Purification and Functional Analysis of BPIFA2

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Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another qualification in this, or any other university or Institute of Learning.

Dedication

I would like to dedicate this thesis to the man who helped me find my love for learning and discovery. A great man, who taught me that if you want to succeed you have to work hard, keep trying and never give up.

Thank you, I miss you!

"For Grandpa"

Acknowledgements

Abstract

Short PLUNC 2, recently renamed BPIFA2, is predominantly expressed in the serous acinar cells and interlobular ducts of the major salivary glands and secreted abundantly into saliva. The original hypothesis that the structure of BPIFA2 is similar to that of the N-terminal of BPI and LBP led to the suggestion that it would also play a role in the innate immune defence of the oral cavity and upper airway. The function of BPIFA2 has not, however, been fully elucidated and thus the aim of this thesis was to develop a protocol for the purification of BPIFA2 from whole saliva, in its native form, to fully determine if it does have similar functions to BPI and LBP. Based on the current literature, a number of purification methods were assessed including precipitation, column chromatography and electrophoresis. Native polyacrylamide gel electrophoresis and electro-elution gave the highest yields of pure protein, which was then used in a variety of functional assays including binding, growth inhibition, bacterial killing, agglutination and biofilm disruption. A novelty of this study was that a range of bacteria were used including gram-positive and gram-negative bacteria and commensal and non-commensal oral bacteria. In addition, Der p 7, a dust mite allergen also shown to have structural similarities to the N-terminal domain of BPI and LBP, was used to develop an assay to examine the effect of BPIFA2 on the TLR-4 pathway in the presence of LPS. Although the allergen was initially used as a positive control for the assay system we were able to show for the first time that Der p 7 can mimic the action of LBP in the CD14-MD2-TLR4 pathway in response to gram-negative bacterial LPS.

The most significant and novel finding of this thesis was the effect of BPIFA2 on gram-positive bacteria, particularly S. mutans, a known causative agent of dental caries. Reduced bacterial viability, increased agglutination and altered biofilm quality were all observed in the presence of BPIFA2. These results suggest a role for BPIFA2 in innate immunity, not against gram-negative bacteria as originally hypothesised, but against gram-positive bacteria.

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Abbreviations

APS	Ammonium persulfate
BA	Columbia blood agar
BASE	Breast cancer and salivary gland expression
BHI	Brain heart infusion
BPI	Bactericidal/permeability increasing protein
BPIF	BPI fold containing family
BPIFA1	BPI fold containing family A, member 1 (formally Short PLUNC 1)
BPIFA2	BPI fold containing family A, member 2 (formally Short PLUNC 2)
BPIFA2A-D	BPI fold containing family A, member 2A-D, (formally BSP30a-d)
BPIFA2E	BPI fold containing family A, member 2E (formally rodent PSP)
BPIFB1	BPI fold containing family B, member 1 (formally Long PLUNC 1)
BPIL	Bactericidal permeability increasing potein
BSP30a-d	Bovine salivary protein 30a-d
CD14	Cluster of differentiation 14
CETP	Cholesterol ester transfer protein
CHO	Chinese hamster ovary cell line
Der p	*Dermatophagoides pterontssinus* allergens
E8	rgpA rgpB double mutant *P. gingivalis* W50 (arginine specific gingipain deficient)
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FA	Fastidious anaerobe agar
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate 1
GST	Glutathione-S-transferase
H2SO4	Sulphuric acid
hBD	Human beta-defensins

HDL	High density lipoproteins
HEK 293	Human embryonic kidney cell line
hNP	Human neutrophil proteins
HPLC	High-performance liquid chromatography
hPLUNC	Human palate lung and nasal epithelium clone
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-10	Interleukin 10
Il-1β	Interleukin 1-beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IMAC	Immobilized metal ion affinity chromatography
IMS	Industrial methylated spirit
K1A	kgp mutant *P. gingivalis* W50 (Lysine specific gingipain deficient)
LBP	Lipopolysaccharide-binding protein
LPLUNC	Long palate lung and nasal epithelium clone
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LUNX	Lung specific X protein
MBP	Maltose binding protein
mCD14	Membrane bound cluster of differentiation 14
MD-2	Myeloid differential protein 2
mPLUNC	Mouse palate lung and nasal epithelium clone
MyD88	Myeloid differentiation primary response gene 88
NaCl	Sodium chloride
NAM	N-acetylmuramic acid
nBPIFA2	Native BPI fold containing family A, member 2
NF-κB	Nuclear factor-kappa B
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PLTP	Phospholipid transfer protein

PLUNC	Palate lung and nasal epithelium clone
PRRs	Pattern-recognition receptors
PSP	Parotid secretory protein
PtdIns(3,4,5)P3	Phosphatidylinositol-3,4,5-trisphosphate
PtdIns(4)P	Phosphatidylinositol-4-phosphate
PtdIns(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
rBPIFA2	BPI fold containing family A, member 2
RPMI-1640	Roswell Park Memorial Institute medium 1640
S2	Drosophila Schneider 2 cell line
sCD14	Soluble cluster of differentiation 14
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLPI	Secretory leukocyte proteinase inhibitor
SPLUNC	Short palate lung and nasal epithelium clone
SPURT	Secretory protein of the upper respiratory tract
TBS	Tris-buffered saline
TBST	Tris-buffered saline tween
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor-beta-1
THP-1	Acute monocytic leukaemia cell line
TLR-2	Toll-like receptor 2
TLR-4	Toll-like receptor 4
TNF-α	Tumour necrosis Factor-alpha
W50	Wild type *P. gingivalis*

Chapter 1.   Introduction

1.1 Oral Cavity, Salivary Glands and Saliva

1.1.1   Oral Cavity

The principle structure of the oral cavity is defined by the jaw bone which holds a collection of teeth and contains the tongue, a very strong and sensitive muscle. The oral cavity extends back towards the throat to the pharynx where the oral cavity separates into the respiratory and digestive tracts (Atkinson and White, 1992).

Each region of the oral cavity contains different epithelium dependent upon the function of that region. For example the hard palate and gingiva are composed of keratinised epithelium which protects them from the regular stress of mastication. Other areas, lined with non-keratinised epithelium, are loosely connected to underlying tissues allowing a range of movement including chewing and speech. These regions include the floor of the mouth and buccal regions. The third functional area of the oral cavity is the specialist tissue. This is primarily the tongue which is lined with a collection of both keratinised and non-keratinised epithelium tightly connected to the lingual muscles (Ten Cate, 1998).

1.1.2   Salivary Glands

In and around the oral cavity there are collections of glands categorised into two types, minor and major, functioning to supply the oral cavity with a constant supply of saliva. There are approximately 600-1000 minor salivary glands located just beneath the epithelial layer, throughout the whole of the oral cavity (Ten Cate, 1998).

There are three major salivary glands which are grouped into pairs. The parotid glands, found below and forward of the ears with ducts that expel saliva into the back of the oral cavity, have a tree-like structure (Tucker, 2007). It is the largest of the three major glands weighing approximately 14-28g (Ten Cate, 1998). The submandibular glands are the second largest of the glands and are located at the back of the jaw on the floor of the oral cavity, their ducts run along the floor of the mouth exiting under the tongue (Ten Cate, 1998). The smallest of the major glands, the sublingual glands, can be found between the tongue and teeth on the floor of the mouth, secreting saliva onto the floor of the mouth. Both the submandibular and sublingual glands, which weigh approximately 10-15g and 2g respectively, are compact and are described as having a 'bunch of grape' like appearance (Figure 1-1) (Tucker, 2007).
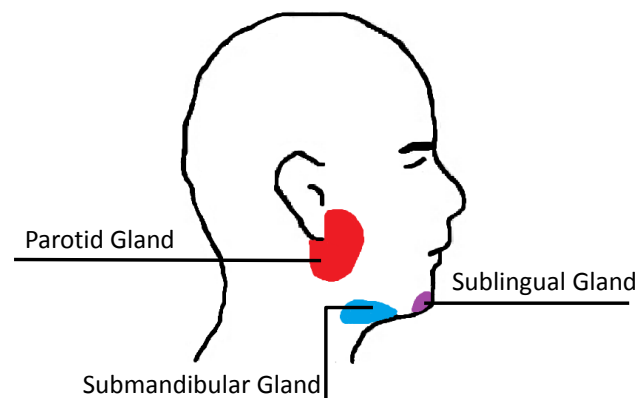
Figure 1-1: Location of the Major Salivary Glands

The major salivary glands are made up of three types of cell; acinar, duct and myoepithelial cells. The acinar cells make up the largest part of the salivary glands and are surrounded by blood vessels, nerves and connective tissue (Ten Cate, 1998). The acinar cells are classified into 2 types: serous cells, which are large

granular pyramidal cells with spherical nuclei that produce a watery saliva of high volume containing secretory products such as enzymes and immune components (Stevens and Lowe, 1997) or mucous cells, which have a clear cytoplasm and flattened nucleus and produce a lower volume of saliva, high in carbohydrates and proteins (Ten Cate, 1998). The parotid gland is made up of only serous acini and so secretes serous saliva. The sublingual gland and minor glands are mostly made up of mucous acini and so secrete mucous saliva and the submandibular gland is made up of a mixture of serous and mucous acini and so secretes seromucous saliva (Ten Cate, 1998). Each lobe of acinar cells drains into a branch-like duct system. The duct cells are separated into 3 cell types: the first are the intercalated cells, whose function is to link the acinar cells to the duct system; the second are the striated cells, which work to regulate the loss of electrolytes through the saliva and re-absorb sodium back into the body; the gland ends with the excretory cells, which secrete potassium into the saliva whilst re-absorbing sodium, here the saliva is released into the oral cavity (Humphrey and Williamson, 2001). The whole gland structure is surrounded by long processes called myoepithelial cells which contract and squeeze the acinar cells when a stimulus is present to expel the saliva from the lobes into the ducts for secretion into the oral cavity (Humphrey and Williamson, 2001). The production of saliva is a continuous, automatic process. Approximately 0.5 ml min^{-1} of saliva is released without stimulation, controlled via the salivary center in the brain stem, which is reduced to approxiomately 0.1 ml min^{-1} when sleeping and during high stress situations. The key stimuli of saliva production are chewing, taste and smell, producing saliva with a very similar protein profile due to the processing of the senses in the brain prior to sending the message to the

salivary center to increase saliva production. Interestingly, unlike the major salivary glands, the minor salivary glands have shown little variation in flow rate in response to these stimuli (Carpenter, 2013). The salivary glands are surrounded by both sympathetic and parasympathetic nerves, which act synergistically to control the flow of saliva by increasing the contraction of the myoepithelial cells (Rhoades and Bell, 2012). The parasympathetic nervous system controls high volume saliva with a low concentration of proteins, whilst the sympthetic nervous system produces protein rich saliva of low volume (Carpenter, 2013).

1.1.3   Saliva

Approximately 1000-1500mL of saliva is secreted per day (Zalewska et al., 2000) and is necessary to provide the oral cavity with a moist environment to aid mastication, swallowing, communication, digestion, local immune defence and the maintenance of pH through buffering.

Due to the composition of cells within each gland, different responses can be achieved, dependent upon the presence or absence of stimulation. Unstimulated whole saliva consists of 20% parotid saliva, 65% submandibular saliva, 7-8% sublingual saliva and the rest from minor glands. When saliva is stimulated whole saliva then consists of over 50% parotid gland saliva. During sleep saliva output is reduced to almost zero (Humphrey and Williamson, 2001). However it is not only the salivary glands that contribute to whole saliva. Contributions are made from alternative regions of the oral cavity such as the oral mucosal epithelial cells, gingival epithelial cells, keratinocytes, neutrophils, macrophages and the gingival crevicular fluid (Gorr, 2009). For example, over 199 proteins have been identified in

gingival crevicular fluid in healthy patients, 57% of which have also been identified in plasma. The remaining 47% were recognised as unique to GCF (Carneiro et al 2012). Some of these contributions represent significant levels of protein in whole saliva, for example a significant proportion of lactoferrin originates from the gingival crevicular fluid. Although some oral proteins are unique to the salivary glands, such as histatin and mucin, many of the proteins secreted in glandular saliva are also expressed in other regions of the oral cavity, including statherin, also found in gingival crevicular fluid; salivary agglutinin, also expressed by macrophages and SLPI expressed by the salivary glands, mucosal epithelium and keratinocytes (Gorr, 2009). In addition to these anti-microbial proteins the oral epithelium also contributes a number of cytokines and chemokines to whole saliva (Ghosh et al, 2012).

1.1.4   Saliva Function

Saliva has a number of functions to ensure the oral cavity remains healthy. It contains high concentrations of free calcium and phosphate which promote the remineralisation of the tooth enamel. Urea, the carbonic acid-bicarbonate system and the phosphate buffer system present in saliva act to buffer the pH of saliva maintaining pH levels between 6 and 7. Saliva also contributes to the initial stages of digestion as 40-50% of salivary protein is α-amylase, produced by the parotid glands (80%) and the submandibular glands (20%), which begins the breakdown of starch into simple sugars and limit dextrins (de Almeida et al., 2008). Mucins are a family of complex glycoproteins present in the mucous saliva of the submandibular and sublingual glands, which aid in the lubrication of the oral cavity. This lubrication

protects the oral cavity from physical stresses of mastication and dehydration. As saliva is produced constantly, it also provides a washing action within the oral cavity and, along with the act of swallowing, washes away food debris and dilutes sugars (de Almeida et al., 2008).

In addition to all of these functions saliva also plays a very important role in oral health. The oral cavity is exposed to a great number of bacteria which, if given the chance, would negatively affect the health of the oral cavity and the rest of the body. Functions mentioned previously also aid in the anti-microbial nature of saliva. For example, by increasing the pH of the oral environment the conditions are no longer optimal for growth of some bacteria (e.g. mutans streptococci) so they compete less effectively, the constant production of saliva and swallowing washes away planktonic bacteria within the oral cavity reducing their opportunity to adhere to surfaces and invade the oral tissues (Staines et al., 1993). In addition to the mechanical effects of flow, saliva also contains over 45 antimicrobial proteins and peptides (Gorr and Abdolhosseini, 2011) including mucins, salivary agglutinin, β-defensins, histatins, cystatins, secretory leukocyte proteinase inhibitor (SLPI), lactoferrin, lysozyme, peroxidases and secretory immunoglobulin A (IgA) (Llena-Puy, 2006, Gorr and Abdolhosseini, 2011)

The initial line of defence against oral bacteria takes the form of salivary mucins and salivary agglutinin, which bind bacteria via lectin-like-carbohydrate interactions causing their aggregation and so preventing the bacteria from adhering to, colonising or invading the oral structures (Staines et al., 1993). The mucins are high molecular weight glycoproteins and can be found in two distinct forms, MG1,

derived from the MUC5B gene and MG2 from the MUC7 gene (Amerongen and Veerman, 2002). MG1, expressed by the mucous acini of the submandibular and sublingual salivary glands, exists in 3 different subtypes and lubricates the oral epithelium by forming a gel like substance. It is this gel like coating covering the oral epithelium which provides the barrier against bacteria. MG1 has been shown to bind *Haemophilus parainfluenzae* and *Helicobacter pylori*, whereas MG2, a smaller molecule, expressed by the serous acini binds to many more bacteria including many oral commensal species (e.g. *Streptococcus gordonii, Streptococcus sanguis, Streptococcus mitis, Actinobacillus actinomycetemcomitans)* and the non-oral species *Pseudomonas aeruginosa* and *Escherichia coli* (Amerongen and Veerman, 2002).

Salivary agglutinin, like MG2, is expressed in the serous acini as a heavily glycosylated protein and is often associated with other salivary proteins (de Almeida et al., 2008). Like MG2, agglutinin binds to many commensal species (Amerongen and Veerman, 2002). It has also been demonstrated that salivary agglutinin increases phagocytosis by neutrophils and macrophages via the lectin pathway (Leito et al., 2011).

β-defensins, Histatins and Statherin are cationic peptides which have been shown, amongst other functions, to lyse bacterial cell envelopes and increase their phagocytosis. These peptides work alongside other salivary anti-microbials, including lactoferrin and lysozyme (Hancock and Diamond, 2000).

Defensins are small peptides, which act against both gram-positive and gram-negative bacteria. Based on their size and the spacing of the disulphide bonds,

defensins are categorised into alpha and beta. Alpha-defensins are mainly expressed in neutrophils and saliva and β-defensins are expressed in epithelial cells of mucous membranes including the uterus, pancreas, kidney, oral cavity, lung and nasal passages (Gorr, 2009). Three α-defensins, human neutrophil proteins (hNP 1-3) and three β-defensins, human beta-defensins (hBD 1-3) are found in whole saliva. Other hBDs (-4, -5 and -6) have not been detected in saliva (Abiko et al., 2003). β-defensins have a number of innate immune functions including: lysis of bacteria; inhibition of the binding of viruses to the host cells and the increased chemotaxis of neutrophils and monocytes. β-defensins also enhance adaptive immunity by binding to chemokine receptor 6 on memory T-cells and immature dendritic cells leading to maturation and induction of co-stimulatory molecule expression. β-defensins have also been shown to act in an anti-inflammatory manner by binding to hemagglutinin B of *Porphyromonas gingivalis* thus reducing the interaction between the bacteria, keratinocytes and dendritic cells, leading to an inhibition of inflammatory cytokine stimulation (Diamond and Ryan, 2011).

Histatins are a group of small cationic proteins of 3-5kDa found mostly in parotid saliva, as histatin 1 and histatin 3. Histatin 5 (3kDa), a product of proteolytic cleavage of histatin 3, has been shown to have a number of indirect antimicrobial functions including metal ion chelation, neutralisation of bacterial lipopolysaccharide (LPS) and inhibition of proteinases (Amerongen and Veerman, 2002). Histatins are also able to act directly on microorganisms such as *Streptococcus mutans* and *Candida albicans*, by integrating into their cytoplasmic

membrane, increasing permeability and causing inhibition of the growth and/or death (Amerongen and Veerman, 2002).

Cystatins are a family of cysteine protease inhibitors mainly affecting peptidases belonging to the papain and legumain families. They therefore protect the host from the effects of bacterial proteases, such as *Porphyromonas gingivalis* gingipains and host proteases, such as lysosomal cathepsins (Amerongen and Veerman, 2002, Baron et al., 1999). Fourteen functional and two pseudogenes for human cystatin have been identified, the products of seven of these (A, B, C, D, S, SA and SN) being constitutively expressed in saliva (Gorr and Abdolhosseini, 2011).

SLPI is a small protein (11.7kDa), first discovered in airway lining fluid, expressed in all of the major and minor salivary glands (Amerongen and Veerman, 2002, Williams et al., 2006). The most defined function of SLPI is its anti-protease activity, protecting the host from excess proteases, released by neutrophils such as elastase and cathepsin G. Although, it is slowly emerging that SLPI could also play an active role in the innate immune system with anti-microbial functions against both gram-positive and -negative bacteria, viruses and fungi (Moreau et al., 2008, Williams et al., 2006).

Lactoferrin is an 80kDa protein produced by all of the salivary glands (Amerongen et al., 2004) and neutrophils (Edgerton and Koshlukova, 2000). It is a member of the transferrin family of iron-binding proteins and acts in an antimicrobial fashion by binding ferric iron (Komine et al., 2007). Nearly all bacteria require iron for growth and so the sequestration of iron prevents the growth of many bacteria. The N-terminal region of lactoferrin, lactoferricin, has shown antibacterial activity distinct

from the iron chelating nature of the parent protein, and protects against fungi, viruses, gram-negative and gram-positive bacteria (Komine et al., 2007). The mechanism by which it does this is unknown, however it has been suggested that the interaction of lactoferricin with the bacterial membrane causes the formation of pores, thus leading to cell death (Chen et al., 2009). Lactoferrin also has LPS neutralising activity by binding to the lipid A portion and competing with LBP and preventing the formation of the LBP-CD14-TLR4 complex resulting in a reduced inflammatory response (Komine et al., 2007, Elass-Rochard et al., 1998).

Lysozyme, a 14kDa protein, is produced by the major salivary glands (Amerongen et al., 2004). It is also known as muramidase as it kills bacteria by breaking down the peptidoglycan component of the cell wall making it susceptible to osmotic lysis (Amerongen et al., 2004). The presence of the highly protective lipopolysaccharide layer in gram-negative bacteria reduces access of lysozyme resulting in them being less susceptible to lysozyme than gram-positive bacteria. Lysozyme also causes aggregation of bacteria resulting in reduced adhesion to oral structures (Cole et al., 2002).

Peroxidase and myeloperoxidase are salivary enzymes produced by the salivary gland and immune cells such as neutrophils and other leukocytes which enter the oral cavity tissues in response to inflammatory stimuli. The main function of peroxidases is to catalyse the oxidation of thiocyanate and chloride ions by hydrogen peroxide producing the antibacterial agents, hypothiocyanate and hypochlorite (Amerongen and Veerman, 2002). Peroxidases protect against bacteria and fungi by targeting transport proteins and sulfhydryl groups, leading to

an inhibition of growth and metabolism (Edgerton and Koshlukova, 2000). They also have some anti-viral activity.

Secretory IgA is an immunoglobulin produced by a subset of plasma cells (IgA+). Since it is in a dimeric form in saliva, it is able to aggregate bacteria and so remove them by reducing their adherence to oral structures (Humphrey and Williamson, 2001). IgA+ plasma cells collect around the acini of the major salivary glands, mainly the sublingual glands. Two classes of IgA are secreted into saliva, IgA1 and IgA2, via the intercalated sections of the ducts and directly through the serous acini. This requires complexion with the polymeric immunoglobulin receptor to facilitate transport across the acinar epithelium and a breakdown product of the receptor, secretory component, remains bound to the IgA released into the lumen. IgA2 is more resistant to bacterial proteases making it a much more stable molecule than IgA1 (Brandtzaeg, 2007). IgG and IgM are also present in saliva however these are thought to come from gingival secretions and not saliva. The IgG and IgM levels in whole saliva are very small in comparison to the level of IgA (de Almeida et al., 2008).

