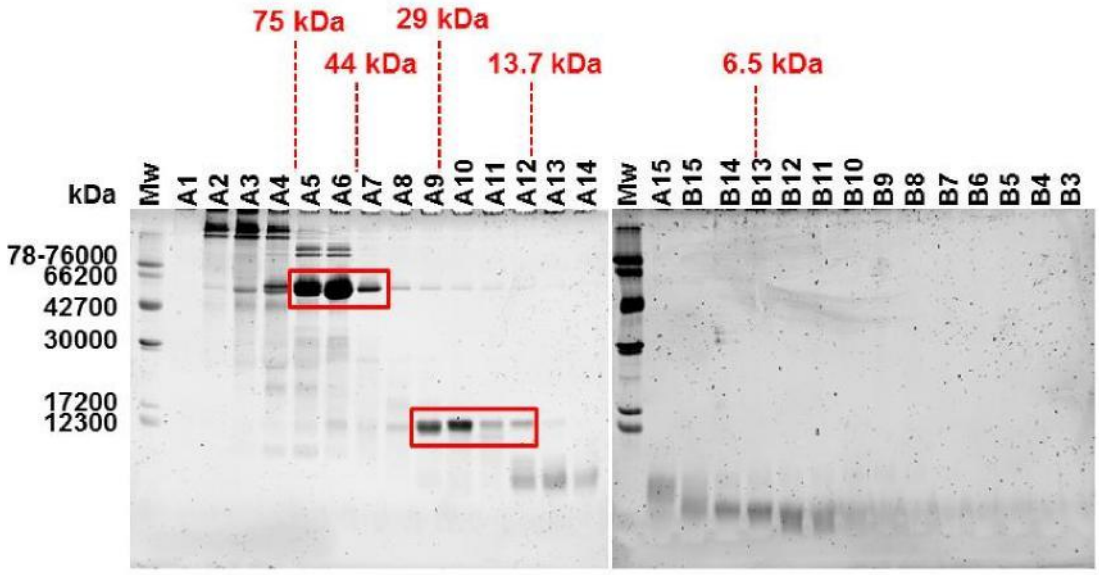


Figure 19. SDS-PAGE of salivary protein fractions purified by size exclusion chromatography under denaturing conditions. The 60 and 14 kDa proteins are now well separated under denaturing conditions (shown in red boxes).

It is clear that the 60 kDa and 14 kDa proteins that previously co-eluted during non-denaturing chromatography (shown in boxes in Figure 14) were now well separated under denaturing conditions (shown in boxes in Figure 19).

It is noteworthy that the inclusion of urea “focused” the salivary proteins into tighter fractions. For example, in the absence of urea (Figure 14) the elution of the 60 and 14 kDa proteins (shown in red boxes) was drawn out over more than ten fractions whereas in the presence of urea these proteins were eluted in far fewer fractions (shown in red boxes in Figure 19). The drawing out of the elution process in the absence of urea strongly suggests that the elution time was increased due to the interaction of the proteins with the column matrix. This interaction was eliminated in the presence of urea and the proteins eluted in much sharper peaks as they were not being retarded by interaction with the column matrix. In summary, proteins eluted in the absence of urea appear to be fractionated based on their size and the

degree to which they interacted with the column. Urea eliminated the interaction of proteins with the column resulting in a protein separation based on size alone.

3.1.2.3 Protective effect of the purified fractions against acid demineralisation of synthetic HAP powder

Fractions with similar molecular weight (Figure 19) were combined into groups (see Figure 20) and these groups were then tested for their protection against acid demineralisation. Figure 20 shows the amount of phosphate dissolved in acid for each purified protein fraction incubated with synthetic HAP, whole saliva and control sample after 10mM acetic acid pH 3.30 challenge. With no adsorbed saliva there was a considerable amount of phosphate dissolution. The presence of adsorbed whole saliva provided a reduction in dissolution, of about 8%, compared to the control, as did some of the purified fractions but these reductions were not statistically significant (in contrast to earlier data shown in Figure 12).

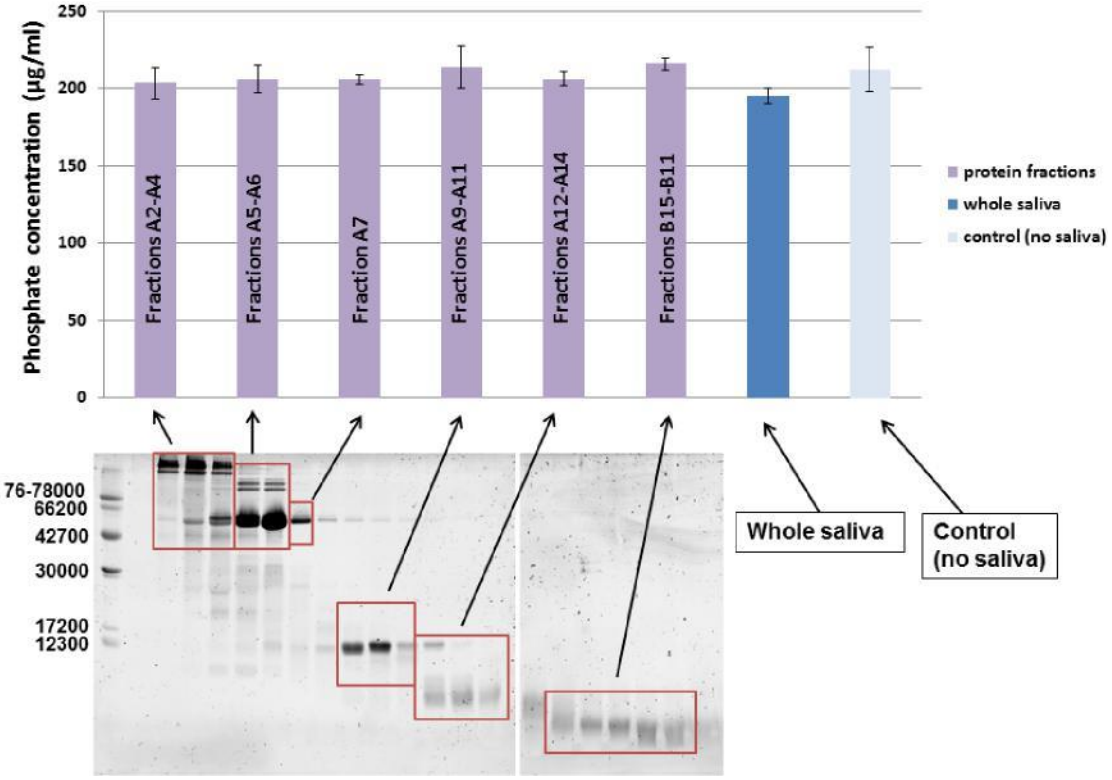


Figure 20. Release of phosphate for protein fractions, whole saliva and control samples, after 10mM acetic acid pH 3.30 challenge to synthetic HAP. Mean of 3 repeats (± SD) with n=6 for each repeat. None of the pooled fractions or the whole saliva control provided significant protection against acid demineralisation.

The protection from whole saliva shown in Figure 20 was poor and clearly not statistically significant, but it was interesting that the level of phosphate released from the control sample was lower this time compared to the control sample in previous experiments of acetic acid challenge (Figure 12). The concentration of released phosphate for the whole saliva sample observed in both experiments (Figure 12 and Figure 20) was similar, around 200 µg/mL, however the phosphate concentration of the control sample was different. The concentration of the dissolved phosphate in Figure 20 was smaller and the protective effect of whole saliva was not statistically significant. The difference in the phosphate concentration of the control

sample in the two experiments can be explained by the fact that the HAP powder (control sample) was treated with Tris in the second experiment (results in Figure 20) while in the first experiment (results in Figure 12), hydroxyapatite was used as a dry powder. In the second experiment, (results in Figure 20) HAP was incubated with 50mM Tris pH 7.4 at 37°C (similar to the test samples being incubated with whole saliva) as described in the Materials and Methods (section 2.2.1.2). In Figure 21, it is shown that the concentration of phosphate dissolved was higher (300µg/mL) when dry synthetic HAP was used and lower (240µg/mL) when Tris treated HAP was used.

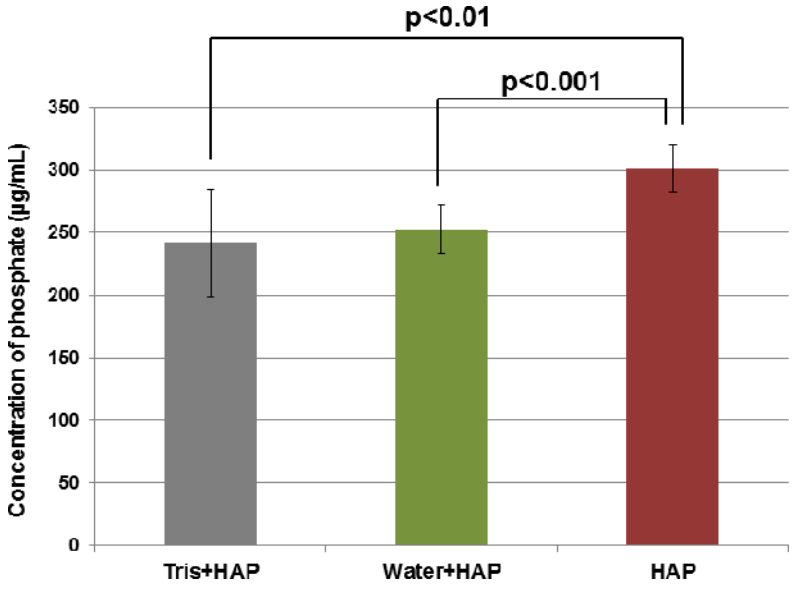


Figure 21. Release of phosphate after 10mM acetic acid pH 3.30 challenge to synthetic HAP, Tris treated HAP and water treated HAP (n=8 for each group, ±SD). Incubation of synthetic HAP with 50mM Tris pH 7.4 or water results in less phosphate dissolution compared to dry synthetic HAP.

3.1.2.4 Purification of a defined fraction consisting of two proteins

The previous results of size exclusion chromatography obtained under non-denaturing conditions showed the 14 and 60 kDa proteins co-eluting, most likely due to the interaction of the proteins and the Superdex 75 column (3.1.2.1 and 3.1.2.2). A typical set of fractions is shown in the SDS PAGE gel of Figure 22a (this gel is actually reproduced here from Figure 14) and the co-eluting proteins in question are clearly visible in lanes A12-B11. Since these fractions are dominated by the 60 and 14 kDa proteins, it was hypothesised that pooling the fractions, and then subjecting them to re-chromatography in the presence of urea would result in a good separation of the two proteins in order to aid their identification.

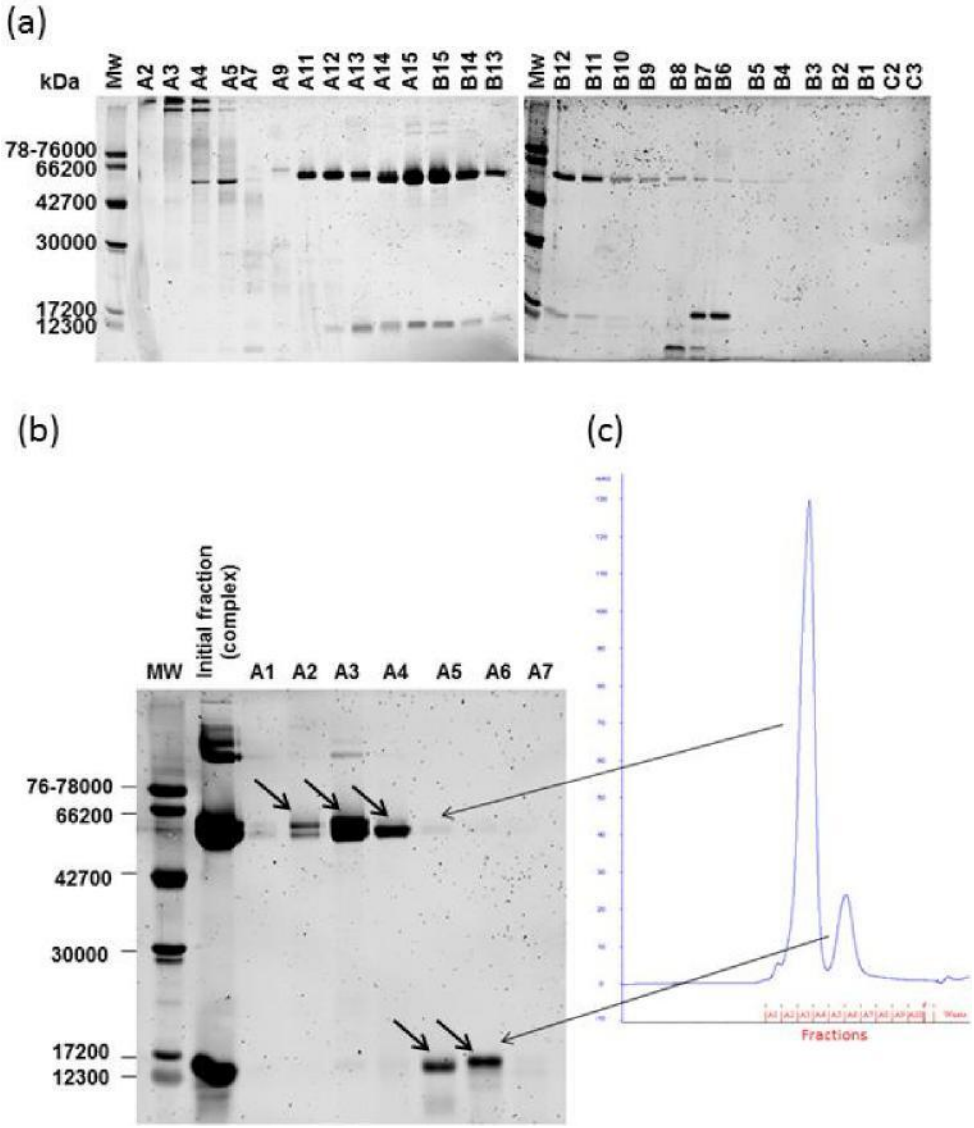


Figure 22. Separation of the 60 and 14 kDa proteins. (a) SDS-PAGE analysis of salivary protein fractions purified by size exclusion chromatography under non-denaturing conditions (b) SDS-PAGE analysis of the pooled fractions containing the co-eluting proteins (lane 1) and each protein purified individually in the presence of urea (fractions A1-A7), (c) size exclusion chromatography in the presence of urea of the pooled fraction containing the 60 and 14 kDa.

Figure 22c shows the re-chromatography with urea of the fractions containing the 14 and 60 kDa proteins. Two peaks were obtained and when they were analysed by SDS PAGE, in Figure 22b, it was clear that an excellent separation of the initial fraction had been achieved and the 14 and 55-60 kDa proteins (arrowed) were purified. However, what is more

interesting, examination of the gel in Figure 22b revealed that the 55-60 kDa protein was clearly two separate proteins (lane A2) and the 14 kDa also appeared to be present at two slightly differing molecular weights (lanes A5 and A6). This is not immediately apparent in the gel in Figure 22a where the proteins were spread over many fractions and any slight variation in molecular weight was difficult to discern.

3.1.2.5 Identification of the two proteins

The purification of the proteins together in Figure 22a led to further investigation about their binding to enamel and their potential protective effect against acid demineralisation. Identifying the two components was the initial step and was accomplished by two different methods.

3.1.2.5.1 Identification of the proteins by Western blot analysis

After the protein fraction was separated by SDS-PAGE, the identical gels were stained with silver stain, Coomassie blue and Oriole fluorescent gel stain (Figure 23). It was noticed that Coomassie blue did not stain the low molecular weight proteins as well as the other two gel stains. Identical gels were transferred onto nitrocellulose membrane for Western blotting. The membrane in Figure 24b was probed with rabbit anti-human α-amylase antibody and then goat anti-rabbit IgG peroxidase. There were two bands detected, close to each other, with similar molecular weights of 59 kDa and 56 kDa. Therefore, the two protein bands were thought to be the glycosylated and non-glycosylated isoforms of salivary α-amylase (Keller et al., 1971, Yao et al., 2003). The membrane in Figure 24c was probed with anti-cystatin SN mouse antibody and then rabbit anti-mouse IgG peroxidase. A band of approximately 14 kDa was detected, the size of which cystatin is considered to run on an electrophoretic analysis (Yao et al., 2001, Carpenter, 2003).

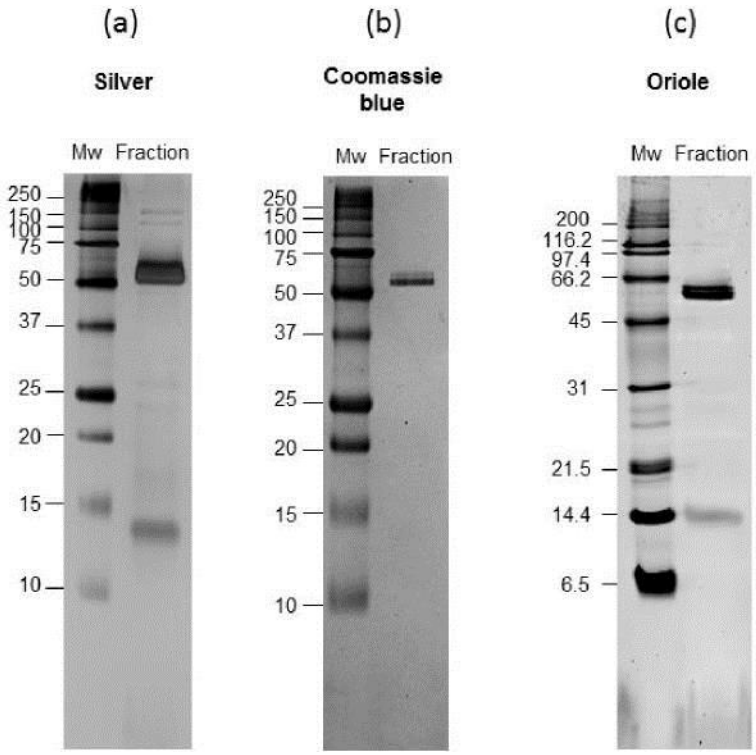


Figure 23. SDS-PAGE of the fraction of the 60 and 14 kDa proteins stained with (a) silver stain, (b) Coomassie blue and (c) Oriole fluorescent stain. Note that Coomassie blue did not stain well the low molecular weight proteins.

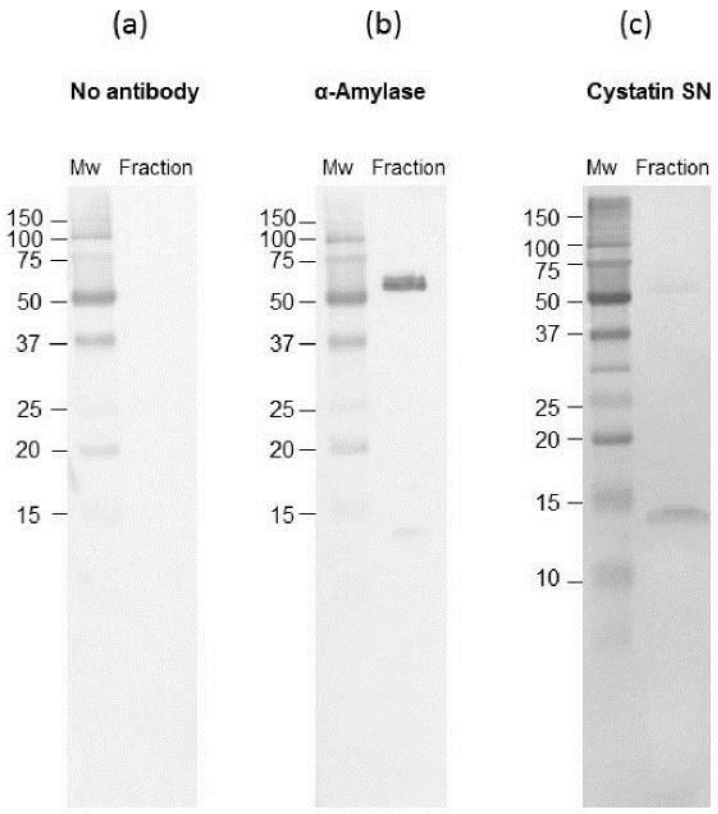


Figure 24. Western blot of the fraction of 60 and 14 kDa proteins probed with (a) no primary antibody as negative control, (b) anti α-amylase antibody and (c) anti cystatin SN antibody. No bands were detected in membrane a. Two protein bands with molecular weights of 59 and 56 kDa were detected in membrane b, thought to be the glycosylated and non-glycosylated isoforms of salivary α-amylase. A protein band of 14 kDa was detected in membrane c, considered to be cystatin.

3.1.2.5.2 Identification of the proteins by mass spectrometry

The two proteins (Figure 23) that were characterised by Western blot as α-amylase and cystatin were further analysed by mass spectrometry. The bands at around 14 and 60 kDa were cut out of the silver stained gel (Figure 23a) with a razor blade and were sent to the Cambridge Centre for Proteomics to be analysed. The gel bands were processed there, including destaining, reduction (DTT), alkylation and enzymatic digestion with trypsin overnight. After digestion, the sample was loaded onto an autosampler for

automated LC-MS/MS analysis. Separation of peptides was carried out by reverse-phase chromatography and a mass spectrometer was used to obtain all m/z (mass-to-charge ratio) values of eluting ions. The MS/MS data were submitted to the Mascot search algorithm (software) and searched against the Uniprot human database to identify potential matches. In other words, the protein bands of interest were cleaved into small peptides and the molecular masses of the peptides were measured and then compared to a database of known protein sequences. The results were statistically analysed to find the best match.

The ions score given from Mascot is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions that scored greater than 28 indicated a match or at least extensive homology ($p<0.05$).

The 14 kDa protein band was identified as cystatin-S (with an overall protein Mascot score: 470). The amino acid sequences of the peptide fragments obtained by mass spectrometry that match this protein are shown in Table 7. Table 7 shows only the peptides with score higher than 28.

Table 7. Amino acid sequences of the peptide fragments obtained by mass spectrometry analysis of the 14 kDa protein band, matching a known salivary protein, cystatin-S.

Score	Peptide
49	R.MSLVNSR.C + Deamidated (NQ)
32	R.RPLQVLR.A
52	K.ATEDEYYR.R
36	R.ALHFAISEYNK.A
101	R.EQTFGGVNYFFDVEVGR.T
70	R.IIPGGIYDADLNDEWVQR.A
31	K.SQPNLDTCAFHEQPELQK.K

The 60 kDa protein band was identified as α-amylase (overall protein Mascot score: 2247) and the amino acid sequences of the matched peptides with a high score (>28) are shown in Table 8.

Table 8. Amino acid sequences of the peptide fragments obtained by mass spectrometry analysis of the 60 kDa protein band, matching α-amylase.

Score	Peptide
47	R.KWNGEK.M + Deamidated (NO)
39	R.NMVNFR.N + Deamidated (NO)
38	K.LGTVIRK.W
49	R.CNNVGVR.I + Deamidated (NO)
40	R.YOPVSYK.L
55	K.IYVSDDGK.A
77	R.SGNEDEFR.N
81	K.INGNCTGIK.I + 2 Deamidated (NO)
42	K.HMWPGDIK.G + Oxidation (M)
78	K.SSDYFGNGR.V + Deamidated (NO)
74	R.LSGLLDLALGK.D
33	R.OIRNMVNFR.N + Deamidated (NO)
61	R.TSIVHLFEWR.W
68	R.WVDIALECER.Y
68	R.ALVFVDNHDNOR.G
86	K.NWGEGWGFMPSDR.A
36	K.SSDYFGNGRVTEFK.Y + Deamidated (NO)
111	R.GHGAGGASILTFWDAR.L
72	R.LSGLLDLALGKDYVR.S
55	K.MAVGFMLAHPYGFTR.V
81	K.DVNDWVGPPNDNGVTK.E + Deamidated (NO)
123	K.TGSGDIENYNDATOVR.D
68	R.DFPAVPYSGWDFNDGK.C

89	K.IAEYMNHLIDIGVAGFR.I + Oxidation (M)
67	K.CKTGSGDIENYNDATQVR.D
98	K.AHFSISNSAEDPFIAIHAESK.L
65	K.EVTINPDTTCGNDWVCEHR.W + Deamidated (NQ)
65	K.AHFSISNSAEDPFIAIHAESKL.-
62	R.YFENGKDVNDWVGPPNDNGVTK.E + 2 Deamidated (NQ)
132	R.NVVDGQPFTNWYDNGSNQVAFGR.G
33	K.GFGGVQVSPPNENVAIHNPFRPWWER.Y + 2 Deamidated (NQ)
78	R.IYVDAVINHMCGNAVSAGTSSTCGSYFNPGSR.D + Deamidated (NQ)
57	K.LHNLNSNWFPEGSKPFIYQEVIDLGGEPIK.S

Together with the Western blotting data (section 3.1.2.5.1), the mass spectrometry data confirmed that the 14 kDa protein band corresponded to human salivary cystatin-S and the 60 kDa protein band to human α-amylase.

3.1.2.6 Adsorption of α-amylase and cystatin to synthetic HAP powder

The adsorption of the two proteins, α-amylase and cystatin, to synthetic HAP, from a mixture containing both proteins (Figure 22a) or separately as purified components (Figure 22b) was investigated next. Pooled protein fractions (Figure 22) of α-amylase, cystatin, and α-amylase/cystatin (1mL) were incubated with 5mg synthetic HAP in each case, for 1hour at 37°C, shaking. The mixture was then centrifuged for 10 minutes and the supernatant which contained the unbound to HAP proteins was loaded and run on SDS-PAGE. The pellet was washed with 50mM Tris, pH 7.4, and by adding 300μl phosphate buffer (100mM, pH 7.4) the proteins bound to HAP were desorbed and also loaded and run on SDS-PAGE. Figure 25 shows the SDS-PAGE analysis of the unbound and bound proteins.

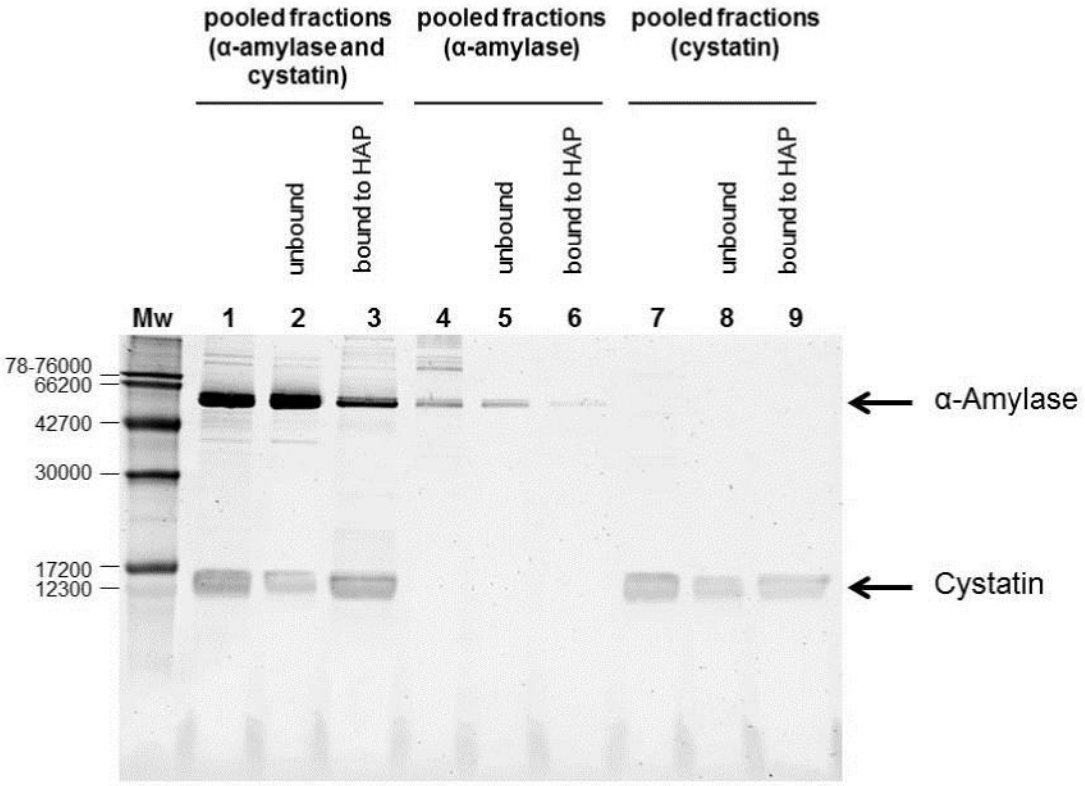


Figure 25. SDS-PAGE of α-amylase and cystatin, unbound and bound to synthetic HAP. Both α-amylase and cystatin were bound to HAP although the proportion of cystatin bound was greater than that of α-amylase (lanes 1 to 3). The smaller isoform of α-amylase was selectively bound to HAP (lanes 3 and 6).