These proteins and peptides do not act in isolation, but work together to protect the oral cavity. Many of these antimicrobial proteins and peptides can be found in association with each other to elicit a range of defence functions against microbes in the oral cavity.

1.2 PLUNC Proteins

The Palate Lung and Nasal Epithelium Clone (PLUNC) gene was initially identified in the developing palate of the mouse embryo in 1999 (Weston et al., 1999). An increase in the expression of PLUNC RNA was noted between days 13 and 14 of gestation, around the time that the palatal shelf elevates and fuses. As with the developing mouse, PLUNC expression in the adult mouse was seen in the nasal collumella, turbinates and nasal passage. In addition, strong expression was identified in the outer epithelial layers of the respiratory passages; continuing through the trachea and the left and right bronchioles reducing significantly and becoming sporadic at the next bronchiole branch, with expression becoming absent in the distal regions of the lung (the terminal bronchioles, respiratory bronchioles and alveoli) (Weston et al., 1999).

The murine PLUNC (mPLUNC) gene is part of an 834 base pair (bp) open reading frame, consisting of a 55bp 5'-untranslated region (UTR), a 207bp 3'-UTR and a translated region of 255 base pairs which was predicted to encode a protein of 28,618Da (Weston et al., 1999). Analysis of the amino acid sequence showed homologies to two murine salivary proteins: von Ebner minor salivary gland protein and parotid secretory protein (PSP) precursor. Significant homology between mPLUNC and PSP precursor was seen and out of the first 15 amino acids of mPLUNC, murine PSP shared 14 identical or conserved amino acids. Similar homology was seen between other species with bovine PSP sharing 12 identical or conserved amino acids with mPLUNC (Weston et al., 1999). Further analysis using PROSITE, a database containing protein domains, families and functional sites,

identified many common functional motifs including, phosphorylation sites for protein kinase C and casein kinase II N-glycosylation sites, a leucine zipper and a novel motif consisting of a repeating sequence, Gly-(Leu/Pro/Gln)-(Pro/leu)-Leu-Pro-Leu x4 (Weston et al., 1999).

1.3 Human PLUNC and the PLUNC family

Human PLUNC (hPLUNC) cDNA consists of a sequence of 1020bp within a single open reading frame, which encodes a 256 amino acid protein rich in leucine (Bingle and Bingle, 2000). Further sequence analysis of hPLUNC identified 9 exons spanning 7.3kb, the first and last exons non-coding, with the TATAA box located 40bp 5' of the end of exon 1. The key difference between hPLUNC and mPLUNC is the absence of the repeat sequence (Gly-(Leu/Pro/Gln)-(Pro/leu)-Leu-Pro-Leu x4) from hPLUNC (Bingle and Bingle, 2000). This suggests that it is not necessary for the structural or functional activity of the protein.

Since its initial identification in 1999, PLUNC has collected a number of names and was renamed Short PLUNC 1 (SPLUNC1) following the identification and characterisation of further PLUNCs. SPLUNC1 is also known as SPURT (Secretory Protein of the upper respiratory tract) (Di et al., 2003) and Lung specific X protein (LUNX) (Iwao et al., 2001). Initial investigations identified seven further proteins encoded by a 300kb region on chromosome 20q11 (Bingle and Craven, 2002). This novel family of proteins were characterised into 2 groups dependent on their amino acid content, the original PLUNC (256 amino acids), SPLUNC1, was grouped with SPLUNC2 (249 amino acids; also known as Parotid Secretory Protein, PSP) and SPLUNC3 (253 amino acids), whose genes contain 9 exons with the first and final

exons being redundant. The long PLUNC group consisted of Long PLUNC 1 (LPLUNC1) (484 amino acids), LPLUNC2 (458 amino acids), LPLUNC3 (463 amino acids) and LPLUNC4 (>469 amino acids), whose genes contain 16 exons (Bingle and Craven, 2002). Over subsequent years the PLUNC family grew to include the completed form of the hLPLUNC4 sequence and the discovery of a human pseudogene LPLUNC5 (Bingle et al., 2004). Two 'novel' genes, BPI-Like (BPIL)1 and BPIL3 were identified, with BPIL1 coinciding with the LPLUNC2 gene and BPIL3 identifying a new gene within the PLUNC cluster, LPLUNC6 (Mulero et al., 2002). An additional SPLUNC was later identified in humans and labelled BASE (Breast Cancer and Salivary gland Expression) or SPLUNC4 (Egland et al., 2003). However, this gene is now described as a, "dying gene" (Bingle et al., 2011b) based on a frame shift caused by the loss of 1 nucleotide in exon 6, which leads to a premature stop codon, different to those seen in chimpanzees, gorillas and rhesus monkeys (Bingle et al., 2011b). Although this shortened protein has been shown to be expressed in breast cancer and salivary glands (Egland et al., 2003), it is believed that the premature stop codon leads to the absence of approximately 50 amino acids and the crucial cysteine residue required for the structurally significant disulphide bond present in every other member of the family.

Currently, the human PLUNC family has been shown to consist of eight transcribed genes and three pseudogenes, the third pseudogene being Vomeromodulin, a 591 amino acid protein expressed in rodents but not humans (Figure 1-2) (Bingle et al., 2011b).
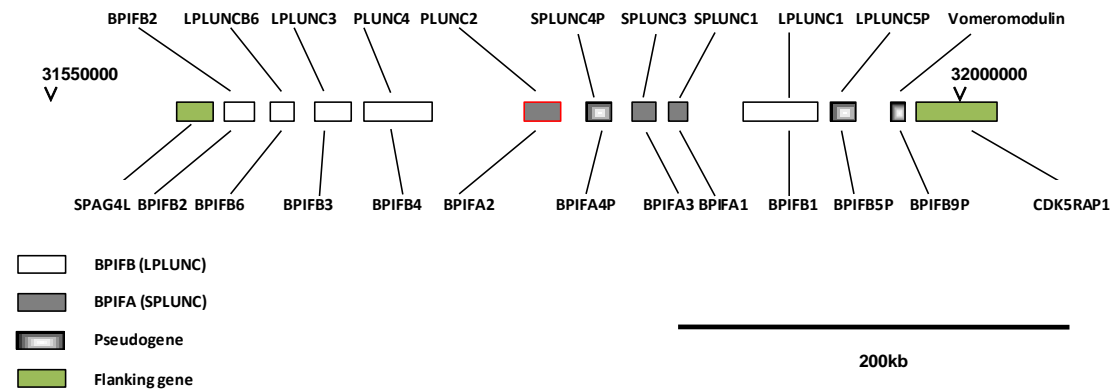
Figure 1-2: Organisation of the human *PLUNC/BPI Fold containing family (BPIF)* gene loci

The human *PLUNC/BPIF* gene locus, located on chromosome 20, spans a region of approximately 300kb and contains three pseudogenes, identified by shaded boxes, three *SPLUNC/BPIFA* genes, represented by grey boxes and five *LPLUNC/BPIFB* genes indicated by white boxes. The PLUNC/*BPIF* region is flanked by two unrelated genes, shown in green. A full explanation of the nomenclature is given in section 1.6.

1.4 Expression of PLUNC Proteins

The best-defined PLUNC expression profiles are for SPLUNC1, SPLUNC2 and LPLUNC1. Expression of hSPLUNC1 has been shown to be similar to that of mSPLUNC1, with expression identified in the trachea and nasopharyngeal epithelium (Bingle and Bingle, 2000).

SPLUNC1 has been shown to be exclusively expressed in the mucous acinar cells of the submandibular and sublingual salivary glands, no expression has been found in the parotid gland possibly due to presence of only serous acinar cells (Bingle et al., 2009). In contract, SPLUNC2 expression was only seen in the serous acinar cells and interlobular ducts of the major salivary glands, and no expression was found in the mucous acinar cells (Figure 1-3). The minor salivary glands were seen to express both SPLUNC1 and SPLUNC2, which is consistent with the presence of both serous and mucous acinar cells in these glands (Bingle et al., 2009).

LPLUNC1 expression has been localised to the oropharynx, nasopharynx and human respiratory tract. It is expressed by seromucous tubules in submucosal glands, the maxillary sinus and strongly by the surface goblet cells and minor mucosal glands of the respiratory tract. At higher magnifications it was established that LPLUNC1 is expressed by serous cells and not mucous cells of the submucosal glands (Bingle et al., 2010).

Parotid

Submandibular

Sublingual

SPLUNC1/BPIFA1   SPLUNC2/BPIFA2   X100
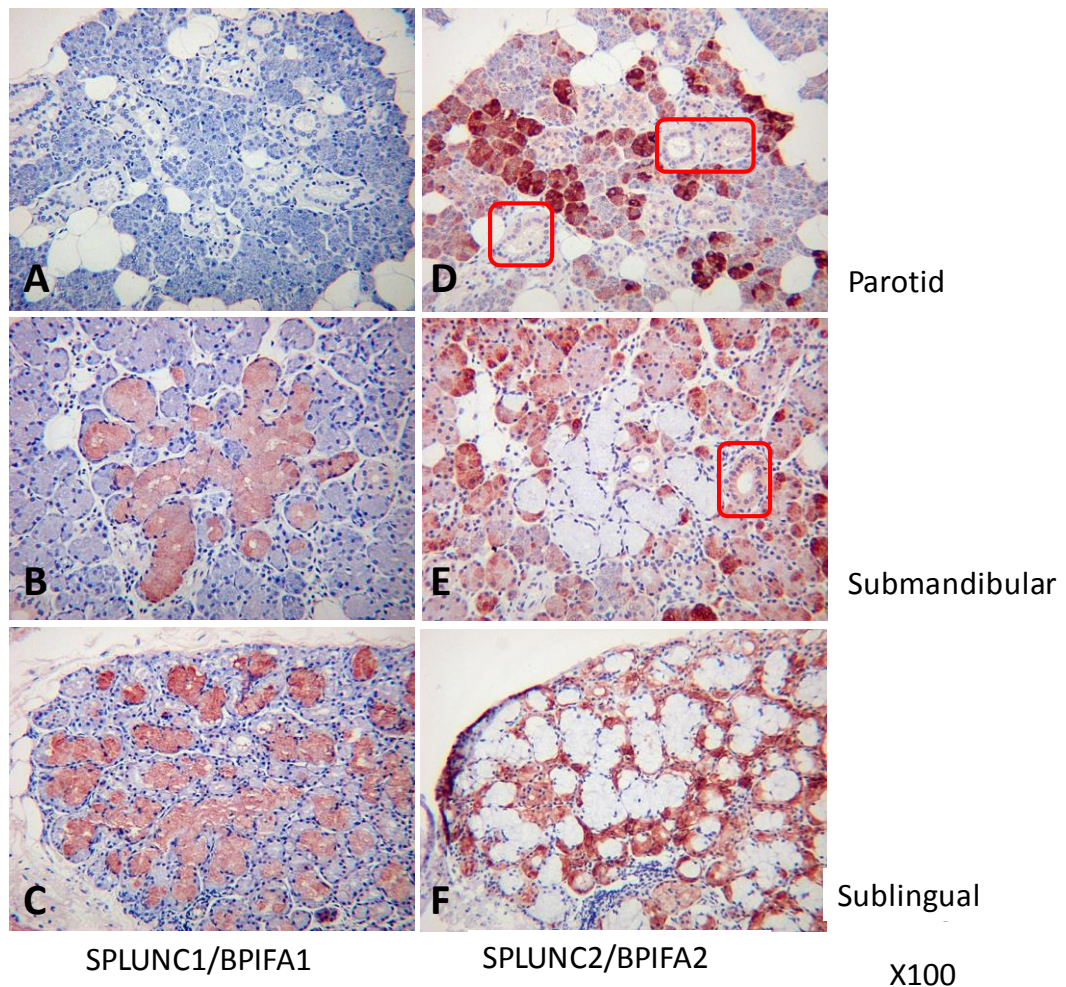
(Adapted from Bingle et al., 2009)

Figure 1-3 Distribution of SPLUNC1/BPIFA1 and SPLUNC2/BPIFA2 in the major salivary glands.

Immunohistochemistry of the parotid (A, D), submandibular (B, E) and sublingual glands (C, F) using a polyclonal SPLUNC1/BPIFA1 antibody (A, B, C) and a SPLUNC2/BPIFA2 antibody (D, E, F) shows the differences in expression of SPLUNC1/BPIFA1 and SPLUNC2/BPIFA2 in the major salivary glands. SPLUNC1/BPIFA1 expression is isolated to the mucous acini present in both the submandibular (B) and sublingual glands (C), negative staining was observed in the parotid gland (A), made up of primarily serous acinar cells. In contrast, SPLUNC2/BPIFA2 staining was positive in the serous acinar cells present in all three of the major salivary glands (D, E, F). In addition SPLUNC2/BPIFA2 expression was observed in the striated and intercalated intralobular ducts (highlighted) of the parotid gland (D) and the submandibular glands (E). A full explanation of the nomenclature is given in section 1.6.

No SPLUNC1 expression has been detected in human peripheral lung tissue (Geetha et al., 2005, Bingle and Bingle, 2000) indicating that SPLUNC1 expression is restricted to the upper airway and oral cavity and is unlikely to be involved in lung functions such as gaseous exchange.

1.5   BPI-LBP-CETP-PLTP protein family

The BPI-LBP-CETP-PLTP family consists of lipid binding and transporting proteins including Bactericidal Permeability Increasing protein (BPI), found in granules of polymorphonuclear neutrophils; Lipopolysaccharide-binding protein (LBP), Cholesteryl Ester Transfer protein (CETP) and Phospholipid Transfer protein (PLTP) produced in the hepatocytes of the liver and released into the blood stream for circulation. With the exception of CETP the genes for these proteins can be found on chromosome 20q11. CETP, the most dissimilar, is found on chromosome 16.

PLUNC proteins are strongly related to the BPI-LBP-CETP-PLTP protein family and are also found on chromosome 20. They are classed as a subfamily within this protein family and are described as being the largest branch of the BPI-LBP-CETP-PLTP protein family (Bingle et al., 2010). In addition to the gene location, PLUNCs have been identified as a member of this family based on a combination of several characteristics such as sequence homology and predicted structure. Structural comparison of each of the family members, BPI, LBP, CETP, PLTP and PLUNC, identified key residues that are conserved between all of them and all show the same conservation of two cysteine residues which form a critical disulphide bond (Beamer et al., 1997). The predicted structure of the PLUNCs suggests that the SPLUNCs contain the N-terminal domain of BPI and the LPLUNCs contain both the N-terminal and the C-terminal domains of BPI (Bingle and Craven, 2002). All members of this family appear to have the ability to bind to and transfer lipid molecules (Bingle and Craven, 2003). The structural similarity led to the hypothesis that PLUNCs function in a similar way to BPI and LBP and suggests that PLUNC

proteins are localised forms of the LBP/BPI proteins with a host defence function (Bingle and Craven, 2002). This hypothesis has gathered a significant level of support but as yet compelling functional data remains elusive.

1.5.1 Lipopolysaccharide Binding Protein (LBP)

LBP is synthesised in the hepatocytes of the liver as a 50kDa polypeptide, which is glycosylated and released constitutively into the bloodstream as a 58-60kDa glycoprotein (Zweigner et al., 2006) at a concentration of less than 500ng ml^{-1} (Schumann, 1992). During acute infection with LPS-containing bacteria, this concentration increases to around 50μg ml^{-1}(Schumann, 1992). Production of LBP is however not limited to the hepatocytes. Cells including lung, intestine and gingival epithelium and heart, renal and lung artery muscle cells also produce LBP (Zweigner et al., 2006). Human LBP contains five potential glycosylation sites and four cysteines, two of which form the critical disulphide bond previously mentioned (Schumann et al., 1990). It contains a 25 amino acid hydrophobic signal sequence followed by a 452 amino acid mature protein, with the functional domain located at the N terminal end (Zweigner et al., 2006). LBP has multiple functions, it is an acute phase protein, whereby its transcription is induced by LPS, interleukin (IL)-1 and IL-6 combined which is further stimulated by tumour necrosis factor (TNF)-α. Transcription can be reduced by transforming growth factor (TGF)-β1, an anti-inflammatory cytokine (Zweigner et al., 2006). In addition to these functions LBP performs a classical role in innate immunity.

LPS from both rough and smooth forms of bacteria (lacking the O-glycan component or possessing it, respectively) found in the bloodstream have been

shown to bind to LBP and this complex has affinity for the cellular receptor CD14 (Schumann et al., 1990). LBP binds to the Lipid A portion of LPS, which leads to the monomerisation of LPS. LBP then transports the monomerised LPS molecule to either membrane bound CD14 (mCD14), on monocytes/macrophages and neutrophils, or soluble CD14 (sCD14) (Zweigner et al., 2006). The ability to transport LPS to both mCD14 and sCD14 allows for both CD14+ cells (monocytes and macrophages) and CD14- cells, (endothelial and epithelial), to be activated in the presence of bacterial LPS. Tobias et al (1995) showed that the main difference between soluble and membrane bound CD14 is that the LBP molecule bound to sCD14 dissociates from the complex compared to mCD14 bound LBP which remains associated with the activating cell (Hailman et al., 1994). It has been shown that the ability of CD14 to interact with LPS is low in the absence of LBP (Hailman et al., 1994) implying that this protein is necessary for an optimum immune reaction to gram-negative bacteria to occur. The LPS-(sCD14/mCD14-LBP) complex is then able to interact with the extra cellular domain of toll-like receptor (TLR)-4 and Myeloid Differential Protein (MD) 2 simultaneously, creating a CD14-TLR4-LBP-LPS complex (Akira et al., 2006). It has been shown that a deficiency of MD2 leads to a reduced responsiveness to LPS indicating that this molecule is important in the TLR-4 pathway (Takeda et al., 2003). The association of each component of this complex leads to the interaction of the intracellular toll-interleukin receptor domain of TLR-4 with an intracellular protein, MyD88 (Raetz and Whitfield, 2002), and following a cytoplasmic signalling cascade, NF-κB is activated which up-regulates the expression of various inflammatory genes including the IL-8 gene (Nakanaga et al., 2007). This interleukin leads to an increased endothelial adhesiveness of phagocytic cells,

including dendritic cells, monocytes and polymorphonuclear neutrophil leukocytes (Iwasaki and Medzhitov, 2004). Upon detecting these inflammatory signals, these cells roll along the endothelium of the blood vessels, adhere and migrate into the inflamed tissue. The activated phagocytes begin engulfing the pathogens and the resulting phagolysosomes (small intracellular compartments, containing enzymes and anti-microbial peptides), fuse together leading to the killing and breaking down of the pathogen. This in turn causes the pH of the phagolysosome to fall providing an optimum pH for enzymatic activity and the killing potential of the cell (Davies et al., 1998). During the process of phagocytosis a variety of cytokines are released including IL-1β, IL-6 and TNF-α. These act to increase vascular permeability and increase the expression of adhesion molecules to assist in the recruitment of more inflammatory cells (Davies et al., 1998).

1.5.2 Bactericidal permeability-increasing protein (BPI)

BPI is approximately 55kDa in size and is expressed in human monocytes, fibroblasts, eosinophils (Canny and Levy, 2008) and all mucosal epithelia (Srivastava et al., 2007). The primary structure of BPI is approximately 45% identical to that of LBP and is transcribed from a gene adjacent to the LBP gene on chromosome 20 (Schultz et al., 2007). Expression of this cationic antimicrobial polypeptide has been identified in a number of species including humans (Uniprot accession number P1723), mice (accession number Q67E05), rats (accession number Q6AXU0), rabbits (accession number Q28739), cows (accession number P17453), ducks (accession number R0JLF2) and frogs (accession number B0BMR6).

BPI is described as having pseudo 2-fold symmetry in an elongated boomerang form, with two functional domains at the C-terminus and the N-terminus connected by a β sheet of approximately 21 amino acids (Beamer et al., 1997). As mentioned previously, in this protein and other family members, there is a critical disulphide bond between residues 135 and 175 (in BPI), which links the edge of the central β sheet to one of the terminal α helices. Deletion or mutation of either of the critical cysteine residues leads to the inactivity of BPI highlighting the importance of this conservation within the family (Beamer, 2003). Although the two BPI domains are similar and both are able to bind a phospholipid (Canny and Levy, 2008), there are significant differences, for example the N-terminal cleft has a slightly larger opening than that in the C-terminal domain. Also the C-terminus is a neutral domain compared to the N-terminus, which is cationic (Beamer et al., 1997) and rich in lysine (Canny and Levy, 2008). These differences may explain the differences in function seen between the two domains.

BPI is able to transfer lipid molecules through aqueous environments by orientating the lipid molecule in the clefts so that the acyl carbon chains (the hydrophobic region) of the lipid are deep inside the cleft with the phospholipid head (the hydrophilic region) near to the surface of the opening (Beamer, 2003). Through electrospray mass spectrometry it was seen that a lipid consisting of a phosphatidylcholine head group and 18 carbon acyl tail groups was bound to the BPI clefts. Although the phosphatidylcholine is predominantly found in eukaryotic cells, the structure is similar to that of LPS found in gram-negative bacteria (Beamer, 2003) and a number of bacteria that interact closely with man, including

Streptococcus pneumoniae and Haemophilus influenzae require phosphatidylcholine on their surface to penetrate through epithelia (Sohlenkamp et al., 2003).

Like LBP, BPI is an innate immune molecule which shows high affinity for the gram-negative bacterial LPS component, Lipid A (Schultz et al., 2007). BPI has been shown to have three actions against LPS, it opsonises the LPS, neutralises LPS and acts as a direct antimicrobial. The C-terminal region of BPI can highlight the presence of the bacteria by opsonising and so, trigger the phagocytosis of the bacteria/components by neutrophils. The N-terminal domain binds to the LPS and leads to the neutralisation of the endotoxic activity (Canny and Levy, 2008) or elicits direct antimicrobial activity via CD14+ blood monocytes, without any inflammatory response (Schultz et al., 2007). Direct bactericidal activity has been demonstrated through the increased permeability of the gram-negative bacterial envelope. Permeability was seen immediately after adding BPI to bacterial suspensions (Weiss et al., 1978, Srivastava et al., 2007) and this caused a reduction in the ability of the bacteria to multiply (Weiss et al., 1975). Initially, BPI was thought to act only on gram-negative bacteria after Weiss et al (1978) saw activity against *E. coli* and *Salmonella typhimurium* but not apparently against various gram-positive species or two species of fungus. However, in 2007 Srivastava et al demonstrated that BPI can recognise pneumolysin, a pore forming protein produced by the gram-positive species, *S. pneumoniae*. Indeed, BPI appears to protect against invasive pneumococcal disease by initiating an innate immune response causing the

apoptosis of nasopharyngeal cells in the presence of *S. pneumoniae* and pneumolysin (Srivastava et al., 2007).

1.5.3 Cholesteryl ester transfer protein (CETP) and Phospholipid transfer protein (PLTP)

CETP and PLTP act to transport a number of lipid molecules around the body. CETP removes cholesteryl esters from high density lipoproteins (HDL), which leads to a reduced concentration of HDL in the body. In contrast PLTP acts to remove phospholipids from triglyceride-rich lipoproteins, thus increasing the HDL level in the body (Masson et al., 2009). As the functions of these proteins are not related to interactions between bacterial components and the immune system they will not be discussed further.

1.6 Nomenclature

The growth of the PLUNC protein family and their characterisation by a number of groups has led to a range of aliases for the members of the family, for example, as previously mentioned SPLUNC1, initially known as PLUNC, is also known as LUNX and SPURT; SPLUNC2 is also known as PSP and LPLUNC1 as Von Ebner minor salivary gland protein. The range of names for each member of the family has led to some difficulty in linking research, therefore, it was recently decided that a more systematic name was required to eliminate this confusion surrounding the relationship of family members (Bingle et al., 2011a). The new naming system has been applied to the superfamily containing BPI, LBP, CETP, PLTP and PLUNC, which is now referred to as the BPI-fold containing superfamily. This new naming system is based upon a BPIF root (BPI fold containing family). The short PLUNC proteins are

now described as BPIFA*n*, for example, SPLUNC1, LUNX and SPURT became BPIFA1 and SPLUNC2 and PSP became BPIFA2. The long PLUNC proteins are described as BPIFB*n*, for example, LPLUNC1 became BPIFB1 and LPLUNC2 became BPIFB2. In order to incorporate all of the BPI-fold family, including paralogues the gene names maybe appended with 'A', 'B', 'C' etc, to differentiate between the two lineages. For example bovine BSP30, previously known as BSP30A, BSP30B, BSP30c and BSP30d, have now been renamed BPIFA2A, BPIFA2B, BPIFA2C and BPIFA2D respectively and mouse and rat PSP are now known as BPIFA2E, ensuring that they are identified as expanded members of the BPIFA2 sub-family (Bingle et al., 2011a). The human BPIF proteins can be seen, along with their previous PLUNC root names and any alternative nomenclature in (Table 1.1)

Table 1.1: The human BPIF/PLUNC family and their alternative nomenclature.

BPIF root	PLUNC root	Alternative nomenclature
BPIFA1	SPLUNC1	PLUNC, SPURT, LUNX
BPIFA2	SPLUNC2	PSP
BPIFA3	SPLUNC3	
BPIFA4	SPLUNC4	BASE
BPIFB1	LPLUNC1	C20orf114
BPIFB2	LPLUNC2	BPIL1
BPIFB3	LPLUNC3	
BPIFB4	LPLUNC4	
BPIFB5	LPLUNC5	
BPIFB6	LPLUNC6	BPIL3
BPIFB7	Vomeromodulin	

1.7   BPIF Proteins

During the development of the systematic nomenclature, it was possible to identify BPIF proteins in a number of species rat, mouse, cow, hamster, pig, horse, dog, marmoset, chicken, zebra finch, platypus, opossum, panda, rhesus monkey, xenopus, orang-utan, chimp, cat and chinchilla (Bingle et al., 2011a). In addition expanded members of each subfamily in certain species could be identified and described in relation to the respective protein in other species (Figure 1-4) (Bingle et al 2011a). This analysis has likely identified all distinct mammalian BPIF-containing proteins and confirmed the previous suggestion that BPIF/PLUNC proteins are restricted to the vertebrate lineage. Furthermore, it confirmed that BPIFA proteins are restricted to the therian lineage. The analysis also confirmed that BPIF proteins are also extremely divergent both in terms of paralagous and orthologous relatives. Overall they are amongst the most divergent mammalian protein families with individual paralogues having sequence identities typically below 30% (Bingle et al 2004).
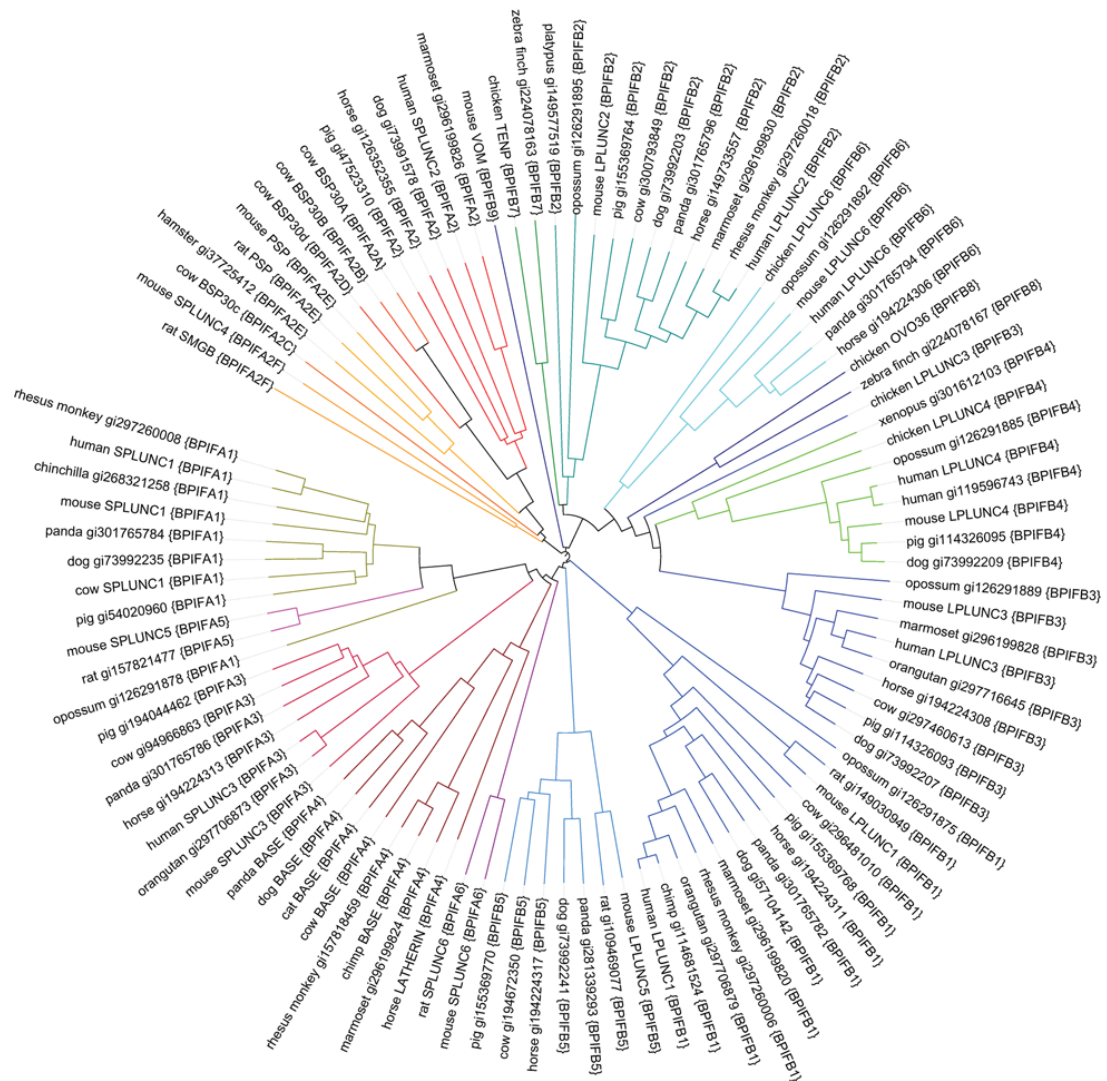
Figure 1-4: Phylogenetic tree of the BPI Like-fold (BPIF) genes

This tree demonstrates the size and diversity of the BPIF family. Constructed using ClustalW and displayed using ITOL by Bingle, Seal and Craven (2011a). This tree demonstrates the usage of the new naming system to identify each distinct member of the BPI fold-like family.

1.8 Short PLUNC 2 (BPIFA2)

BPIFA2 is the major salivary PLUNC protein and is secreted into the saliva at much higher levels than BPIFA1 (SPLUNC1). The mouth provides an accessible model and

saliva provides an abundant source of this native protein for use in functional studies and it is for these reasons that this protein was chosen for the focus of this study. Despite the similarity of BPIF proteins to the host defence proteins, BPI and LBP, and the reported expression of BPIFA2 in gingival keratinocytes in response to the addition of heat killed bacteria along with inflammatory cytokines, such as TNF-α (Shiba et al., 2005), function of the BPIF proteins remain unresolved. Functional studies with human BPIFA2 are very limited.

BPIFA2 has been identified as the human orthologue of the rodent parotid secretory protein (BPIFA2E) even though the protein sequence identity between the two is less than 35% (Bingle et al., 2009). The BPIFA2 gene is found on chromosome 20orf70, which is synteneic to chromosome 2 in the mouse, where mouse BPIFA2E can be found (Geetha et al., 2003). As previously mentioned the BPIF2A2 gene contains 9 exons, transcription of the gene begins with exon 2 and the stop codon is located in exon 8, leaving the first and last exons as non-coding (Bingle et al., 2009). Translation of this gene produces a protein of 249 amino acids with a molecular weight of 27,011Da (Uniprot accession number Q96DR5).

The BPIFA2/SPLUNC2 proteins are the most divergent group of paralogous proteins within the family (Bingle et al 2011). It is clear that this group of genes has undergone a significant degree of divergence during mammalian evolution. This has manifested itself in the development of four distinct homologues within the bovine lineage. This divergence is clearly illustrated in the phylogenetic analysis presented in Figure 1.4.

Human BPIFA2 protein consists of an N-terminal signal sequence (residues 1-16); indicating that BPIFA2 is a secretory protein. The protein is hydrophobic in nature and contains a significant number of leucine/isoleucine residues (26%), The pI of the protein is predicted to be 5.35. As is the case with all BPIF proteins (Bingle et al 2024), it contains two cysteine residues that are predicted to make a single disulphide bond. Although there is no published structure for the protein, threading analysis confirms that the protein will be expected to take on the general β-barrel structure found in other family members (Figure 1-5).
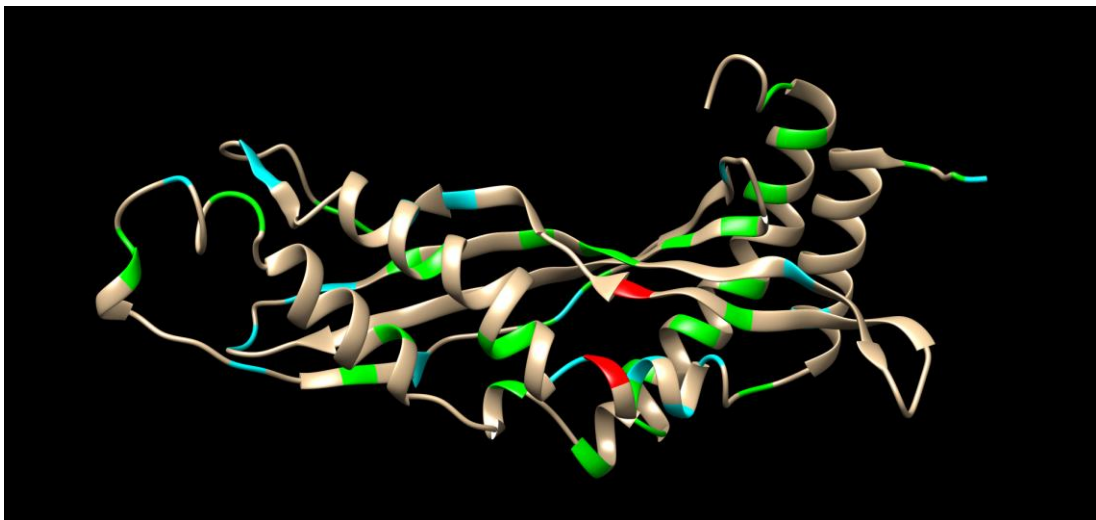
Figure 1-5: The predicted structure of BPIFA2 generated by threading.

Human BPIFA2 was threaded using the Phyre server and the resultant model was rendered using Chimera (http://www.cgl.ucsf.edu/chimera). The model shows the position of the two cysteine residues (red) as well as the position of the hydrophobic residues, Leu (Green) and Ile (Blue). The hydrophobic residues are mostly found within the inner portion of the barrel structure (model generated by Colin Bingle).

The mature BPIFA2 protein (residues 17-249) also contains two N-glycosylation sites on residues 124 and 132 (Gorr et al 2011). Western blotting of saliva for SPLUNC2 identified multiple positive bands, indicating that BPIFA2 is differentially glycosylated to give a variety of sizes (Bingle et al., 2009). Immunohistochemical analysis of the human major salivary glands identified BPIFA2 expression in the

serous acinar cells of the parotid gland, striated and intercalated cells of the intralobular ducts (Bingle et al., 2009). More specifically, positive staining was seen in groups of single, double and triple cells in the collecting ducts of the parotid gland. In addition the protein was seen in the submandibular gland, however antibodies raised to two different epitopes of BPIFA2 produced different staining patterns; Antibody-A (raised to an internal epitope of BPIFA2) showed positivity for the serous acinar cells and the intralobular ducts, as with the parotid gland, however antibody-B (raised to an extreme C-terminal epitope) showed little reactivity in the serous acinar cells of the submandibular glands and none at all in the intralobular ducts. No reactivity was seen in the mucous acini of any gland with either antibody. The sublingual glands followed the pattern of expression seen in the submandibular glands for antibody-A but no staining was seen with the antibody-B (Bingle et al., 2009). The reasons for these different staining patterns is not known but could be caused by alternative splicing of the gene giving a different isoform without the epitope (although there is no experimental support for this, Bingle et al 2009), or some form of post-translational modification may occur that hides the C-terminal epitope. The lumens of collecting ducts also stained positively (Bingle et al., 2009) indicating that BPIFA2 is expressed by the serous acini within the major glands and then secreted via the ducts into saliva.

BPIFA2 protein was also shown to be expressed in some minor salivary glands, with the glands of the vallecular region of the tongue showing the same staining pattern as seen with the parotid gland. Other minor glands, including those in the posterior tongue, followed the pattern seen in the sublingual and submandibular glands.

Minor glands further down the respiratory tract, the respiratory mucosa and tissues outside the oral cavity showed no expression of BPIFA2 (Bingle et al., 2009).

Expression has also been identified in a number of different species including: hamster (BPIFA2E); rat and mouse (BPIFA2E and BPIFA2F); Cows (BPIFA2A-D) and pig and horse (BPIFA2) (Gorr et al., 2011). Indeed, expression of the protein in rodent salivary glands has been studied extensively (Poulson et al., 1986; Laursen et al., 1998; Weston et al., 1999; LeClair et al., 2001).

1.9 Hypothesis and Aims

BPIFA2 is a heavily glycosylated protein secreted from both major and minor salivary glands into saliva; however saliva also contains a great number of other proteins and peptides. The function of the BPIF family of protein has yet to be elucidated, but due to their predicted structure and their similarity in gene location to known LPS binding proteins, BPI and LBP, it is proposed that they function in an antimicrobial manner.

BPI and LBP control the growth and activity of pathogenic bacteria, either by direct binding; opsonisation to facilitate phagocytosis, by minimising the immune response to control levels of inflammation or directly preventing their growth and adhesion. BPIF proteins may share some of these functions.

Hypothesis

Due to the predicted structural similarity between BPIFA2 and the innate immune proteins BPI and LBP, it is hypothesised that BPIFA2 may function in the innate immune system, against gram negative bacterial LPS, either by acting directly

against bacteria in a bactericidal manner, initiating an immune response or as an anti-toxin, by reducing the inflammatory response.

Aims

1. To develop a suitable protocol for the purification of native BPIFA2 protein from stimulated whole saliva and to use this protein for functional assessment.

2. To perform a variety of functional assays designed to determine bacterial binding, killing and growth inhibition.

3. To examine the potential role of the BPIF proteins in controlling the inflammatory response to bacterial LPS.

Chapter 2.   Purification of BPIFA2

2.1 Introduction

The purification of BPIF proteins is essential to their functional characterisation. A number of different methodologies have been attempted in order to achieve successful purification of BPIF proteins. A variety of biological fluids, such as human saliva (Geetha et al., 2003, Abdolhosseini et al., 2012 (BPIFA2)) human tracheobronchial fluids (Campos et al., 2004 (BPIFA1)), horse sweat (Beeley et al., 1986 (Latherin/BPIFA4)) and bovine saliva (Haigh et al., 2008 (BPIFA2 proteins)) have been used as a source of BPIF proteins. The culture of chinchilla nasopharyngeal epithelial cells has also been used as a source of secreted native protein (McGillivary and Bakaletz, 2010 (BPIFA1)). Recombinant protein has also been produced in bacteria (Geetha et al., 2003, Haigh et al., 2008, Khovidhunkit et al., 2005, Gakhar et al., 2010, Bartlett et al., 2008, McDonald et al., 2009, Chu et al., 2007, Abdolhosseini et al., 2012 (BPIFA1, BPIFA2 and BPIFA4)) and mammalian cells (Geetha et al., 2003 (BPIFA2)). In addition BPIF peptides have been designed to combat the issues behind purification of the protein (Geetha et al., 2003, Gorr et al., 2008); however these short peptides (7-13 amino acids) have been selected specifically from the BPIFA2 sequence to coincide with active portions of the BPI protein. Any results seen with these peptides therefore must be viewed with caution, as they may not represent the function of the BPIFA2 protein as a whole. BPIFA2 is a leucine rich hydropobic protein with a pI of 5.35. It is expected to take on the general β-barrel structure found in other family members. Most of the

hydrophobic residues in the molecule cluster within the inner clef of the molecule (Figure 1-5) and the surface charge is evenly distributed across the molecule.

Ethanol precipitation was adopted by Abdolhosseini et al (2012) and Campos et al (2004) in the purification of BPIFA2 and BPIFA1 from human saliva and tracheobronchial secretions respectively, as BPIF proteins remained soluble in a 70-75% ethanol solution whilst many other proteins did not. Campos et al (2004) further purified the BPIF by subjecting it to HPLC. They clearly demonstrated purification of the BPIF proteins from saliva and tracheobronchial secretions with ethanol. Closer examination of the results published by Abdolhosseini et al (2012), however, shows that the 'purified' sample contains a number of lower molecular weight proteins, which could include a number of anti-bacterial proteins such as histatins, statherin and lysozyme all of which may interfere with further functional analysis.

A similar method, adopted by Haigh et al (2007), involved the purification of BPIF orthologues, BPIFA2A and BPIFA2B found in bovine saliva. They precipitated a number of contaminant proteins at 50% isopropanol before adjusting the isopropanol concentration to 63% (for the purification of both BPIFA2A and B) or 65% (for the purification of BPIFA2A only) to precipitate any remaining contaminants. Following purification the proteins were concentrated by lyophilisation before being exposed to ion exchange chromatography. However this method resulted in only 82% and 77% purity of BPIFA2A and B respectively with clear contaminant protein bands present in the BPIFA2B sample at 97kDa, 45kDa and approximately 38kDa on a Coomassie stained SDS-PAGE gel. In addition to this

incomplete purification, the use of lyophilisation to concentrate the protein would increase the probability of recovering a denatured protein.

A final method for the purification of native BPIF protein from biological samples includes the use of anti-PSP immunoaffinity chromatography (Geetha et al., 2003), however, very little work containing this method has been published as a preference to the use of artificial peptides was subsequently adopted by this group (see later in this section).

Bacterial expression of BPIF proteins still appears to be the more favoured method of expression and purification used to date, possibly due to the reduced cost and the ability to produce a large amount of protein at a much faster rate. *Escherichia coli* has been the bacteria of choice for the expression and purification of BPIF proteins with a number of different tags including 6xHIS (Khovidhunkit et al., 2005, McDonald et al., 2009, Haigh et al., 2008), V5 (Geetha et al., 2003), MBP (Gakhar et al., 2010, Bartlett et al., 2008) and GST (Chu et al., 2007). However, as BPIF proteins are believed to be bactericidal, the use of bacteria to express this protein does not seem a highly considered choice. It might be expected that either the expression of BPIF would lead to toxicity of the bacterial expression system, loss of the BPIF protein through bacterial binding or that expression by a bacterial system would interfere with future bactericidal assays. In addition to these considerations, it is known that BPIF proteins are glycosylated; *E. coli* expression systems are unable to naturally glycosylate proteins without co-transfection with glycosylation systems, such as the N-glycosylation system from *Campylobacter jejuni* (Chen, 2012).