Both α-amylase and cystatin were adsorbed from a solution containing both proteins, although the proportion of cystatin adsorbed was greater than for α-amylase (lanes 1-3). The α-amylase band was actually a doublet although a gel of this acrylamide percentage was not optimum for resolving the two α-amylase isoforms of glycosylated and non-glycosylated α-amylase as described in section 3.1.2.5.1. It was apparent by comparing lanes 1 with 3, and 4 with 6 that the smaller non-glycosylated isoform of α-amylase was selectively bound to HAP. The presence of α-amylase had no apparent effect on cystatin binding and *vice versa*.

In summary, when synthetic HAP was used as enamel substrate, whole saliva did not protect against the demineralising effects of 100mM citric acid, pH 2.79, but did provide a significant level of protection against 100mM citric acid, pH 3.50, with all proteins appearing to survive the attack. Whole saliva also reduced the demineralising effects of 10mM acetic acid, pH 3.30, and again all proteins remained adsorbed after the attack. In addition, various salivary protein fractions, purified by size exclusion chromatography under denaturing conditions, offered a very small degree of protection. Two proteins with molecular weights of 60 kDa and 14 kDa were eluted together as a complex under non-denaturing conditions but under denaturing conditions the two proteins were purified as monomers. Mass spectrometry and Western blotting indicated that the two proteins were α-amylase (60 kDa) and cystatin-S (14 kDa). It was also demonstrated that cystatin had strong affinity for synthetic HAP whereas of the two known α-amylase isoforms the non-glycosylated species showed the higher binding affinity. There was no cooperativity in terms of binding between cystatin and α-amylase.

3.2 Protective effect of adsorbed salivary proteins against acid demineralisation of human enamel powder

The previous results were obtained using synthetic HAP to simulate the tooth enamel surface but, in the following experiments, human enamel powder was used in order to validate those results.

3.2.1 Protective effect of adsorbed whole salivary proteins

3.2.1.1 Exposure to 100mM citric acid pH 2.79 and pH 3.50

The same concentrations and pH values of citric acid as used with synthetic HAP were used again in order to test the protective effect of whole salivary proteins when human enamel powder is used. The SDS-PAGE analysis (Figure 26) showed that some previously adsorbed proteins remained adsorbed following a challenge by citric acid at pH 2.79 and 3.50. Some proteins such as those of approximately 51, 34, 24.5 and 20 kDa were initially bound to enamel powder but these specific proteins appear to have been lost after the citric acid pH 2.79 challenge (arrowed in Figure 26a). Likewise, the proteins with molecular weights of 96, 39, 32, 28, 25 and 19.5 kDa were initially bound to enamel but appear to have been lost after the citric acid pH 3.50 attack (arrowed in Figure 26b). Whole salivary proteins reduced the phosphate dissolution significantly ($p<0.01$) by 25%, after exposure to citric acid, pH 2.79; however, the reduction (12%) in mineral loss afforded by these proteins during citric acid, pH 3.50, demineralisation, was not statistically significant (Figure 27 and Figure 28).

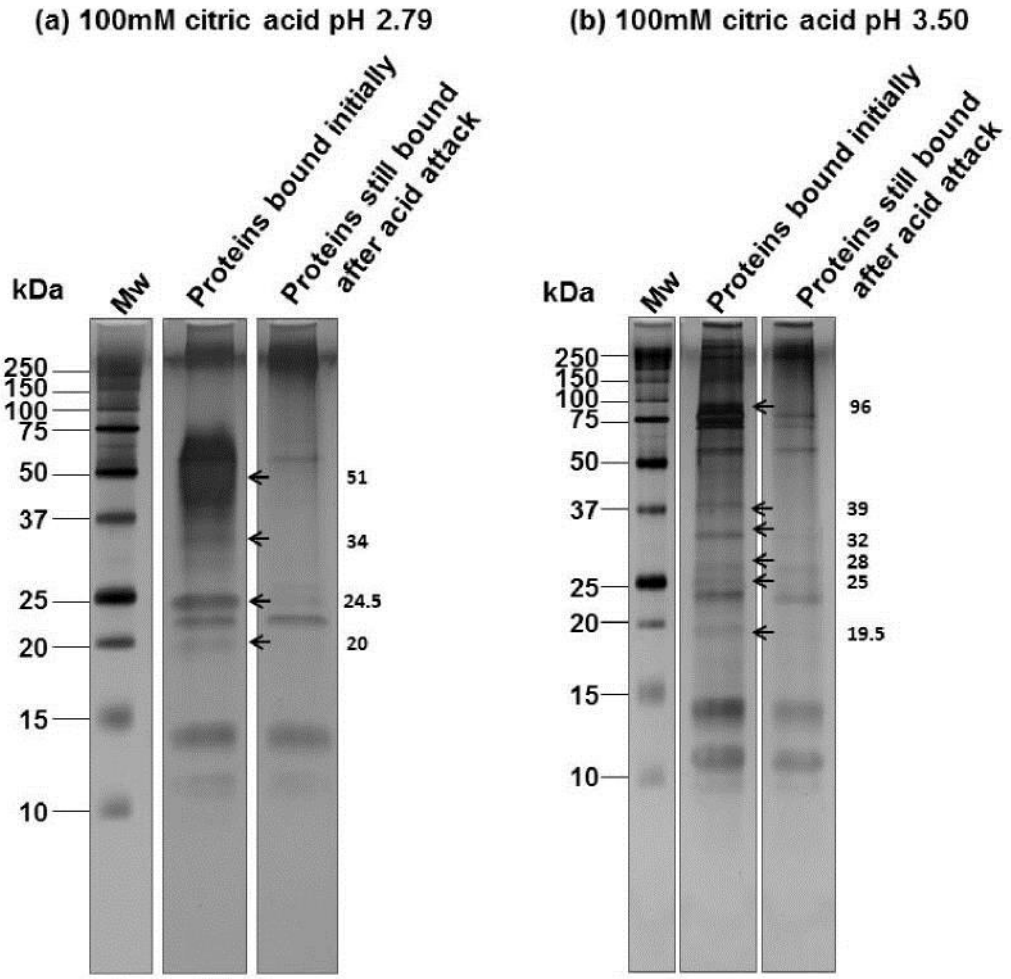


Figure 26. SDS-PAGE analysis of adsorbed salivary proteins to human enamel powder before and after exposure to 100mM citric acid, (a) pH 2.79 and (b) pH 3.50. Note that several proteins (arrowed) were initially bound to enamel but appear to have been lost after the citric acid at pH 2.79 and pH 3.50**.**

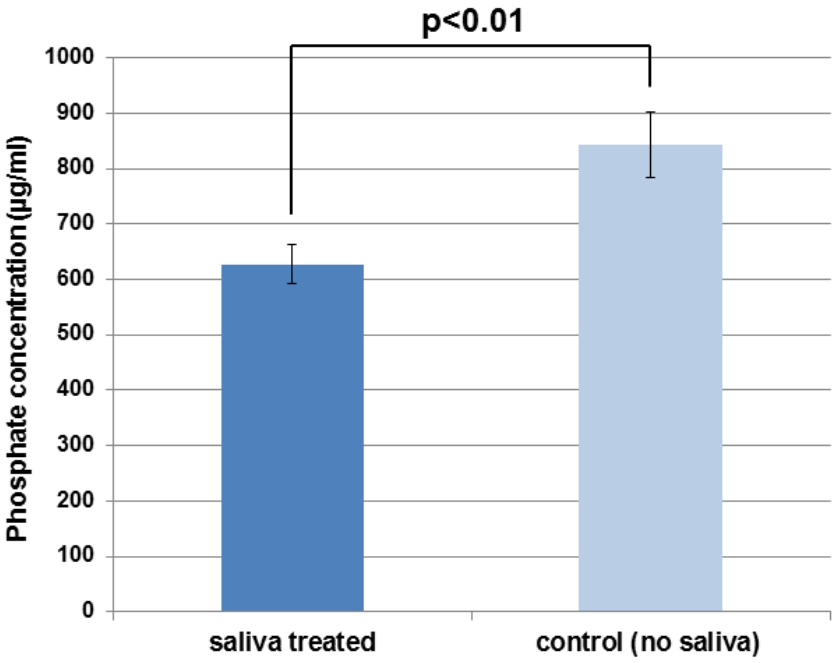


Figure 27. Release of phosphate after 100mM citric acid pH 2.79 challenge to human enamel powder. Mean of 3 repeats (± SD) with n=8 for each repeat. Adsorbed salivary proteins had a significant protective effect.

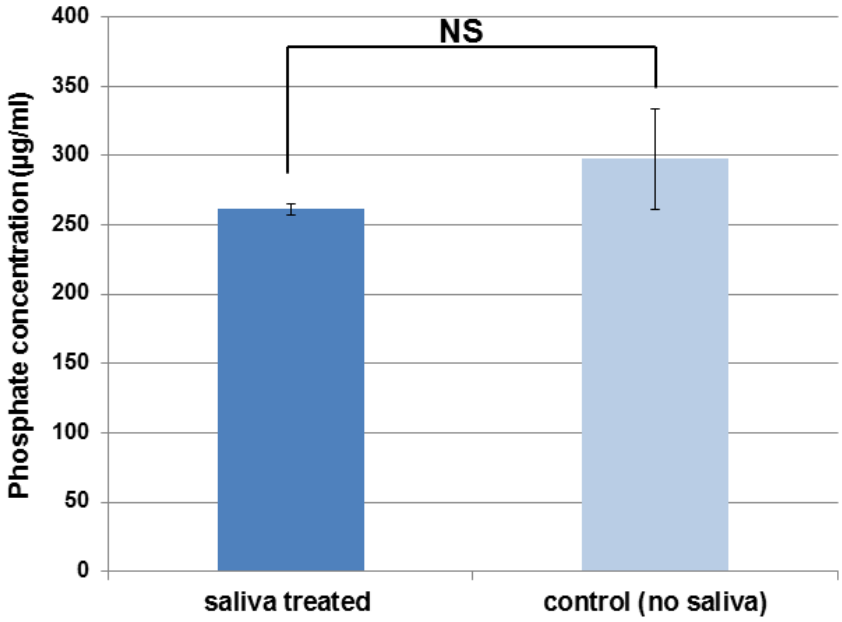


Figure 28. Release of phosphate after 100mM citric acid pH 3.50 challenge to human enamel powder. Mean of 3 repeats (± SD) with n=8 for each repeat. Adsorbed salivary proteins had no significant protective effect.

3.2.1.2 Exposure to 10mM acetic acid pH 3.30

The protective effect of whole salivary proteins against 10mM acetic acid pH 3.30 was then investigated using human enamel powder as enamel substrate. All salivary proteins survived the acid attack and remained bound to the human enamel powder (Figure 29). Measurement of the phosphate released during the acid attack showed that adsorbed salivary proteins significantly decreased the mineral loss by 26%, ($p<0.0001$) compared with the control (Figure 30).

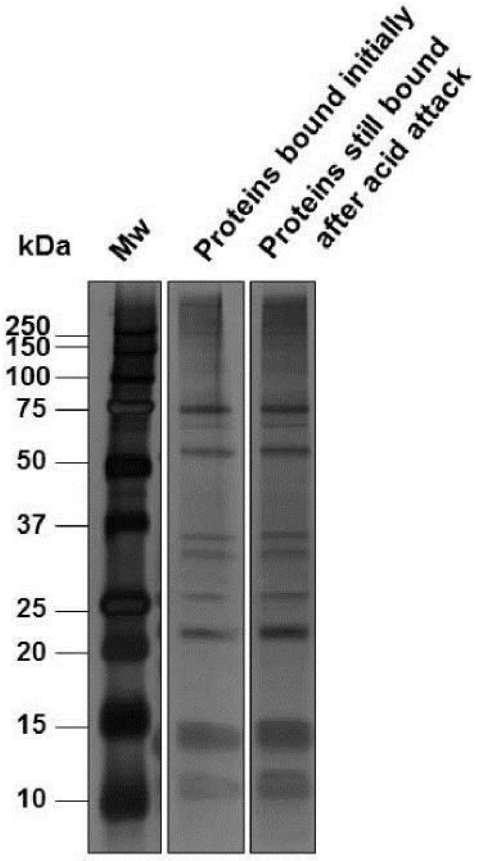


Figure 29. SDS-PAGE analysis of salivary proteins adsorbed to human enamel powder, before and after exposure to 10mM acetic acid pH 3.30. Note that proteins survived acid attack by 10mM acetic acid pH 3.30.

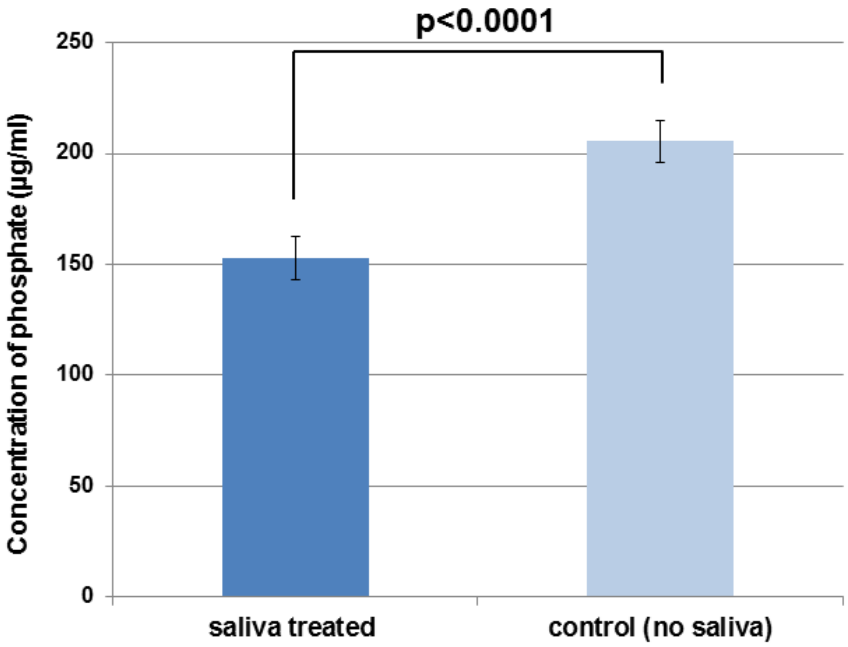


Figure 30. Release of phosphate after 10mM acetic acid pH 3.30 challenge to human enamel powder. Mean of 3 repeats (± SD) with n=8 for each repeat. Adsorbed salivary proteins had a significant protective effect.

3.2.2 Protective effect of purified salivary proteins

Figure 31 shows the protection provided by whole saliva and the various purified saliva fractions after 10mM acetic acid pH 3.30 challenge when saliva fractions were adsorbed to human enamel powder. The fractions used are the same as those used in Figure 20 (protective effect of the fractions when adsorbed to synthetic HAP). The results were broadly similar to the results obtained from when saliva was adsorbed to synthetic HAP (Figure 20) though in this case the reduction in mineral loss of about 12% afforded by pooled fractions A2-A4 did reach significance ($p<0.01$) making this fraction as effective as the whole saliva control.

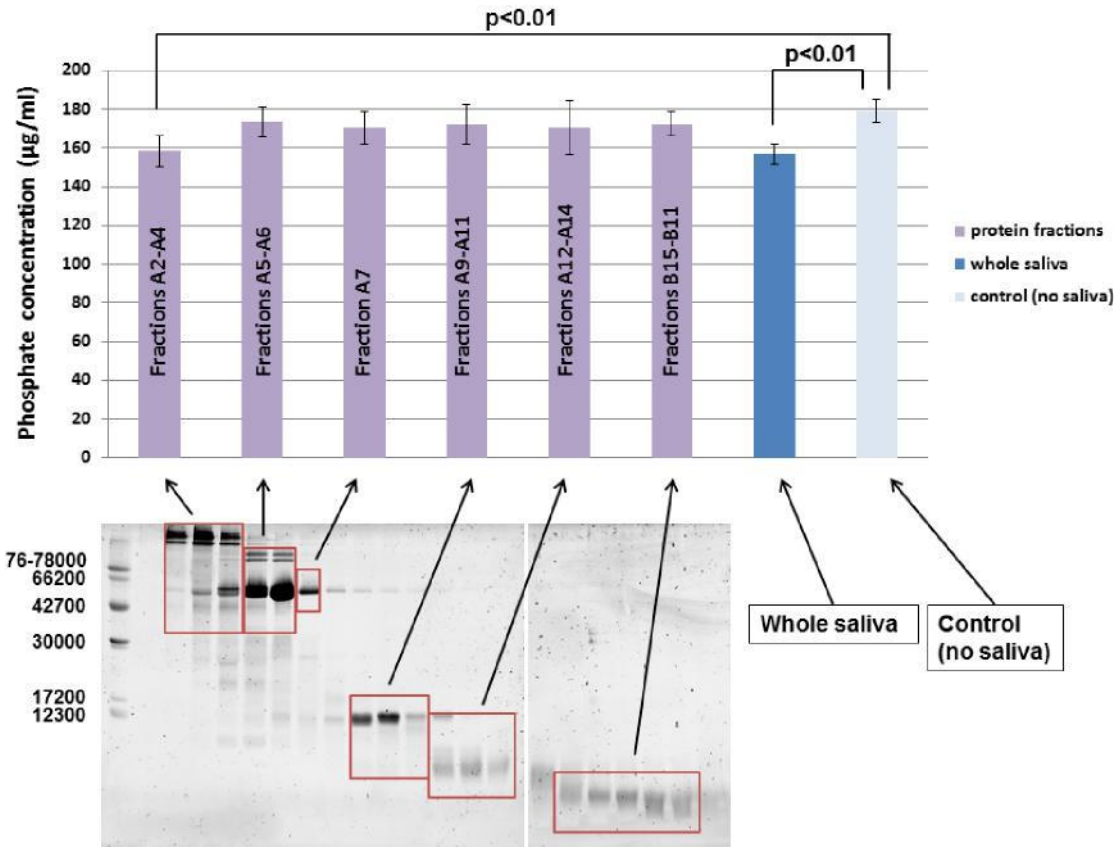


Figure 31. Release of phosphate for protein fractions, whole saliva and control samples, after 10mM acetic acid pH 3.30 challenge to enamel powder. 1 repeat (± SD) with n=4. Pooled fractions A2-A4 reduced the acid demineralisation as significantly ($p<0.01$) as the whole saliva control.

3.2.2.1 Adsorption of α-amylase and cystatin to human enamel powder

The adsorption behaviour of α-amylase and cystatin onto powdered enamel was also explored and the results showed differences from those obtained with synthetic HAP (section 3.1.2.6). α-Amylase and cystatin were incubated as a pooled fraction (Figure 22a), or individually (Figure 22b), with powdered enamel, and the unbound and bound to enamel proteins were analysed by SDS-PAGE (Figure 32). α-Amylase did not bind well to enamel powder in the presence or the absence of cystatin (lanes 3 and 6). On the other hand,

cystatin appears to have been bound well to powdered enamel either with the α-amylase present or not (lanes 3 and 9).

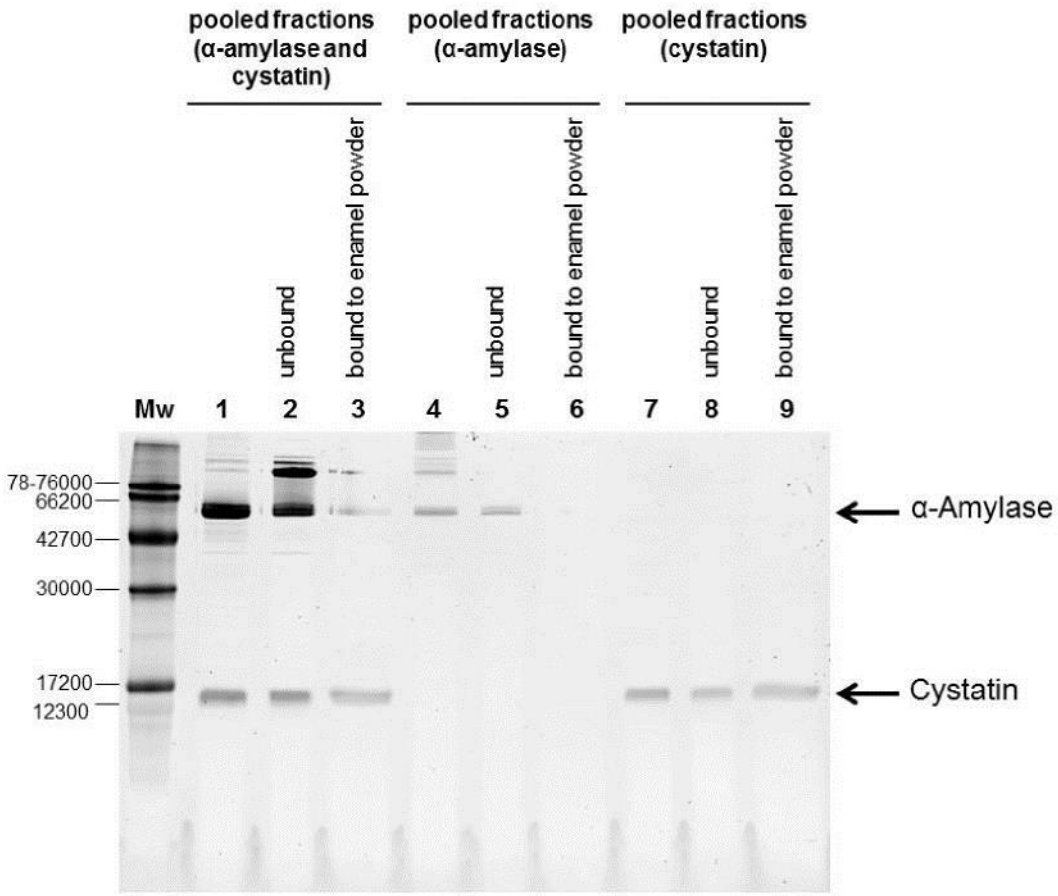


Figure 32. SDS-PAGE of α-amylase and cystatin, unbound and bound to human enamel powder. Note that α-amylase did not adsorb well to enamel powder (lanes 3 and 6). Cystatin was adsorbed to enamel powder in the presence or absence of α-amylase (lanes 3 and 9).

3.2.3 Differential adsorption of whole salivary proteins to synthetic HAP and enamel powder

Clear differences in the protein binding patterns between the enamel powder and the HAP powder were observed (Figure 33) after acetic acid challenge. For example, some proteins, such as the 152, 73, 56, 19 and 16 kDa proteins (arrowed) were selectively adsorbed to synthetic HAP. The whole

saliva that was used to adsorb onto the two powders was collected the same day at the same time. It was important to examine whether differences in the protein adsorption behaviour were critical in terms of the protection against acetic acid exposure offered by the adsorbed proteins.

Figure 30 showed that despite the difference in proteins adsorbed to HAP and enamel powder, the protection level remained similar in both cases. Salivary proteins reduced the mineral loss significantly ($p<0.0001$) by 30% when adsorbed to HAP powder (Figure 12), and by 26% when adsorbed to enamel powder (Figure 30).

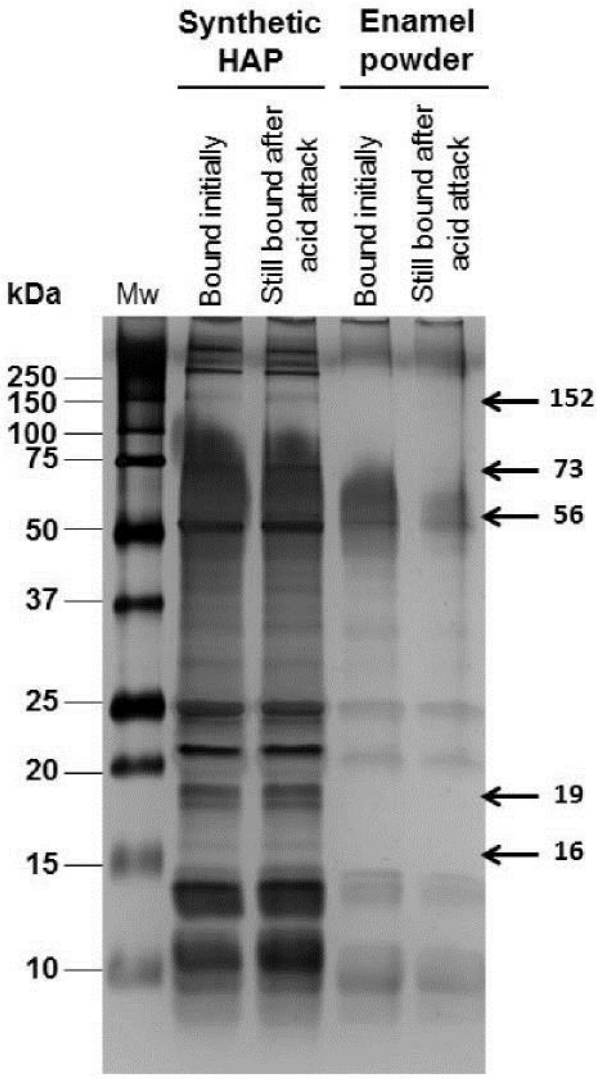


Figure 33. SDS-PAGE analysis of salivary proteins adsorbed to human enamel powder or HAP powder before and after exposure to 10mM acetic acid pH 3.30. Different proteins from whole saliva were adsorbed to synthetic HAP and enamel powder.

In summary, when human enamel powder was used as enamel substrate, whole saliva was shown to offer a significant level of protection against 100mM citric acid pH 2.79 but not against 100mM citric acid pH 3.50. In both cases, not all proteins remained adsorbed, and some salivary proteins appear to have been lost after the acid attack. Whole saliva reduced the mineral loss significantly after attack with 10mM acetic acid pH 3.30 and all

proteins remained adsorbed to enamel after the attack. It was demonstrated that different proteins were adsorbed to synthetic HAP and enamel powder but the level of protection provided after acetic acid demineralisation was similar. Results in adsorption of the fraction containing α-amylase and cystatin, or the two proteins separately, to powdered enamel suggest that cystatin has a strong affinity for synthetic HAP and human enamel powder while α-amylase did not bind well to enamel powder.

3.3 Protective effect of salivary proteins against acid demineralisation of natural human enamel surfaces

The preceding sections reported results on the protective effect of whole salivary proteins and various fractions on synthetic HAP and enamel powder in the face of citric and acetic acid challenges. However, the degree of protection afforded varied from experiment to experiment. This may have been due to inter- and intra- donor variations in the salivary proteome. It was hypothesised that despite inter- and intra- donor variations (Jenzano et al., 1987, Quintana et al., 2009) in the total salivary proteome, evolution will have ensured that the elements of the salivary proteome should be constitutively protective when faced with the natural adsorbent – the natural enamel surface. This and the fact that it is the actual enamel surface that is of most clinical relevance, led to a switch to begin using natural human tooth enamel surfaces to investigate the protective effect of adsorbed salivary proteins. This was somewhat ironic as synthetic HAP and enamel powder were initially chosen as adsorbents as they would be consistent; human enamel is of course subject to biological variation and its composition is affected by many factors such as exposure to fluoride.

The protective effect of salivary proteins using natural enamel surfaces was investigated following the acid demineralisation assay as described in

Materials and Methods (section 2.2.2) but using only 10mM acetic acid, pH 3.30.

3.3.1 Protective effect of adsorbed whole salivary proteins

The experimental system was based on that previously used to examine the effect of copper ions on acid demineralisation of human enamel surfaces (Brookes et al., 2003). In brief, sound human teeth were cut in half longitudinally and all non-natural enamel surfaces covered with nail varnish. Tooth halves were then sequentially dipped in a series of vials containing acid to establish a baseline for mineral loss in each dip. Before the 6th dip, one tooth half was incubated in saliva and salivary proteins adsorbed while the other half remained free of saliva to act as control. Tooth halves were then dipped in a further 14 vials of acid. Phosphate released by demineralisation into each vial was normalised to the amount of phosphate dissolved in acid in vial 1 which was always defined as 100%. Figure 34 shows the phosphate released into the first 5 vials to establish a mineral loss baseline. After 5 acid challenges, whole saliva was adsorbed to the enamel surface and in the subsequent 6th acid exposure there was an apparent increase in acid dissolution but during the next exposure the adsorbed whole salivary proteins reduced the mineral loss significantly by 43% ($p<0.01$). Moreover, the proteins remained protective in the subsequent acid exposures, though the effect was gradually lost. The five acidic challenges, before saliva adsorption, showed that the phosphate dissolution was increased or remained the same after each 30 sec acidic challenge. For this reason, the analysis of the results was made by comparing the level of dissolution in vial 5 (point just before saliva adsorption) with that in vial 7 (point with the biggest reduction in acid dissolution after saliva adsorption).

In contrast, control samples (with no salivary protein adsorption) showed no sudden reduction in the mineral released into the vials (Figure 35).