Although, even this is not likely to yield fully glycosylated protein as would be seen in a mammalian expression system or in its native form.

The use of mammalian cell lines have also been used to express and purify BPIF proteins, either by natural expression and the collection of secretions from human tracheobronchial cell cultures, with purification by ethanol precipitation, (Campos et al., 2004); transfected expression in PC12 and GH4C1 rat cells, with no purification step (Geetha et al., 2003, Abdolhosseini et al., 2012) or chinchilla nasopharynx primary cells with SDS-PAGE protein elution (McGillivary and Bakaletz, 2010). The absence of a purification step (Geetha et al., 2003, Abdolhosseini et al., 2012) may lead to complications with functional analysis due to the presence of a number of mammalian derived proteins. However, this method did show evidence of mammalian post-translational modifications when compared to bacterial expression systems.

The method utilised by McGillivary and Bakaletz (2010) appears to be the least suitable for purification as this involves two lyophilisation steps to concentrate the sample, protein separation by SDS-PAGE, and protein refolding using urea. Of the methods adopted, this has the highest susceptibility to error, as protein folding is not guaranteed and functionality may be affected due to such denaturing steps.

As an alternative to purification of BPIF protein, some groups have used BPIFA2 peptides. These small sections (7-13 amino acids) of the BPIFA2 sequence have been selected and artificially manufactured (Geetha et al., 2003). Geetha et al (2003) and Gorr et al (2008) demonstrated the design of a number of BPIFA2-derived peptides relating to cationic peptides found in BPI and LBP that are

responsible for LPS binding. Although this method reduces the issues surrounding expression and purification of the BPIF proteins, these peptides cannot be fully relied upon for functional analysis as their position within the folded BPIF protein may inhibit their interaction with bacteria leading to them being non-functional regions or the structural arrangement of BPIF may lead to a higher degree of selectivity than would be demonstrated by the presence of these peptides.

A summary of the BPIF protein, source of BPIF protein and purification techniques currently in the literature can be seen in (Table 2.1).

Table 2.1: Purification methods currently described in the literature

BPIF	Source of protein	Purification method	Reference
Human BPIFA2	Human saliva	Anti-PSP immunoaffinity chromatograohy	Geetha et al., 2003
Human BPIFA2	Human saliva	Ethanol Precipitation	Abdolhossieni et al. 2012
Human BPIFA1	Human tracheobronchial fluid from cell culture	Ethanol Precipitation + HPLC	Campos et al., 2004
Equine BPIFA4	Horse sweat	Ion exchange chromatography + gel filtration	Beeley et al., 1986
Bovine BPIFA2A and B	Bovine saliva	Isopropanol precipitation + ion exchange chromatography	Haigh et al., 2008
Chinchilla BPIFA1	Chinchilla nasopharyngeal epithelial cells	SDS-PAGE protein elution	McGillivary and Bakaletz., 2010
Human BPIFA2 Bovine BPIFA2A and B Hamster BPIFA2 Human BPIFA1 Human BPIFA1 Equine BPIFA4 Mouse BPIFA1 Human BPIFA2	Bacterially expressed recombinant	Various tags – 6xHIS, V5, MBP, GST	Geetha et al., 2003, Haigh et al., 2008, Khovidhunkit et al., 2005, Gakhar et al., 2010, Bartlett et al., 2008, McDonald et al., 2009, Chu et al., 2007, Abdolhosseini et al., 2012

In addition to the attempts to study isolated BPIF proteins, proteomic studies have shown that BPIFA2 is a component of the dental pellicle (Siqueira et al 2007). Dental pellicle is the surface film that forms on the teeth and consists mainly of salivary proteins. It functions to assist in the demineralisation and remineralisation of the teeth, regulate lubrication and controls the early colonisation of the tooth

surface by oral bacteria (Siqueira, Custodio and McDonald 2012). The observation that the protein is found in this film suggest that these surface films could also be used as a potential purification strategy.

As the absolute structure of BPIFA2 is still unproven the most suitable method for purification is currently unknown. Our limited knowledge of BPIFA2 structure led us to investigate the potential use of a number of techniques previously reported in the literature for the purification of other BPIF family members. Standard protein purification techniques such as column chromatography and protein precipitation were thus considered alongside a novel technique in the BPIF protein field, electro elution, a simple yet potentially effective method for purification of native protein.

2.2 Aim

The aim of this part of the study was to use stimulated whole saliva, as a source of BPIFA2, and assess a number of different methods to identify the most suitable for purification. As previously mentioned, BPIFA2 is present in abundance in whole saliva, which is easily collected in a rapid and non-invasive way. Attempts were made to minimise any denaturation of the protein to ensure a purified sample containing BPIFA2 in its native condition for use in later functional analysis.

2.3 Materials and Methods

2.3.1 Saliva Collection

Stimulated whole saliva was collected from a healthy volunteer with a typical BPIFA2 expression based on previous work (Bingle et al., 2009; Ethics approval not required). The subject did not eat or drink for at least one hour prior to collection. Saliva was stimulated with uncoated, sugar free chewing gum and 20mLs collected over 5 minutes, in glass universal tubes. The sample was immediately centrifuged at 3500rpm for 15 minutes at 4°C, held on ice and used within 30 minutes of collection.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.3.2.1 Gel Casting

All components of the BIORAD gel casting system were washed thoroughly, the glass plates were cleaned with 70% IMS before assembly.

A 12% (w/v) SDS-Polyacrylamide resolving gel (Appendix 6.1) solution was prepared and 10% ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added just before pouring into the gel casting system and overlaying with isopropanol. After 5-10 minutes, when the gel was set, all traces of isopropanol were removed by washing with distilled water. A 4% stacking gel (Appendix 6.1) solution was prepared and 10% APS and TEMED were added immediately prior to pouring the stacking gel. A 10-well or 15-well comb was added to the stacking gel and this was allowed to polymerise for 5-10 minutes.

The cast gels were then assembled into the BioRad gel running system.

2.3.2.2   Sample preparation

Samples were combined with equal volumes of 2x SDS-sample buffer (Appendix 6.1) and boiled for 5 minutes at 95°C to denature the proteins. Samples were either stored at -20°C or directly loaded onto a 12 % SDS-PAGE gel.

2.3.2.3   Electrophoresis

All samples were electrophoresed alongside a protein standard ladder using the BioRad mini gel system at 100v for 2 hours.

2.3.2.4   Silver stain analysis of SDS-PAGE gels

Protein gels were analysed by silverstain (Silverstain plus, Biorad). The gel was fixed overnight in 40% ethanol, 10% acetic acid and 50% water. Staining was achieved with the silverstain plus kit (BioRad) following manufacturer's instructions (Appendix 6.2). The gels were scanned into a computer with the Lab Scanner Image Master (Amersham) and stored as digital images.

2.3.2.5   Instant Blue (Novexin Ltd, Cambridge, UK) Staining of SDS-PAGE Gels

The instant blue solution was mixed well before use by inverting the bottle 3-4 times. Following electrophoresis the gel was carefully removed from the plate and added to a flat plastic tray containing instant blue solution. The gel was placed onto a rocker for up to 15 minutes at room temperature. The gel was scanned into a computer with the Lab Scanner Image Master (Amersham) and stored as digital images.

2.3.3   Western Blotting

2.3.3.1   Transfer of proteins from SDS-PAGE Gels

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane (Whatman, USA) in an XCell II™ Blot Machine (Invitrogen). All components (2x 3mm blotting paper, 5x sponge, 1x gel sized nitrocellulose membrane) were soaked in transfer buffer (Appendix 6.3) for 5 minutes prior to assembly. The blot stack was assembled with the membrane towards the positive electrode, with no air bubbles and electro-blotting was performed at 30v for 60-90minutes.

2.3.3.2   Dot Blot analysis

5µl of sample was dotted onto a nitrocellulose membrane (Whatman, USA) and allowed to air dry, before being subjected to western blot analysis.

2.3.3.3   Western Blot

Nitrocellulose membranes from SDS-PAGE and dot blots were blocked in 5% (w/v) dried non-fat milk in tris-buffered saline containing 0.05% Tween-20 (TBST) (overnight 4°C or room temperature 60 minutes), washed three times for 5 minutes in TBST and incubated with the appropriate primary antibody (BPIFA2B (1:500; In house antibody generated by Eurogentec) or α-amylase (1:500; Sigma, UK)) in 5% dried non-fat milk in TBST at 4°C, overnight or at room temperature for 60 minutes. The production and validation of the BPIFA2 antibodies has previously been described in detail (Bingle et al 2009). The membranes were washed three times for 5 minutes in TBST and incubated with the corresponding secondary antibody (Anti-rabbit HRP (1:2000; New England Biolabs) in 5% dried non-fat milk in TBST for 30 minutes.

2.3.3.4   Enhanced Chemiluminescence (ECL)

Western blots and dot blots probed with the HRP conjugated secondary antibody were washed twice for 5 minutes in TBST and once for 5 minutes in tris-buffered Saline (TBS). Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, UK) was used following manufacturer's instructions; solutions A and B were mixed in equal parts and allowed to equilibrate for 2 minutes at room temperature. The solution was pipetted onto the membrane ensuring even coverage and left for 5 minutes at room temperature. Excess ECL reagent was removed and the membrane sandwiched between 2 plastic sheets before visualisation either with ECL Film or the G:box, gel documentation and analysis system (Syngene, Cambridge, UK).

2.3.4   BPIFA2 Enrichment

2.3.4.1   Formation of Saliva Film

10ml aliquots of stimulated saliva were pipetted into small glass Petri dishes and left at room temperature for 30-150 minutes. At the air-liquid interface, a film formed. All of the saliva under the film was removed with a glass pipette and added to a second glass plate to allow a second film to form. Upon the removal of the saliva under the second film, each of the films were scraped into 1ml phosphate buffered Saline (PBS) and dialysed against water overnight. The sample was either concentrated by Polyethylene glycol (PEG) for 1 hour or it was added to a 10kDa cut off centrifugal concentrator (~1ml final volume) (10x concentrated). The combined films were analysed by western blot and protein staining.

2.3.5   Purification of BPIFA2

2.3.5.1   Ammonium Sulphate Fractionation

Ammonium Sulphate was added slowly, with stirring, to stimulated saliva to give a final saturation of 30%, pH7.2. The saturated saliva was incubated for 2 hours at 4°C then centrifuged for 20 minutes at 15,000 rpm at 4°C. The supernatant was collected and the pellet was resuspended into 300µl of TBS and stored at -20°C. The remaining saliva was re-saturated at 10% increments (40%, 50%, 60%, 70% and 80%) using the same method. The resuspended pellets were dialysed overnight against 50mM TBS before being analysed by western blotting and Instant Blue protein staining.

2.3.5.2   Ion Exchange Chromatography

Stimulated saliva (1ml) was collected and passed through a Q-sepharose ion exchange column, in TBS pH7.5 (Appendix 6.3), at a flow rate of 1ml/minute. The concentration of NaCl was increased slowly from 0.0M to 0.20M, followed by a rapid increase to 0.45M then a second rapid increase to 1.0M NaCl. Fractions corresponding to peaks were collected and analysed by dot blot. Positive fractions were concentrated by lyophilisation then passed over a second Q-sepharose column, where NaCl concentration was gradually increased from 0 to 0.25M followed by a rapid increase to 1.00M NaCl. Fractions corresponding to peaks were collected and analysed by dot blot and western blotting.

2.3.5.3   Ethanol Precipitation (Campos et al, 2004)

BPIFA2 protein was separated from the majority of the other proteins present in saliva using acetone and ethanol precipitation. 10mls of saliva was concentrated with 2.5 volumes of ice cold acetone (100%) per volume of saliva. After 10 minutes on ice the sample was centrifuged at 16,000rpm for 10 minutes at 4°C and the pellet resuspended in 1ml of PBS. Three volumes of Ethanol (100%) per volume of sample were added and the sample was held on ice for 10 minutes before centrifuging at 16,000rpm for 10 minutes at 4°C. 2.5 volumes of ice cold acetone (100%) per volume of sample was added to the supernatant and held on ice for 10 minutes. The sample was centrifuged again, 16,000rpm for 10 minutes at 4°C, and the precipitated proteins were resuspended in 1ml PBS (Campos et al., 2004). The ethanol precipitated BPIFA2 was analysed by western blot and silver stain analysis.

2.3.5.4   Size Exclusion Chromatography

Following ethanol precipitation, 2 mls of the sample was passed through a size exclusion column (S200-HR) at a flow rate of 0.16ml/minute with PBS. Fractions corresponding to peaks on the chromatogram were analysed by dot blot.

2.3.6   Native Gel Electrophoresis Systems

1-4mls of whole saliva was separated using the BIORAD Large gel system, according to the manufacturer's instructions. All gels cast were 12% (w/v) native acrylamide gels (Appendix 6.5) and resolved at 190V overnight.

2.3.6.1   Protein Elution

Each component of the Bio-Rad protein eluter was soaked in native elution buffer (Appendix 6.5) for 5 minutes prior to assembly following manufacturer's

instructions (Figure Figure 2-1). The elution chamber was filled with native elution buffer. The elution was performed at 200mA over 25 minutes, then the polarity was reversed and the eluter was re-run at 200mA for 30 seconds. The fractions were collected into a glass tube by vacuum and the fractions were analysed by dot blot. Positive fractions were dialysed against water and concentrated by centrifugation with a 10kDa cut off concentrator column.
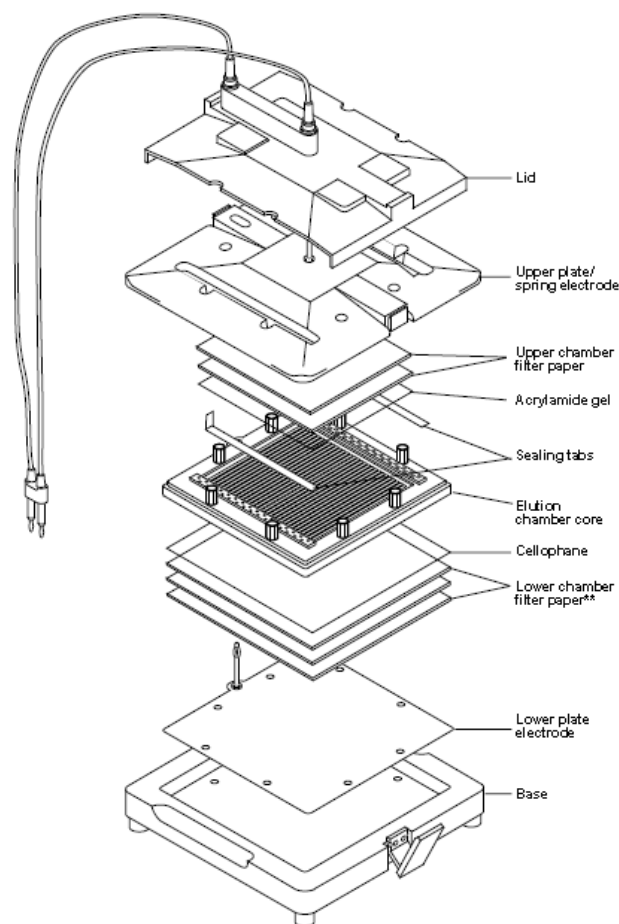
Image taken from Biorad Laboratories, Whole Gel Eluter Instruction Manuel

Figure 2-1: Schematic demonstrating the BioRad electroelution equipment.

Proteins were resolved on a native polyacrylamide gel using the BioRad Large Gel system, the gel was then transferred to the BioRad electroelution apparatus where the proteins were eluted from the gel into the 30 fraction chambers. The fractions were then collected into glass test tubes before being analysed by western blot for BPIFA2 and α-Amylase.

2.3.7 Recombinant BPIFA2

2.3.7.1 S2-Cell expressed BPIFA2

<u>Drosophila Schneider 2 (S2) Cell line</u>

The non-adherent Insect cell line, *Drosophila Schneider* 2 (S2) expressing recombinant BPIFA2, was kindly donated by Dr Lynne Bingle (Appendix 6.4).

<u>Maintenance</u>

The S2 cell lines were maintained in Ex-cell 420 Serum Free Medium for Insect Cells (Sigma, UK) supplemented with 10% heat inactivated foetal calf serum (FCS) and $100UmL^{-1}$ Penicillin/Streptomycin at a density of between $1x10^6$ - $5x10^6$ cells ml^{-1}. On a weekly basis the cells were centrifuged at 1000rpm for 5 minutes and resuspended in fresh media, unless stimulation was taking place.

<u>Expression</u>

S2 cells were centrifuged at 1000rpm for 5 minutes and resuspended at a density of 8-$10x10^6$ cells ml^{-1} in media with or without serum. The S2 cells were stimulated with 500µM filter sterilised copper sulphate either overnight (with serum) or for 15 days (without serum). The S2 cells were then centrifuged at 1000rpm for 5 minutes and the conditioned media was collected into a glass bijou and analysed by dot blot or western blot.

2.3.7.2 NI-NTA bead BPIFA2 purification

Initially, a small column was packed with 2mls of Ni-NTA beads (Qiagen) and conditioned media, from stimulated S2 cells with serum, was added to the column and allowed to run through. The column was washed with 1 column volume of 20mM IMAC buffer (Appendix 6.6), 1 column volume of 25mM IMAC buffer, and

then weakly bound proteins were washed off the column with 2 column volumes of 50mM IMAC buffer. Bound proteins were eluted with 2 column volumes of increasing concentrations of IMAC buffer (150mM, 250mM and 500mM), with an intermittent wash step of 1 column volume of 20mM IMAC buffer.

A second method involved the addition of the Ni-NTA beads to the stimulated S2 media (with FCS) and incubation on a roller overnight at 4°C. The beads were centrifuged at 3500rpm for 10 minutes and any unbound protein was removed. The unbound protein was washed from the beads twice with 4 column volumes of 50mM IMAC buffer before being loaded into a column. The beads were washed a final time with 4 column volumes of 50mM IMAC buffer before the bound proteins were eluted with 2 column volumes of 150mM IMAC buffer followed by 2 column volumes of 250mM IMAC buffer. Following this 3 column volumes of 500mM IMAC buffer were passed over the column to remove any proteins still bound to the beads. The fractions were analysed by western blotting.

2.4 Results

2.4.1 BPIFA2 Enrichment

2.4.1.1 Formation of Saliva Film

As previously highlighted, proteomic studies have shown that BPIFA2 is a significant component of the dental pellicle. These observations, coupled with experimental data using a similar salivary film and shown in a presentation to our department by an external speaker, suggested that there was an enrichment of a protein corresponding to the molecular weight of BPIFA2. This led us to believe that the use of a saliva film would allow us to collect a sample enriched with native protein. Whole saliva was placed in a small glass dish and a film was naturally allowed to form at room temperature. BPIFA2 was present in this film but we noticed that much of the BPIFA2 remained in the lower portion of the sample (Figure 2-2A). This indicated that although BPIFA2 did contribute to the saliva film, we could not use this as a purification method as too much protein was being lost and there was not sufficient enrichment of the protein for use in downstream functional assays. In addition, the long incubation period at room temperature was not ideal and the presence of a number of contaminant proteins (Figure 2-2B) meant that further purification steps would be needed; this process was not used in further purification attempts.
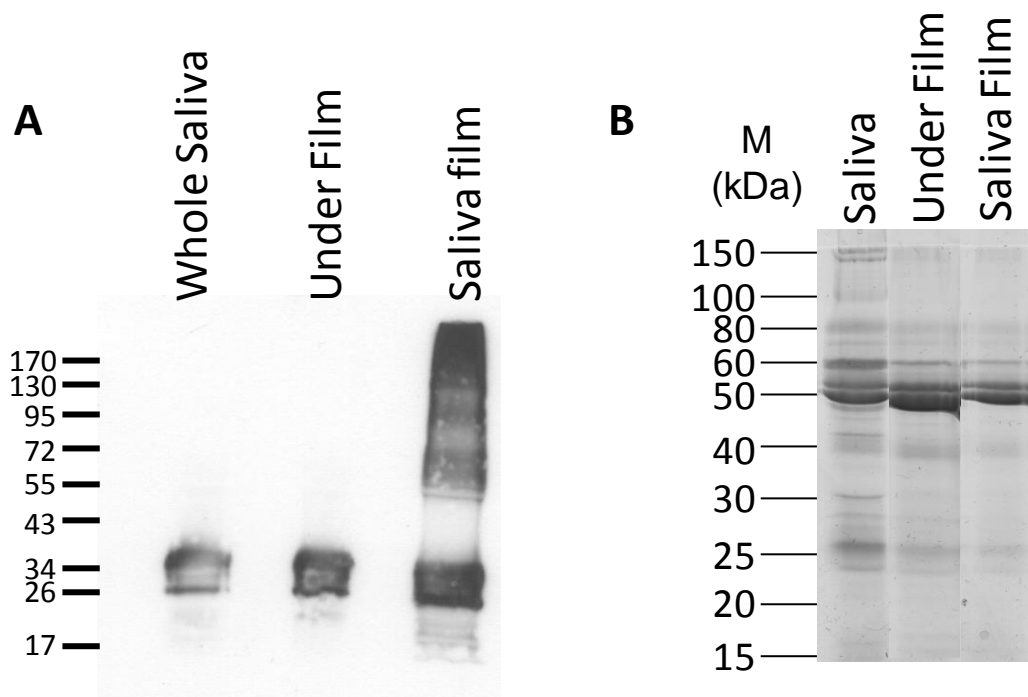
Figure 2-2: Enrichment of BPIFA2 following the formation of a saliva film

2-5mls of saliva was placed into a glass dish and incubated for 30-150 minutes. The film that formed at the air liquid interface was collected, concentrated by Polyethylene glycol or a 10kDa spin concentrator before being analysed by western blot (A) and Instant Blue protein staining (B). BPIFA2 was detected by western blot following concentration of the sample. Although it appeared that the BPIFA2 was enriched in the sample, a large number of contaminant proteins were still present. This method was not used further during the project.

2.4.2   Purification of BPIFA2

2.4.2.1   Ammonium Sulphate Fractionation

Figure 2-3A demonstrates Instant Blue staining of salivary proteins following precipitation with ammonium sulphate. 40-50% ammonium sulphate resulted in significant protein precipitation whilst 70-80% ammonium sulphate resulted in little further precipitation. Western blot analysis of the precipitated proteins for BPIFA2 identified that BPIFA2 precipitated at all of the concentrations of ammonium sulphate (Figure 2-3B).
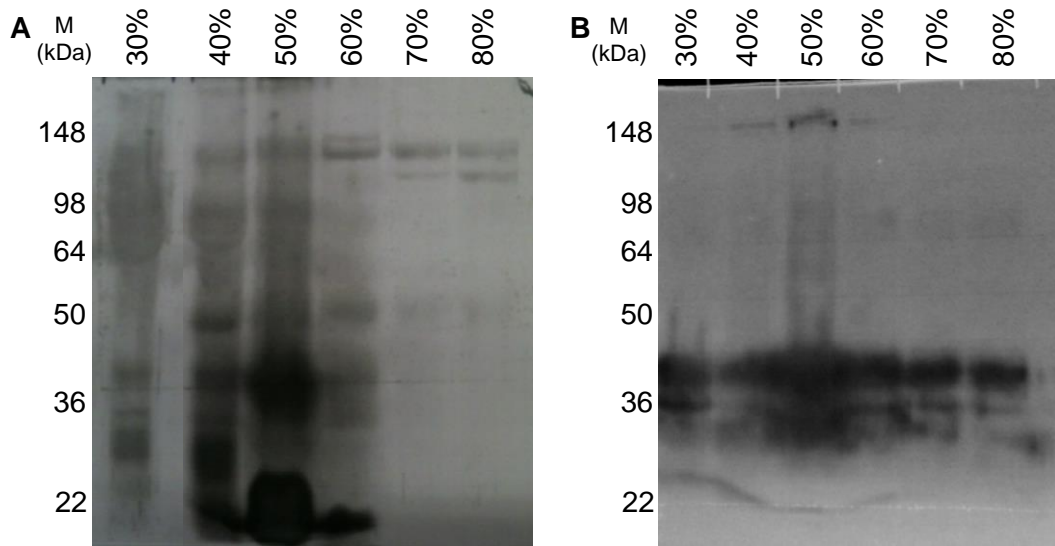
Figure 2-3: Ammonium Sulphate precipitation of BPIFA2 from Saliva

Proteins precipitated by increasing concentrations of ammonium sulphate were analysed by instant blue stained SDS-PAGE gel (A). Precipitation of BPIFA2 was detected by western blot (B). BPIFA2 was seen to be precipitated at every ammonium sulphate concentration.

2.4.2.2 Ion Exchange Chromatography

Approximately half of the salivary proteins in the sample passed through the column without binding and these were washed out prior to the addition of the NaCl buffer. A 280nm absorption peak identified the elution of these proteins between 0mM and 0.2mM NaCl followed by an elution of a higher concentration of proteins at 0.5M NaCl. A small absorption peak was identified following an increase in NaCl concentration to 1.0M. These peaks, corresponding to fractions 2-9, 14-26 and 45-46, were analysed by dot blot for BPIFA2 (Figure 2-4).
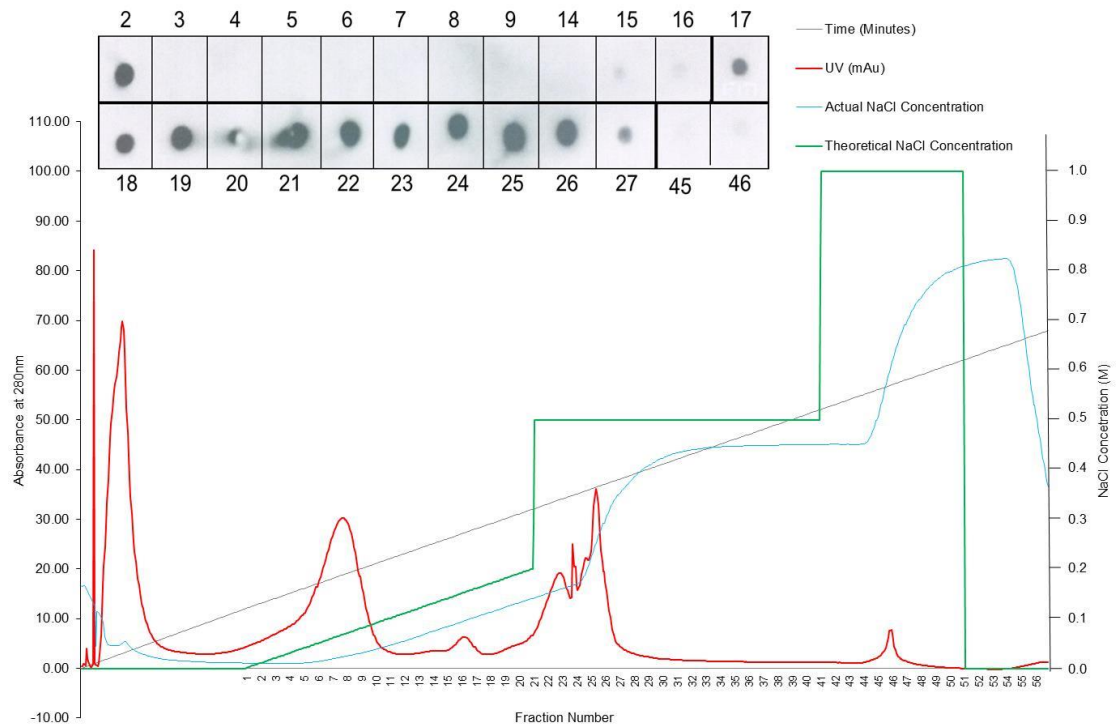
Figure 2-4: Ion exchange chromatography

Peaks identified on the chromatogram corresponding to fractions 2 and 17-27 showed positivity for BPIFA2 following dot blot analysis

Fractions 2 and 17-27 were also positive for BPIFA2. Fraction 17-27 were combined and concentrated by lyophilisation and passed over a second Q-sepharose ion exchange column at a shallower NaCl gradient to further separate any proteins. Protein peaks were identified between fractions 28-32, 38-44 and 61-66. These fractions were analysed by dot blot analysis, which identified fractions 62 and 63 as being positive for BPIFA2. Western blot analysis for BPIFA2 confirmed this positivity (Figure 2-5).
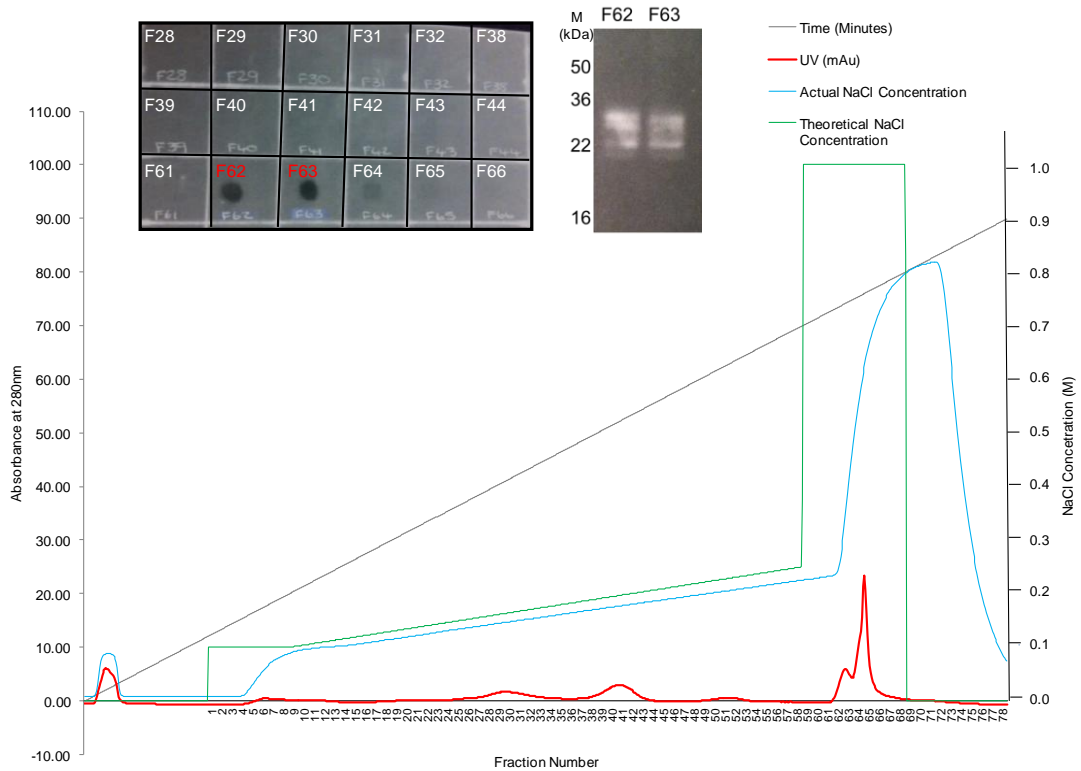
Figure 2-5: Ion exchange chromatography

Fractions 17-27 were combined and concentrated; proteins in the sample were further separated on a second ion exchange Q-sepharose column. Protein peaks identified on the chromatogram corresponding to fractions 28-32, 38-44 and 62-66 were analysed by dot blot analysis. Fractions 62 and 63 showed positivity for BPIFA2, which was confirmed by western blot analysis. Chemiluminescent activity was collected over 5 minutes using the G:box, gel documentation and analysis system (Syngene, Cambridge, UK).

2.4.2.3 Ethanol Precipitation (Campos et al, 2004)

Some BPIFA2 present in whole saliva was solubilised in 75% Ethanol and precipitated proteins were removed by centrifugation. Most proteins present in whole saliva were successfully precipitated and removed as contaminants, however, in addition to BPIFA2, a number of salivary proteins between approximately 10 and 15kDa remained soluble in the 75% ethanol. Disappointingly some of the BPIFA2 was insoluble in 75% ethanol and was lost in the ethanol pellet (Figure 2-6).
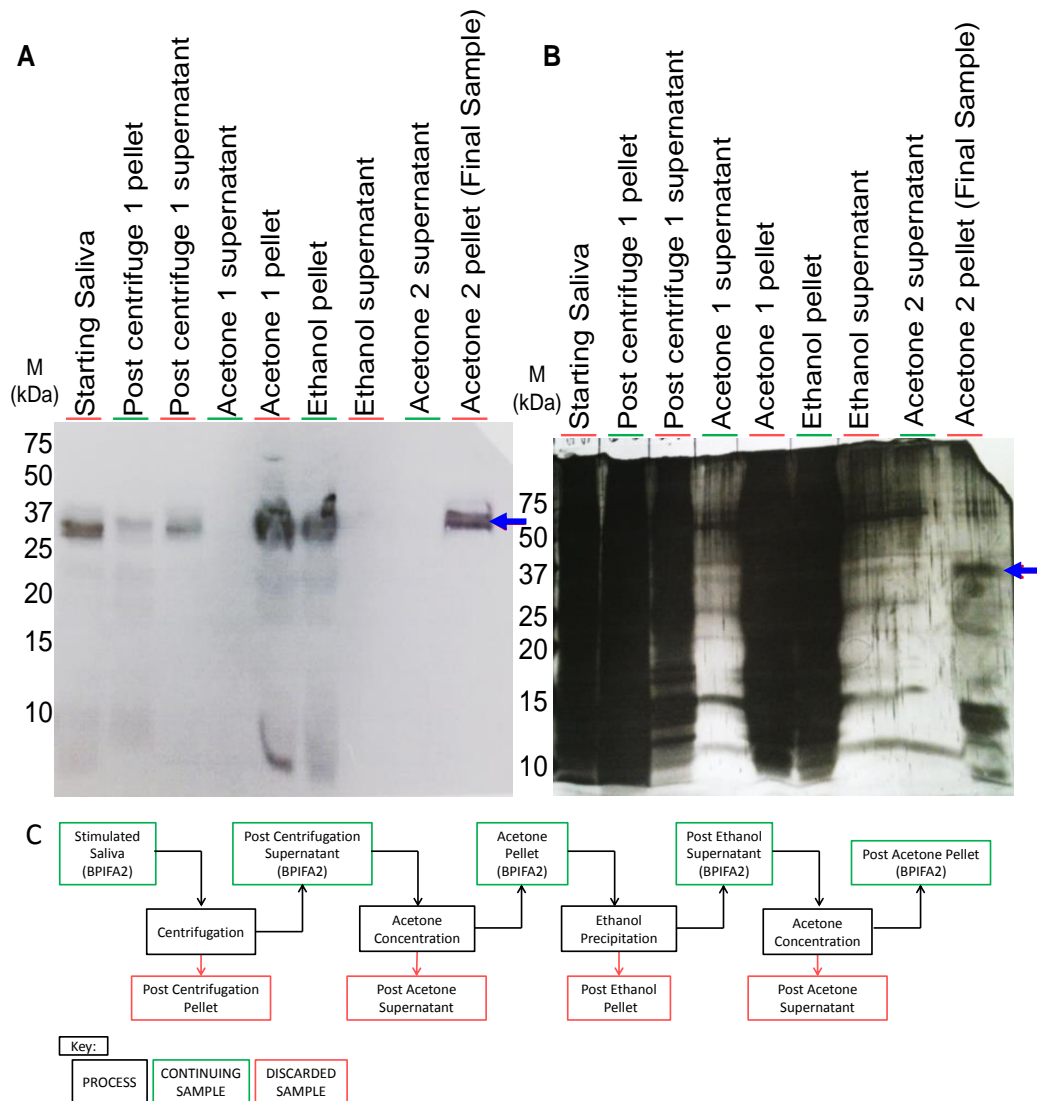
Figure 2-6: Ethanol precipitation of native BPIFA2 from whole saliva

The western blot (A) identified that some BPIFA2 was lost following the initial centrifugation to remove debris from the saliva (Post centrifuge 1 pellet); all BPIFA2 present in the sample was successfully precipitated by acetone. Much of the BPIFA2 was lost following solubilisation with ethanol. The western blot showed that BPIFA2 was absent from the 75% ethanol. The final acetone precipitation led to the concentration of BPIFA2 remaining in the sample, allowing it to be detected by western blot analysis. Silverstain analysis (B) identified that ethanol precipitation reduced the number of salivary proteins in the final sample however purification of BPIFA2 was unsuccessful. Blue arrows highlight the position of BPIFA2. Figure demonstrates representative results from a number of preparations (in excess of 15). A schematic of the Ethanol Precipitation procedure is included for clarity (C). Samples for SDS-PAGE were taken at the points highlighted in green and red. Red arrows indicate where the resulting sample was removed from the procedure and black arrows indicate the progression to the next stage.

2.4.2.4 Size Exclusion Chromatography

BPIFA2 partially purified by ethanol precipitation was further purified using a size exclusion column. Protein peaks were recorded between fractions 7 and 28 (Figure 2-7), these fractions were analysed by dot blot analysis. No BPIFA2 was detected following dot blot analysis, possibly due to the dilution of the sample, from 2mls to 3.5mls per fraction; however concentration of the sample failed to locate any BPIFA2 in the fractions.

Fraction: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
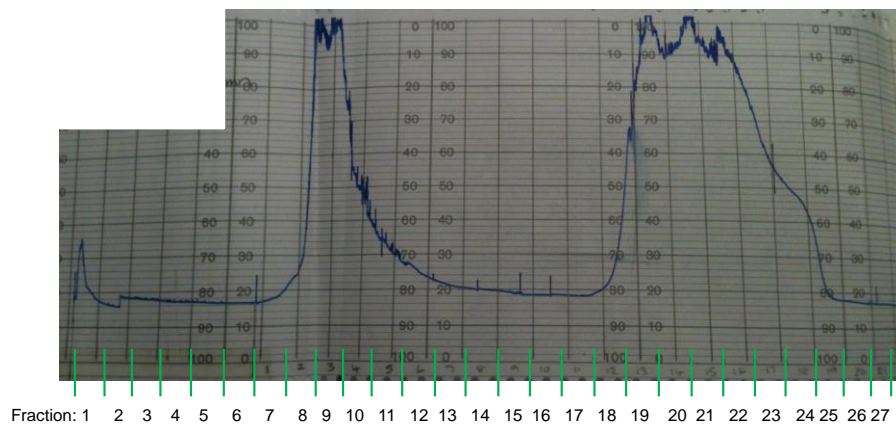
Figure 2-7: Size exclusion chromatography of BPIFA2 purified by the Ethanol Precipitation method.

Peaks were detected between fractions 7-12 and 18-27 and these fractions were analysed by dot blot for presence of BPIFA2, none of the fractions positively detected BPIFA2.

2.4.3   Native Gel Electrophoresis Systems

2.4.3.1   Protein Elution

Salivary proteins were separated using large native polyacrylamide gel electrophoresis (Figure 2-8) before being eluted into 30 small fractions.
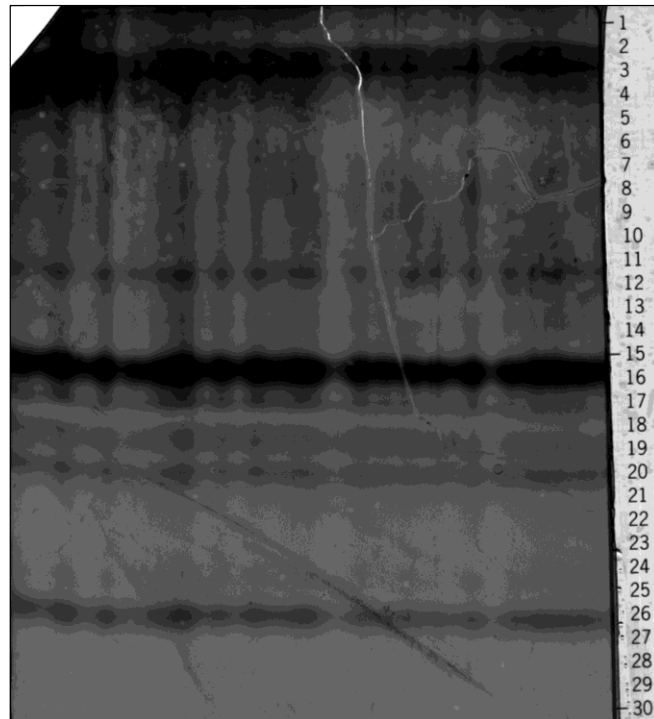
Figure 2-8: Coomassie stained native polyacrylamide gel of salivary proteins before electroelution.

Salivary proteins were separated by native polyacrylamide gel electrophoresis, before being electro-eluted into 30 different fractions of tris-glycine buffer. The gel demonstrates the separation of the salivary proteins throughout the gel.

These fractions were analysed by dot blot and the BPIFA2 positive fractions were collected and combined. The original method identified a contaminant band of around 50kDa, this was later identified as α-amylase. Dot blot analysis of each of the fractions for BPIFA2 and α-amylase identified that not all of the BPIFA2 positive

fractions were contaminated with α-amylase and so by discarding the α-amylase-containing fractions, pure BPIFA2 could be collected.

Western blot analysis identified that BPIFA2 fractions could be consistently collected without α-amylase contamination (Figure 2-9A). Further preparations identified that this method consistently produced pure BPIFA2. Instant Blue protein staining confirmed that BPIFA2 was successfully purified from whole saliva using this method (Figure 2-9B).
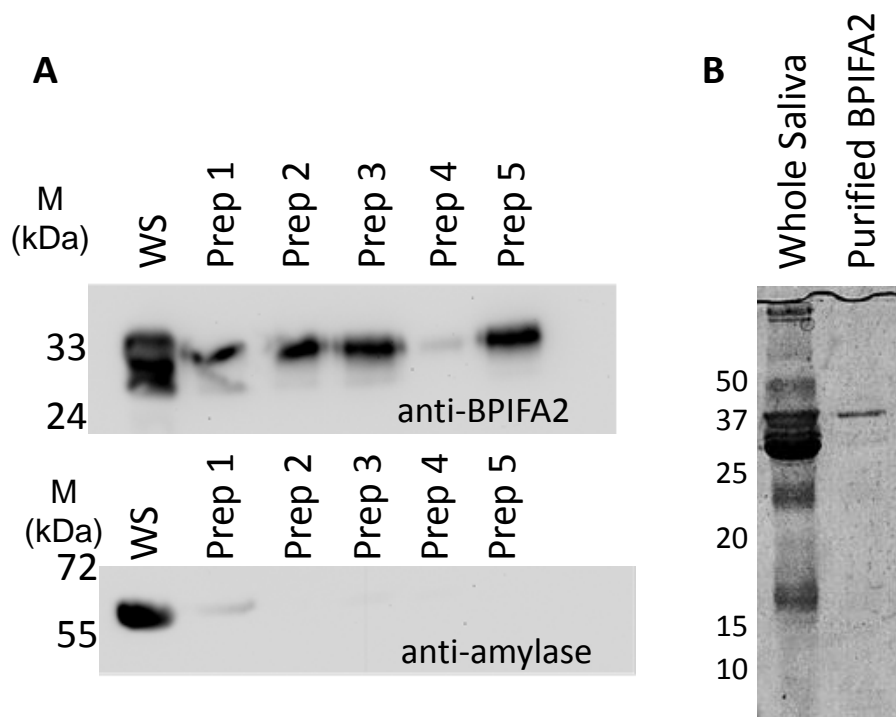
Figure 2-9: Purified nBPIFA2 by protein elution from a large Bio-Rad native polyacrylamide gel electrophoresis

Initial purification attempts highlighted that BPIFA2 samples were contaminated with α-amylase. Consistantly successful purification of BPIFA2 was achieved by the seperation of nBPIFA2 positive; α-amylase negative fractions (A: Prep 2-5) from BPIFA positive; α-amylase positive fractions (A: Prep 1). Whole saliva (WS) was used as a positive control. Purity was assessed using Instant Blue protein staining (B) which demonstrated the purity of the BPIFA2 sample. Preparations shown are representative of the many performed (>20).