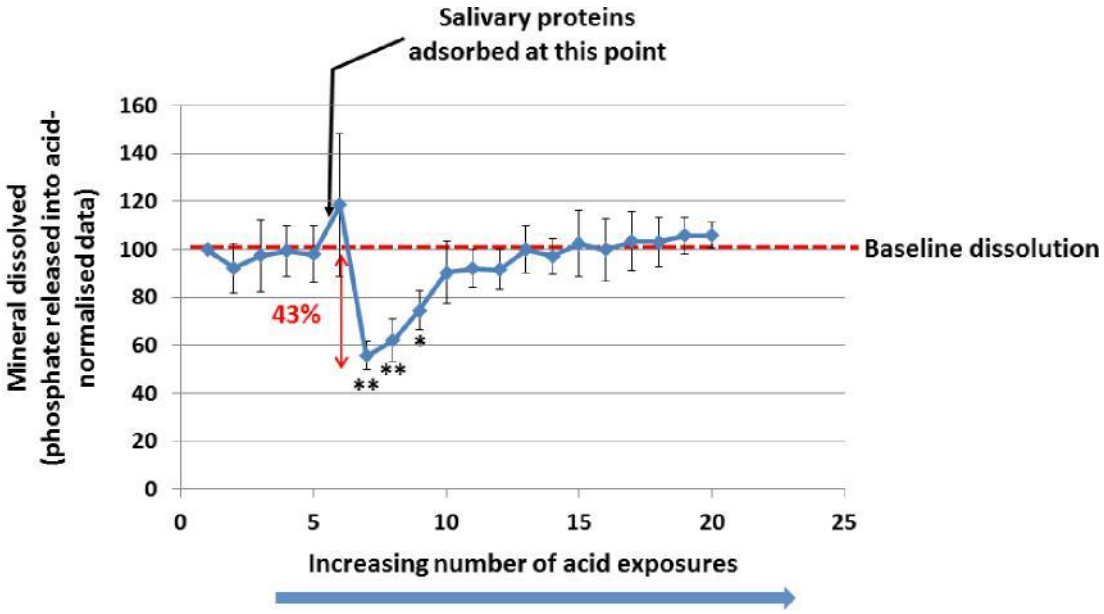


Figure 34. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after saliva adsorption. Mean of 5 volunteers (± SD) with n=3 for each volunteer. (*p<0.05; **p<0.01). Data were normalised to the amount of phosphate dissolved in the first acid exposure. The adsorption of whole saliva resulted in the significant reduction of the acid demineralisation and the proteins remained protective for the next acid challenges though the protective effect was gradually lost.

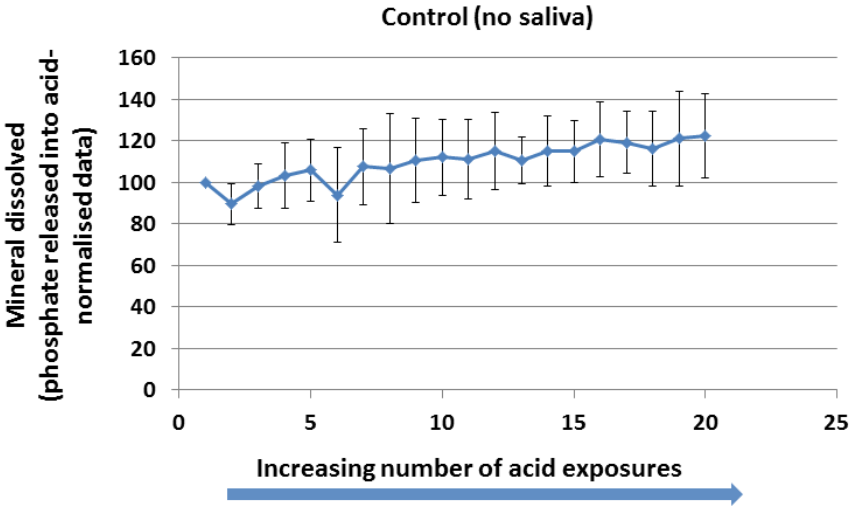


Figure 35. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, for control samples (no saliva adsorption). Mean of 5 repeats (± SD) with n=3 for each repeat. Data were normalised to the amount of phosphate dissolved in the first acid exposure. No sudden reduction of the mineral loss was observed in control samples.

The apparent increase in phosphate released during the first acid challenge following saliva adsorption (data point 6, Figure 34) suggested that saliva adsorption had actually caused more mineral to be dissolved. However, saliva is supersaturated in phosphate and calcium ions with respect to HAP and salivary phosphate may be loosely bound to the enamel surface or bound to adsorbed salivary proteins. If this phosphate was labile under acidic conditions it would be released during the 6th acid exposure giving the apparent increase in phosphate dissolved in acid immediately after saliva adsorption. To test this possibility, 1.5 mL of whole saliva was concentrated to 200µL using an Amicon ultra-centrifugal filter device with 4-5000 MW cut off (Merck Millipore, Darmstadt, Germany). The sample was then made up to 1.5mL volume with 50mM Tris pH 7.4 so that any small ions present in the saliva were diluted prior to exposure of the enamel surface to the saliva. The

phosphate concentration of the saliva sample was measured before and after the dialysis to ensure that the dialysis procedure was effective (Figure 36).

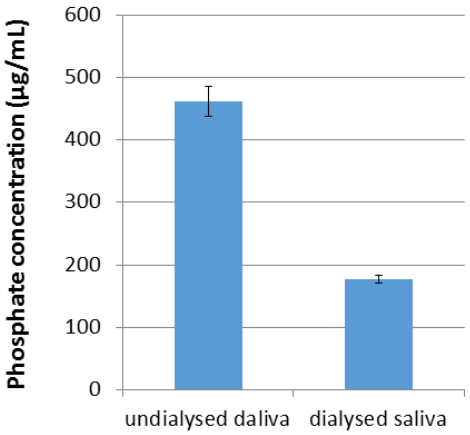


Figure 36. Measurement of the phosphate concentration in whole saliva before and after dialysis. Note that the phosphate level was significantly ($p<0.05$) decreased by 60% in dialysed saliva.

The ion depleted saliva was then compared to normal whole saliva in the dipping experiment in order to see whether the phosphate increase would be still obvious on the first acid challenge (point 6) after saliva adsorption. Teeth treated with 50mM Tris, pH 7.4, instead of saliva served as controls. Calcium release into the acid was also measured. The green plot line in Figure 37 shows the phosphate and calcium lost by the control tooth and, as expected, there was a steady increase in mineral dissolved in each dip. The blue plot line shows the mineral dissolved in each dip from a tooth treated with whole saliva prior to the 6th acid exposure. There was an obvious spike of phosphate and calcium released during the 6th exposure similar to that seen in Figure 34. The red plot line shows the mineral dissolved in each dip from a tooth treated with ion depleted saliva prior to the 6th acid exposure. In this case, rather than seeing a peak in mineral loss, there was a reduction in

mineral loss during the 6th challenge. These data strongly suggest that salivary phosphate and calcium bind to the enamel surface or the adsorbed salivary proteins and give an artefactual apparent increase in mineral dissolution during acid challenge 6 immediately after adsorption of salivary proteins.

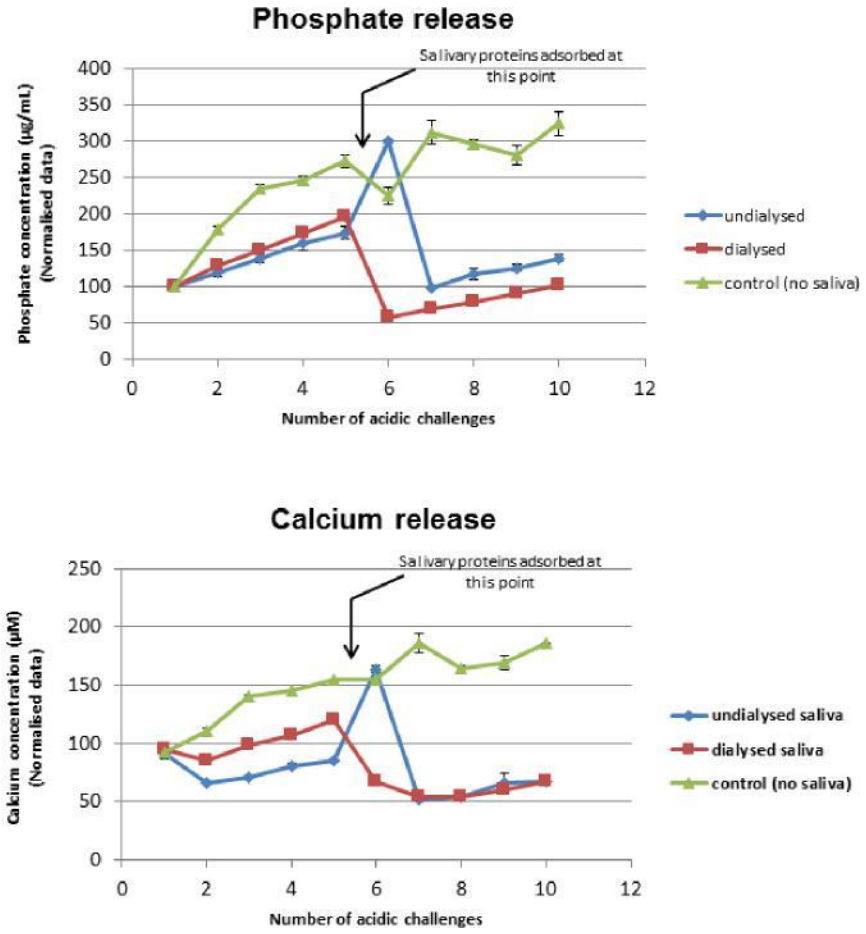


Figure 37. Measurement of phosphate and calcium dissolved in each acetic acid (pH 3.30) challenge, before and after exposure to whole saliva, dialysed saliva and 50mM Tris pH 7.4 for control samples, in order to investigate the spike of phosphate released during the 6th exposure. The spike of phosphate and calcium in the 6th acid exposure was not apparent when ion depleted saliva was adsorbed to the enamel surface.

In addition to this, another possibility was that salivary proteins released during the first acid challenge after saliva adsorption could be phosphorylated. This phosphate could have been hydrolysed by the sulphuric acid used in the phosphomolybdate assay, thereby further contributing to the apparent release of phosphate seen in the first acid exposure following adsorption of the salivary proteins. To test this possibility, salivary proteins released into acid after each acid challenge, were analysed by SDS-PAGE and selectively stained for phosphoproteins. Proteins lost in the first acid challenge after saliva adsorption included many phosphorylated proteins (Figure 38) suggesting that they could also contribute to the elevated phosphate levels measured in vial 6. Given these findings it was concluded that the phosphate peak that sometimes appeared in vial 6 was artefactual and could be ignored.

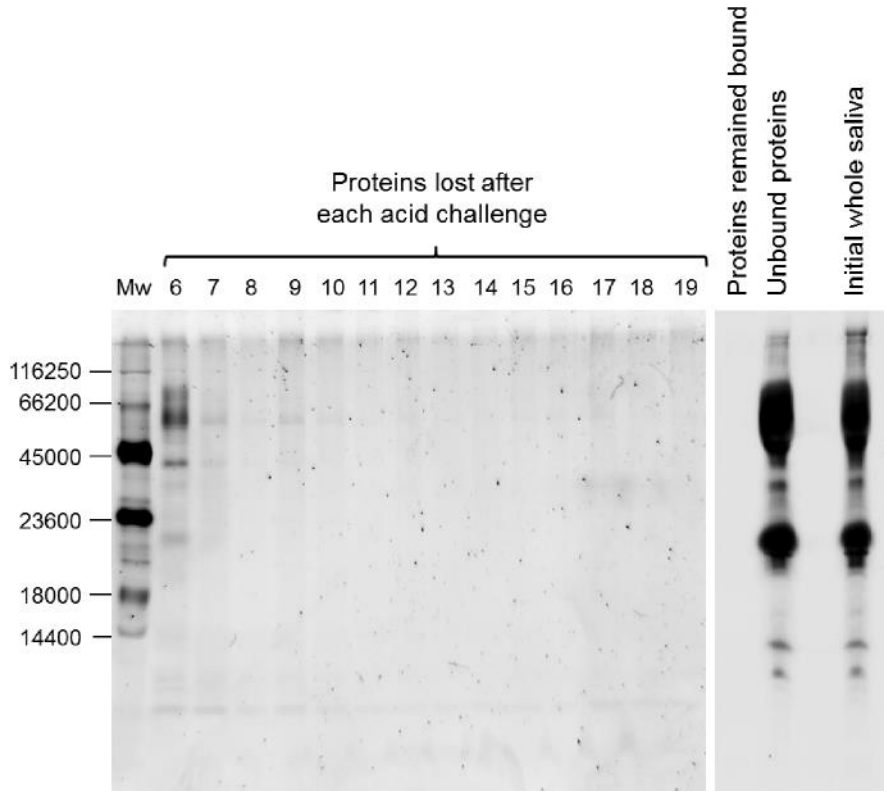


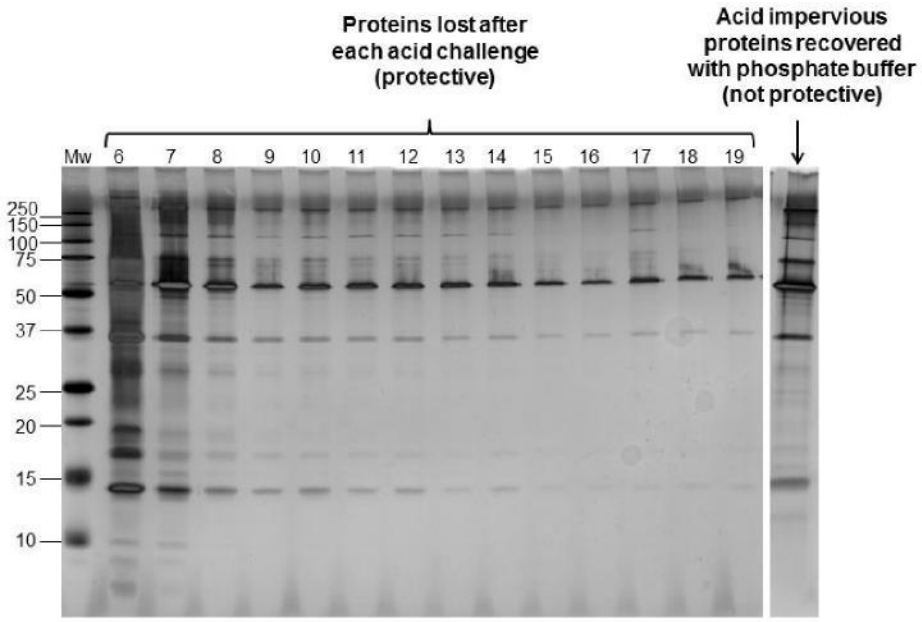
Figure 38. SDS-PAGE analysis of salivary proteins lost in each acetic acid challenge, proteins remaining bound to the enamel surface after acid exposures and unbound proteins, stained for phosphoproteins. The apparent elevation of phosphate released into vial 6 may be partly explained by phosphate hydrolysed in the phosphomolybdate assay from these phosphoproteins, suggesting that the apparent increase in mineral loss in vial 6 may be artefactual.

3.3.2 Characterising the adsorbed salivary proteins released during each acid challenge and those remaining adsorbed to the enamel

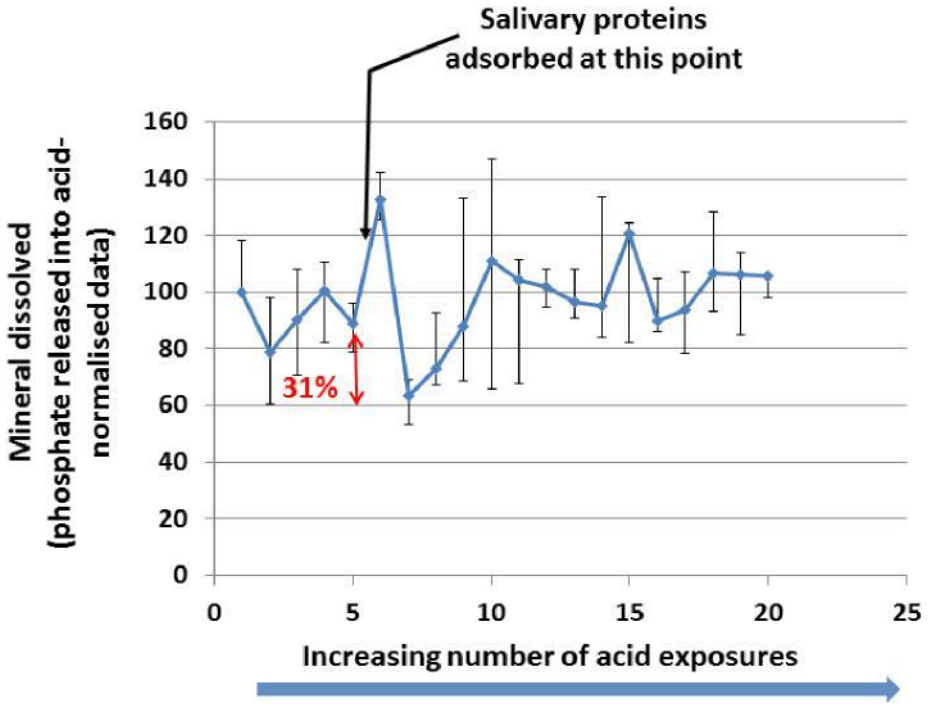
When whole salivary proteins were adsorbed to natural enamel surfaces a high degree of protection against acid attack was observed. However it is clear that the protective effect was gradually decreased and finally lost on subsequent acid challenges (Figure 34).

The proteins lost in each acid challenge (Figure 34) were analysed by SDS-PAGE (Figure 39a). Many of the adsorbed salivary proteins were lost during

the first acid exposure (vial 6). After that, a gradual desorption of proteins was observed in the subsequent acid challenges until eventually very little protein was lost in the later challenges. The loss of these proteins corresponded with the loss of protection (Figure 39b), thus it was hypothesised that these proteins lost during the acid attack were protective. Even after the 19th acid challenge there was still a considerable amount of protein associated with the enamel that could be recovered by desorbing with phosphate buffer but this protein, though impervious to the acid, provided no protection.


Proteins lost after each acid challenge (protective)
Acid impervious proteins recovered with phosphate buffer (not protective)
Mw
6
7
8
9
10
11
12
13
14
15
16
17
18
19
250
150
100
75
50
37
25
20
15
10

(a)


Salivary proteins adsorbed at this point
Mineral dissolved (phosphate released into acid-normalised data)
160
140
120
100
80
60
40
20
0
31%
0
5
10
15
20
25
Increasing number of acid exposures

(b)

Figure 39. (a) SDS-PAGE analysis of salivary proteins (from one volunteer) lost in each acetic acid challenge and proteins remaining bound to the enamel surface after acid exposures. Many proteins were lost during the first acid exposure with a gradual loss in subsequent exposures. A large number of proteins, impervious to the acid, remained bound after the 19th challenge but they were no longer protective. **(b) Phosphate dissolved in each vial of acetic acid (pH 3.30), before and after the adsorption of a volunteer's saliva; (± SD) n=3.** Reduction of acid dissolution was observed after saliva adsorption and the proteins remained protective for the next few acid exposures after which the protective effect was lost. The loss of proteins was consistent with the loss of this protective effect. Note the peak of phosphate loss immediately following adsorption of saliva is artefactual and is not related to mineral dissolution (see Figure 37).

3.3.2.1 Inter-individual variation of whole saliva composition

It was hypothesised that the proteins released into acid were protective and therefore an attempt to identify some of these proteins was made. However, the inter-individual variation in whole saliva is an important aspect that needed to be considered first. Figure 40 shows the proteins released into acid and the proteins remaining bound to enamel originating from the saliva of five volunteers. It is clear that the protein composition differed amongst volunteers but common proteins were observed in more than one volunteer. For example, a protein with molecular weight of 55 kDa appeared to be lost in volunteers 1, 3 and 4 (red arrows). A 35 kDa protein was released into acid in volunteers 2, 3, 4 and 5 and a smaller protein of 14 kDa also appeared to be lost into acid in volunteers 1, 2 and 4 (red arrows). What is more, the results showed that the proteins remaining bound to enamel after 19 acid challenges; the non-protective proteins (recovered by desorption with phosphate buffer as described in section 2.2.2.5) also varied between individuals. The black arrows in Figure 40 show proteins that appeared to remain bound to enamel in more than one individual, such as a 50 kDa protein which was found in all five volunteers, and 25 kDa proteins which appeared in volunteers 1, 2 and 5.

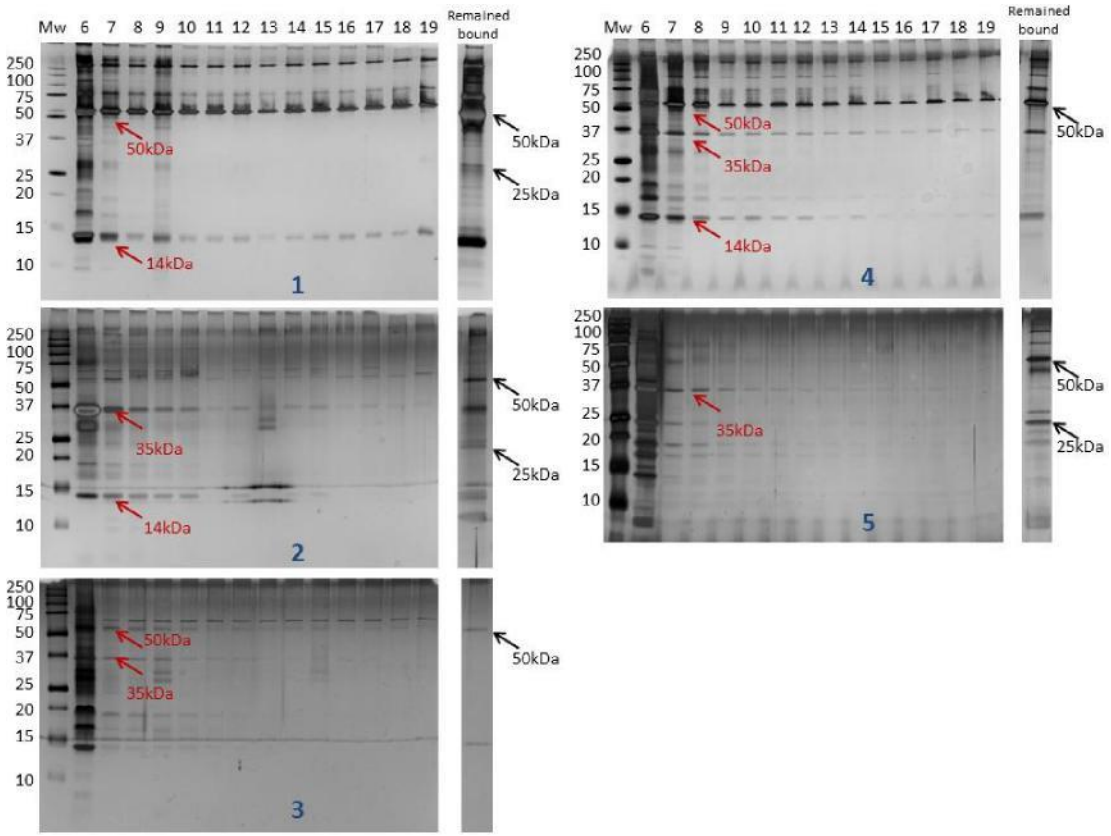


Figure 40. SDS-PAGE analysis of salivary proteins, from five volunteers, lost in each acetic acid challenge (6th to 19th) and proteins remaining bound to the enamel surface after acid exposures. Protein patterns differ among individuals but there are proteins released into acid (red arrows) or remained bound to the enamel (black arrows) that are common to more than one volunteer.

3.3.2.2 Identification of adsorbed proteins released and retained during acid challenges by mass spectrometry

Although the adsorbed proteins from different volunteers could vary (Figure 40), it was clear there were some proteins that were common to more than one volunteer.

The aim was to use mass spectrometry to identify the proteins that were hypothesised to act in a protective manner (i.e. those adsorbed proteins whose loss corresponds with loss of protection). It was not practical to analyse all samples from all volunteers so whole saliva from one volunteer

was used. In order to obtain an adequate amount of protein for mass spectrometry analysis, the dipping experiment was carried out using five tooth halves (instead of one) and 5mL of whole saliva (see section 3.3.1). Figure 41 shows the salivary proteins lost during the 7th, 8th and 9th acid challenges, where the adsorbed proteins are considered to be protective, and the proteins that remained bound to enamel after 19 acid challenges which were impervious to acid attack but no longer protective. The gel bands (labelled red numbers) were excised from the gel and analysed by mass spectrometry for identification.

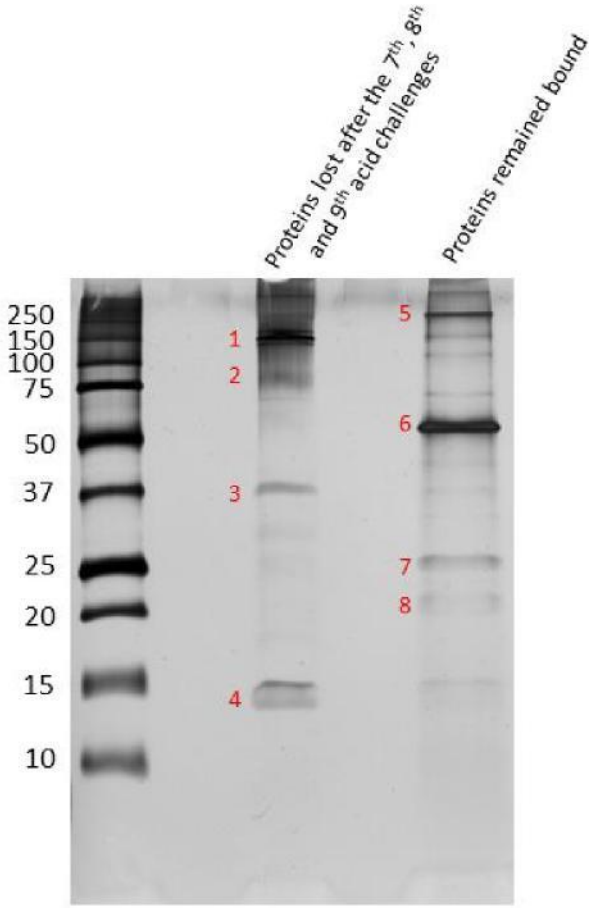


Figure 41. SDS-PAGE analysis of salivary proteins (from one volunteer) released during the 7th, 8th and 9th acetic acid challenges and those remaining adsorbed to enamel after 19 challenges. Eight protein bands (red numbers) were cut and analysed by mass spectrometry. It was hypothesized that the first four proteins (1-4) were protective and the last four proteins (5-8) were not protective.

The protein samples were processed and analysed at the Cambridge Centre for Proteomics as described in section 3.1.2.5.2 and the Mascot results for the proteins lost during acid challenges (gel bands 1-4, Figure 41) are summarised in Table 9. The table includes three proteins that could be within each gel band. The overall protein score is the summation of the individual peptide scores and peptides with a score greater than 28 are indicative of a positive identification; hence, only peptide with scores >28 are shown in Table 9.

The three potential proteins within the gel band '1' of 153 kDa (Figure 41) are a fragment of dihydropyrimidinase-like 2 variant with overall protein Mascot score: 185 and Mass: 62688, the collagen alpha-2 (I) chain with overall protein Mascot score: 127 and Mass: 129586, and the collagen, type I, alpha 1, isoform CRA_a with overall protein Mascot score: 91 and Mass: 85144.

According to Mascot results, the three candidate proteins for the gel band '2' of 80 kDa are the collagen alpha-2 (I) chain with overall protein Mascot score: 121 and Mass: 129586, the protein S100-A8 with overall protein score: 41 and Mass: 10885 and the isoform 6 of Sarcolemmal membrane-associated protein with overall protein score: 31 and Mass:58008.

The three possible proteins within the gel band '3' of about 38 kDa are the protein S100-A8 with overall protein Mascot score: 136 and Mass: 10885, lysozyme with protein score: 101 and Mass: 15661 and protein S100 with a protein score: 73 and Mass: 13259.

Finally, the potential proteins within the gel band '4' of 15 kDa are lysozyme with an overall protein Mascot score: 492 and Mass: 16982, the profiling 1, isoform CRA_b with protein score: 212 and Mass: 11497, and the protein S100 with protein score: 117 and Mass: 13259 (Table 9).

Table 9. Proteins lost during acid challenges identified within each gel band. Amino acid sequences of the peptide fragments obtained by mass spectrometry for each gel band, matching known proteins.