2.4.4   Recombinant BPIFA2

2.4.4.1   S2-Cell expressed BPIFA2

Expression of rBPIFA2 in S2 cells was achieved by stimulating transfected cells with

500 µM copper sulphate in the presence and absence of FCS. For further

purification of BPIFA2 by Ni-NTA beads, expression of BPIFA2 had to be performed

in the presence of FCS as serum free media stripped the nickel from the column. For

the media which was not purified by the Ni-NTA beads, BPIFA2 was expressed into

serum-free media to reduce the number of mammalian proteins present in the

sample. Cells that were stimulated in the presence of FCS expressed detectable

concentrations following 24 hour stimulation (Figure 2-10A), however in the

absence of FCS detectable levels of rBPIFA2 were only achieved after a 15 day

stimulation (Figure 2-10B).
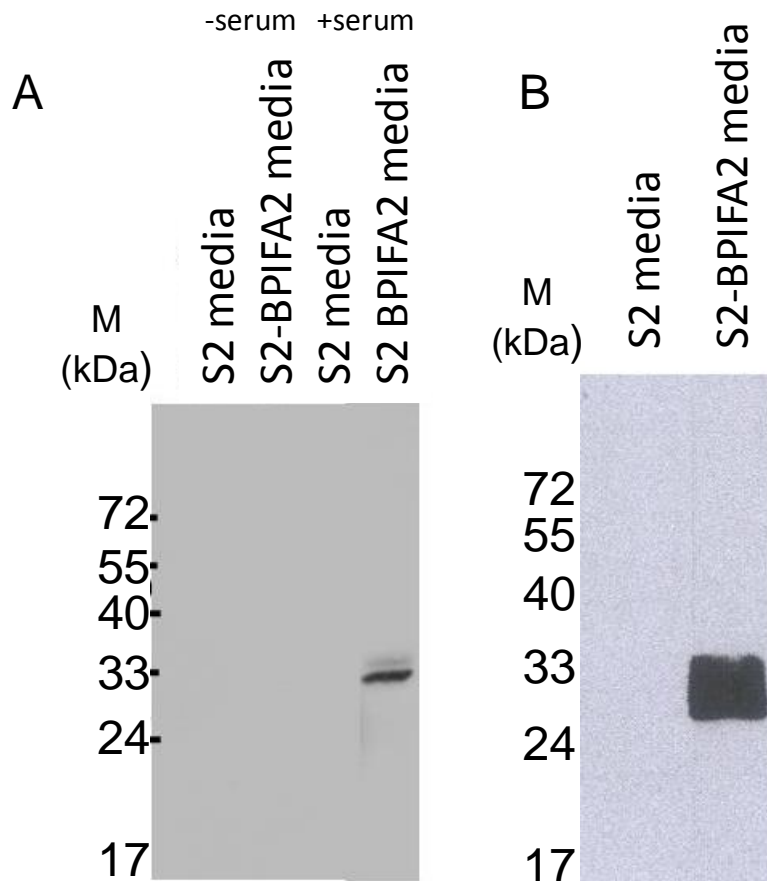
Figure 2-10: rBPIFA2 expression in Drosophila Schneider (S2) cells

Stimulation of the BPIFA2 transfected S2 cells in the presence of serum showed detectable levels of BPIFA2 after 24 hours (A), in the absence of serum a 15 day stimulation was required before levels were high enough to be detected (B). S2 cells not transfected with BPIFA2 did not show any positivity for BPIFA2 on stimulation.

2.4.4.2 NI-NTA bead BPIFA2 purification

Purification of rBPIFA2 from S2 cell expression was attempted using Ni-NTA agarose

beads. The 6xHIS tag on the rBPIFA2 allowed for the purification using this method.

Original attempts at purification by adding the stimulated media directly to a

packed column led to the elution of rBPIFA2 protein at all of the wash stages (Figure

2-11), indicating that the concentration of rBPIFA2 protein was overloading the

column and insufficient washing was taking place.
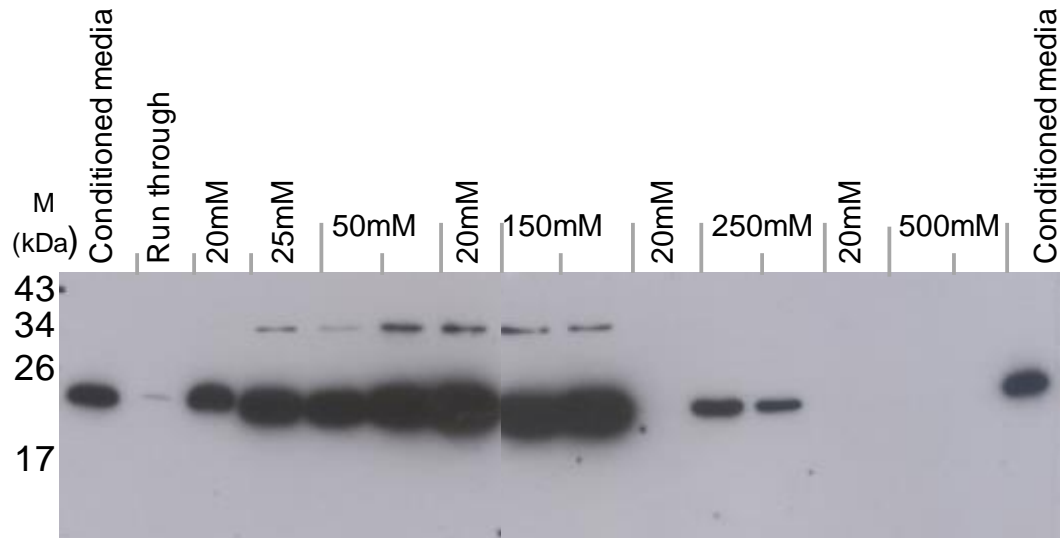
Figure 2-11: Purification by sequential elution of rBPIFA2 from Ni-NTA beads

Passing conditioned media over a packed Ni-NTA column led to the elution of rBPIFA2 at every concentration of IMAC Buffer (samples of sequential fractions subjected to western blotting). Blot is representative of a number of repeated attempts (>5).

It was decided that the stimulated media should be batch adsorbed to the beads by placing them in a glass bijou bottle on a roller at 4°C overnight, and washing thoroughly by centrifugation before being packed into the column. This led to a much improved purification method with rBPIFA2 elution taking place at 150mM IMAC buffer (Figure 2-12).
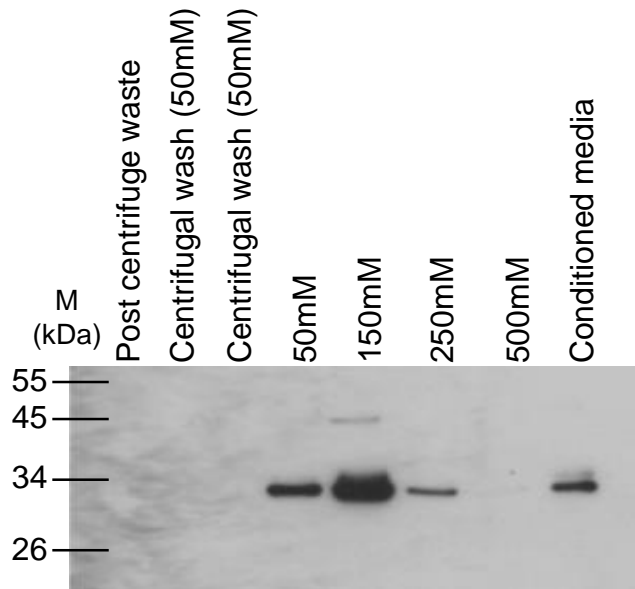
Figure 2-12: Purification of rBPIFA2 with Ni-NTA beads following an overnight incubation and centrifugal washing step

Incubating Ni-NTA beads with the conditioned media of BPIFA2 transfected S2 cells and washing via centrifugation before packing the beads into a column lead to improved protein elution when compared to traditional column methods. rBPIFA2 eluted at 150mM IMAC buffer. (Samples of sequential fractions subjected to western blotting) Blot is representative of a number of repeated attempts (>5).

2.5 Discussion

The presence of BPIFA2 in saliva allows for its collection in a rapid and simple way. However, the presence of a number of other proteins in saliva, particularly antimicrobial proteins, means that BPIFA2 must be purified before it can be used in functional studies. The aim of this study was to assess the currently utilised methods of purification and, if necessary, develop an alternative method in order to produce a pure form of BPIFA2.

The use of pure BPIFA2 protein is beneficial as it removes the risk of contaminating proteins interfering with any later functional studies. Also, purification of native BPIF proteins is the only way to ensure true post-translational modifications of the protein, such as glycosylation, are present. Several studies have described the purification of BPIF proteins from natural sources (Abdolhosseini et al., 2012, Geetha et al., 2003, McGillivary and Bakaletz, 2010, Campos et al., 2004), utilising a number of methods, including immuno-affinity chromatography (Geetha et al., 2003), ethanol precipitation (Abdolhosseini et al., 2012, Campos et al., 2004) and electroelution from an SDS-PAGE gel coupled with column chromatography and re-naturation of the BPIFA1 (McGillivary and Bakaletz, 2010).

The immuno-affinity chromatography method of purification appears to be quite successful in some laboratories, with the purification of native BPIFA2 from human saliva. In this study, ion exchange chromatography was used to purify BPIFA2 from whole saliva. Ion-exchange chromatography was also used by Beeley, et al (1986) to purify a protein, latherin (BPIFA4), from horse sweat. Beeley et al (1986) demonstrated successful purification of latherin using ion exchange

chromatography following gel filtration. Although BPIFA2 was collected into just 2 fractions following two ion exchange runs, there was a concern that a significant proportion of BPIFA2 protein would be lost from such a long process leading to the need for a large sample size.

Abdolhosseini et al (2012) and Campos et al (2004) reported that BPIFA2 and BPIFA1 were soluble in ethanol whilst other contaminating proteins were not and thus had used this as a means of purifying the proteins from whole saliva and from airway lining fluid respectively. We adapted the methods of these published studies and out early results suggested this would indeed be a suitable purification method as protein staining indicated out BPIFA2 preparation was free of contaminating proteins. Unfortunately, closer examination of the SDS-PAGE gel using a silver staining method highlighted the presence of small contaminating proteins (10-15kDa), similar to the contaminants identified by Abdolhosseini et al (2012). As previously mentioned, these proteins could be antibacterial proteins, cystatin, histatin, statherin and/or lysozyme. N-terminal sequencing did in fact identify cyctatin in our preparation (unpublished data from BSc Dissertation). In addition, following western blot analysis of the ethanol precipitation fractions it was evident that much of the BPIFA2 protein was lost in the ethanol precipitate pellet. The use of size exclusion chromatography in order to remove the contaminant proteins lead to significant reduction in the concentration of BPIFA2 protein to undetectable levels. Concentration of the fractions did not aid in the detection of BPIFA2 in the fractions, this could have been due to loss of the protein through interactions with the stationary phase of the column, if this was the case a high concentration of

BPIFA2, and so a very large starting volume, would be required with no guarantee of pure, functional BPIFA2 protein. It was believed that BPIFA2 could be enriched by the formation of a saliva film at an air-liquid interface; this method of enrichment of BPIFA2 had not been described previously. It was hoped that the enrichment of BPIFA2 would result in the removal of the small contaminating proteins prior to ethanol precipitation. Initial results indicated that these small proteins had been successfully removed; however, trace amounts were present which became apparent on further concentration. In addition to these problems, proteins have been shown to denature in the presence of ethanol at temperatures of above 0°C (Zellner et al., 2005), also human serum albumin has been reported to change structurally following incubation with ethanol at concentrations as low as 10%, which becomes more pronounced as incubation time increases (Lin et al., 2004). Therefore the use of 75% ethanol to purify BPIFA2 from saliva and the periods where BPIFA2 is held at 4°C, provide ample opportunity for conformational change and even denaturation of the BPIFA2 protein and may render the purified BPIFA2 non-functional. For these reasons formation of a saliva film followed by ethanol purification was concluded not to be a suitable method for purification.

Ammonium sulphate fractionation had not previously been described as a method to purify BPIF proteins; however it is a common method for the initial enrichment of many other proteins. Unfortunately BPIFA2 failed to partition into a particular fraction or fractions of ammonium sulphate.

The most successful method of BPIFA2 purification from whole saliva involved the separation of salivary protein using native polyacrylamide gel electrophoresis,

coupled with electro-elution. Previous attempts to purify BPIFA1 from cultured primary chinchilla nasopharynx cell secretions involved separation of proteins by SDS-PAGE in the absence of β-mercaptoethanol, excision of bands, electro-elution, dialysis and lyophilisation. The resuspended sample was then passed through a detergent removal column, denatured and renatured using increasing concentrations of urea. The re-natured protein was then further dialysed; lyophilised and resuspended before being subject to function assays. This method, although reported to be successful, contains a number of opportunities for the incorrect folding, permanent denaturation and/or loss of the protein. It was the aim of this study to develop a method with a minimal number of these opportunities. Therefore, purification under native conditions was attempted. Initial attempts involving the pooling of all BPIFA2 positive fractions lead to the co-purification of BPIFA2 and amylase from the gel, but by identifying the BPIFA2 fractions that lacked contaminating amylase and discarding those, pure BPIFA2 could be collected. The purified BPIFA2 required dialysis to reduce the salt concentration prior to functional analysis but no further process was required, reducing the chance of denaturation or deformation of the final protein, unlike the method described by McGillivary and Bakaletz (2010).

Quantification of the native purified BPIFA2 was attempted, using a BioRad protein quantification kit and using western blot and densitometry to compare with a commercial BPIFA2 sample, however, results were highly variable (results not shown). As native concentrations of BPIFA2 in saliva have not yet been determined, and as the functional investigations were in an early stage of development, it was

decided that the importance would be placed upon the collection of as much purified protein as possible for use in functional assays. Thus the absolute concentration and/or yield of protein from any given sample were not a priority. Future development of the project, including yield of the purified protein and a more restricted functional assessment is planned based on the findings of this project.

A novel observation in this project is the interaction between BPIFA2 and laboratory plastics. A number of purification methods failed to result in detectable levels of BPIFA2 due to this interaction. BPIFA2 has shown itself to be a 'sticky' protein, strongly adhering to many surfaces, which may indicate some functional significance.

We intended to use purified recombinant BPIFA2, produced in S2 cells for functional comparison. Other groups have described recombinant BPIF protein production in E. coli and although the use of E. coli would have allowed for rapid and low cost production of BPIFA2 it was thought that the use of bacteria to produce a potentially antibacterial protein may lead to production problems or issues with functional studies, in addition to the absence of eukaryotic glycosylation. Consequently an attempt was made to stably transfect the BPIFA2 gene into mammalian CHO cells to allow fully glycosylated protein production, however yields from these cells were almost undetectable, resulting in insufficient protein for future studies (results not shown). S2 cells, cultured at room temperature, without CO_2, are able to produce glycosylated protein, which allowed for BPIFA2 to be produced close to its native form. Previous expression levels were

reported to be up to 22mg/L (Johanson et al., 1995) making this expression system preferential over bacterial and other mammalian systems. For the purification of recombinant BPIFA2 from the culture medium by Ni-NTA beads, the cells were stimulated in the presence of FCS. It was reported in the Drosophila expression system literature that the absence of FCS caused the nickel ion to strip from the beads, therefore expression of proteins using this system and purification on Ni-NTA beads in the absence of FCS would have been unsuccessful. FCS was required in order to successfully purify the rBPIFA2. As previously mentioned, in the presence of FCS the S2 cells secreted detectable levels of BPIFA2 protein after just 24 hours, however the use of FCS introduced a number of contaminating proteins to the conditioned media. Furthermore, purification of the BPIFA2 protein was successful when desorbing with 150mM IMAC buffer, however, unfortunately residual imidazole, even at very low concentrations caused significant killing of a number of bacteria in initial assays (results not shown). It was decided that, even with extensive dialysis to remove the imidazole, there was a high chance that any bacterial killing seen could be due to the presence of imidazole confounding interpretation of a function for the BPIFA2 protein. Commercial rBPIFA2 was considered, however, it was deemed too expensive for the volumes required in our functional assays. It was decided therefore, that the conditioned media, in the absence of FCS would be used in further functional assays comparing with conditioned media from untransfected S2 cells as a control. The major disadvantages of this were the 15 day culture required to achieve detectable levels of BPIFA2 in the media and the presence of numerous contaminant proteins.

A suitable method of purification of the recombinant BPIFA2 would need to be developed, maybe similar to that identified for native BPIFA2, which does not lead to problems with later functional analysis. This would then allow true comparisons to be made between rBPIFA2 and purified nBPIFA2.

A number of methods have been assessed in order to successfully purify native BPIFA2 from whole saliva including ammonium sulphate fractionation, ethanol precipitation, ion exchange and size exclusion chromatography and native gel electrophoresis with protein elution. The main failing of many of the methods, including column chromatography and ethanol precipitation, was the repeated loss of the protein to undetectable levels, indicating that an extremely large starting volume would be required in order to purify sufficient protein for functional assays. The most successful method was native gel electrophoresis with protein elution. This method, although initially resulting in amylase contamination, resulted in detectable pure nBPIFA2 and unlike many other methods attempted required no chemicals that could result in conformational change or denaturation of the protein. This method is novel in the BPIF protein field and demonstrates a simple but effective method for native BPIFA2 purification.

Chapter 3.   Functional Analysis of BPIFA2

3.1   Introduction

Since the discovery of the first BPIF protein, BPIFA1 in 1999, the function of the BPIF family of proteins has remained a mystery. Many theories have been suggested; however no conclusive proof has yet been published.

The *BPIFA1* gene was initially identified in the developing palate of the murine embryo, with a defined expression pattern evident around the time of palatal shelf elevation and fusion. Although this expression was noted to occur during development it was not believed to play a role in these stages of morphogenesis of the murine palate, and was suggested to play a more regulatory role in the development of the nasal collumella, sinus cavities and common nasal passage. Expression analysis of the adult mouse identified continued expression of the BPIFA1 gene in the trachea and bronchial passages (Weston et al., 1999) suggesting that the function of this protein extends further than regulation of morphogenesis during gestation. The diminished expression of the BPIFA1 gene in the distal regions of the bronchial passages strongly indicates that the BPIFA1 protein is unlikely to function in gas exchange in the alveolus (Weston et al., 1999, Bingle and Bingle, 2000), however since the initial discovery this has been a frequently suggested function. It has recently been proposed that one of the functions of the BPIF proteins is to act in a surfactant-like manner by reducing surface tension following studies with recombinant human BPIFA1 protein (Bartlett et al., 2011, Gakhar et al., 2010) and recombinant Latherin; an equine member of the human BPIF family expressed in the saliva and sweat (McDonald et al., 2009). However, this study

failed to explain the abundance of Latherin in saliva, since surfactant activity is not as important in the upper airway and mouth as it is in the lungs. What is more likely is their second suggestion, that Latherin, and so other members of the BPIF protein family, affect the ability of bacteria to attach to host cells either via coating the host epithelium or by inhibiting bacterial surface proteins (McDonald et al., 2009).

As previously mentioned, BPIF proteins are relatives of the innate immune protein family containing BPI and LBP, whose primary role is to bind to bacterial LPS to increase or hinder the immune response. The predicted structural similarity between the BPIF proteins, BPI and LBP further strengthens the initial suggestion of BPIF-bacterial interaction being a primary function of the BPIF family. BPI and LBP bind to bacterial LPS via the functional domain predicted to be present in each of the BPIF proteins, leading to the belief that BPIF proteins also have the ability to bind to bacterial LPS. Different techniques have been utilised in an attempt to demonstrate the ability of BPIF protein binding to bacterial LPS; ethanol precipitated BPIF proteins from human and bovine saliva failed to show binding to LPS (Geetha et al., 2003, Campos et al., 2004, Haigh et al., 2008), however following purification of human BPIFA2 by the same ethanol precipitation technique, Abdolhosseini et al (2012) identified binding to sepharose beads coated with *P. aeruginosa* LPS, which was further confirmed using recombinant BPIFA2. Other methods include the adsorption of LPS to polystyrene tubes followed by incubation with BPIFA1 in whole nasal lavage fluid (Ghafouri et al., 2004), which identified the binding of BPIFA1 to *E. coli* LPS and agglutination and adhesion techniques with synthetic peptides derived from BPIFA2 (Geetha et al., 2005). These peptides

showed positive binding to *P. aeruginosa* and *E. coli* LPS. Geetha (2005) also demonstrated that pre-incubation of bacterial LPS with these BPIFA2 peptides lead to an inhibition of the binding of LPS to LBP. Later studies identified that the presence of the synthetic peptides leads to an inhibition of *P. aeruginosa* adhesion (biofilm) (Gorr et al., 2008), as did a study involving recombinant BPIF protein (Gakhar et al., 2010).

In addition to bacterial binding, various other functions have been studied including growth inhibition, bacterial killing and agglutination, where varied responses have been reported. Contrasting data has been published regarding the function of BPIF proteins, some groups report that BPIF proteins inhibit the growth of bacteria such as *Mycoplasma pneumoniae* (recombinant mouse BPIFA1) and *P. aeruginosa* (purified native and recombinant BPIFA2A and BPIFA2B) (Haigh et al., 2008, Chu et al., 2007) though published work using BPIFA2 peptides showed no inhibition (Gorr et al., 2008). Similarly, the data surrounding the bactericidal function of BPIF proteins is not in agreement; purified chinchilla BPIFA1 and recombinant BPIFA2 protein reduced the viability of *Haemophilus influenzae* and *P. aeruginosa* respectively (McGillivary and Bakaletz, 2010, Geetha et al., 2005, Geetha et al., 2003). This is, however, disputed by other workers, who have reported no bactericidal activity with recombinant hamster BPIFA2, recombinant human BPIFA1 or BPIFA2 peptides (Bartlett et al., 2008, Khovidhunkit et al., 2005, Gorr et al., 2008).

3.2   Hypothesis and Aim

Hypothesis

The predicted structure of BPIF proteins identifies similarities with both BPI and LBP, which are involved in the innate immune response. We hypothesise that the function of BPIFA2 is related to the innate immune response, either directly, by binding to bacteria and increasing or inhibiting the inflammatory responses, or indirectly by preventing bacterial adhesion and growth.

Aim

As the research surrounding BPIFA function is quite contradictory there is no consensus regarding the function. Many studies have involved the use of *P. aeruginosa* but few studies have described the effect of BPIFA2 on other bacteria. The aim of the work reported in this chapter was to examine a variety of functions of BPIFA2, including: bacterial binding ability; inhibition of growth; bacterial killing and agglutination against a number of different bacteria including oral commensal bacteria (*S. gordonii* and *S. mutans*), oral pathogens (*P. gingivalis* and *T. forsythia*), non-oral commensal bacteria (*P. aeruginosa* and *E. coli*), a respiratory commensal organism (*S. aureus*) and a respiratory pathogen (β-haemolytic streptococcus).

3.3   Materials and Methods

3.3.1   Bacteria

Table 3.1: Bacterial strains

Bacteria (strain)	Mutation	Antibiotic Resistance	Origin
Streptococcus gordonii (Challis)	-	-	Lyophilised stocks within the School of Clinical Dentistry, Sheffield, UK
Streptococcus mutans (Ingbritt)	-	-	
Staphylococcus aureus (Oxford)	-	-	
Escherichia coli (U125643)	-	-	
Porphyromonas gingivalis(W50)	-	-	
B-haemolytic streptococcus (Clinical strain)	-	-	Hallamshire Hospital Microbiology Diagnostic Service, Sheffield, UK
Pseudomonas aeruginosa (Clinical strain)	-	-	
Haemophilus influenza (Clinical strain)	-	-	
Porphyromonas gingivalis mutant (E8)	$\Delta rgp:ArgpB$ (parent strain W50)	10µg ml^{-1} erythromycin	M. Curtis, Barts and The London School of Medicine and Dentistry, London UK
Porphyromonas gingivalis mutant (K1A)	Δkgp (parent strain W50)	10µg ml^{-1} erythromycin	
Porphyromonas gingivalis mutant (EK18)	$\Delta rgpA:rgpB$ Δkgp (parent strain W50)	10µg ml^{-1} erythromycin 20µg ml^{-1} chloramphenicol	J. Higham University of Sheffield, UK
Tanerella forsythia (ATCC 43037)	-	-	S. Roy University of Sheffield

3.3.2   Culture of Bacteria

All growth media was prepared according to the manufacturer's instructions using distilled water and sterilised by autoclaving at 15 psi (121°C) for 15 minutes on liquid cycle.

S. gordonii, S. pyogenes and *S. mutans* were maintained on Columbia blood agar base (Oxoid, Hampshire, UK) supplemented with 5% (v/v) horse blood (Oxoid, Hampshire, UK) (referred to from here as BA) at 37°C in CO_2 (5% CO_2/95% air). Liquid cultures were grown overnight in Brain Heart Infusion (BHI) broth (Oxoid, Hampshire, UK) supplemented with Yeast Extract (5mg ml^{-1}) (Oxoid, Hampshire, UK) in CO_2 (5% CO_2/95% air) at 37°C.

S. aureus, P. aeruginosa and *E. coli* were maintained on BA at 37°C in aerobic conditions. Liquid cultures were grown overnight aerobically at 37°C in BHI broth supplemented with Yeast Extract (5mg ml^{-1}).

P. gingivalis strains were maintained on Fastidious anaerobe (FA) agar (LabM Limited, Lancashire, UK) containing 5% horse blood, anaerobically (80% N_2, 10% H_2, 10% CO_2) (miniMACS Anaerobic Workstation, Don Whitley Scientific, UK) for 48 hours. Liquid cultures were grown anaerobically in BHI broth supplemented with Yeast Extract (5mg ml^{-1}), Haemin (5µg ml^{-1}) (Sigma, UK), Cysteine-hydrochloride (0.5mg ml^{-1}) (ICN Biomedicals Inc, Basingstoke, UK) and Vitamin K (1µg ml^{-1}) (Sigma, UK) overnight. Mutant strains of *P. gingivalis* were grown in these conditions with the addition of the required antibiotics (Table 3.1).

T. forsythia was maintained on FA agar supplemented with 5% horse blood, NAM

(0.17mM) (Sigma, UK) and gentamicin (15µg ml^{-1}) (Sigma, UK). Liquid stocks were

grown in tryptic soy broth (TSB) supplemented with Yeast Extract (5mg ml^{-1}),

Haemin (1mg ml^{-1}), Vitamin K (1mg ml^{-1}), *N*-acetylmuramic acid (NAM) (1µg ml^{-1})

and gentamicin (15µg ml^{-1})

For use in all assays, all bacteria were grown in liquid cultures overnight.

3.3.3 Treatments

Table 3.2: Functional Study treatments

Treatment	Origin (See Chapter 2. Purification of BPIFA2)
Native (n)BPIFA2	Native gel elution
Native Elution buffer (control)	See appendix. (6.5 - Protein Purification)
Ethanol precipitated native BPIFA2	Ethanol Precipitation
Native (Enriched) BPIF	Film enrichment
Saliva	Healthy volunteer
Recombinant (r)BPIFA2	BPIFA2 transfected S2 cell conditioned media
S2 cell conditioned media (control)	Untransfected S2 cell conditioned media
PBS	-
dH$_2$O	-

3.3.4 BPIFA2 binding to bacteria

The A$_{(600)}$ of the overnight culture of *P. aeruginosa, E. coli, P. gingivalis* (W50, E8,

K1A)*, S. gordonii, S. mutans, T. forsythia* and a β-haemolytic streptococcus was

measured and 1ml of bacteria with OD1 was removed per treatment. The bacteria

were washed in 1ml PBS twice by centrifugation (13,000rpm for 5 minutes). The

bacteria were incubated in 50µl of saliva, purified nBPIFA2 or PBS for 1 hour at

37°C. The bacteria were centrifuged (13,000rpm for 5 minutes) and the supernatant

removed. The bacteria were washed with 1 ml PBS by centrifugation before being

transferred to a new microfuge tube. The bacteria were washed twice before SDS

lysis buffer was added and the bacteria boiled at 95°C for 5 minutes.

Binding of BPIFA2 to the bacteria was established by western blot analysis (2.3.3).

3.3.5   Growth Curve

Cuvettes were sterilised by submersion in 100% ethanol overnight followed by a wash with sterile water and air drying in sterile conditions.

The $A_{(600)}$ of the overnight culture was measured and a bacterial suspension of OD0.1 in 2 x BHI was added to a sterile cuvette containing an equal volume of either filter sterilised purified nBPIFA2 or sterile dH_2O. The cuvette was sealed with parafilm before being secured within a 25mL tube with sponges. Bacteria were incubated as described in 3.3.2 above for 60 minutes on a spyra-mixer before the bacteria were pipetted and the $A_{(600)}$ was recorded. Readings were taken every 60 minutes until the bacteria reached stationary growth phase.

3.3.6   Bacterial Killing

3.3.6.1   Paper Discs

A sterile cotton bud was soaked in an overnight culture of each bacterium and streaked onto a suitable agar plate (3.3.2) in a minimum of three different directions. Discs of filter paper (5mm diameter) were sterilised by autoclaving, dried, then soaked in either rBPIFA2, purified nBPIFA2, saliva, PBS or Spectinomycin (50mg ml^{-1}) and then placed onto the streaked plate. The bacteria were incubated overnight as described in 3.3.2 and any zone of inhibition measured.

3.3.6.2   Direct

A sterile cotton bud was soaked in an overnight culture of each bacterium and streaked onto a suitable agar plate (3.3.2) in a minimum of 3 different directions.

5µl of each treatment (rBPIFA2, purified nBPIFA2, Saliva, PBS or Spectinomycin (50mg ml^{-1}) was dotted onto each lawn. Bacteria were incubated as in 3.3.2 overnight and the zone of clearance was measured.

3.3.7   Bacterial Killing 2

The A$_{(600)}$ of the overnight culture was measured and bacteria were added to either purified nBPIFA2, PBS or Mutanolysin (10µg ml^{-1}) at an OD of 0.05. These were incubated in as in 3.3.2 for 3 hours before being serially diluted 1:10 and 5µl of this dilution dotted onto agar plates in triplicate. The plates were incubated overnight as in 3.3.2 and any colonies formed were counted.

3.3.8   Agglutination

<u>Set up</u>

S. mutans, S. gordonii, P. aeruginosa, S. aureus, E. coli, P. gingivalis (W50, E8, K1A and EK18) and β-haemolytic streptococcus were cultured overnight, as described in 3.3.2, in liquid cultures, counted and adjusted to 2x10^9 ml^{-1}. 1mL of this adjusted suspension was washed in PBS by centrifugation (13,000rpm for 5 minutes). The bacteria were resuspended in 1.5mls PBS containing 2µL fluorescein isothiocyanate 1 (FITC) to fluorescently label the bacteria and incubated, in the dark, for 15 minutes at 4°C with gentle agitation. Following incubation the bacteria were washed four times with 1.5 mls PBS to remove excess FITC label and resuspended in 1mL PBS. 200µL was added to the first well of a polystyrene, U-bottomed 96 well plate. The bacteria were then serially diluted 1:2 and incubated in darkness at 4°C overnight. Photographs were taken using the Syngene G:box, Gel Documentation

and Analysis System (Syngene, Cambridge, UK) to establish optimum bacterial numbers for the agglutination assay.

<u>Assay</u>

The agglutination was performed with bacteria with and without FITC staining to ensure that the FITC labelling did not affected the results. All bacteria were subjected to the same processes, with the omission of FITC from the non-stained bacteria.

An overnight culture of bacteria was adjusted to 5×10^8 ml^{-1}. 1mL of this adjusted suspension was washed in PBS by centrifugation (13,000rpm for 5 minutes) and the bacteria resuspended in 1.5mls PBS containing 2µL FITC (or not in the case of the unstained bacteria). This was incubated, in darkness, for 15 minutes at 4°C with gentle agitation. Following incubation the bacteria were washed four times with 1.5 mls PBS to remove excess FITC label and resuspended in 1mL PBS. 100µL per treatment was added to microfuge tubes, centrifuged (13,000rpm for 5 minutes) and resuspended in 100µL of purified nBPIFA2, rBPIFA2, S2 media, Saliva or PBS. This was added to U-bottomed 96 well plates (Greiner, UK) and incubated in darkness at 4°C overnight. Photographs were taken using the Syngene G:box, Gel Documentation and Analysis System (Syngene, Cambridge, UK) to determine if any agglutination occurred.

3.3.9 Biofilm Disruption

Purified nBPIFA2 was added to polystyrene 96 well plates and incubated overnight to allow the BPIFA2 to bind to the plastic wells. The wells were washed with PBS

and *P. aeruginosa, S. gordonii, T. forsythia* or *S. mutans* added to the wells at an OD of 0.05. Biofilms were allowed to form over 48-96 hours before the planktonic bacteria were removed and each well washed two to three times with PBS. Biofilms were then stained with 0.1% Crystal Violet at room temperature for 10 minutes. The crystal violet was removed and each well was washed 4-5 times with PBS. Photographs were taken of each biofilm.

3.3.10 Protein-Lipid Overlay Assay

Membrane Lipid Strips (Echelon, Salt Lake City, UT) were blocked with TBS-Tween 20 (0.25% Tween-20) containing 1% (w/v) non-fat dry milk overnight at 4°C. They were then incubated for 1 hour at room temperature with rBPIFA2, conditioned media from untransfected S2 cells, enriched BPIFA2 (film method) or the positive control, PI(4,5)P$_2$ Grip™ (0.5µg ml^{-1}). The membrane lipid strips were washed three times for 10 minutes each with TBS-Tween and incubated for 1 hour at room temperature with primary antibody (Anti-BPIFA2-B (1:500) or Anti-GST (Sigma, UK) (1:2000)). The wash step was repeated before the lipid strips were incubated for 30 minutes at room temperature with the appropriate secondary antibody (Anti-Rabbit HRP (1:2000) for Anti-BPIFA2-B and Anti-mouse HRP (1:2000) for Anti-GST). The lipid strips were washed twice in TBS-Tween followed by a final wash with TBS. The binding of BPIFA2 to the membrane lipids was detected using ECL (Thermo Fisher Scientific Inc, USA).

3.4 Results

3.4.1 Bacterial Binding

To establish if BPIFA2 binds to bacteria in a targeted manner, a number of different bacteria were mixed with whole saliva incubated for 1 hour at 37°C before being extensively washed and analysed by western blot.

With the exception of *P. gingivalis*, all bacteria tested bound BPIFA2 from saliva (Figure 3-1A). Repeating the binding assay with the *P. gingivalis* mutants showed that in the absence of lysine specific gingipains, BPIFA2 bound to and could be recovered from these bacterial cells (Figure 3-1B).

Although this method shows binding of BPIFA2 to a number of different bacteria it does not determine whether BPIFA2 is directly binding to the bacteria or whether it is binding indirectly by virtue of being complexed with another salivary protein, such as amylase or mucin, which is directly binding to the bacteria.

BPIFA2 was purified by native gel elution and incubated with each bacterium. Purified nBPIFA2 showed no binding to *S. gordonii, P. aeruginosa* or *E. coli*. Binding was seen with *S. mutans* and β-haemolytic streptococcus. *S. aureus* showed some non-specific binding of the anti-BPIFA2 antibody so BPIFA2 binding could not be determined using this method. No false positives were seen with the PBS controls (Figure 3-1A).
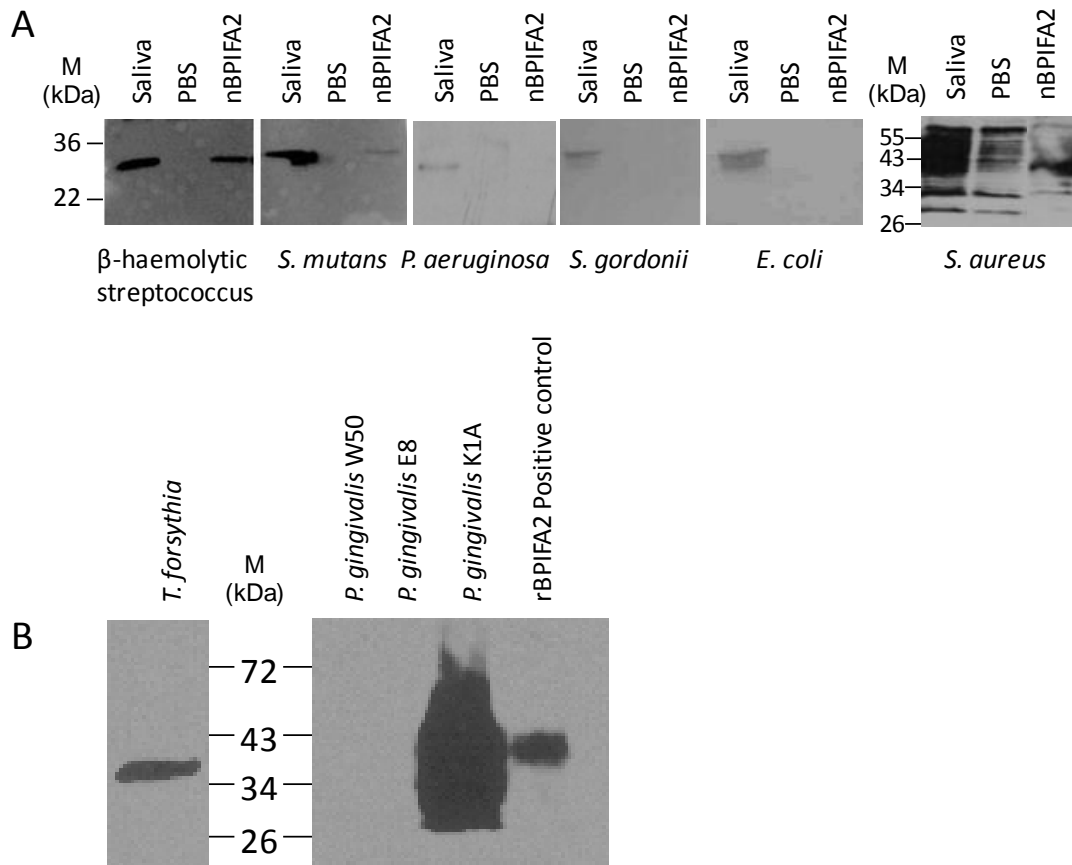
Figure 3-1: Binding of BPIFA2 in whole saliva and purified nBPIFA2 with bacteria

A 1ml (OD 1) aliquot of an overnight culture of *P. aeruginosa, E. coli, P. gingivalis* (W50, E8, K1A)*, S. gordonii, S. mutans,* β-haemolytic streptococcus and *T. forsythia* was removed and washed before being incubated with whole saliva or purified nBPIFA2 for 1 hour at 37°C. The bacteria were washed before being added to SDS lysis buffer and subjected to western blot analysis to assess binding of BPIFA2.

Incubation of many of the species (A) for 1 hour at 37°C with whole saliva showed binding of BPIFA2, however incubation with purified nBPIFA2 only showed positive binding with β-haemolytic streptococcus*,* and *S. mutans*. No BPIFA2 binding was seen with *S. gordonii, P. aeruginosa* or *E. coli*. Non-specific binding was observed with *S. aureus*. No positivity was detected with the PBS control.

Following incubation with whole saliva, binding was seen with all bacteria tested with the exception of *P. gingivalis* (B). Here *P. gingivalis* (W50) showed no binding to BPIFA2, along with the arginine gingipain mutant (E8). However removal of the lysine specific gingipain (K1A) lead to detection of bound BPIFA2 to the bacteria.

rBPIFA2 collected from cultured *Drosophila Schneider* 2 (S2) was used as a positive control.

Western blots shown here are representative of a number of repeats (>10).

3.4.2   Growth Curve

Growth curves using purified nBPIFA2 were used to collect initial data regarding the the effect of BPIFA2 on the growth of a number of bacteria. As physiological concentrations of BPIFA2 have yet to be determined we decided that for our initial investigations we would focus on the effect of the presence or absence of BPIFA2 by using a single, unkown concentration of purified nBPIFA2, and not a range of concentrations. It is intended that future studies of functional analysis will include a concentration curve in line with physiological levels once this has been determined.

Normal growth of *S. mutans* (Figure 3-2A) had an average doubling time of 47.6 minutes under the conditions employed here and in the presence of BPIFA2 this doubling time was only slightly increased to 62 minutes giving only a 1.3-fold increase in doubling time. *S. gordonii* growth in the absence of BPIFA2 (Figure 3-2B) had a doubling time of 45 minutes and this, in the presence of BPIFA2 was inhibited throughout the time course, such that the doubling time in the presence of BPIFA2 was twice that seen with the normal control (90 minutes). Interestingly the maximum cell density of *S. gordonii* in the presence of BPIFA2 was reduced considerably in comparison with the control.

Growth of *E. coli* (Figure 3-3A) and *P. aeruginosa* (Figure 3-3B) were also minimally affected by the presence of BPIFA2, with an increase in doubling time of just 1.4-fold and 1.42-fold respectively. The maximum cell densities were only slightly reduced for both bacteria in the presence of BPIFA2.