Protein	Score	Peptide
Gel band 1 (153kDa by SDS PAGE)		
Dihydropyrimidinase-like 2 variant (fragment)	**185**	
	47	K.VFNLYPR.K
	40	R.GSPLVVISQGK.I
	52	R.MVIPGGIDVHTR.F + Oxidation (M)
	47	K.DHGVNSFLVYMAFK.D + Oxidation (M)
Collagen alpha-2 (I) chain	**127**	
	50	R.GVVGPQGAR.G
	43	R.GPSGPQGIR.G
	33	R.GPAGPSGPAGK.D
Collagen, type I, alpha 1, isoform CRA_a	**91**	
	41	R.GPAGPQGPR.G
	50	K.QGPSGASGER.G
Gel band 2 (80kDa)		
Collagen alpha-2 (I) chain	**121**	
	43	R.GVVGPQGAR.G
	50	R.GPSGPQGIR.G
	28	R.GPAGPSGPAGK.D
Protein S100-A8	**41**	
	41	K.LLETECPQYIR.K
Isoform 6 of Sarcolemmal membrane-associated protein	**31**	

	31	R.LTALQVR.L
Gel band 3 (38kDa)		
Protein S100-A8	**136**	
	35	K.GADVWFK.E
	48	K.ALNSIIDVYHK.Y
	53	K.LLETECPQYIR.K
Lysozyme	**101**	
	49	R.LGMDGYR.G
	52	R.STDYGIFQINSR.Y
Protein S100	**73**	
	38	K.DLQNFLK.K
	35	K.LGHPDTLNQGEFK.E
Gel band 4 (15kDa)		
Lysozyme	**492**	
	35	R.AWVAWR.N
	48	R.LGMDGYR.G + Oxidation (M)
	30	R.QYVQGCGV.-
	36	R.YWCNDGK.T
	32	K.RLGMDGYR.G
	50	R.ATNYNAGDR.S
	40	K.WESGYNTR.A
	62	R.GISLANWMCLAK.W + Oxidation (M)
	101	R.STDYGIFQINSR.Y + Deamidated (NQ)
	59	K.TPGAVNACHLSCSALLQDNIADAVACAK.R
Profiling 1, isoform CRA_b	**212**	
	36	K.TDKTLVLLMGK.E
	58	K.STGGAPTFNVTVTK.T

	86	R.SSFYVNGLTLGGQK.C + Deamidated (NQ)
	31	R.DSLLQDGEFSMDLR.T + Oxidation (M)
Protein S100	**114**	
	31	K.DLQNFLK.K
	39	K.LGHPDTLNQGEFK.E
	44	R.NIETIINTFHQYSVK.L

The proteins remained bound to enamel after 19 acid challenges (gel bands 5-8, Figure 41) were also analysed by mass spectrometry and the Mascot results are summarised in Table 10. According to the Mascot results, three potential proteins within the gel band ‘5’ of 240 kDa (second lane, Figure 41) are the protein FAM227A with overall protein Mascot score: 41 and Mass: 75903, a fragment of the proteasome activator complex subunit 4 with protein score: 33 and Mass: 130054 and the myosin, heavy polypeptide, non-muscle, isoform CRA_a with protein score: 31 and Mass: 227646. However, only one peptide fragment was found matching these proteins which may not be sufficient for identification.

For gel band ‘6’ of 55 kDa, the Mascot results suggested the following three proteins within the band, the cDNA FLJ54371, highly similar to serum albumin with protein score: 1178 and Mass: 72370, the serpin peptidase inhibitor clade A with protein score: 127 and Mass: 46850 and a fragment of dihydropyrimidinase-like 2 variant with protein score: 109 and Mass: 62688.

Two potential proteins within gel band ‘7’ of 26 kDa are the protein S100 with protein score: 121 and Mass: 13259 and the PRSS3 protein with protein score: 81 and Mass: 27040.

Finally, two candidate proteins within the band ‘8’ of 21.5 kDa are the protein S100-A8 with overall protein score: 183 and Mass: 10885 and the protein S100 with overall protein score: 160 and Mass: 13259.

Table 10. Proteins remained bound to enamel after 20 acid challenges identified within each gel band. Amino acid sequences of the peptide fragments obtained by mass spectrometry for each gel band, matching known proteins.

Protein	Score	Peptide
Gel band 5 (240kDa)		
Protein FAM227A	**41**	
	41	K.IADINLR.T
Proteasome activator complex subunit 4 (fragment)	**33**	
	33	R.LLINLLK.K
Myosin, heavy polypeptide 9, non-muscle, isoform CRA_a	**31**	
	31	K.VIQYLAYVASSHK.S
Gel band 6 (55kDa)		
cDNA FLJ54371, highly similar to Serum albumin	**1178**	
	48	K.AACLLPK.L
	42	K.LVTDLTK.V
	39	K.YLYEIAR.R
	44	K.LCTVATLR.E
	28	K.DDNPNLPR.L
	53	K.FQNALLVR.Y + Deamidated (NQ)
	21	K.TYETTLEK.C
	66	K.QTALVELVK.H
	35	K.LVAASQAALGL.-
	49	K.SLHTLFGDK.L
	26	K.KYLYEIAR.R
	77	K.KQTALVELVK.H

	64	K.LVNEVTEFAK.T
	32	R.FKDLGEENFK.A
	32	R.HPDYSVVLLLR.L
	63	K.AVMDDFAAFVEK.C
	44	R.RHPDYSVVLLLR.L
	41	K.VPQVSTPTLVEVSR.N
	66	K.KVPQVSTPTLVEVSR.N
	56	K.QNCELFEQLGEYK.F
	56	R.RPCFSALEVDETYVPK.E
	37	K.VFDEFKPLVEEPQNLIK.Q
	32	K.EFNAETFTFHADICTLSEK.E
	49	K.EFNAETFTFHADICTLSEKER.Q
	58	R.LVRPEVDVMCTAFHDNEETFLK.K
Serpin peptidase inhibitor, clade A (Alpha-1 antiproteinase, antitrypsin)	**127**	
	31	K.QINDYVEK.G
	56	K.SVLGQLGITK.V
	39	K.LSITGTYDLK.S
Dihydropyriminidinase-like 2 variant (fragment)	**109**	
	46	K.VFNLYPR.K
	40	R.GSPLVVISQGK.I
Gel band 7 (26kDa)		
Protein S100	**121**	
	44	K.DLQNFLK.K
	38	K.LGHPDTLNQGEFK.E
	39	R.NIETIINTFHQYSVK.L

PRSS3 protein	**81**	
	42	K.NKPGVYTK.V
	39	K.TLNNDIMLIK.L + Deamidated (NQ); Oxidation (M)
Gel band 8 (21.5kDa)		
Protein S100-A8	**183**	
	35	K.GADVWFK.E
	30	-.MLTELEK.A + Oxidation (M)
	56	K.ALNSIIDVYHK.Y
	62	K.LLETECPQYIR.K
Protein S100	**160**	
	36	K.DLQNFLK.K
	44	K.VIEHIMEDLDTNADK.Q
	28	R.NIETIINTFHQYSVK.L

Tentative identification of some proteins can be made from analysing the Mascot results. The 38 kDa gel band (band 3, Figure 41 and Table 9) may correspond to a dimer of the protein S100-A8 (10.885 kDa) identified within the band. The 15 kDa gel band (band 4) can be confidently identified as lysozyme (16.982 kDa) since it is the protein with the highest protein score, 10 peptide fragments from the protein band were matched and the molecular weight of the unknown protein is in agreement with that of lysozyme. Therefore, protein S100-A8 and lysozyme that were lost during acid challenges could be two protective proteins.

3.3.3 Protective effect of purified salivary proteins against acid demineralisation of human natural enamel surfaces

3.3.3.1 Adsorption of α-amylase and cystatin to natural enamel surfaces and protective effect against acid demineralisation

The pooled fraction containing mainly α-amylase and cystatin, purified by size exclusion chromatography under non-denaturing conditions (Figure 22a), as well as the fractions of the two proteins separately, purified by size exclusion re-chromatography under denaturing conditions (Figure 22b), were investigated for their adsorption to natural enamel surfaces and their potential protective effect from acid attack.

It was shown in previous sections (3.1.2.6 and 3.2.2.1) that both α-amylase and cystatin were bound to HAP although the proportion of α-amylase bound was smaller than that of cystatin. It had also been shown that α-amylase did not bind well to enamel powder in the presence or the absence of cystatin while cystatin seems to have been bound well to powdered enamel.

The adsorption behaviour of the two proteins, together or separately, to natural enamel surfaces was also explored. Pooled protein fractions of α-amylase and cystatin together or individual purified proteins were incubated with a tooth half for 10 minutes at 37°C. After the incubation, the protein solution which contained the unbound proteins was analysed by SDS-PAGE. The tooth half was rinsed with distilled water and the adsorbed proteins desorbed with 200µl of phosphate buffer (100mM, pH 7.4) and analysed by SDS-PAGE. The resulting SDS-PAGE gel shown in Figure 42 indicated that only a small quantity of α-amylase adsorbed to enamel when it was with or without cystatin. On the other hand, cystatin, which was shown previously (Figure 25 and Figure 32) to bind well to synthetic HAP and powdered enamel, did not appear to bind to natural enamel surface either in the presence of α-amylase (lane 3) or on its own (lane 9) (Figure 42).

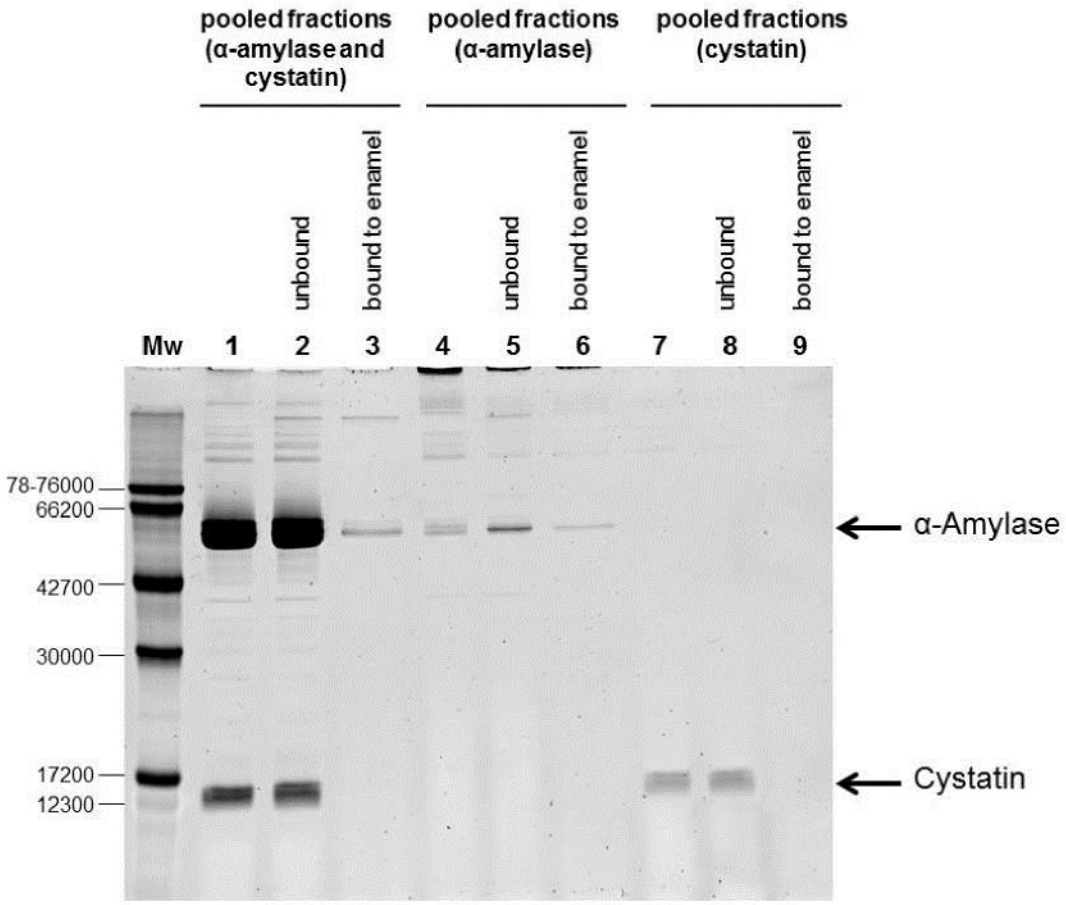


Figure 42. SDS-PAGE of α-amylase and cystatin, unbound and bound to human natural enamel surfaces. Only a small amount of α-amylase seems to adsorb to enamel in the presence of cystatin (lanes 1 to 3) or in the absence of cystatin (lanes 4 to 6). However, note that cystatin does not bind to enamel in the presence or the absence of α-amylase (lanes 3 and 9).

The protective effect of the mixed fraction of α-amylase and cystatin (at the same concentration as it is in saliva) was also investigated by carrying out the dipping experiment where half of a tooth was sequentially dipped into five vials of acetic acid (10mM, pH 3.30) and the fraction of the two proteins was adsorbed to the tooth before the 6th acid exposure. Fourteen more acid challenges were performed and the acid demineralisation was determined by the measurement of phosphate released into acid. The results of the phosphate measurement revealed that the fraction of α-amylase and cystatin

reduced the mineral loss by 17% but the protective effect was not significant and was not retained at the next acid exposure (Figure 43).

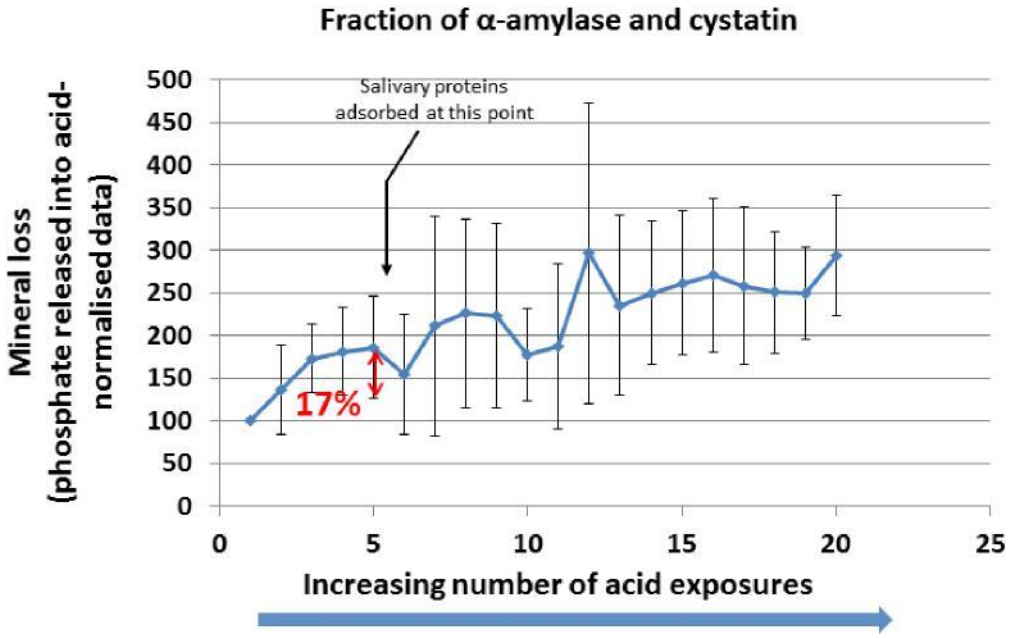


Figure 43. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of the pooled fraction of α-amylase and cystatin. Mean of 3 repeats (± SD). The fraction of the two proteins reduced the mineral loss straight after the adsorption, but only by 17% and the protective effect was not statistically significant and did not last until the next acid exposure.

3.3.3.2 Protective effect of saliva protein fractions purified by size exclusion chromatography under denaturing conditions when adsorbed to natural enamel surfaces

Different methods of protein purification were used in order to obtain protein fractions and test their protective effect against acid demineralisation on natural enamel surfaces. Protein fractions purified by size exclusion chromatography under denaturing conditions, as shown in Figure 20 and Figure 31, were first tested for their protective properties using synthetic

HAP and powdered enamel (sections 3.1.2.3 and 3.2.2). The results showed that apart from the pooled fractions A2-A4, which reduced the mineral loss by about 12% when adsorbed to powdered enamel, none of the protein fractions were capable of reducing the mineral loss. This might be explained because the purification was carried out under denaturing conditions which would have destroyed the native conformation of the proteins and possibly their function too. In order to test this hypothesis, whole salivary protein was adsorbed to the natural enamel surface plus or minus 6M urea (a strong denaturing agent) and the teeth were subjected to the dipping assay to measure the protective effect of the adsorbed proteins.

The results showed that salivary protein adsorbed from whole saliva in the presence of 6M urea, reduced acid demineralisation by 25% but this was not statistically significant (Figure 45). When adsorbed in the absence of urea, salivary proteins provided a larger degree of protection (40% reduction in mineral loss) (Figure 44), a similar level to that observed previously (Figure 34). However this effect was also not statistically significant, probably due to the large error bars associated with this experiment. These may be a reflection of the variability of the teeth used and, thus, the way each tooth surface adsorbs protective proteins and loses mineral. Owing to the limited availability of suitable teeth from the tissue bank it was not possible to carry out dipping experiments using large numbers of teeth and, therefore, due to the small n values, the error bars were sometimes large. This might explain the fact that although the protection from whole saliva was relatively high, with a 40% reduction in mineral loss (Figure 44), it was not statistically significant.

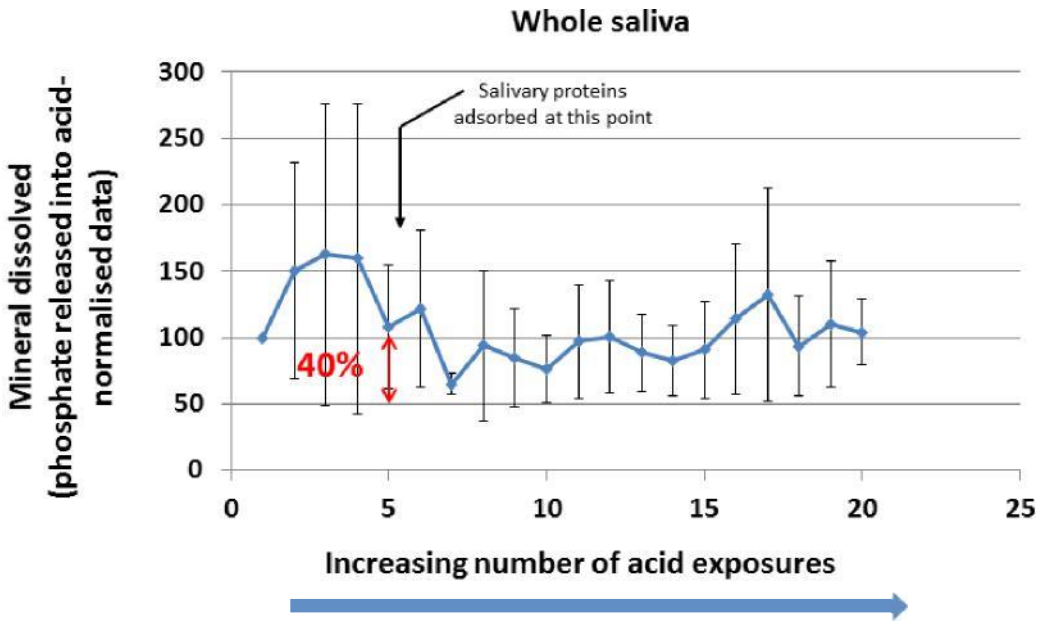


Figure 44. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after whole saliva adsorption. Mean of 3 repeats (± SD). Whole saliva reduced the mineral loss by 40% but the protective effect was not statistically significant.

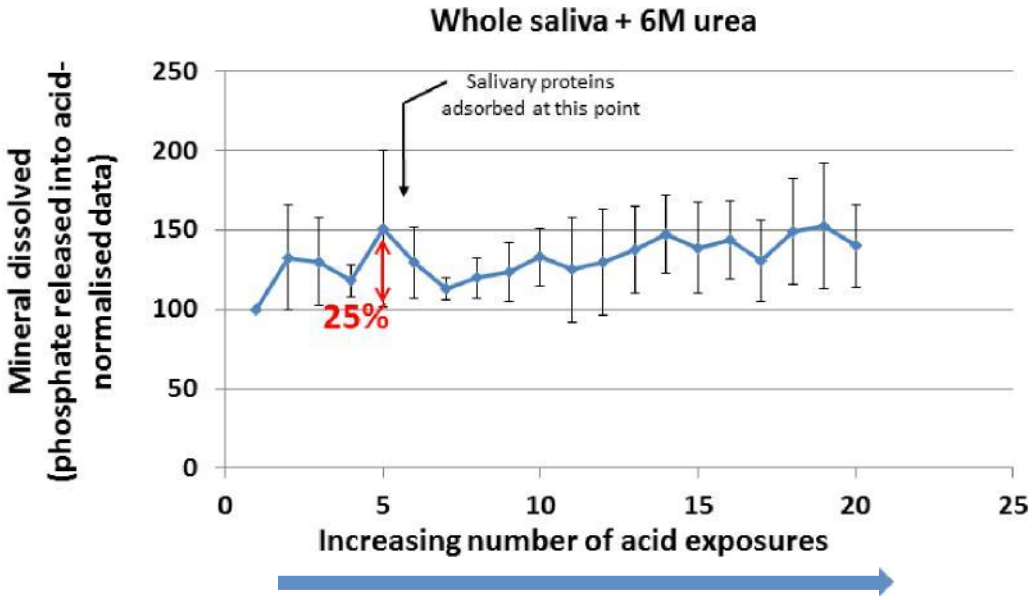


Figure 45. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of 6M urea treated whole saliva. Mean of 3 repeats (± SD). Urea treated saliva reduced the mineral loss by 25% but the protective effect was not statistically significant.

3.3.3.3 Saliva protein fractions purified in native state by HAP chromatography

From the data presented in Figure 45, it appeared that salivary proteins purified under denaturing conditions did not maintain their protective properties against acid-induced demineralisation so there was a need for a method to purify salivary proteins in their native state. One such method is HAP chromatography. Salivary proteins were run through a HAP column in 50mM Tris pH 7.4 (buffer A). Under these conditions proteins are most likely to adsorb onto the HAP column. Unabsorbed proteins simply wash through the column. Adsorbed proteins are then competitively desorbed and eluted from the column with 50mM Tris, 500mM phosphate buffer, pH 7.4 (buffer B).

Figure 46 shows a peak of unbound proteins which were eluting from the column when the elution buffer was 100% buffer A (0mM phosphate) at a run volume of ~3.5mL. When the elution buffer was switched to 100% buffer B (500mM phosphate), all the proteins bound to HAP column were desorbed and eluted at a run volume of ~16mL. The red conductivity plot shows the increase in conductivity as the phosphate ion concentration in the eluent increased from 0 to 500mM. The first peak in the chromatogram contained the flow through proteins that did not bind to the HAP column and the second, much smaller, peak contained all the proteins that were bound to HAP and eluted by the phosphate buffer.

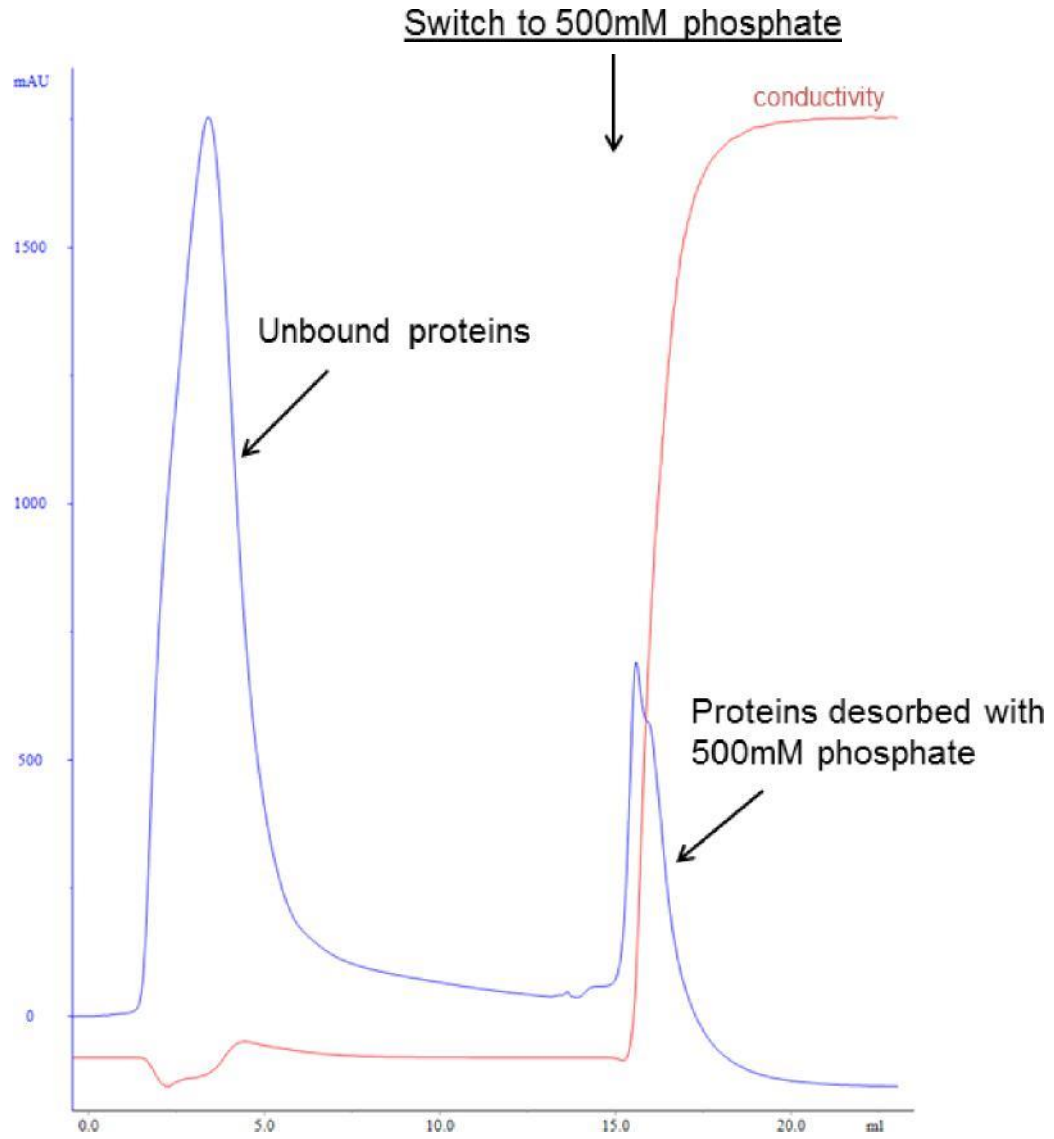


Figure 46. HAP chromatography of whole saliva. Unbound proteins were eluted with buffer A: 50mM Tris pH 7.4 and the proteins bound to HAP column were eluted with buffer B: 50mM Tris, 500mM phosphate buffer, pH 7.4. The red plot shows the increase in conductivity as the concentration of phosphate increased from 0 to 500mM.

The unbound proteins were first tested for any protective properties against acid demineralisation of enamel. It is obvious, (Figure 47), that the adsorption of unbound proteins to enamel surfaces prior to exposure 6, did not provide any protection during subsequent exposures. The error bars in Figure 47 appear to get bigger in the later dips. The reason for this is unclear

but may include damage to the nail varnish covering the cut enamel surface and root during the acid exposures, allowing acid to attack an increased area of exposed mineral. Regardless of the large error bars associated with later exposures it was clear that the proteins adsorbed prior to exposure 6 did not reduce mineral loss.

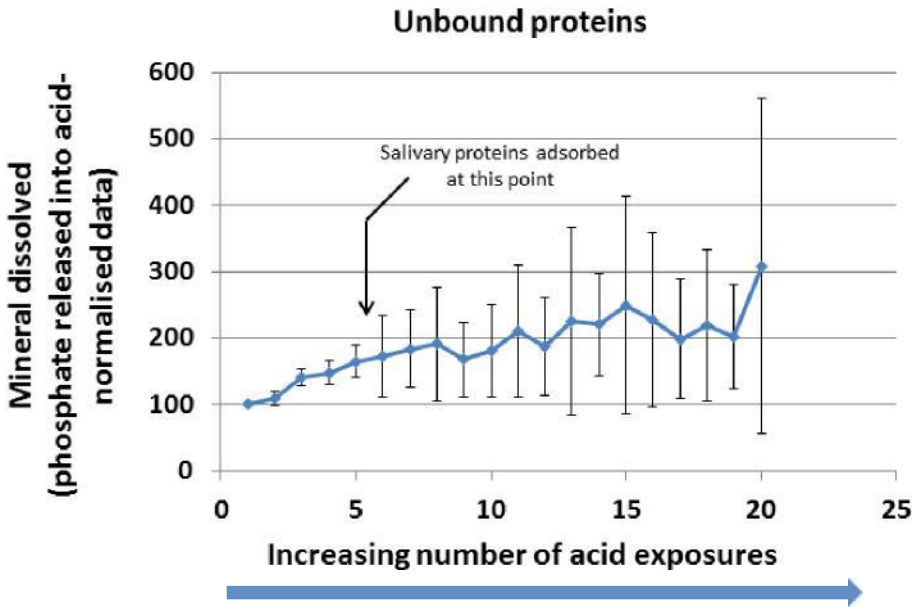


Figure 47. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of the unbound to HAP column proteins. Mean of 3 repeats (± SD). The proteins that did not bind to HAP column did not reduce the mineral loss.

In contrast, the proteins that were bound to the HAP column and desorbed by 500mM phosphate buffer offered a significant degree of protection against acid (Figure 48). These proteins reduced the phosphate dissolution significantly by 32% ($p<0.05$) at the first acid exposure after salivary protein desorption (point 6). In the next acid challenges, the reduction in mineral loss remained significant ($p<0.05$, $p<0.01$, $p<0.001$) until eventually the protective effect was lost.

The proteins lost in each acid challenge (from the bound to HAP column proteins that offered protection) were analysed by SDS-PAGE (Figure 49) and the most noteworthy protein was a low molecular weight protein (around 14 kDa) lost in the first four acid challenges. The loss of this particular protein, in this case, as well as the loss of another protein with a molecular weight around 55 kDa, mirrored the loss of protection against acid demineralisation (Figure 48). After all twenty acid challenges, two proteins at the same molecular weight remained bound to the enamel surface, although they were no longer protective. The gel was stained with Oriole Fluorescent Gel Stain, which is not as sensitive as silver stain, so perhaps more proteins were lost in the first acid challenges and the fluorescent gel stain was not sufficiently sensitive to detect them.

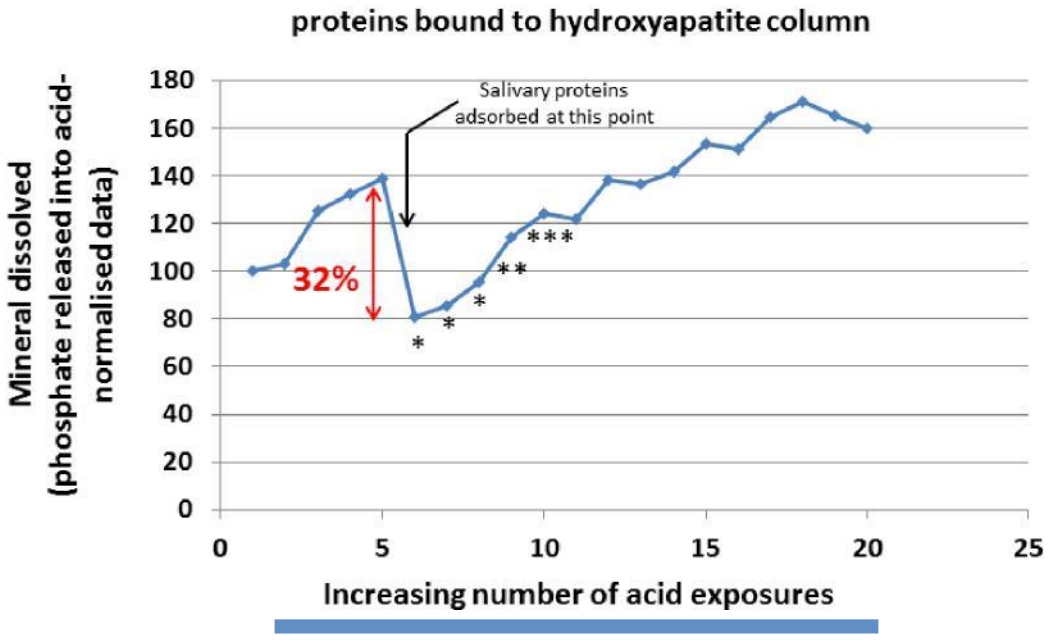


Figure 48. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of all proteins bound to HAP column and desorbed with 500mM phosphate buffer (n=1). (*p<0.05; **p<0.01; ***p<0.001). The proteins that were bound to the HAP column reduced the mineral loss significantly by 32% and the protective effect remained significant for the subsequent acid challenges.

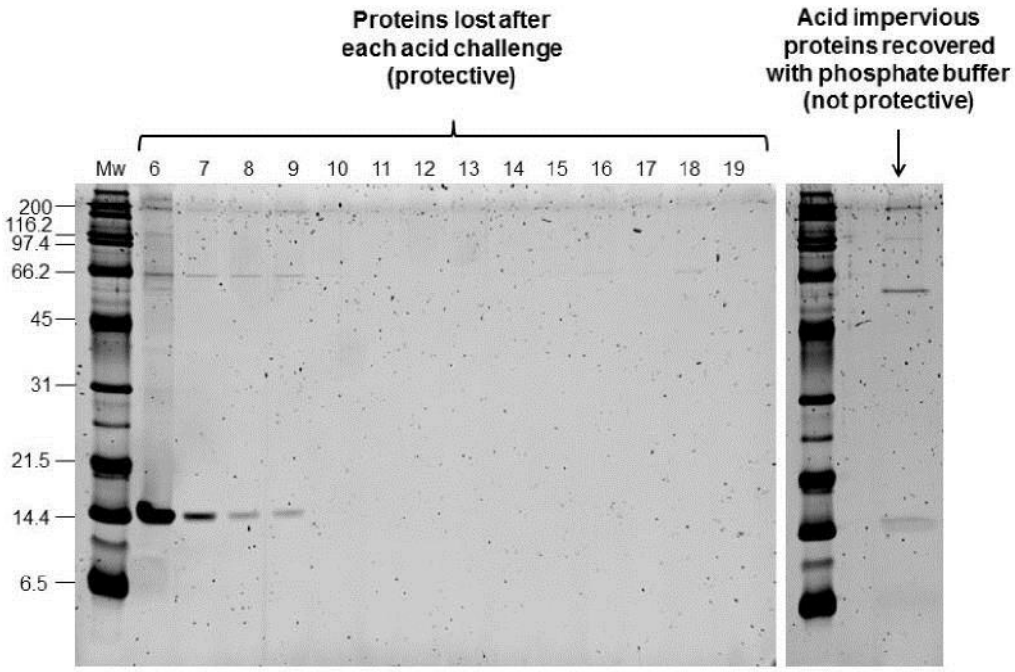


Figure 49. SDS-PAGE analysis of HAP column bound proteins. Proteins lost in each acetic acid challenge and proteins remaining bound to the enamel surface after acid exposures. Note that a protein of approximately 55 kDa and a protein of 14 kDa were lost in the first four acid challenges.

These results revealed that the proteins retained by the HAP column represented a subset of the total salivary protein (about 15%) that were protective. Proteins comprising the column flow through, which did not bind to the HAP column, did not offer protection against acid demineralisation. Further purification of salivary proteins was therefore focused on the subset that contained the protective proteins.

3.3.3.4 Purification of salivary proteins by HAP chromatography using a linear phosphate gradient

The same two buffers (buffer A: 50mM Tris pH 7.4 and buffer B: 50mM Tris, 500mM phosphate buffer, pH 7.4) were used to generate a linear gradient

from 0 to 500mM phosphate buffer to desorb proteins bound to the HAP column in order of their tendency to be desorbed by an increasing concentration of phosphate; in effect, an increasing order of their binding affinity to the column. Proteins with a low affinity for the column were desorbed and eluted early in the gradient at low phosphate concentration while strongly adsorbed proteins were eluted later as the phosphate concentration approached 500mM. Whole saliva was loaded on to the column in buffer A and the non-adsorbed proteins washed through. The first peak in the chromatogram, (Figure 50), contained the proteins that did not adsorb to the HAP column. These proteins were not protective against acid demineralisation of enamel (Figure 47) and it was decided that these proteins would not be collected for any further investigation. Once the peak of the unbound proteins was fully eluted, the composition of the eluting buffer was gradually changed by slowly increasing the proportion of buffer B. This resulted in the phosphate concentration increasing linearly over time from 0 to 500mM which resulted in the gradual desorption of the adsorbed proteins from the HAP column. These proteins were collected in fractions A1-A15 (Figure 50). These fractions included all of the proteins of the much smaller peak in Figure 46, which contained 15% of the total protein and provided protection against acid demineralisation (Figure 48). Aliquots of the unbound proteins and the various protein fractions eluted with phosphate buffer were analysed by SDS-PAGE (Figure 51). The first lane shows the molecular weight standards (10-250 kDa), the second lane shows the large number of the unbound proteins and the next lanes show the protein fractions A1-A15 that were desorbed and eluted from the HAP column by the phosphate gradient. Salivary proteins, loosely bound to the HAP column, were eluted first (lanes towards the left hand side of the gel) at a relatively low phosphate concentration. Salivary proteins more strongly bound to the HAP column, were eluted last (lanes towards the right hand side of the gel) requiring a higher concentration of phosphate in order for them to be desorbed.

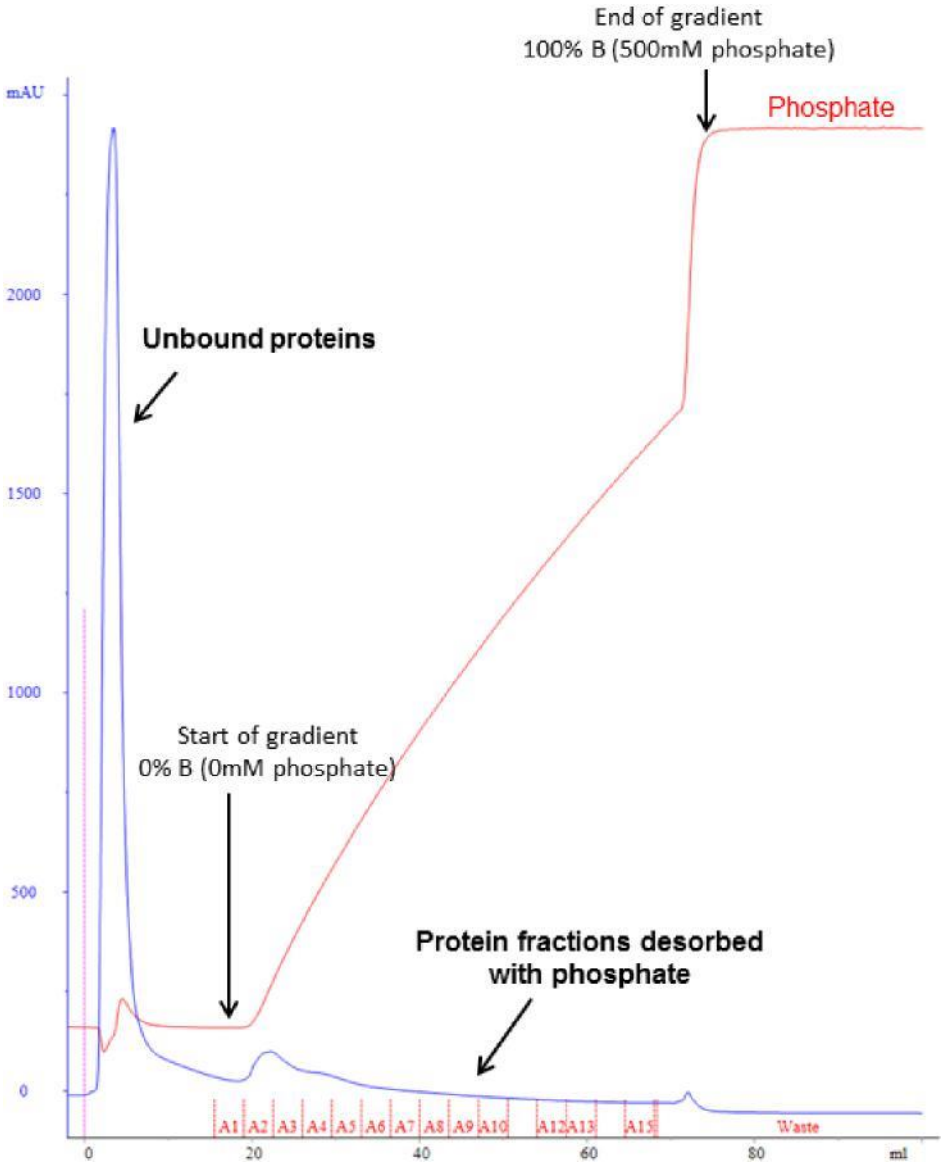


Figure 50. HAP chromatography of whole saliva using a 0-500mM phosphate linear gradient. Unbound proteins were eluted with Buffer A: 50mM Tris pH 7.4 and proteins bound to HAP column were gradually desorbed and eluted with the increase of Buffer B: 50mM Tris, 500mM phosphate buffer, pH 7.4.

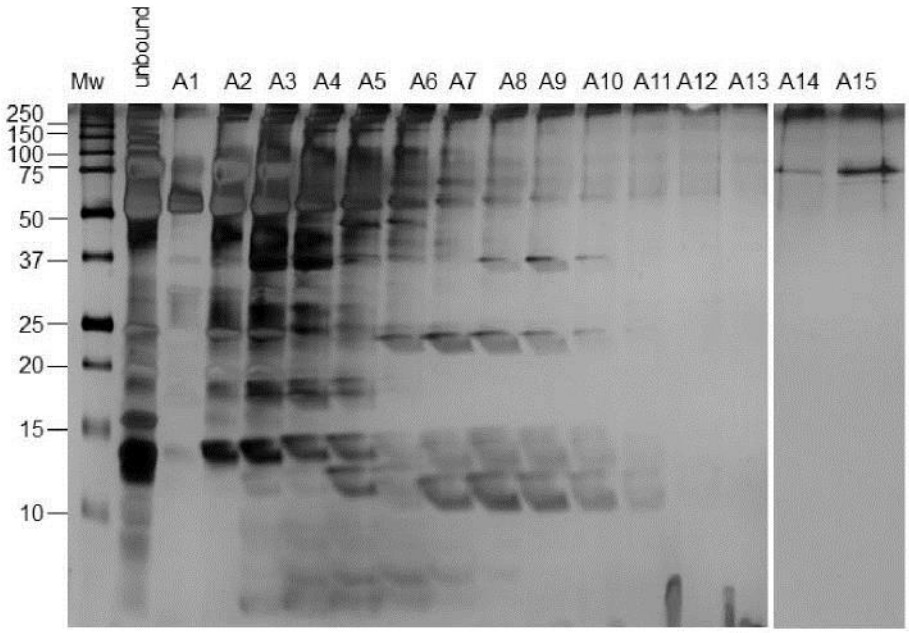


Figure 51. SDS-PAGE of salivary protein fractions purified by HAP chromatography using a 0-500mM phosphate linear gradient. Lanes towards the left hand side of the gel contained proteins loosely bound to the HAP column and lanes towards the right hand side of the gel show proteins more strongly bound to the HAP column.

The protein fractions were divided into two groups, then desalted using a desalting column equilibrated with 0.15M ammonium hydrogen carbonate, and the two groups were finally tested for their protective properties. The first group of protein fractions, A1-A6, reduced the mineral loss by 34% (Figure 52). The level of protection was reduced almost by half in the next acid challenge but the protective effect remained evident for the next few acid challenges and gradually disappeared.

The second group of protein fractions, A7-A10, was tested only once for its protective properties and it was shown that it reduced the acid demineralisation by about 30%, but the protective effect was lost over the next two acid challenges (Figure 53). The protection provided by these fractions seemed to be only temporary.

Protein fractions A1-A6 and A7-A10 were pooled and the level of protection was then tested. These ten fractions included all the protective proteins

purified by HAP chromatography because not many proteins were eluted after fraction A10 (Figure 51). A 75 kDa protein was only eluted in fractions A14 and A15, but since this protein was mainly present in the first fractions, A11-A15 fractions were not included in the protective pooled fractions to be tested.

Indeed, the fractions A1-A10 provided a high level of protection (52% reduction in mineral loss) and the protective effect remained apparent for at least the next ten acid challenges (Figure 54). The reduction in acid demineralisation appeared in acid challenge six, straight after the adsorption of the pooled A1-A10 protein fractions.

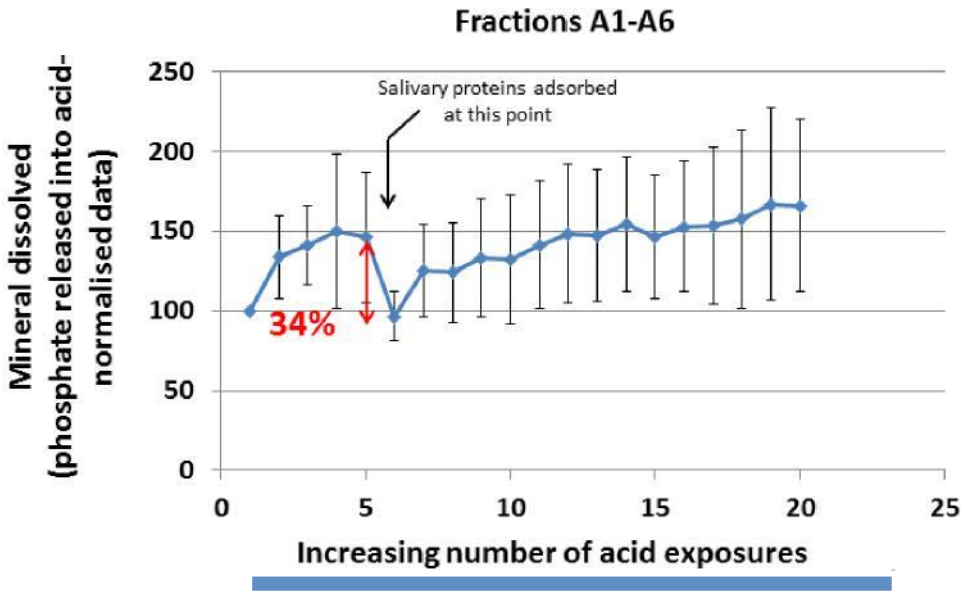


Figure 52. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of the first group of protein fractions (A1-A6) purified with phosphate linear gradient. Mean of 3 repeats (± SD). Salivary proteins in fractions A1-A6 reduced the mineral loss by 34% but the protective effect was not statistically significant.

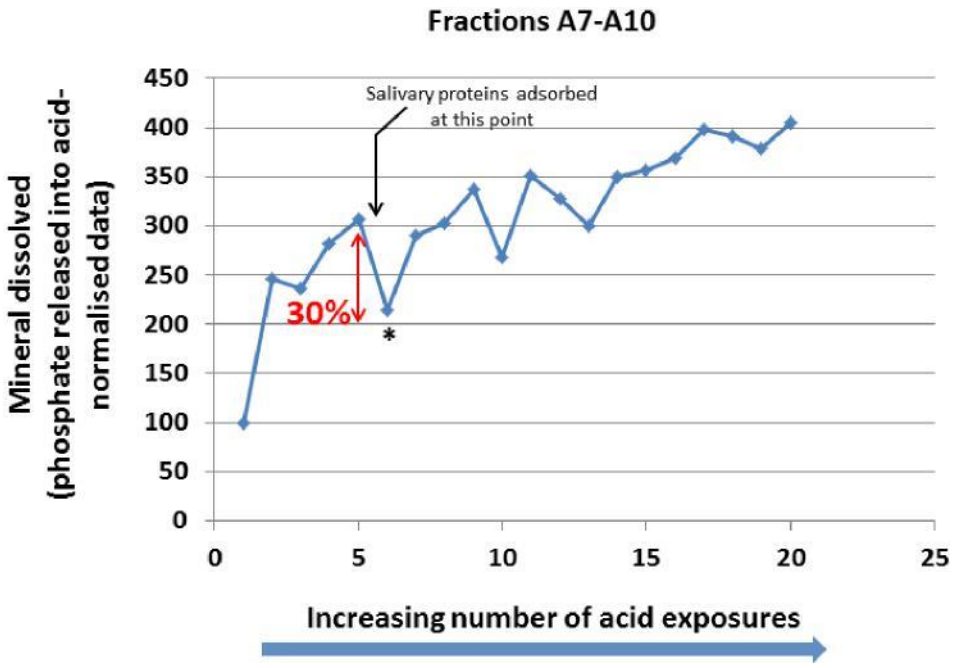


Figure 53. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of the second group of protein fractions (A7-A10) purified with phosphate linear gradient (n=1). (*p<0.05). Salivary proteins in fractions A7-A10 reduced the mineral loss significantly by 30%, but the protective effect lasted only for the next acid exposure.

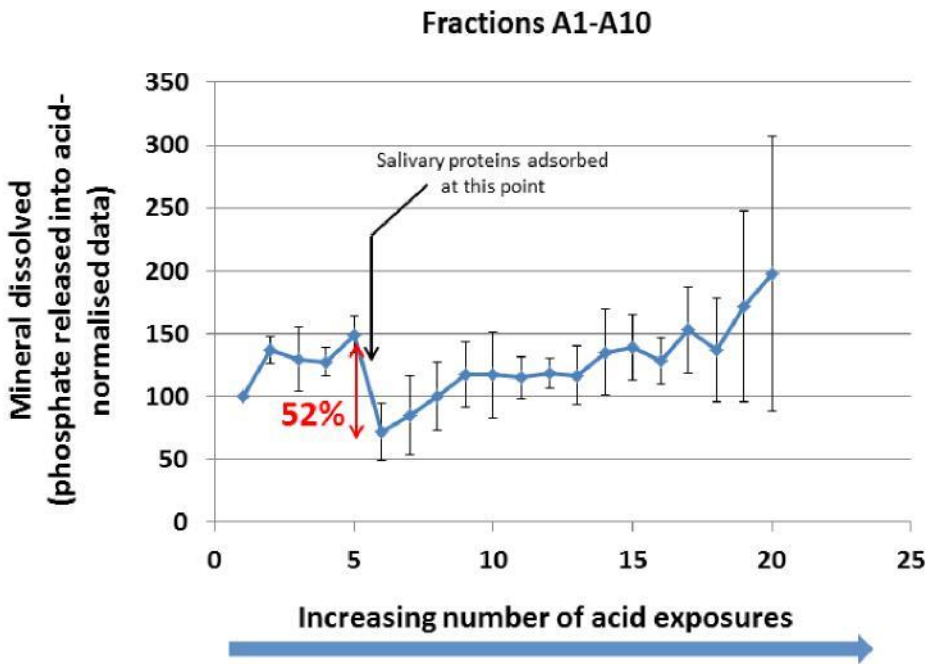


Figure 54. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of A1-A10 protein fractions purified with phosphate linear gradient. Mean of 3 repeats (± SD). Salivary proteins in fractions A1-A10 reduced the mineral loss by 52% and the protective effect was not statistically significant but remained apparent for the next ten acid exposures.

3.3.3.5 Fractionation of salivary proteins using isoelectric focusing

Purification of salivary proteins by HAP chromatography resulted in the identification of a subset of proteins that were responsible for protection of enamel against acid demineralisation. Further purification of this subset by different methods, such as isoelectric focusing (IEF), was needed in order to identify specific protective proteins or protein complexes.

Whole saliva was added to a HAP column, and salivary proteins were purified using a phosphate linear gradient as shown previously (Figure 50 and Figure 51). The eluted fractions A1-A10, shown to be protective (Figure 54), were then further separated according to their isoelectric point. The ten IEF protein fractions eluted from the MicroRotofor were loaded onto a gel to be analysed by SDS-PAGE. The isoelectric point (pI) of each fraction was

estimated from the pH range of the ampholytes used for the fractionation. Proteins in each IEF fraction were visible when loaded onto SDS-PAGE gel and separated according to their molecular weight (Figure 55). The gel was stained with Coomassie blue (compatible with MicroRotofor kit), but some protein bands were not obvious as this is not a very sensitive staining method. A protein of around 55 kDa seemed to be present in fractions 3 to 10 indicating a range of isoelectric points for this protein (pH 5-10). There was also a big area of staining in the lower right hand corner of the gel which shows low molecular weight proteins but due to the high concentration of ampholytes in the protein sample, the resolution of the gel was reduced and the bands were not clear.

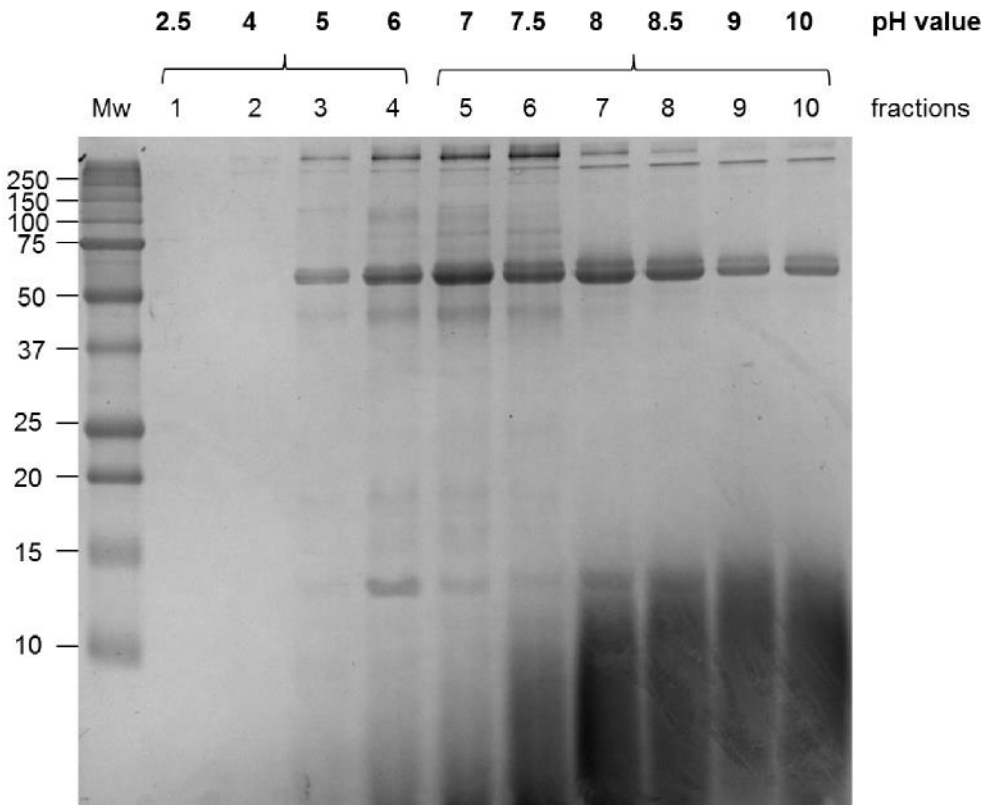


Figure 55. Fractionation of salivary proteins by isoelectric focusing. SDS-PAGE of IEF protein fractions stained with Coomassie blue. A protein of around 55 kDa appears in fractions 3 to 10 indicating a range of isoelectric points for this protein, and a considerable amount of low molecular weight proteins also appear in a number of fractions towards the right hand side of the gel but they are not well resolved into discrete bands due to the high concentration of ampholytes remaining in the sample.

The IEF protein fractions were divided into two groups, one containing the predominately high molecular weight proteins with low isoelectric point (pH 3 to pH 6) and the other containing more low molecular weight proteins with higher isoelectric points (pH 7 to pH 10). The two groups were then tested for their protective properties against acid demineralisation of the enamel. Proteins with a low isoelectric point (pH 3-6) did not reduce the phosphate dissolution (Figure 56) while proteins with a high isoelectric point (pH 7-10) decreased the amount of phosphate loss by 49% after their adsorption to enamel (Figure 57). The decrease of the phosphate loss was evident from the first acid challenge after the adsorption of the proteins (point 6) and the protective effect lasted only for the next two acid challenges. The proteins

with high pI seemed to offer a high degree of protection but the protective effect was only temporary. It should be mentioned that the pH values of the IEF fractions were not measured so the estimation of the pH values was based on the pH profile of a MicroRotofor run when pH 3-10 Bio-Lyte ampholytes were used.

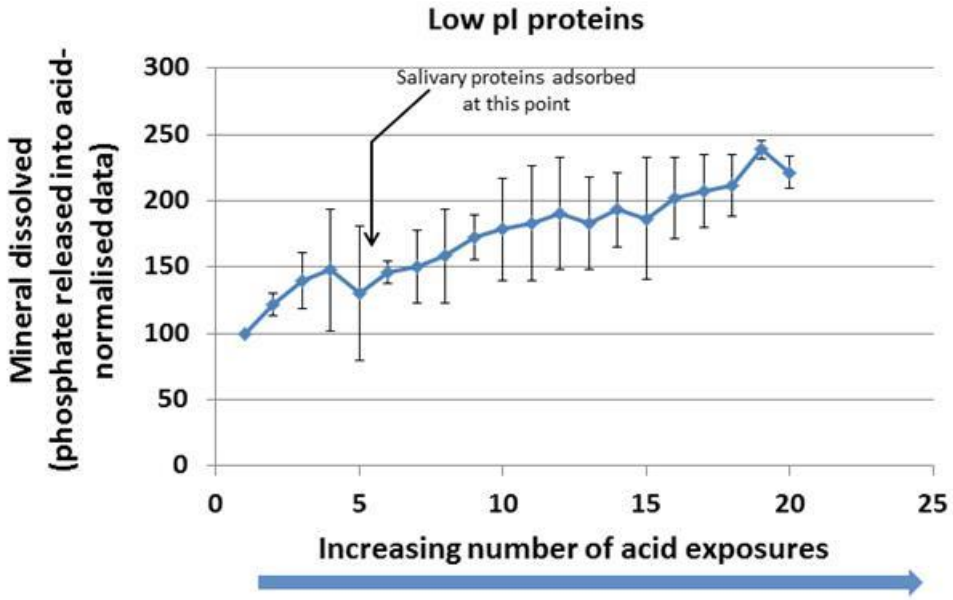


Figure 56. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of IEF protein fractions with low isoelectric point. Mean of 2 repeats (± SD).
Protein fractions with low pI did not reduce the mineral loss.

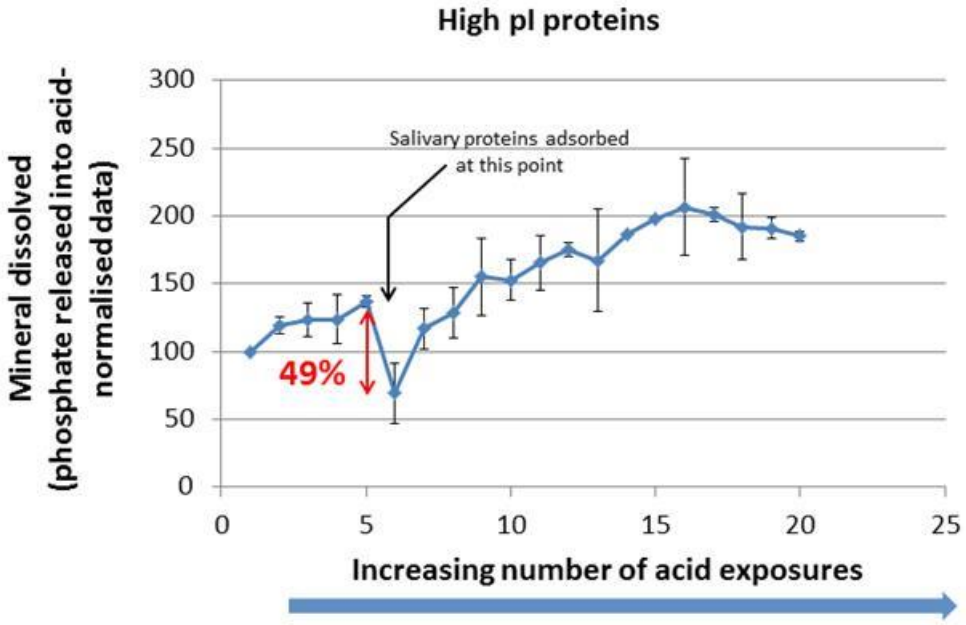


Figure 57. Concentration of phosphate dissolved in each acetic acid (pH 3.30) exposure, before and after adsorption of IEF protein fractions with a high isoelectric point. Mean of 2 repeats (± SD). Protein fractions with high pI reduced the mineral loss by 49% but the protective effect (not statistically significant) was apparent only for the next two acid exposures.

Chapter 4
Discussion

The protection against acid demineralisation of dental enamel provided by whole saliva or purified salivary proteins has been investigated using synthetic HAP powder, human enamel powder and natural enamel surfaces as substrate materials. Dental enamel has a complex structure and consists primarily of substituted HAP (Weatherell, 1975). Pure HAP can be represented by the empirical formula $Ca_{10}(PO_4)_6(OH)_2$. However, the biological hydroxyapatite lattice can accommodate many substituent ions e.g. CO_3^{2-}, F^-, Cl^-, K^+, Na^+, Fe^{2+}, Zn^{2+}, Sr^{2+} and Mg^{2+} and phosphate in the lattice may be protonated. When a substitution occurs, charge neutrality must be maintained and a wide range of stoichiometries may be envisaged:

e.g. $Ca_9(Mg)(PO_4)_6$ (OH)(F); $Ca_9(PO_4)_5(HPO_4)$ (OH); $Ca_9(PO_4)_5(CO_3)$ (OH);

Clearly, no single formula can be written to represent the true stoichiometry of a bulk biological mineralised tissue (Featherstone, 2000, Wopenka and Pasteris, 2005). Nevertheless, synthetic HAP in the form of powder, discs or blocks is commonly used in dental research as a model for dental enamel (Barbour et al., 2005, Shah et al., 2011). Synthetic HAP is readily available, inexpensive and consistent therefore, researchers prefer to use HAP powder to emulate the dental enamel, which in contrast is more difficult to obtain, its use requires ethical approval and is prone to biological variation. Given this, it would appear desirable that data obtained using synthetic HAP is subsequently validated using natural enamel surfaces. From its inception, the aim of this study was to examine the protective effect provided by whole saliva and purified salivary proteins against enamel demineralisation using synthetic HAP powder to establish techniques and obtain initial data prior to validating the findings against natural enamel powder and finally natural tooth surfaces. This experimental design not only saved using valuable natural enamel powder and natural enamel but also provided a much needed comparison between synthetic HAP, human enamel powder and natural enamel surfaces and their response to salivary protein adsorption and subsequent demineralisation on an acid challenge. What is more,

although many studies have investigated and reported the protective effect of specific salivary or non salivary proteins (Kielbassa et al., 2005, Siqueira et al., 2010, Shah et al., 2011) the present work appears to be the first systematic approach to attempt to identify protective species from the full range of whole salivary proteins.

In the first part of this chapter the results from the experiments carried out using synthetic HAP will be discussed. Salivary proteins are known to reduce HAP dissolution in acid (Hannig et al., 2004, Wetton et al., 2007) but the mechanism by which salivary proteins protect against acid demineralisation is not clear. The second part will be the discussion of the results obtained by using human enamel powder and in the third part results from the use of natural enamel surfaces will be discussed. Finally, there will be a general discussion including important points of the study.

As a navigational aid the overall structure and inter-relationships of sections 4.1, 4.2 and 4.3 and their subsections are shown as flow diagrams at the beginning of each section.

4.1 Protective effect of adsorbed salivary proteins against acid demineralisation of synthetic HAP powder

The flow diagram of the structure of this section is shown in Figure 58.

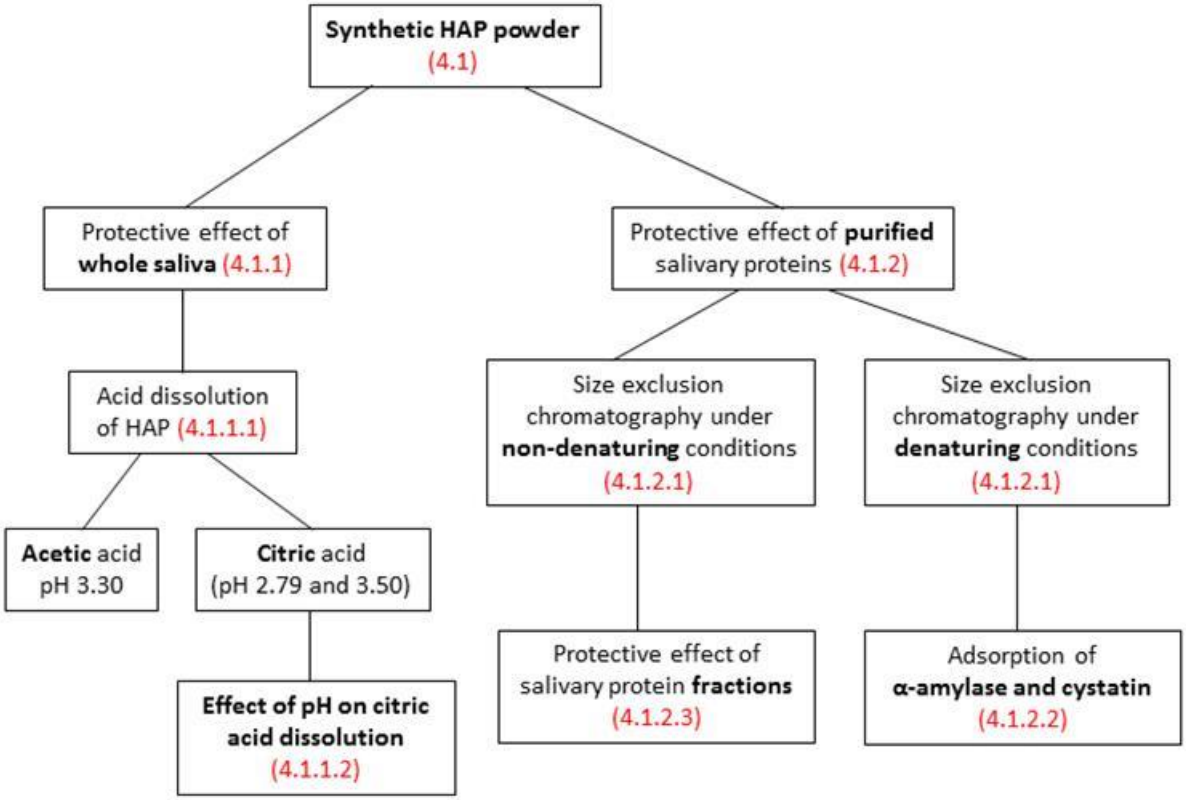


Figure 58. Flow diagram of the structure of section 4.1 (Discussion of the results obtained from synthetic HAP).

4.1.1 Protective effect of whole salivary proteins adsorbed to synthetic HAP powder

From the results presented in section (section 3.1.1) it was shown that when synthetic HAP was challenged with 10mM acetic acid pH 3.30, the adsorbed salivary proteins reduced the demineralisation significantly by 30% and

several proteins seemed to be acetic acid immobile (Figure 11 and Figure 12).

The protective effect of adsorbed salivary proteins against citric acid demineralisation of synthetic HAP seemed to be pH dependent. The adsorbed salivary proteins did not reduce the HAP dissolution on exposure to 100mM citric acid (pH 2.79) compared to the control (no adsorbed salivary proteins). In contrast however, salivary proteins provided protection against acid demineralisation of HAP at a higher pH value of citric acid (pH 3.50) typical of that associated with orange juice and fruit beverages (Figure 9 and Figure 10).

From the SDS-PAGE analysis it was clear that all adsorbed proteins survived the 100mM citric acid pH 3.50 attack and remained associated with the HAP surface (Figure 8). Thus, it was hypothesised that the proteins responsible for providing the protection were amongst the proteins that remained adsorbed to the HAP after the acid exposure. However, after the citric acid pH 2.79 challenge some proteins such as the 23 and 25 kDa proteins were lost or lower concentrations appeared to remain bound and, although many proteins remained bound to HAP, they were clearly not protective (Figure 8). As alluded to above, pH 2.79 may simply have been too acidic for the protective mechanism to function even if proteins remained bound during the challenge.

The mechanism by which salivary proteins protect the HAP against acid dissolution is not clear however some possibilities and hypotheses are discussed next.

4.1.1.1 Acid dissolution of HAP and protective role of proteins

HAP is in equilibrium with its component ions and at neutral pH and in accordance with Le Chatelier's principle this equilibrium is far to the left:

$Ca_{10}(PO_4)_6(OH)_2 \Leftrightarrow 10Ca^{2+} + 6PO_4^{3-} + 2OH^-$

However, acid can move the equilibrium to the right by reacting with hydroxide and phosphate ions and removing them from the equilibrium,

causing more component ions to be released from the HAP to maintain the equilibrium and, thus, more HAP dissolves (Figure 59). In addition, protons can protonate phosphate ions in the HAP lattice. This reduces the negative charge on the phosphate and causes a charge imbalance which destabilises the lattice. It is hypothesised that a possible way by which adsorbed salivary proteins inhibit HAP dissolution is by protecting the HAP surface. Adsorbed salivary proteins could block the access of acid to the mineral surface and, thus, inhibit the reaction between the acid and HAP components. It is widely suggested that defects in the HAP lattice, so called screw dislocations, are focal points for acid attack and they represent the initial points of dissolution (Arends and Jongebloed, 1977). Proteins adsorbed over these defects may therefore sterically protect these sensitive sites from protons. Alternatively, adsorbed salivary proteins could prevent the lattice phosphates being protonated and in this way avoid charge imbalance or could cause stabilisation of the lattice in cases where phosphates have already been protonated. One could envisage a scenario where a bound protein helps to delocalise charge and so relieve the strain caused by the charge imbalance.

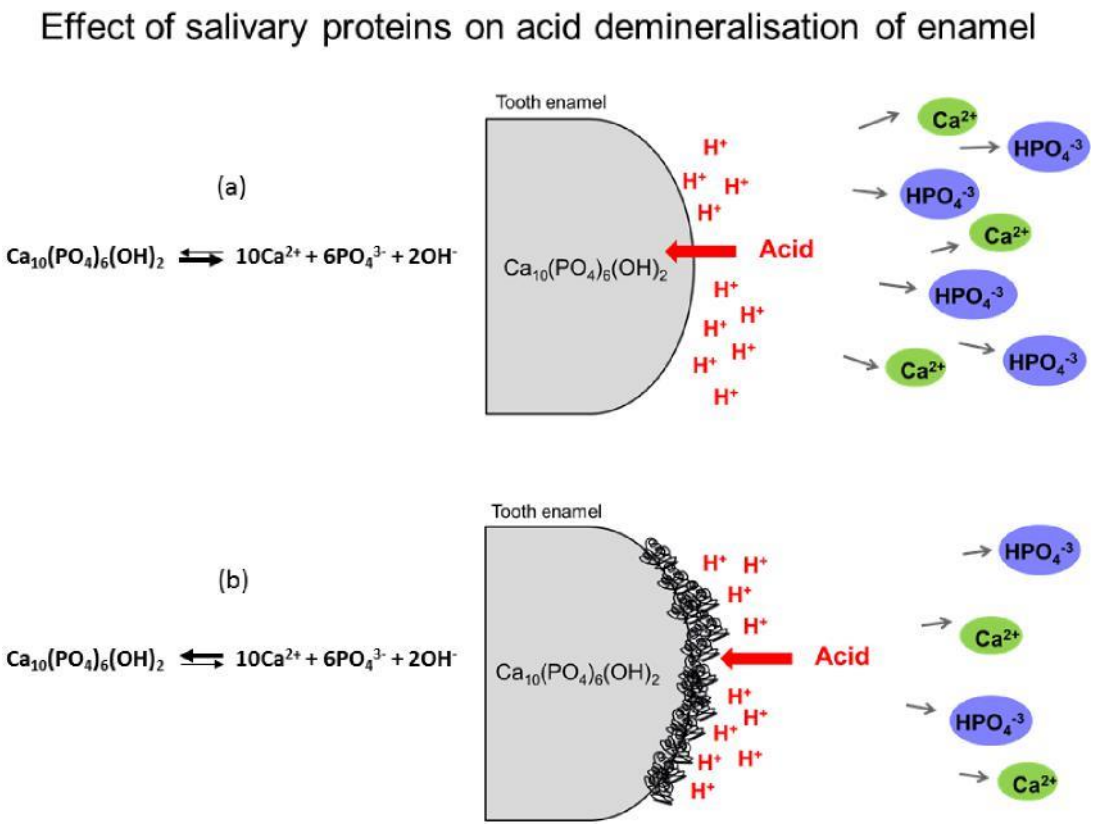


Figure 59. Effect of acid and proteins on HAP dissolution. HAP is in equilibrium with its component ions and at neutral pH and this equilibrium is to the left (Le Chatelier's principle). (a) Acid can shift this equilibrium to the right since protons react with hydroxide and phosphate ions and remove them from the equilibrium. It also protonates the phosphate in the enamel causing more component ions to be released from the HAP and HAP dissolves. (b) Salivary proteins are adsorbed and protect the HAP surface. This protein pellicle attenuates the effect from the acid resulting in less HAP dissolution.

4.1.1.2 Effect of pH on citric acid dissolution of synthetic HAP and protection by proteins

The results from the citric acid dissolution of HAP can be explained considering the effect that different pH values of citric acid have on the HAP surface and the proteins. As described above, citric acid would affect the direct dissolution of HAP by protonation of the HAP surface and by Le Chatelier's principle removal of common ions from the equilibrium. However, citrate can also act as a chelator.

As a chelator, citric acid would bind calcium in solution and according to Le Chatelier's principle more HAP would dissolve. Using the pK_a values of citric acid (pK_{a1}=3.13, pK_{a2}=4.76 and pK_{a3}=6.4) and the Henderson-Hasselbach equation, $pH= pK_a+\log([A^-]/[HA]$, it can be estimated that the predominant form of citric acid at pH 2.79 is AH_3 and a small amount of AH_2^-. However, citric acid at pH 3.50 exists as approximately 50% in AH_3 form and 50% in AH_2^-, as well as a small amount of AH^{2-}. This indicates that citric acid may start to have chelating properties at pH 3.50 as 50% of the acid molecules will be exhibiting two negatively charged carboxylate groups although this species is not considered as a strong chelator as the triply ionised form that exhibits three charged carboxylate groups at higher pH.

The charge carried by ionisable carboxylated side chains of glutamic and aspartic acids contained within the salivary proteins, hence the protein adsorption to HAP, is also affected by the pH of the surrounding solution. Given the pK_a values for side chain carboxylate groups on glutamic acid and aspartic acid, shifting from 2.79 to 3.5 may alter their protonation state (charge). It is known that the acidic proteins, rich in glutamic and aspartic acid bind to the calcium sites of HAP through their carboxylate ions (Gorbunoff and Timasheff, 1984). It is possible that at the higher pH of citric acid, pH 3.50, where the concentration of the carboxylate ions is increased and the ratio $[COO^-]/[COOH]$ is increased (in accordance with the Henderson-Hasselbalch equation), the acidic proteins with their negatively charged carboxyl groups bind more to the positively charged calcium sites of HAP surface and protect against dissolution. When the pH of citric acid is lower at pH 2.79, the ratio $[COO^-]/[COOH]$ is decreased, and therefore the uncharged carboxyl groups of proteins may not bind to the calcium sites of HAP surface so readily and proteins do not protect. According to the Henderson-Hasselbalch equation, the ratio of $[COO^-]$ to [COOH] depends on the pK_a, the acid dissociation constant, of the glutamic and aspartic side chain carboxylate groups. For example the ionisable groups of aspartic acid and glutamic acid are uncharged (protonated) below their pK_a and negatively charged (ionised) above their pK_a. However, the pK_a of the amino acids within a protein can be influenced by the inductive effect of peptide bonds

and neighbouring amino acids. Given this, it is difficult to predict the exact ionisation state of glutamic and aspartic side chain carboxylate groups in a protein and how the charge is affected on changing the pH from 2.79 to 3.50 (even though the pK_a values for the side chains of these free amino acids are readily available in any biochemistry text book).

4.1.2 Protective effect of purified salivary proteins adsorbed to synthetic HAP powder

4.1.2.1 Purification of salivary proteins under non-denaturing and denaturing conditions

The strategy was to separate salivary proteins into fractions based on their molecular size using size exclusion and test the protective effect of each fraction in an attempt to identify the protective protein(s). Although salivary proteins were expected to be separated based on their size following a size exclusion chromatography method described by Baron et al. (1999), the results in section 3.1.2.1 revealed that the 14 and 60 kDa proteins were co-eluting under non-denaturing conditions. The elution of 14 and 60 kDa proteins in the same fractions, which was not reported from Baron et al., suggested that either the two proteins existed as a complex under non-denaturing conditions or they interacted with the column matrix which retarded their progress through the column and resulted in a separation that was no longer based on purely molecular size but included an element of affinity chromatography. Interested in the purification of the salivary cystatins, Baron et al. (1999) used human submandibular sublingual saliva, meaning that many proteins such as the abundant amylase with molecular weight of around 60 kDa, were excluded from their sample. What is more, they used a Sephacryl column which consists of cross-linked allyl dextran and N, N'-methylene bisacrylamide, whereas in the present study a Superdex column was used which is composed of dextran and highly cross-

linked agarose. Various proteins may interact or not with the different columns prepacked with different media.

Although the concentration of sodium chloride in the mobile phase was increased (from 50mM to 150mM) in order to prevent the non-specific binding of the proteins to the column matrix, under non-denaturing conditions (as recommended by the manufacturer of the column) the 14 and 60 kDa proteins still eluted together. Even in a mobile phase containing 150mM NaCl, the proteins seem to still spend a lot of time bound to the column rather than in the mobile phase. However, under denaturing conditions, in the presence of 6M urea, the 14 and 60 kDa proteins were well separated based on their size. It is therefore likely that urea disrupted the hydrogen bonds between the proteins and the column, the proteins spent more time in the mobile phase and eluted according to their size as expected.

In addition to this, urea dissociates any protein complexes which results in the elution of proteins as monomers. The comparison among the size exclusion chromatography of standard proteins (Figure 17) and whole saliva (Figure 18) under the same denaturing conditions, and the size exclusion chromatography of whole saliva under non-denaturing conditions (Figure 13) indicates that both 14 and 60 kDa proteins had delayed elution in the absence of urea. More specifically, the 14 kDa protein started eluting in fraction A12 in the absence of urea (Figure 14) while in the presence of urea its elution started at fraction A9 (Figure 19). The 60 kDa protein started eluting in fraction A11 (Figure 14) in the absence of urea while in the presence of urea its elution started at fraction A5 (Figure 19). The fact that the elution of both proteins was delayed under non-denaturing conditions could be explained by both proteins interacting with the column. However, had the 60 and 14 kDa proteins been a naturally occurring complex, under denaturing conditions they would have eluted as monomers at the expected elution volumes. Evidence from the results of the current study suggests that they do elute at the expected volumes. Therefore, the possibility of the two proteins existing as a complex cannot be ruled out. Further research is

needed to establish whether the 14 and 60 kDa proteins are only interacting with the protein matrix or whether they are also forming a complex under non-denaturing conditions (or indeed, interact with the column matrix as a complex).

The 14 and 60 kDa proteins were identified by western blot analysis and mass spectrometry as cystatin and α-amylase. Interestingly, the “60 kDa” protein was observed (Figure 23 and Figure 24) to be two separate proteins with similar molecular weight of 59 and 56 kDa which are in agreement with the two isoforms of α-amylase corresponding to the glycosylated and the non-glycosylated isoforms of the enzyme.

4.1.2.2 Adsorption of α-amylase and cystatin to synthetic HAP powder

With regard to the adsorption of α-amylase and cystatin to HAP, the proportion of cystatin adsorbed was greater than that of α-amylase. Interestingly, the smaller non-glycosylated isoform of α-amylase seemed to selectively bind over the larger glycosylated isoform (Figure 25). Both α-amylase and cystatin are members of the acquired enamel pellicle (Jensen et al., 1992) but it is important to note that protein interactions are very important for the adsorption to HAP and formation of the pellicle. Perhaps a drawback of the method used to investigate the adsorption of the α-amylase and cystatin in the current study is that their adsorption was tested in the absence of any other proteins or saliva components and this could affect their binding capacity to HAP. Results from an investigation into protein adsorption to HAP by Yin et al. (2006) suggested that the amount of a specific protein bound to HAP may depend on the amount of other proteins, also adsorbed to HAP. It was shown that salivary statherin and egg yolk phosvitin enhanced the adsorption of histatin 5 to HAP (Yin et al., 2006). However, approaches of this kind raise the question of how clinically relevant the results are when only a limited number of proteins are included especially when some of them are non salivary in origin.

4.1.2.3 Protective effect of the purified (by size exclusion chromatography) salivary protein fractions adsorbed to synthetic HAP powder

The protein fractions purified by size exclusion chromatography (Figure 20) did not reduce significantly the dissolution of HAP. The level of protection offered by adsorbed whole saliva and purified salivary protein fractions was very modest (around 8% reduction in mineral loss) and the need for validation of the results led to the use of human enamel powder. The degree of protection provided by whole saliva here (8%) was smaller compared to the one shown in Figure 12 (30%). This difference, as described in the Results (section 3.1.2.3), is explained by the fact that the level of phosphate released from the control sample in the initial experiments (figure 12) was lower compared with the control sample in this experiment (Figure 20) where the protection from whole saliva was shown to be around 8%. The HAP powder was treated with Tris in this experiment (similar to the test samples being incubated with whole saliva) while in the first experiments it was used as a dry powder. This may be regarded as a criticism of the methodology used as the introduction of a step in which the control (no saliva treatment) is incubated in Tris - rather than left as dry powder – has made it more difficult to compare the results obtained between experiments.

Regarding the small level of protection provided by purified protein fractions, explanations will be discussed in greater detail in section 4.3.2.

4.2 Protective effect of adsorbed salivary proteins against acid demineralisation of human enamel powder

The flow diagram of the structure of this section is shown in Figure 60.

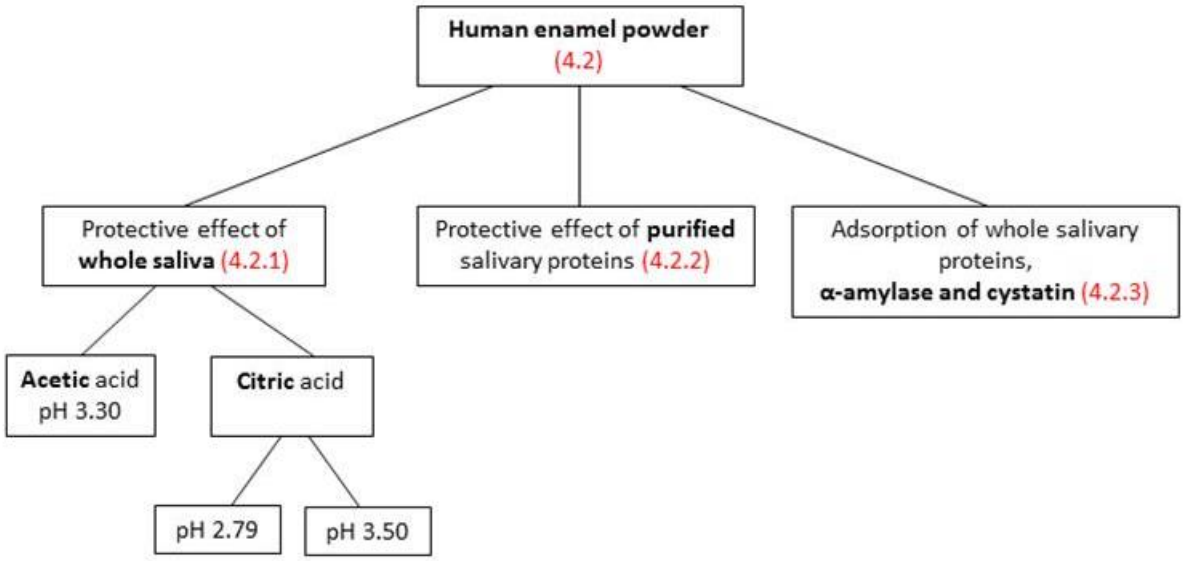


Figure 60. Flow diagram of the structure of section 4.2 (discussion of the results obtained from human enamel powder).

4.2.1 Protective effect of whole salivary proteins adsorbed to human enamel powder

The response of human enamel powder with adsorbed whole salivary proteins to a 100mM citric acid challenge differed from that of synthetic HAP with adsorbed whole salivary proteins. Whole saliva reduced the phosphate dissolution from enamel powder during a citric acid challenge at both pH 2.79 and 3.50. In contrast, adsorbed whole salivary proteins only provided protection for synthetic HAP at the higher pH. This is an important finding

given the common usage of synthetic HAP in dental research. Interestingly, the protection provided to enamel powder was bigger and statistically significant at lower pH value (pH 2.79) of citric acid compared to the higher pH (pH 3.50) at which the protection was smaller and not statistically significant (25% compared to 12% section 3.2.1.1). What is more, not all adsorbed proteins seemed to survive after citric acid attack. It is difficult to explain these results but they might be related to the binding affinity of the salivary proteins being dependent on the exact chemical composition of the adsorbent surface. As discussed in the opening section of the Discussion, natural HAP is chemically different to synthetic HAP due to heteroionic substitution in the crystal lattice which may affect protein adsorption at a given pH. Another possibility is that surface rugosity (roughness) at the nanometre level impacts on protein adsorption to materials. Protein adsorption increases with rugosity, regardless of the increased surface area resulting from the increased rugosity (Scopelliti et al., 2010). The synthetic powder was prepared commercially while the enamel powder was prepared in house and surface rugosity may differ due to production processes such as milling etc.

It is interesting that salivary proteins only protected synthetic HAP from acid attack at the higher pH of 3.5 whereas salivary proteins protected enamel powder at pH 3.5 and 2.79. Most fruit and vegetables contain citric acid and the pH of commonly eaten fruits ranges typically from 2 to 4 (FDA, 2015). For example, lemons are pH 2.2 - 2.4; tangerines are pH 4; grapefruits are pH 3 - 3.3; apples are ~pH 3.5 and pineapples are pH 3.3 - 5.2. From the data obtained here it can be hypothesised that saliva has evolved so as to protect enamel against the acidic fruits; especially fruits that are sweet and more likely to be eaten. This evolutionary process appears to have generated a set of protective proteins that are adapted to protect enamel even at a low pH such as 2.79. The fact that the proteins fail to protect synthetic HAP at this low pH suggests that evolution has produced protective salivary protein(s) whose operational tolerance is limited to natural enamel. It would be interesting to examine the saliva of species who feed

exclusively on acidic fruits to see if evolution has provided them with saliva that is particularly adapted to inhibiting enamel dissolution.

Whole saliva was also shown to significantly protect the enamel against 10mM acetic acid, pH 3.30, attack and all proteins survived the acid attack. The table below summarises the results obtained from the investigation of the protective effect against acid demineralisation of synthetic HAP powder and human enamel powder.

Table 11. Protective effect of whole saliva against acid demineralisation of synthetic HAP powder and human enamel powder

	Synthetic HAP powder	**Human enamel powder**
100mM citric acid pH 2.79	No reduction in mineral loss Proteins such as the 25 and 23 kDa did not survive acid attack	25% reduction in mineral loss ($p<0.01$) Proteins such as the 51, 34, 24.5 and 20 kDa did not survive acid attack
100mM citric acid pH 3.50	20% reduction in mineral loss ($p<0.01$) All proteins survived acid attack	12% reduction in mineral loss (NS) Proteins such as the 96, 39, 32, 28, 25 and 19.5 kDa did not survive acid attack
10mM acetic acid pH 3.30	30% reduction in mineral loss ($p<0.0001$) All proteins survived acid attack	26% reduction in mineral loss ($p<0.0001$) All proteins survived acid attack

4.2.2 Protective effect of the purified (by size exclusion chromatography) salivary protein fractions adsorbed to human enamel powder

Regarding the effect of purified salivary protein fractions against acetic acid demineralisation, similar results to HAP powder of moderate protection were observed with enamel powder. The fraction containing the high molecular weight proteins reduced the acid dissolution significantly. It is questionable whether the level of protection seen (12%), although statistically significant, is clinically relevant. It is not easy to assess the exact degree of protection provided by whole saliva or specific salivary proteins to enamel surfaces because it always depends on the individual's saliva composition and enamel surface. In the oral environment it will also depend on the nature of the plaque associated with the enamel surface and it is again difficult to assess the effect of small amounts of protection on the relative cariogenicity of this plaque.

The results of this study are consistent with those of other studies and suggest that whole saliva protects the enamel against acid demineralisation (Featherstone et al., 1993, Hannig et al., 2003, Hara et al., 2006). However, there are many variable parameters among all the studies investigating the protective effect of saliva in acid erosion, which does not allow the direct comparison of the protective levels. Examples of these parameters are the tooth substrate, the time of exposure to saliva, the time of acid exposure, the concentration and pH of the acid and the environment the study is carried out at (*in vitro*, *in vivo*, *in situ*).

It was suggested that powdered enamel is not appropriate for the investigation of the initial stages of acid demineralisation of enamel because the surface loss cannot be measured (Young and Tenuta, 2011). However, other methods for determining the level of acid demineralisation can be performed such as the measurement of phosphate dissolution carried out in the current study.

4.2.3 Differential adsorption of whole salivary proteins, α-amylase and cystatin to synthetic HAP and human enamel powder

It is interesting that the binding affinity of α-amylase to human enamel powder was found to be lower compared to its affinity to synthetic HAP. Very small amounts (if any) of α-amylase were found to be adsorbed to enamel powder when α-amylase was in a fraction containing both α-amylase and cystatin. It is somewhat surprising that no α-amylase was adsorbed to enamel powder when α-amylase was in a fraction alone. Another important finding was that cystatin was bound to synthetic HAP and human enamel powder when it was in a mixture with α-amylase or as cystatin alone.

There are two likely explanations for the different adsorption behaviour of α-amylase to synthetic HAP and enamel powder. The effect of the surface area and rugosity of the two powders, as well as the presence of heteroionic substituents in enamel powder, can influence the binding of the proteins to each surface. Small particle size exhibits a bigger specific surface area and subsequently an increased amount of surface available for protein binding. In a study using bovine enamel powder, it was demonstrated that the amount of casein bound to enamel was increased as the particle size of the enamel powder got smaller (Pearce and Bibby, 1966). The differential adsorption of whole salivary proteins to synthetic HAP and human enamel powder shown in the results (Figure 33) can also be explained by possible differences in the morphology and chemistry of the two powders. The presence of organic impurities and inorganic trace elements in the human enamel powder may indicate that it simulates natural tooth surfaces better than synthetic HAP. However, synthetic HAP is usually preferred in the literature for its greater reproducibility.

4.3 Protective effect of adsorbed salivary proteins against acid demineralisation of natural enamel surfaces

The flow diagram of the structure of section 4.3 is shown in Figure 61.

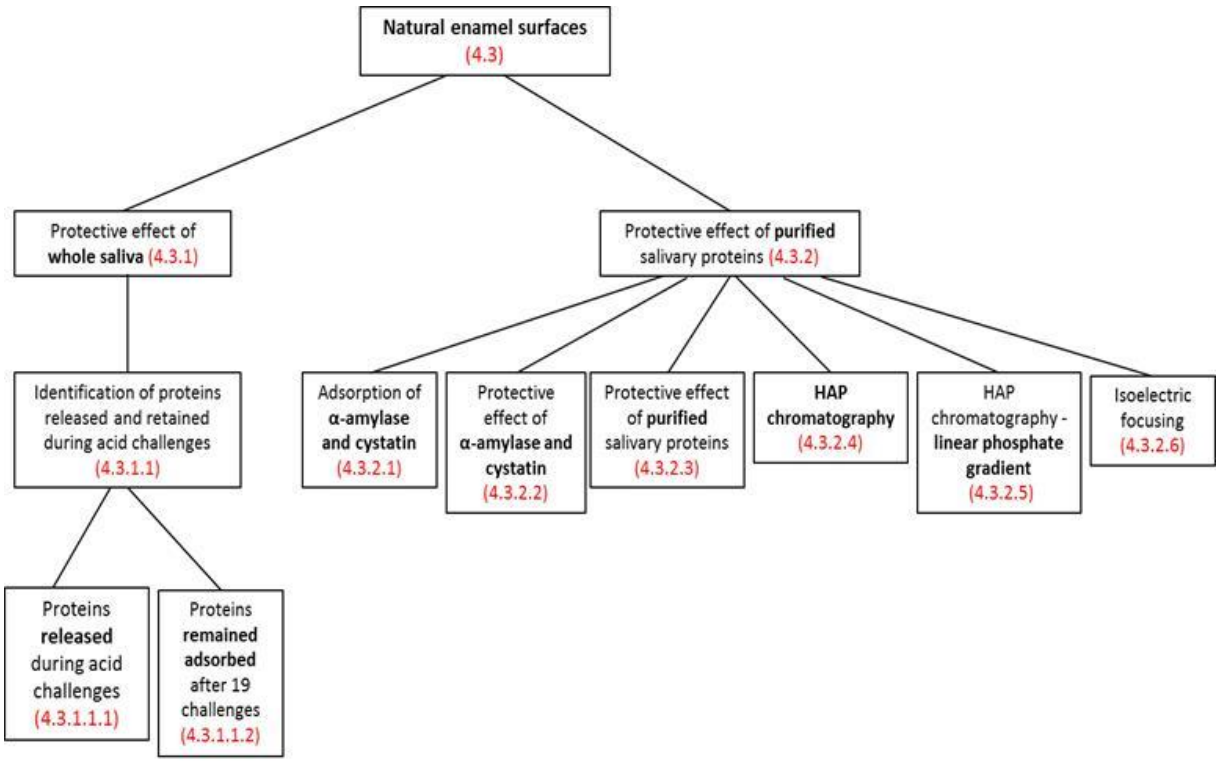


Figure 61. Flow diagram of the structure of section 4.3 (discussion of the results obtained from natural enamel surfaces).

4.3.1 Protective effect of adsorbed whole salivary proteins

In the last part of this study the effect of salivary proteins against acid demineralisation was investigated using natural human enamel surfaces. There is a large volume of published data on the protective role of saliva against acid demineralisation of enamel. In agreement with previous research, and after the protective effect of saliva was demonstrated using powdered substrates, the current study also demonstrated that whole saliva provides significant protection against acid demineralisation of natural

enamel surfaces (Figure 34). The level of protection was high (43%), which could be clinically relevant compared with the moderate degree of protection (10-30%) offered by salivary proteins in powdered substrates. These findings emphasise the importance of using natural enamel surfaces to investigate the effect of saliva on acid erosion and should be a major consideration in designing experiments.

Baumann et al. (2015), extracted the mature “enamel proteins” (presumably including any salivary or dietary proteins that had penetrated any surface porosities in the enamel) from human teeth. They found that deproteinated enamel was more susceptible to acid erosion by citric acid (1% (w/v), pH 3.6). The authors suggested that the mature enamel proteins may play a role in salivary protein adsorption, although no evidence was provided to support this notion (Baumann et al., 2015). It is clear however, from the data presented in this thesis, that adsorbed salivary proteins greatly enhance any protective effect provided by the mature enamel proteins as the teeth used in this thesis were not subject to deproteinisation and yet still benefited from significant protection following adsorption of salivary proteins. That mature enamel proteins may play a protective role against acid attack further supports the contention raised here that actual enamel surfaces are the substrates of choice over powdered minerals.

The most important finding reported in this thesis was that the protective effect of whole salivary proteins remained significant even in the face of repeated acid (10mM acetic acid, pH 3.30) challenges (Figure 34). The method used in this study is therefore useful for investigating the duration of the protective effect against multiple episodes of acid attacks. However, the protective effect was not permanent as the salivary proteins were gradually lost along with the protective effect, with increasing numbers of acid challenges. Although a large number of proteins remained bound to enamel after 19 challenges, it was shown that they were not protective; the protective proteins being lost during the initial acid attacks.

4.3.1.1 Identification of adsorbed proteins released and retained during acid challenges

It was hypothesised that the proteins released during the initial acid challenges may be the ones providing protection against acid demineralisation since their loss corresponded with the observed loss of protection. The acid impervious proteins remaining bound to enamel after 19 acid challenges may include proteins that were protective but they no longer protect, or proteins that were not protective to begin with. It was shown that the patterns of protein loss varied among individuals. However, there was a number of proteins such as the 50, 35 and 14 kDa proteins which were found in more than one individual. A few studies have demonstrated significant variability of salivary patterns among individuals (Millea et al., 2007, Quintana et al., 2009). The current study demonstrated the variation of adsorbed salivary proteins to the enamel surface which may be associated, not only, with the inter-individual variability of salivary proteins, but also, the variability of the tooth surfaces. The mineral composition of enamel and the tooth surface can vary among individuals, therefore, it is important that in research based on the protein adsorption to enamel the variability of tooth enamel surfaces is taken into consideration. In a recent study, Carvalho and Lussi showed that the acid erosion of enamel differs between different types of teeth (Carvalho and Lussi, 2015). In the current study, both human molars and premolars were used which added one more variable to the equation.

4.3.1.1.1 Proteins released during acetic acid challenges

The proteins lost in the initial acid attacks (where loss of such proteins corresponded to loss of protection) were characterised by mass spectrometry, as it was hypothesised that these are the protective species.

From the results obtained by mass spectrometry, two of the proteins released during the acid challenges were identified. According to the Mascot results (section 3.3.2.2) the possible identification of the 38 kDa gel band was the protein S100-A8, known also as calgranulin A, which has a molecular weight of 10885 Da. Another possible match for the 38 kDa gel

band was the protein S100 which has a molecular weight of 13259 kDa. This result is in agreement with previous studies that identified the protein S100-A8 in the human acquired enamel pellicle (Yao et al., 2003, Siqueira et al., 2007). The formation of a homo-oligomer or hetero-oligomer with another member of the S100 protein family may explain the difference in the molecular weight between the SDS-PAGE gel band (38 kDa) and the identified protein (10.8 kDa). The S100-A8 protein is a member of the S100 protein family of calcium-binding proteins which tend to form oligomers characterised by non-covalent bonds. S100 proteins and S100-A8 have been implicated in several functions including their involvement in the regulation of inflammation, cell proliferation and differentiation (Vogl et al., 2006, Donato et al., 2013).

Based on the Mascot results a second protein band running at 15 kDa on SDS-PAGE was putatively identified as lysozyme (16.9 kDa). Lysozyme has also been identified in the human acquired enamel pellicle in previous studies (Yao et al., 2001, Yao et al., 2003, Siqueira et al., 2007). Lysozyme is an enzyme known for its ability to kill gram-positive bacteria by hydrolysing the glycosidic bonds of the bacterial cell wall peptidoglycan. It has also been reported that it displays antiviral activity and antimicrobial function against gram-negative bacteria and fungi (Fabian et al., 2012). The identification of lysozyme between the proteins released during acid challenges in this study suggests that lysozyme may also contribute to the protective effect of the enamel against acid demineralisation of the enamel.

4.3.1.1.2 Proteins remained adsorbed to enamel after 19 acid challenges

The proteins that remained bound after 19 acid challenges provided no protection against acid attack. According to the Mascot results, the possible identification of the 55 kDa gel band was the cDNA FLJ54371, highly similar to serum albumin with an overall protein score of 1178. Serum albumin has been identified in human acquired enamel pellicle (Yao et al., 2003, Siqueira et al., 2007). The 26 kDa protein band was identified as protein S100 with molecular weight of 13258 kDa and the 21.5 kDa as protein S100-A8 with

molecular weight of 10885 kDa. Both 26 and 21.5 kDa protein bands may represent homodimers of protein S100 and protein S100-A8 respectively. S100 and S100-A8 were also identified in the group of proteins lost in the initial acid challenges that were hypothesised to be protective. It is possible that these proteins were not protective even during the initial acid attacks or they were protective as part of some other complex.

4.3.2 Protective effect of adsorbed purified salivary proteins

4.3.2.1 Adsorption of α-amylase and cystatin to natural enamel surfaces

Distinct differences in the adsorption behaviour of the α-amylase and cystatin to synthetic HAP, human enamel powder and natural enamel surfaces were observed. Cystatin was shown to adsorb to both synthetic HAP (Figure 25) and human enamel powder (Figure 32) in the presence or the absence of α-amylase, whereas no cystatin was adsorbed to natural enamel surfaces with or without α-amylase (Figure 42). Furthermore, only a small amount of α-amylase was bound to enamel when it was in a mixture with cystatin, or α-amylase alone (Figure 42). The results of this study emphasise the important differences in protein binding to the enamel depending on the different enamel substrate used for the investigation. From the SDS-PAGE analysis (Figure 42) it seems that the smaller non-glycosylated isoform of amylase was selectively adsorbed. A further study with more focus on the adsorption of the α-amylase onto natural enamel surfaces, investigating whether one of the two α-amylase isoforms has the selective binding affinity for enamel, would be very interesting.

It is critical to note that there are important differences between powdered substrates and natural enamel surfaces which would affect the adsorption of proteins. The fact that the enamel is not chemically homogenous means that powdering enamel results is an ‘average’ enamel sample. The fluoride rich

surface, which is more acid resistant and a more powerful adsorbent, is lost in the powdered enamel. The enamel crystals in the interior of the enamel are less stable due to the higher concentrations of carbonate and magnesium and lower concentration of fluoride. These changes may affect the interactions between the enamel surface and saliva (Robinson et al., 1995).

What is more, mature enamel proteins which may also play an important role in the electrostatic interactions between enamel and salivary proteins, are missing completely from synthetic HAP and are disarranged in the powdered enamel (Baumann et al., 2015).

In summary, the distinct distribution of the enamel components (inorganic and organic) no longer exists in powdered enamel. These factors may explain the bigger level of protection of natural enamel surfaces by whole saliva compared with human enamel powder or synthetic HAP, as well as the different adsorption behaviour of salivary proteins, such as α-amylase and cystatin.

4.3.2.2 Protective effect of the mixed fraction of α-amylase and cystatin

The fraction containing α-amylase and cystatin was shown to reduce the mineral loss only during the first acid challenge after adsorption (Figure 43). The 17% of protection provided by α-amylase and cystatin was not significant and did not last for the next acid challenge.

This result may be explained by the fact that protein interactions can affect the adsorption and function of the proteins. For instance, α-amylase was reported to form heterotypic complexes with mucin MG1 and histatin 1 (Iontcheva et al., 1997, Siqueira et al., 2012b). Functional assays were performed by Siqueira and his colleagues to investigate whether the function of the α-amylase and that of histatin 1 would remain the same when they existed as a complex. They demonstrated that the antifungal activity of histatin 1 was reduced by 43% when it was complexed with amylase.

However, the starch hydrolysis activity of amylase/histatin 1 complex was similar to the activity of amylase alone. Furthermore, the formation of the amylase/histatin 1 complex was shown to provide better protection against proteolysis of the two partners compared to amylase or histatin 1 alone (Siqueira et al., 2012b).

Taken together, when the teeth are exposed to a mixture of saliva and acid solution, the adsorption of α-amylase and cystatin to enamel and the potential protective effect against acid demineralisation may be influenced or even be dependent on their interactions with other proteins. Studies investigating the protective potential of purified proteins are obviously missing interactions with other salivary proteins. The advantage of the method used here involving the adsorption of all salivary proteins and their subsequent loss during repeated acid challenges was an attempt to overcome this problem.

4.3.2.3 Protective effect of saliva protein fractions purified by size exclusion chromatography under denaturing conditions

This study has shown that protein fractions purified by size exclusion chromatography under denaturing conditions did not provide protection against acid demineralisation of the enamel. A probable explanation for this result may be the loss of the quaternary, tertiary and secondary structure of the proteins and possible conformational change due to the treatment with the chaotropic urea. As a result of denaturation, proteins which are no longer in their native state may lose their ability to adsorb to enamel due to the destruction of specific domains that recognise the enamel surface or recognise other proteins initially adsorbed to enamel.

Another possible explanation for the inability of purified protein fractions to provide protection against acid demineralisation is that the salivary proteins may need to interact with other proteins or saliva ions in order to adsorb to enamel and protect. The important role of salivary ions in relation to enamel demineralisation was recently demonstrated by Martins et al. (2013) who compared the level of protection provided by undialysed saliva with that

offered by dialysed saliva. It was shown that natural saliva provided a higher degree of protection compared to undialysed saliva which was also protective. The loss of calcium and phosphate from dialysed saliva would reduce the level of supersaturation and leave enamel more vulnerable to dissolution according to Le Chatelier's principle (as described in section 4.1.1.1). The main criticism of this approach, however, is that the enamel demineralisation assay was carried out for a period of 12 days. These conditions may provide information about long term acid exposure but it would have been more useful if the effect of short-term acid exposure was included (Martins et al., 2013). Approaches of this kind, using extended periods of enamel demineralisation when investigating the effect of salivary proteins fail to consider the fact that salivary proteins may get washed away after some time of acid exposure. Hara et al., suggested that after 10 minutes of demineralisation, adsorbed proteins may be released from the enamel (Hara et al., 2006). This is in agreement with the results of the current study which has shown clearly that initially adsorbed salivary proteins are lost during short-term (30 sec) acid challenges.

4.3.2.4 Purification of salivary proteins in native state by HAP chromatography and protective effect of purified fractions

In order to avoid the problems associated with purification methods employing denaturing conditions, HAP affinity chromatography under non-denaturing conditions was employed.

The first protein fraction to elute from the column by HAP chromatography was the flow through proteins that did not bind to the HAP column (Figure 46). Although this fraction contained a large amount of salivary proteins, it did not provide any protection from acid demineralisation of the enamel (Figure 47). In contrast, the second protein fraction eluted with 500mM phosphate buffer (pH 7.4) which contained all proteins that were adsorbed to the HAP column, reduced the acid dissolution significantly by 32% and the protective effect remained for the subsequent acid challenges (Figure 48). These results revealed that a subset of whole salivary proteins comprising

about 15% of the total protein (i.e. the proteins that adsorbed to the HAP column) are protective.

Comparing the two results, it can be seen that proteins with no binding affinity for HAP are not able to protect the HAP from acid dissolution. This is in agreement with the hypothesis of proteins protecting the HAP from acid by adsorbing to the HAP surface. It is important to note that in this study HAP chromatography was not used as a simulation of the protein adsorption onto enamel but as a protein purification method *per se*.

It is also interesting that when the bound proteins which seemed to protect the enamel were analysed by SDS-PAGE, it was shown that a protein of approximately 55-60 kDa and a 14 kDa protein were lost during the first acid challenges after the protein adsorption but the same proteins seemed to remain bound after 19 acid challenges (Figure 49). The protective effect of the proteins bound to the HAP column was lost after 19 acid challenges presumably due to their desorption. Another possible explanation for this might be that the proteins were not desorbed but remained bound (as shown with the 55 and 14 kDa proteins) but with a different non-protective conformation. Further research should be done to investigate whether the 55-60 and 14 kDa proteins that appeared to be bound to the HAP column and the tooth surface are in fact α-amylase and cystatin. It would be interesting to investigate their adsorption and protection to natural enamel surfaces when they are in a mixture with other proteins that bind to and protect the enamel.

4.3.2.5 Further purification of the protective subset of proteins by HAP chromatography using a linear phosphate gradient

Further purification of the protective subset of proteins was indeed achieved by HAP chromatography using a linear phosphate gradient. The group of fractions containing proteins loosely bound to the HAP column was shown to reduce the acid demineralisation of enamel by 34% and the protective effect remained for the subsequent acid challenges (Figure 52), while the group of fractions containing the strongly bound proteins reduced the acid

demineralisation by 30% but the protective effect was temporary (Figure 53). These results may raise questions about the relationship between the adsorption of the proteins to the HAP column and their protective potential once adsorbed to enamel surfaces. However, it is important to bear in mind that proteins were purified based on their binding ability to the HAP column and their tendency to be desorbed by phosphate buffer. Additionally, the binding ability of proteins to the HAP column at neutral pH (pH 7.4) may differ from their binding ability to enamel surfaces at acidic pH (acetic acid pH 3.30 challenges), due to the charge on the proteins being dependent on the pH. Most importantly, it was shown that the mixture of the two groups of fractions (i.e. proteins loosely and strongly bound to the HAP column) was more protective (reduced the mineral loss by 52%) and the protective effect was evident for the next ten acid challenges (Figure 54). This finding further supports the idea of protein interactions being important for protein adsorption and protection.

4.3.2.6 Isoelectric focusing for further fractionation of the protective subset of salivary proteins

Isoelectric focusing was another method used to further purify the protective subset of salivary proteins. The group of fractions containing proteins with low isoelectric point (pH 3 to pH 6) did not reduce the mineral loss (Figure 56) while the group of fractions with proteins of high isoelectric point (pH 7 to pH 10) were shown to protect the enamel against acid demineralisation (Figure 57). Although proteins with high isoelectric point reduced the mineral loss by 49%, the protective effect was temporary. A possible explanation for this result may be the effect of the protein charge at acidic pH conditions. Proteins with high isoelectric point have a net positive charge at neutral pH (normal mouth conditions) and at pH 3.30 (10mM acetic acid challenges) which means that they are able to interact with the negatively charged phosphate sites of the enamel, adsorb and protect the enamel from acid dissolution. On the other hand, proteins with low isoelectric point at around 3, have no net charge at pH 3.30 which means decreased protein adsorption

to enamel and subsequent decreased protection. However, proteins with a low isoelectric point at a pH range between 4 to 6 are positively charged at pH 3.30 and should theoretically protect in a similar manner to the proteins with high isoelectric point. The fact that the protein group with low isoelectric point did not reduce the acid demineralisation may also be explained by the small amount of proteins belonging to this group compared to the group of high isoelectric point (Figure 55). These results reveal the need for further investigation into the effect of the isoelectric point of proteins on protein adsorption and protection.

4.4 General discussion

In this thesis an attempt has been made to identify salivary proteins that are able to inhibit acid demineralisation of enamel. The protective effect of whole saliva was investigated as saliva is mixed in the mouth. The present study was designed to determine the effect of salivary proteins on the acid demineralisation of enamel and to characterise specific proteins that provide protection. The comparison of the outcomes from the investigation of the effect of whole saliva on demineralisation of powdered substrates and natural enamel surfaces highlighted the importance of using natural enamel surfaces for studies involving protein adsorption and the protective effects of adsorbed proteins.

There is a need for standardisation of the conditions used for studying the acid demineralisation of human enamel with relation to salivary proteins. Comparisons of the present results to data in the literature are not easy because there is a large number of different methodologies used to determine the effects of saliva on acid demineralisation of enamel. A number of factors, such as the type of saliva, mineral substrate, time allowed for saliva adsorption and formation of acquired enamel pellicle, type of acid and time of acid exposure, differ among studies, which makes the comparison of results almost impossible. Young and Tenuta discussed the same problem in their study and also suggested guidelines for good methodology in initial erosion models (Young and Tenuta, 2011).

Different protein purification methods provided important insights into the adsorption of specific protein fractions onto enamel surfaces and their protective potential. A subset of salivary proteins was revealed by HAP chromatography (Figure 48) that are protective. HAP chromatography identified a large proportion of whole salivary proteins which did not bind to HAP column and did not show any protection. In future investigations, it might be possible to identify all proteins belonging to this protective subgroup by mass spectrometry and compare them with the list of proteins identified previously in human acquired enamel pellicle (Siqueira et al., 2007). The categorisation of the human acquired enamel proteins by

Siqueira et al. (Table 5) provides information on the contribution of the proteins to the protective effect against acid demineralisation. For instance, in regard to their chemical properties, one third of the total protein has binding affinity for calcium or phosphate, and this result could be associated with increased adsorption of proteins to an enamel surface and subsequent protection of enamel from acid. With regard to the biological function of the acquired enamel pellicle proteins, the authors reported that 15.5% of the total protein is involved in remineralisation and this result is also associated with the protection of enamel from acid demineralisation. Therefore, it would be very interesting to investigate what percentage of the proteins present in the human acquired enamel pellicle actually contribute to the protection of the human enamel from demineralising. Certainly, the results in this thesis have identified proteins that remain strongly adsorbed to enamel surfaces following 19 acid challenges (Figure 34 and Figure 39) but these proteins show no protective properties whatsoever. This emphasises the fact that just because a protein adsorbs to the enamel surface this does not guarantee it will protect against acid attack.

4.5 Conclusions and future directions

Saliva plays a major role in the oral health and provides the tooth enamel with natural protection against acid erosion. The characterisation of specific salivary proteins that inhibit the acid demineralisation of enamel and understanding the mechanisms by which these proteins function is an important area of research where there is abundant room for further progress.

The outcomes of this research project can be summarised in the following points:

- Whole saliva reduced the acid demineralisation of synthetic HAP, powdered human enamel and natural enamel surfaces. This is the first study that compared the effect of salivary proteins on demineralisation of powdered substrates and natural enamel surfaces. The level of protection found was higher, hence more clinically relevant, when natural enamel surfaces were used for the investigation. Therefore, the results of this study suggest that powdered substrates may not be appropriate for the investigation of the effect of salivary proteins on enamel demineralisation.

- Salivary proteins lost during acid challenges corresponded to the loss of the protective effect. It has been hypothesised that these proteins may be protective. What is more, a large number of proteins appeared to be acid impervious, remaining adsorbed to enamel surface after 19 acid challenges but they were no longer protective. Proteins S100-A8 or S100 and lysozyme were identified among the proteins released into acid. Proteins S100-A8 and S100 were also identified among the acid impervious proteins.

- HAP chromatography revealed that only a subset of salivary proteins, containing about 15% of the total protein, are protective. Further fractionation of this subset reduced the level of protection provided by the new fractions, indicating that the protective effect is most likely a

synergistic effect of various salivary proteins that interact with each other and the enamel surface in order to achieve significant inhibition of the acid demineralisation.

- The adsorption behaviour of α-amylase and cystatin to enamel was different for the different enamel substrates. The smaller non-glycosylated isoform of α-amylase seems to be selectively bound to enamel. Cystatin seemed to have great binding affinity for synthetic HAP and human enamel powder, however, it did not adsorb to natural enamel surfaces when it was in a mixture with α-amylase or cystatin alone.

Future work for this project would include investigation of the effect of salivary proteins on enamel demineralisation caused by different acids such as lactic acid (responsible for tooth caries) and phosphoric acid (the acid in cola type drinks). Future research would also include further fractionation of the protective subset of salivary proteins in order to identify specific salivary proteins. However, a review of the literature and results from this study suggest that the protective mechanism is complicated and may require the presence of different salivary proteins, protein interactions and protein complexes.

More research on the adsorption behaviour of α-amylase onto enamel surfaces also needs to be undertaken before the association between the selective adsorption of one isoform of α-amylase and the potential protective activity is more clearly understood.

The effect of the isoelectric point of salivary proteins on their adsorption to enamel surfaces and the potential protective properties could also be further assessed.

Further studies on identification of salivary proteins from a bigger number of subjects or from various target groups, such as caries-susceptible, caries-free and xerostomia patients is also recommended.

List of Abbreviations

AMPs: antimicrobial peptides

AI: amelogenesis imperfecta

CA-125: cancer antigen 125

2-DE: two-dimensional gel electrophoresis

DTT: dithiothreitol

ER: endoplasmic reticulum

HAP: hydroxyapatite

IEF: isoelectric focusing

Ig: immunoglobulin

KLK4: kallikrein-4

Ksp: solubility product constant

LC-MS: liquid chromatography – mass spectrometry

MMP20: matrix metalloproteinase-20

M2BP: Mac-2 binding protein

MRP14: migration inhibitory factor-related protein 14

MS: mass spectrometry

MUC5B: mucin 5B (MG1)

MUC7: mucin-7 (MG2)

NS: non-significant (statistically)

OSCC: oral squamous cell carcinoma

PCR: polymerase chain reaction

PRPs: proline-rich proteins

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: scanning electron microscopy

SMH: surface microhardness

TBS-T: Tris-buffered saline, Tween 20

TEM: transmission electron microscopy

Appendix A: Participant information sheets

INVESTIGATING THE BIOLOGICAL PROPERTIES AND COMPOSITION OF SALIVA

General Protocol

Recruitment: Potential volunteers will be contacted verbally or by email (see appendix 1). Either the PI or other LDI staff/post graduate students associated with the research will contact volunteers (the Principle Investigator is currently the post graduate research tutor in LDI and as such would not personally recruit current LDI post graduate research students).

Obtaining Consent: Potential volunteers will be provided with a participant information sheet (appendix 2) briefly explaining the research being carried out, inclusion/exclusion criteria and what would be expected of them should they agree to volunteer. Once an individual has elected to volunteer, they will complete a written consent form (appendix 3).

Collecting Saliva: At a prearranged time, volunteers will report to Oral Biology where they will be seated in a quiet room and given a glass of mineral water to drink. After 10 minutes they will be given a piece of paraffin wax and instructed to chew the wax in order to stimulate salivary flow. They will be asked to collect the saliva by drooling into a plastic container. During this period a member of staff associated with the project will be on hand to provide any assistance required (e.g. ensuring first aid is provided in the event of choking). The saliva sample will be labelled with the collection date, time and a unique identification number recorded on the consent form (consent forms will be numbered incrementally). Whole saliva will be used immediately or stored frozen for a maximum of 24 hours prior to use. Non-viable proteins isolated from the saliva may be kept beyond this period for further analysis.

Disposal of samples: All solid salivary residues and liquid samples <1ml will be disposed of in the clinical waste while larger liquid residues will be washed to waste down the laboratory sink with suitably diluted house hold bleach.

Version 3
04/09/12

INVESTIGATING THE BIOLOGICAL PROPERTIES AND COMPOSITION OF SALIVA

Participant information sheet

What are we asking you to do?

We are inviting you to participate in research being carried out at LDI on salivary proteins. We are looking for healthy volunteers willing to donate samples of their saliva. Donation is painless but we ask that you refrain from eating or drinking anything but water for 1 hour prior to donation. Donation involves drinking a glass of mineral water and 10 minutes later chewing tasteless wax to stimulate your salivary flow which you collect by drooling into a plastic container. The whole process will take about 20 minutes. We will not ask about your medical history other than to confirm you are in good general health, that you do not consider yourself to have any saliva related problems (e.g. dry mouth) and whether you smoke or not. In some cases, we may ask volunteers to donate again at a later date but volunteers can simply decline if they wish. The only personal details we will store are your name, the date you donated saliva and the brief medical history described above. At no time will your personal details be passed to any third party.

Why are we interested in your saliva?

Saliva contains several hundred protein molecules. We are interested in how specific salivary proteins protect the teeth from harmful acids derived from food or bacteria living on teeth. We aim to identify which salivary proteins protect against acid attack and understand how they are able to provide this protection. Ultimately, we may be able to apply this knowledge to design novel molecules that could help to protect patients' teeth.

Saliva also contains many proteins that stimulate cell growth and tissue repair (this why many animals and humans instinctively lick or suck wounds). As yet, no one has exploited these regenerative properties in the fields of tissue engineering and regenerative medicine and so our aim is to test the effects of salivary proteins on various cell types currently used in tissue engineering to see whether such proteins can help engineered tissues to grow.

Finally, saliva can vary from person to person and one possible long term outcome of this research is that we will be able identify people who are lacking in specific salivary proteins that might predispose them to oral diseases such as caries, erosion or even cancer. Being able to identify such people would allow preventative measures to be enacted as soon as possible in order to prevent disease (note - the study described here will not provide this information).

What do you need to do to volunteer?

Once you have decided you would like to help with our research by donating saliva, it is simply a matter of signing a consent form to show you are willing to take part and that you are aware of what is involved. The consent form will be stored securely in LDI (this is the only personal information we will hold). Signing the form does not commit you in any way – you are always free to withdraw your consent at any time without reason. To consent, please contact XXXXXXXX (email;tel) and they will arrange a mutually convenient time for you to sign the form. They will also be happy to answer any questions you may have.

Version 3
04/09/12

Appendix 1

Initial email used to contact potential volunteers.

Dear xxxxx,

LDI is carrying out research on the properties and functions of salivary proteins. We are looking for healthy volunteers who do not suffer from dry mouth to help with this research by donating a saliva sample (both non-smokers and smokers are welcome to volunteer). Donating saliva involves rinsing the mouth out with mineral water, chewing tasteless paraffin wax and drooling into a plastic container. This will take about 20 minutes. We will ask you to refrain from eating or drinking anything but water for 1hour prior to donating your saliva. You would be free to withdraw from donation at any point. Your saliva would not be stored for more than 24 hours and would be disposed of through clinical waste. However, protein components isolated from your saliva may be kept for further analysis. If you are willing to help please reply to this email and we can send you the participant information sheet and answer any questions you may have.

Regards

xxxxxxxxxxxxx

Email: [insert email address]

Tel: [insert phone number]

Version 3
04/09/12

CONSENT FORM

Project title: Investigating the Biological Properties and Composition of Saliva

Tick here

I confirm that I have read and understand the participant's information sheet..... ☐

I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily..... ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason..... ☐

I believe my general health is satisfactory..... ☐

I believe I have no problems with my saliva (e.g. dry mouth)..... ☐

I am a smoker.....yes/no

Volunteer's name	Signature	Date

Person taking consent	Signature	Date

Consent Form Number .....

Version 3
04/09/12

Appendix B: Oral presentations

Protective effect of salivary proteins against acid dissolution of enamel. Nikoletta Pechlivani, Deirdre Devine, Alan Mighell, Philip Marsh, Steven Brookes. Faculty of Medicine and Health postgraduate conference, June 2014 (2nd place)

Caries affects a third of adults (2010 Adult Dental Survey) and has negative effects on quality of life. Saliva protects enamel against acid demineralisation but it is unclear which proteins are involved or their mechanism of action.

Objectives: Investigate salivary protein adsorption and protection using powdered substrates and natural enamel. Characterise protective proteins.

Methods: Salivary proteins were adsorbed to hydroxyapatite powder, enamel powder and human enamel surfaces and exposed to acid. Demineralisation was determined by spectrophotometric assay of phosphate dissolved. Proteins were characterised by SDS-PAGE. Proteins were further fractionated by size-exclusion and/or hydroxyapatite chromatography to identify specific protective molecules.

Results: Salivary proteins offered modest protection when adsorbed to powdered substrates but reduced demineralisation dramatically (by ~43% ($p<0.0001$)) when adsorbed to enamel. Repeated acid challenges gradually desorbed the protective proteins which corresponded to a loss of protection. Hydroxyapatite chromatography of whole saliva revealed that protective proteins comprise a subset amounting to ~15% of the total protein.

Conclusions: The use of natural enamel surfaces is important when investigating the protective effect of salivary proteins. Protective species in saliva comprise a subset of the total proteins. Understanding the mechanism of protection will inform the development of prophylactic/therapeutic peptides for clinical use.

(Acknowledgements: CASE studentship funded by BBSRC and GSK)

Protective effect of salivary proteins against acid demineralisation of enamel. Nikoletta Pechlivani, Deirdre Devine, Alan Mighell, Philip Marsh, David Bradshaw, Nisha Patel, Steven Brookes. Leeds School of Dentistry postgraduate research day, July 2014 (1st place)

Salivary proteins form an initial pellicle that protects enamel against acid demineralisation but it is unclear which specific proteins are involved or their mechanism of action.

Objectives: 1) Investigate salivary protein adsorption and protection using powdered synthetic hydroxyapatite, powdered enamel and natural enamel surfaces. 2) Characterise the protective salivary proteins.

Methods: Salivary proteins were adsorbed to hydroxyapatite powder, enamel powder and human enamel surfaces and exposed to acid. Demineralisation was determined by spectrophotometric assay of phosphate dissolved. Proteins were characterised by SDS-PAGE. Proteins were further fractionated by size-exclusion and/or hydroxyapatite chromatography to identify specific protective molecules.

Results: Salivary proteins offered modest protection when adsorbed to powdered substrates but reduced demineralisation dramatically (by ~43% ($p<0.0001$)) when adsorbed to enamel. Repeated acid challenges gradually desorbed the protective proteins which corresponded to a loss of protection. Hydroxyapatite chromatography of whole saliva revealed that a subset of proteins comprising ~15% of the total protein were protective. Treatment with chaotrophic agents abolishes the protective effects suggesting protection is dependent on protein conformation or complex formation.

Conclusions: The results emphasise the importance of using natural enamel surfaces when investigating the protective effect of salivary proteins. The protective species in saliva comprise a minor subset of the total proteins that are protective in their native conformation. A better understanding of how salivary proteins protect against demineralisation will aid the design of therapeutic saliva substitutes and potentially allow for caries/erosion susceptible individuals to be identified based on unfavourable polymorphisms of their salivary proteome.

(Acknowledgements: CASE studentship funded by BBSRC and GSK)

Appendix C: Poster presentations

Protective effect of salivary proteins against acid demineralisation of enamel. Nikoletta Pechlivani, Deirdre Devine, Alan Mighell, Philip Marsh, Steven Brookes. Leeds School of Dentistry postgraduate research day, July 2013 (1st place)

A selective adsorption of proteins occurs when hydroxyapatite, the main component of dental enamel is exposed to saliva. Salivary proteins form an initial pellicle capable of protecting the enamel against acid demineralisation but it not known which specific proteins are involved. An understanding of pellicle formation and function will aid the design of therapeutic saliva substitutes.

Objectives: Characterise initially adsorbed enamel pellicle proteins that inhibit the acid dissolution of hydroxyapatite and to investigate the role of protein-protein interactions in pellicle formation.

Methods: Salivary proteins were purified by size exclusion chromatography. Whole saliva and/or purified salivary proteins were adsorbed to hydroxyapatite powder, human enamel powder and real human enamel surfaces which were then exposed to 10mM acetic acid pH 3.3. Demineralisation was determined by measuring phosphate dissolved from the hydroxyapatite using a spectrophotometric assay. Adsorbed proteins were characterised by SDS PAGE and western blotting.

Results: Human enamel powder and hydroxyapatite powder behaved differently with respect to salivary protein adsorption but adsorbed proteins in both cases offered the same degree of protection (10% reduction in mineral loss). However, the degree of protection achieved using real enamel surfaces was more impressive (50% reduction in mineral loss). Cystatin SN was identified as a potential protective adsorbent. Amylase formed a complex with cystatin in solution but adsorption of cystatin is not dependent on interaction with amylase .

Conclusions: Adsorbed salivary proteins inhibit hydroxyapatite dissolution. Protein interactions and the formation of complexes could have a significant impact on protein adsorption and the protective potential. The identification of protective protein domains and analysis of the mechanisms by which proteins provide protection will be critical for the design of therapeutic peptides used by xerostomia patients.

(Acknowledgements: CASE studentship funded by BBSRC and GSK)

Leeds Dental Institute
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Effect of salivary proteins on human enamel demineralisation

Nikoletta Pechlivani, Steven Brookes, Deirdre Devine, Alan Mighell, Philip Marsh.
Leeds Dental Institute, University of Leeds, Leeds, LS2 9LU

Introduction

A selective adsorption of proteins occurs when hydroxyapatite, the main component of dental enamel is exposed to saliva. Salivary proteins form an initial pellicle capable of protecting the enamel against acid demineralisation but it is unknown which specific proteins are involved. A better understanding of pellicle formation and function will aid the design of therapeutic saliva substitutes [1].

Aims

- Characterise initially adsorbed salivary proteins that inhibit the acid dissolution of hydroxyapatite.
- Investigate the role of protein-protein interactions in pellicle formation.

Long term translational targets:

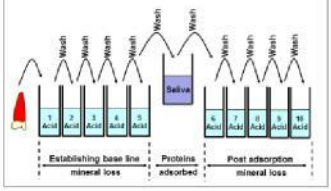
- Design protective peptides for therapeutic use.
- Screen for salivary polymorphisms in the general population that are poorly protective allowing for early intervention.

Materials and Methods

Acid demineralisation assay using hydroxyapatite and enamel powder

Hydroxyapatite or enamel powder +/- saliva
↓
1 hour at 37 °C, shaking
↓
Centrifuge and wash pellet with Tris pH 7.4
↓
Challenge with 10mM acetic acid pH 3.3
↓
Phosphomolybdate assay to determine phosphate dissolved (as a measure of demineralisation)
Desorb proteins with phosphate buffer and analyse by SDS PAGE

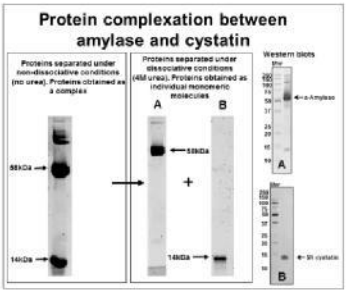
Acid demineralisation assay using human tooth enamel surfaces



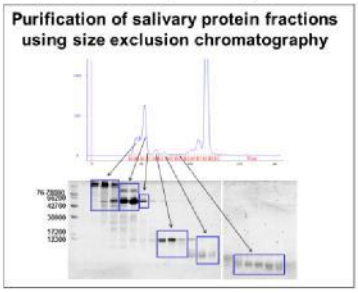
Protein purification:
We purified salivary proteins using size exclusion chromatography, a method that separates molecules according to their size.

Results

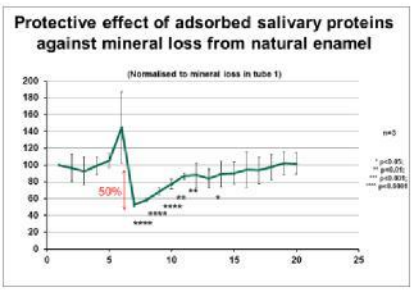
Protein complexation between amylase and cystatin



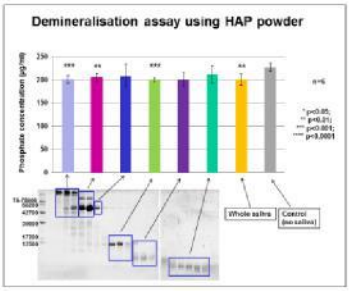
Purification of salivary protein fractions using size exclusion chromatography



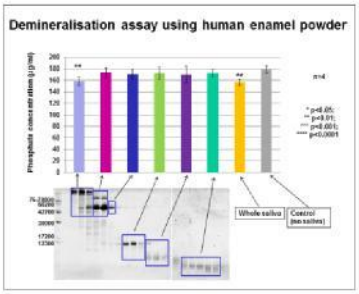
Protective effect of adsorbed salivary proteins against mineral loss from natural enamel



Demineralisation assay using HAP powder

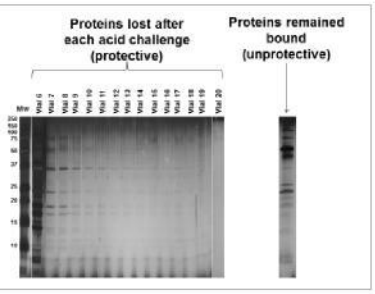


Demineralisation assay using human enamel powder



Proteins lost after each acid challenge (protective)

Proteins remained bound (unprotective)



Conclusions

- Adsorbed salivary proteins inhibit hydroxyapatite dissolution.
- Real human enamel powder and synthetic hydroxyapatite powder behave differently with respect to salivary protein adsorption but adsorbed proteins in both cases offer the same degree of protection (10% reduction in mineral loss). However, the degree of protection achieved using real enamel surfaces is more impressive (50% reduction in mineral loss).
- Some proteins (i.e. protective proteins) are gradually lost during the acid challenges. With their loss there is an increase in acid demineralisation. Most of the proteins remain adsorbed to enamel surface even after 15 acid challeges although these proteins offer no protection against acid demineralisation.
- Amylase and cystatin form a complex. Protein interactions and the formation of complexes might have a significant impact on protein adsorption and the protective potential.

References
1.Bennick, A., M. Cannon, and G. Madapallimattam. *The nature of the hydroxyapatite-binding site in salivary acidic proline-rich proteins.* Biochem J, 1979. 183(1): p. 115-26.

The Leeds Teaching Hospitals NHS
NHS Trust

Protective Effect of Salivary Proteins on Human Enamel Demineralisation. Nikoletta Pechlivani, Deirdre Devine, Alan Mighell, Philip Marsh, David Bradshaw, Nisha Patel, Steven Brookes. International Association of Dental Research Pan European Regional Congress, Dubrovnik, Croatia, September 2014

Salivary proteins form an initial pellicle that protects enamel against acid demineralisation but it is unclear which specific proteins are involved or their mechanism of action.

Objectives: 1) Investigate salivary protein adsorption and protection using powdered synthetic hydroxyapatite, powdered enamel and natural enamel surfaces. 2) Characterise the protective salivary proteins.

Methods: Whole salivary proteins from human volunteers were adsorbed to synthetic hydroxyapatite powder, human enamel powder and actual human enamel surfaces and challenged with 10mM acetic acid. Demineralisation was determined by spectrophotometric assay of phosphate released into the acid. Adsorbed proteins were characterised by SDS-PAGE. Salivary proteins were fractionated by size exclusion and/or hydroxyapatite chromatography and the protective properties of the fractions investigated as described above.

Results: Whole salivary proteins offered no significant protection when adsorbed to synthetic hydroxyapatite powder but reduced demineralisation by ~12% ($p<0.01$) when adsorbed to human enamel powder. Even greater protection was achieved using human enamel surfaces with a ~43% reduction in demineralisation ($p<0.0001$) but repeated acid challenges gradually desorbed the protective proteins which corresponded to a loss of protection. Hydroxyapatite chromatography of whole saliva revealed that a subset of proteins comprising ~15% of the total protein were protective. Further fractionation of this subset is underway to identify the specific protective species. Treatment with chaotrophic agents abolishes the protective effects suggesting protection is dependent on protein conformation or complex formation.

Conclusions: The results emphasise the importance of using natural enamel surfaces when investigating the protective effect of salivary proteins. The protective species in saliva comprise a minor subset of the total proteins that are protective in their native conformation. A better understanding of how salivary proteins protect against demineralisation will aid the design of therapeutic saliva substitutes and potentially allow for caries/erosion susceptible individuals to be identified based on unfavourable polymorphisms of their salivary proteome.

(Acknowledgements: CASE studentship funded by BBSRC and GSK).

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Protective effect of salivary proteins on human enamel demineralisation

Nikoletta Pechlivani [1], Deirdre Devine [1], Alan Mighell [1], Philip D Marsh [1], David Bradshaw [2], Nisha Patel [2], Steven Brookes [1].
[1] Dept of Oral Biology, School of Dentistry, University of Leeds, UK; [2] GlaxoSmithKline UK.

Introduction

A selective adsorption of proteins occurs when hydroxyapatite, the main component of dental enamel, is exposed to saliva. Salivary proteins form an initial pellicle capable of protecting the enamel against acid demineralisation but it is unclear which specific proteins are involved. A better understanding of pellicle formation and function will aid the design of therapeutic saliva substitutes [1].

Aims

- Investigate salivary protein adsorption and protection using powdered synthetic hydroxyapatite, powdered enamel and natural enamel surfaces.
- Characterise the protective salivary proteins.

Long term translational targets:

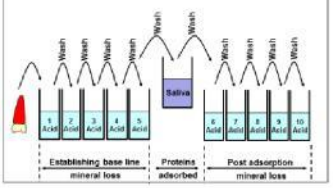
- Design protective peptides for therapeutic use.
- Screen for salivary polymorphisms in the general population that are poorly protective allowing for early intervention.

Materials and Methods

Acid demineralisation assay using hydroxyapatite and enamel powder

Hydroxyapatite or enamel powder +/- saliva
↓
1 hour at 37 °C, shaking
↓
Centrifuge and wash pellet with Tris pH 7.4
↓
Challenge with 10mM acetic acid pH 3.3
↓
Phosphomolybdate assay to determine phosphate dissolved (as a measure of demineralisation) | Desorb proteins with phosphate buffer and analyse by SDS PAGE

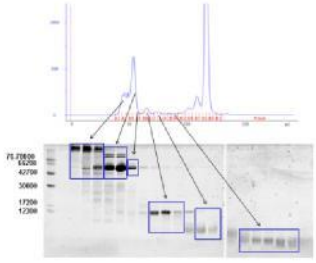
Acid demineralisation assay using human tooth enamel surfaces



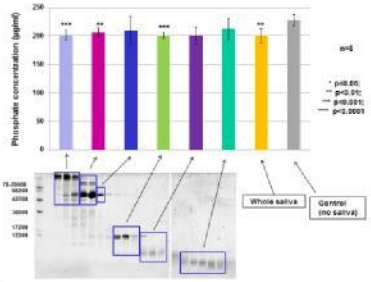
Protein purification:
Salivary proteins were purified using size exclusion chromatography and hydroxyapatite chromatography.

Results

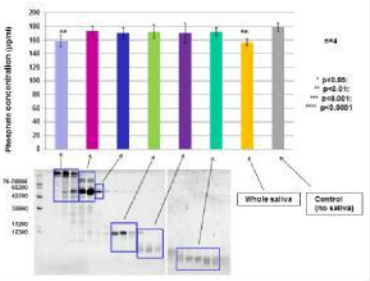
Purification of salivary proteins by size exclusion chromatography



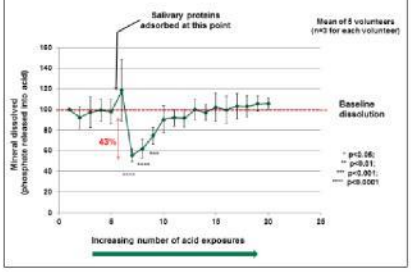
Demineralisation assay using hydroxyapatite powder



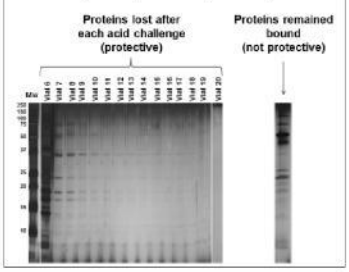
Demineralisation assay using human enamel powder



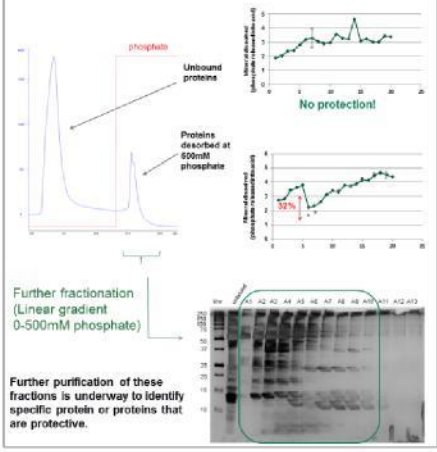
Protective effect of adsorbed salivary proteins against mineral loss from natural enamel



SDS PAGE of proteins lost in each acid challenge (i.e. the protective proteins)



Hydroxyapatite chromatography



Further purification of these fractions is underway to identify specific protein or proteins that are protective.

Conclusions

- It is important to use natural enamel surfaces when investigating the protective effect of salivary proteins.
- The protective species in saliva comprise a subset of proteins comprising 15% of the total protein and they are protective in their native conformation.
- Further fractionation of this subset is underway to identify the specific protective species.

References
1 Bennick, A., M. Cannon, and G. Madapallimattam, *The nature of the hydroxyapatite-binding site in salivary acidic proline-rich proteins.* Biochem J, 1979. 183(1): p. 115-26.

Acknowledgements: CASE studentship funded by BBSRC and GSK

The Leeds Teaching Hospitals NHS
NHS Trust

Protective Salivary Proteins Against Human Enamel Demineralisation. Nikoletta Pechlivani, Deirdre Devine, Alan Mighell, Philip Marsh, David Bradshaw, Nisha Patel, Steven Brookes. International Association of Dental Research world congress, Boston, Mass., USA, March 2015

The initial adsorption of salivary proteins to enamel is critical for the protection of the enamel against acid demineralisation but, it is unclear which specific proteins are involved in protection or the mechanism by which they act.

Objectives: Purify salivary proteins and systematically characterise proteins that are protective against acid demineralisation of the enamel.

Methods: Whole salivary proteins from human volunteers were adsorbed to natural human enamel surfaces and challenged with 10mM acetic acid. Salivary proteins were also fractionated by methods including hydroxyapatite chromatography, size exclusion chromatography and isoelectric focusing. Adsorbed proteins were characterised by SDS-PAGE and spectrophotometric assay of phosphate released into the acid determined acid dissolution.

Results: Whole salivary proteins reduced acid demineralisation by 43% ($p<0.0001$); however, repeated acid challenges gradually desorbed the protective proteins which correlated with a loss of protection. After 20 acid challenges some proteins remained adsorbed but these evidently had no protective value. Size exclusion and hydroxyapatite chromatography revealed that a subset of proteins, containing about 15% of the total protein, was protective. Treatment with denaturing agents suggested that protection was dependent on protein conformation or complex formation. Further fractionation of this subset by isoelectric focusing and the use of a Bio-Gel P-60 column showed that proteins with high pI were active and the presence of the low molecular weight proteins were essential for protection.

Conclusions: The protective species in saliva comprise a minor subset of the total proteins that function when they are in their native conformation. The formation of complexes could have a significant impact on the protective potential. Identification of protective proteins and protein domains and a better understanding of how salivary proteins protect will aid the design of therapeutic saliva substitutes. Polymorphism screening could also be used to identify caries/erosion susceptible individuals.

(Acknowledgements: CASE studentship funded by BBSRC and GSK).

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Protective salivary proteins against human enamel demineralisation

Nikoletta Pechlivani [1], Deirdre Devine [1], Alan Mighell [1], Philip D Marsh [1], David Bradshaw [2], Nisha Patel [2], Steven Brookes [1].
[1] Division of Oral Biology, School of Dentistry, University of Leeds, UK;
[2] GlaxoSmithKline Consumer Healthcare, Weybridge, UK.

Introduction

A selective adsorption of proteins occurs when hydroxyapatite, the main component of dental enamel, is exposed to saliva. Salivary proteins form an initial pellicle capable of protecting the enamel against acid demineralisation but it is unclear which specific proteins are involved. A better understanding of pellicle formation and function will aid the design of therapeutic saliva substitutes [1].

Aims

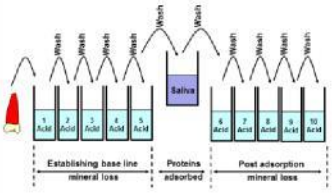
- Investigate salivary protein adsorption and protection using natural enamel surfaces.
- Characterise the protective salivary proteins.

Long term translational targets:

- Design protective peptides for therapeutic use.
- Screen for salivary polymorphisms in the general population that are poorly protective allowing for early intervention.

Materials and Methods

Acid demineralisation assay using human tooth enamel surfaces



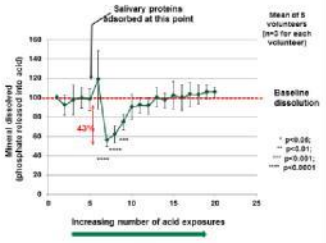
Protein purification
Proteins were purified using:
- size exclusion chromatography
- hydroxyapatite chromatography
- isoelectric focusing

Spectrophotometric assay
of phosphate released into the acid determined acid dissolution.

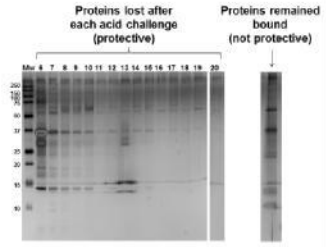
SDS-PAGE analysis
was used to characterise proteins.

Results

Protective effect of adsorbed salivary proteins against mineral loss from natural enamel

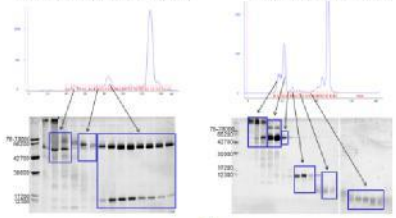


SDS PAGE of proteins lost in each acid challenge (i.e. the protective proteins)



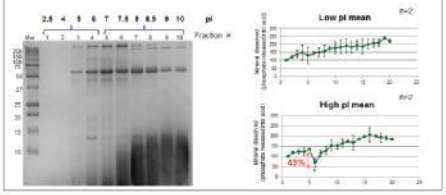
Purification of salivary proteins by size exclusion chromatography

Non denaturing conditions | Denaturing conditions

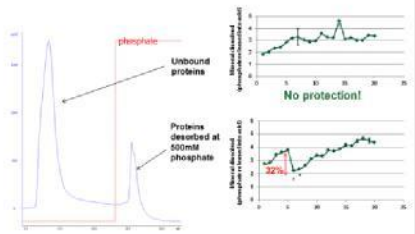


Denatured protein fractions do not protect against acid demineralisation

Purification of salivary proteins by isoelectric focusing

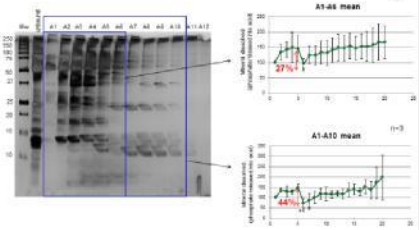


Purification of salivary proteins by hydroxyapatite chromatography



A subset of proteins comprising about 15% of total protein are protective

Further fractionation of protective subset (Linear gradient 0-500mM phosphate)



Conclusions

- Whole salivary proteins reduce acid demineralisation significantly.
- The protective species in saliva comprise a subset of proteins, containing 15% of the total protein, and they are protective in their native conformation.
- Proteins with high isoelectric point (pI) are more active and the presence of low molecular weight proteins results in higher degree of protection.
- Further fractionation of the protective subsets is underway to identify the specific protective species.

References
1.Bennick, A., M. Cannon, and G. Madapallimattam, *The nature of the hydroxyapatite-binding site in salivary acidic proline-rich proteins.* Biochem J, 1979. 183(1): p. 115-26.

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