As a preliminary investigation, and due to limited volumes of purified BPIFA2 protein, this assay was only completed once. As a result no valid comment on the significance of our results can be made and any interpretation of results must be done with caution.
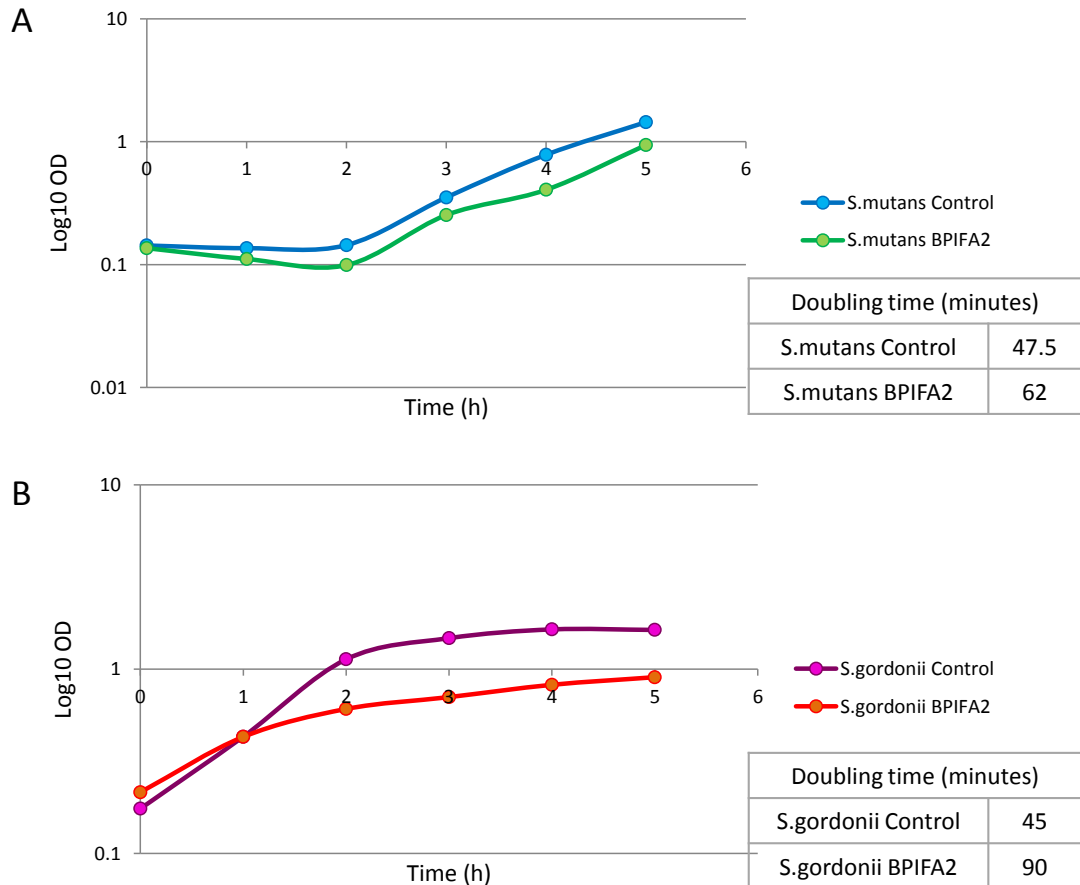
n=1

Figure 3-2: Preliminary results of the growth of *S. mutans* (A) and *S. gordonii* (B) in the presence and absence of purified nBPIFA2

S. mutans and *S. gordonii* were resuspened at an OD of 0.1 in 2 x BHI broth. PBS or purified nBPIFA2 was added at 1:1 and the $OD_{(600)}$ was measured at time 0. The cuvette was sealed with parafilm, secured within a 25mL tube and incubated on a spyra-mixer in CO_2 for 60 minutes before being removed, mixed by pipetting and the $OD_{(600)}$ recorded this was repeated until the stationary phase was evident.

The presence of BPIFA2 increased the doubling time of the *S. mutans* 1.3-fold compared the control sample. The final maximum cell density was slightly reduced following 5 hours incubation.

The presence of BPIFA2 considerably reduced the doubling time and the maximum cell density of the *S. gordonii* compared the control sample following 5 hours of growth. In the presence of BPIFA2 the doubling time of *S. gordonii* increased 2-fold.

Doubling time (minutes)	
E.coli Control	35.4
E.coli BPIFA2	50.1

Doubling time (minutes)	
P.aeruginosa Control	69
P.aeruginosa BPIFA2	97.8
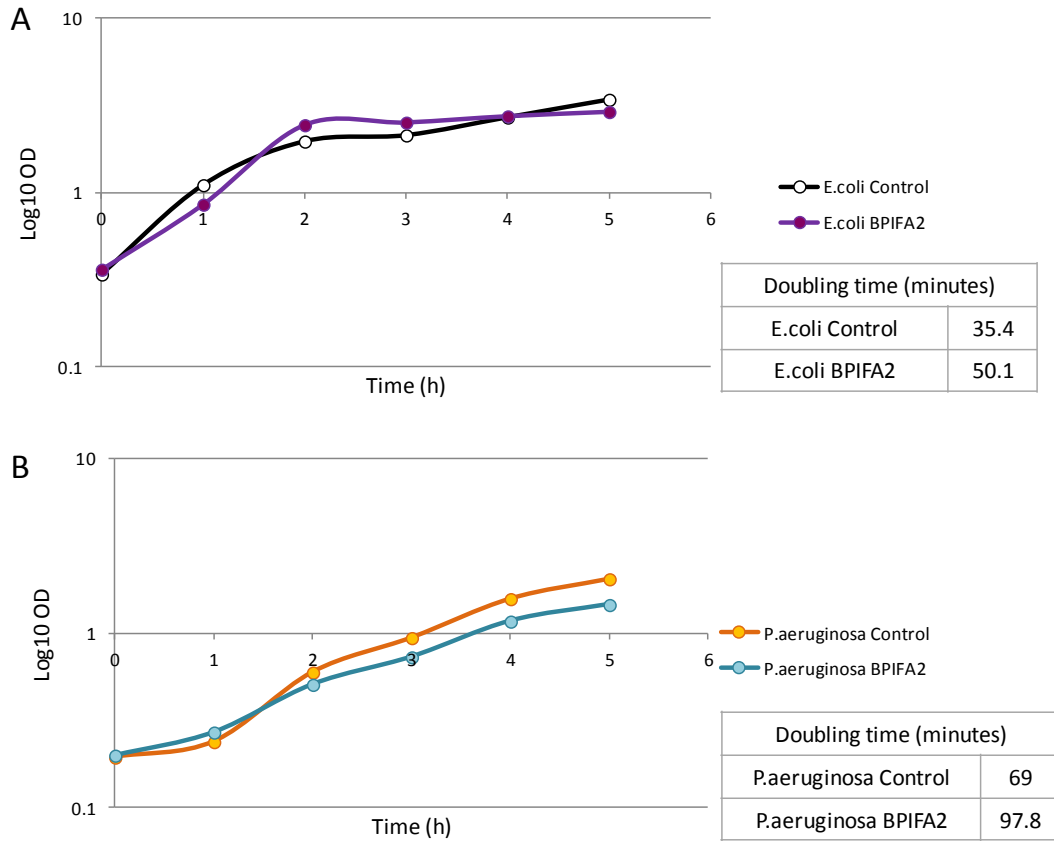
n=1

Figure 3-3: Preliminary results of the growth of *E. coli* (A) and *P. aeruginosa* (B) in the presence and absence of purified nBPIFA2

E. coli and *P. aeruginosa* were resuspened at an OD of 0.1 in 2 x BHI broth. PBS or purified nBPIFA2 was added at 1:1 and the $OD_{(600)}$ was measured for time 0. The cuvette was sealed with parafilm, secured within a 25mL tube and incubated on a spyra-mixer in CO_2 for 60 minutes before being removed, mixed by pipetting and the $OD_{(600)}$ recorded this was repeated until the stationary phase was evident.

The presence of BPIFA2 only slightly affected the growth of both *E. coli* and *P. aeruginosa* resulting in a 1.4-fold and a 1.42-fold increase in doubling time respectively compared to the control.

3.4.3   Bacterial Killing

To determine whether the presence of BPIFA2 was causing a reduction in the viability of the cells unpurified rBPIFA2-, purified nBPIFA2-, saliva-, spectinomycin- (50mg ml^{-1}) or PBS-soaked paper disks were added to agar plates streaked with *S. gordonii, E. coli, S. mutans, S. aureus,* β-haemolytic streptococcus or *P. aeruginosa*.

Spectinomycin was selected as a positive control as it is a well-researched antibiotic known to have antimicrobial activity against both gram-negative and gram-positive bacteria. Additionally, in our assays it showed a clear zone of inhibition in both agar killing assays and demonstrated bactericidal activity. A zone of inhibition was seen surrounding the disks soaked in spectinomycin (50mg ml^{-1}) for all bacteria and no zone of inhibition was seen surrounding the PBS soaked paper disk. However, no zone of inhibition was observed around the discs soaked in purified nBPIFA2, rBPIFA2 or saliva (Figure 3-4).

To assess whether this lack of killing was due to the inability of the samples to diffuse through the agar, 5μl of unpurified rBPIFA2, purified nBPIFA2, saliva, spectinomycin (50mg ml^{-1}) or PBS were dotted directly onto an agar plates streaked with the same bacterial species. As before, spectinomycin (500μg ml^{-1}) produced a zone of inhibition, whilst the PBS, rBPIFA2, purified nBPIFA2 and saliva did not (Figure 3-5).
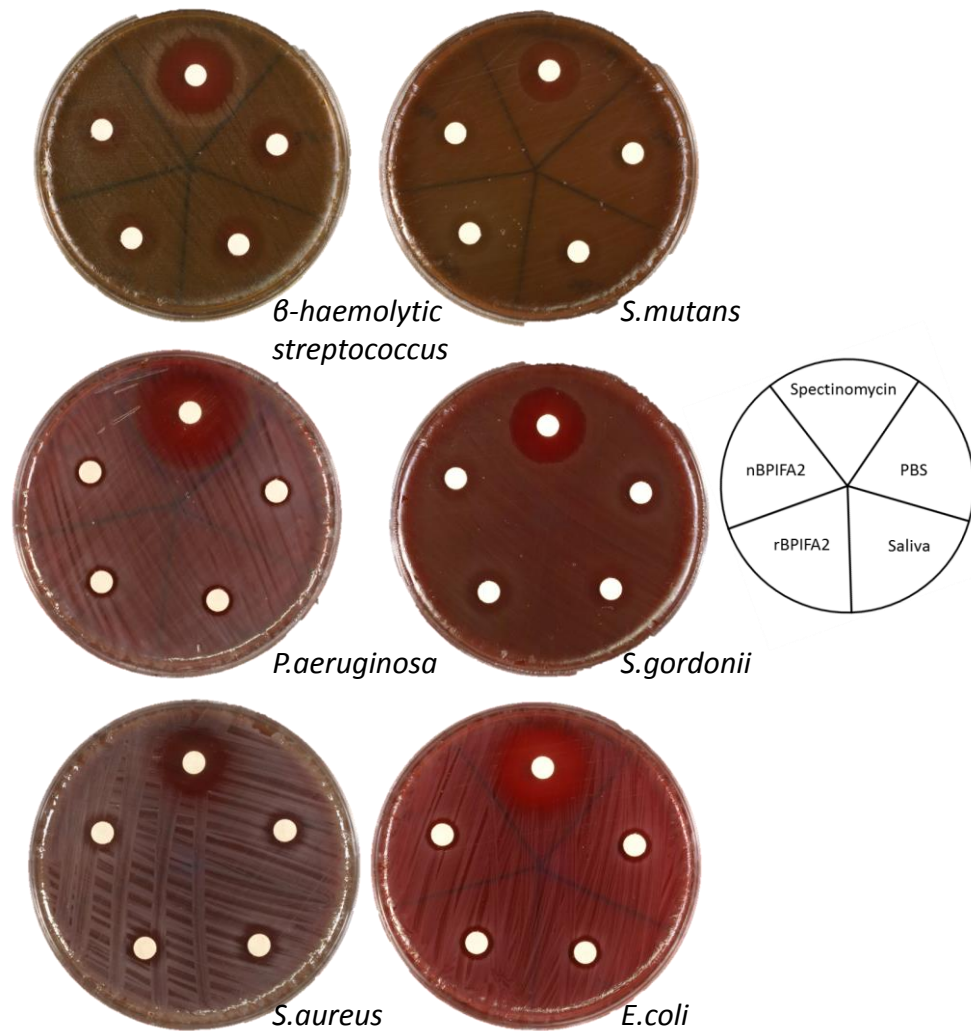
Figure 3-4: Zone of inhibition assay – paper discs

β-haemolytic streptococcus*, S. mutans, P. aeruginosa, S. gordonii, S. aureus and E. coli* were streaked onto an agar plates. Sterile paper discs were soaked in rBPIFA2, purified nBPIFA2, saliva, spectinomycin or PBS and added to the plates. Following incubation (3.3.2) any zone of inhibition was measured. A clear zone of inhibition was present with the antibiotic positive control; however no zone of inhibition, in excess of that seen with the PBS control was seen with any of the other treatments. Pictures are representative of 4 repeats.
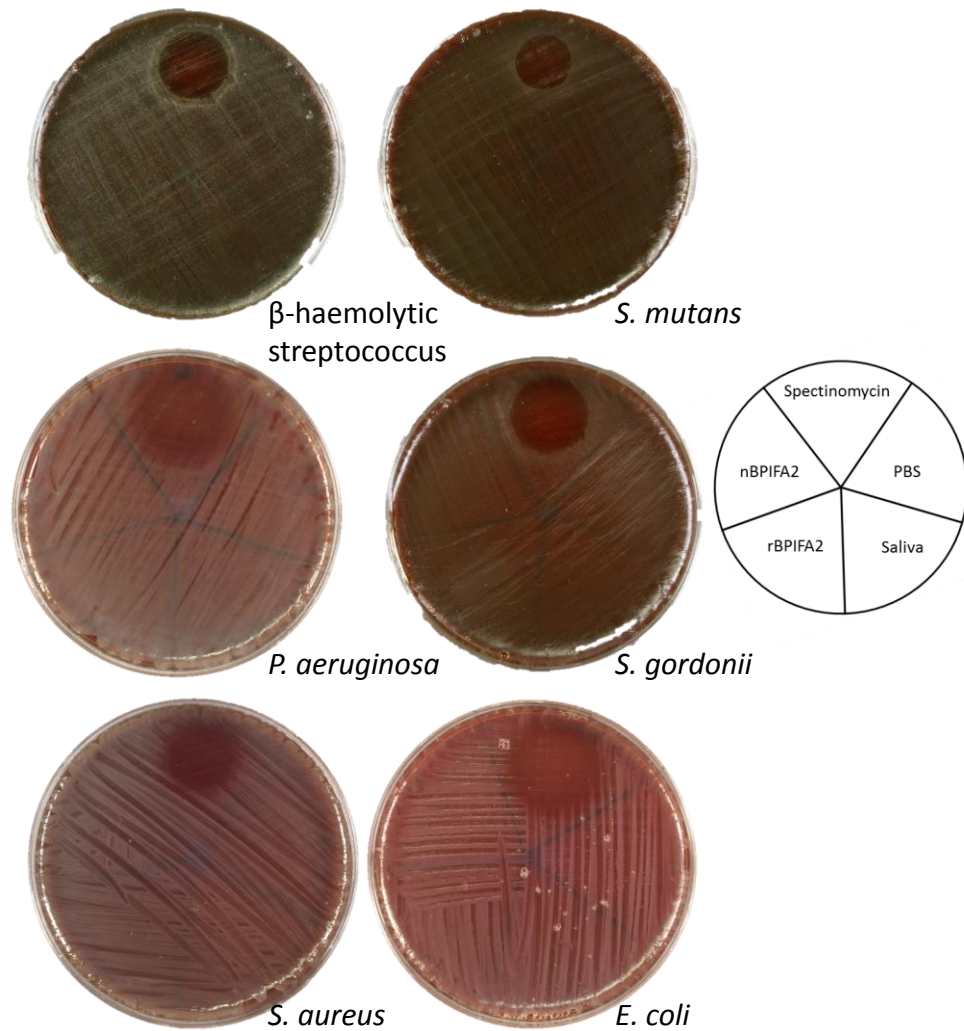
Figure 3-5: Zone of inhibition assay - Direct application

β-haemolytic streptococcus, *S. mutans, P. aeruginosa, S. gordonii, S. aureus and E. coli* were streaked onto an agar plates. rBPIFA2, purified nBPIFA2, saliva, spectinomycin or PBS were directly added to the plates and allowed to soak into the agar. Following incubation (3.3.2) any zone of inhibition was measured. No zone of inhibition was observed with the negative control, and clear zones of inhibition were observed with the antibiotic positive control, however no zone of inhibition was seen with rBPIFA2, purified nBPIFA2 or saliva for any of the bacteria. Pictures are representative of 4 separate experiments.

3.4.4 Bacterial Killing 2

To establish whether BPIFA2 possesses the ability to kill bacteria in a broth culture, purified nBPIFA2 was incubated with *S. mutans* and *S. gordonii* for 3 hours before being serially diluted and spotted onto agar plates for colony counts to determine the number of viable bacteria per ml.

No difference in the bacterial numbers was seen with *S. gordonii* in the presence of BPIFA2 compared with the PBS negative control. For both bacteria, the presence of mutanolysin ($10\mu g$ ml^{-1}) reduced the viable bacterial count by approximately a third compared to the PBS control. In contrast a 75% reduction in viable *S. mutans* was seen in the presence of purified nBPIFA2 when compared to the PBS control and approximately 30% less than the positive control (Figure 3-6).
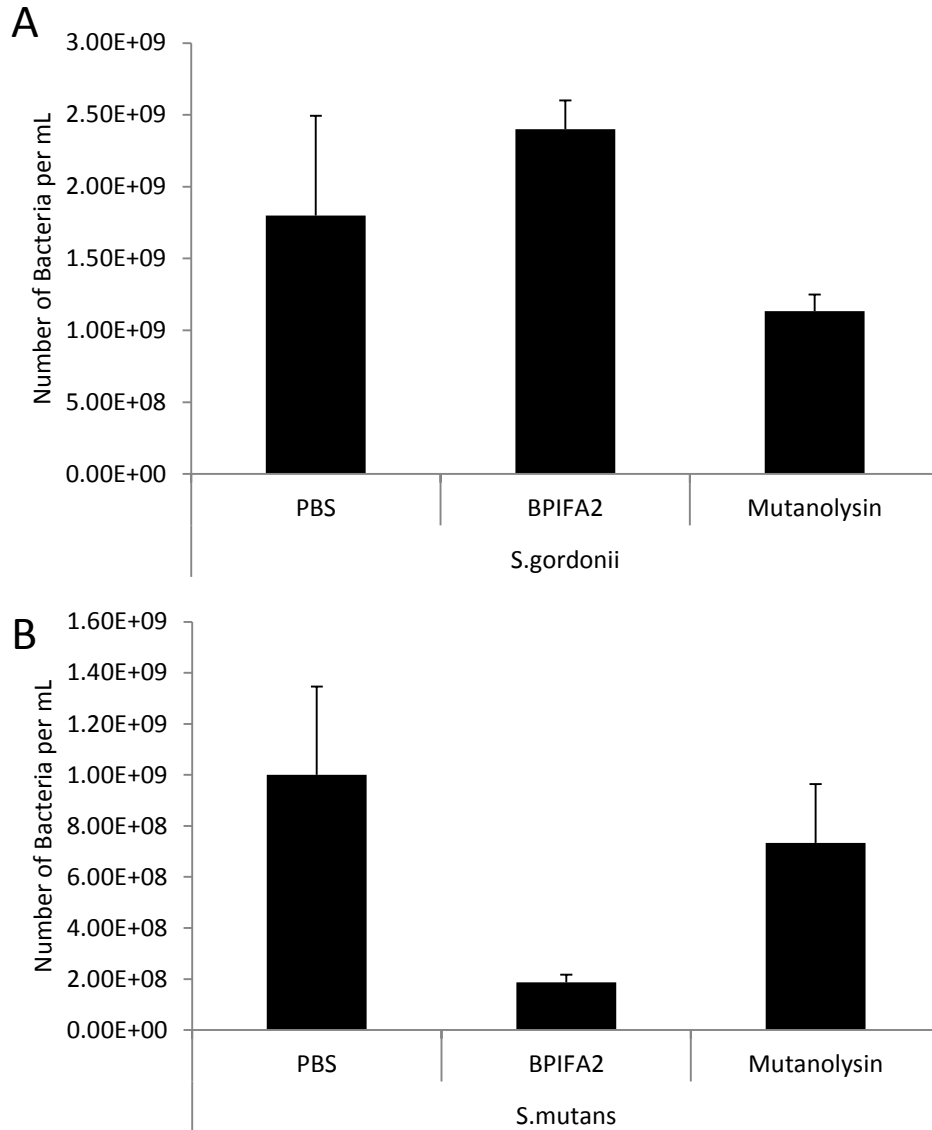
N=3

Figure 3-6: Killing of bacteria with purified nBPIFA2

From an overnight bacterial broth, bacteria were added to nBPIFA2, PBS or antibiotic, mutanolysin ($10\mu g$ ml^{-1}) at an $OD_{(600)}$ of 0.05. The bacteria were incubated (3.3.2) for 3 hours before being serially diluted in PBS and $5\mu l$ dotted onto BA plates in triplicate. Plates were incubated overnight (3.3.2) and the number of colonies were counted and the number of bacteria per ml was calculated.

No change in viability of *S. gordonii* (A) was seen in the presence of purified nBPIFA2.

S. mutans (B) showed a reduction in viability in the presence of purified nBPIFA2 of approximately 4 times less than that seen with mutanolysin and 5 times less than the PBS control. Data presented is the mean of 3 repeats in triplicate, error bars are standard error of the mean.

3.4.5   Agglutination

Bacteria were labelled with FITC and incubated overnight in native elution buffer, purified nBPIFA2, rBPIFA2, conditioned media from empty S2 cells, saliva or PBS. An indication of no agglutination was determined by the visualisation of a tight button of bacteria at the bottom of the well, whilst an indication of agglutination was determined by presence of a matt or reduction of the size of the button.

Following incubation of *S. mutans* with PBS, conditioned media from S2 cells and native elution buffer a tight button was formed, showing that this strain of *S. mutans* does not autoaggregate in PBS, conditioned media from untransfected S2 cells or native elution buffer. In the presence of saliva and purified nBPIFA2 the button was less dense, suggesting that in the presence of nBPIFA2, *S. mutans* agglutinates. However, in the presence of rBPIFA2 no agglutination was evident.

Neither the β-haemolytic streptococcus nor *S. gordonii* were agglutinated by either rBPIFA2 or purified nBPIFA2, however, they were by whole saliva.

Agglutination of *P. aeruginosa* showed inconclusive results as no tight button of bacteria could be seen following incubation with PBS and native buffer. However faint buttons of bacteria could be seen following treatment with the conditioned S2 cell media, purified nBPIFA2 and rBPIFA2.

The most convincing result was seen with *S. aureus*. Clear tight buttons of bacteria could be seen in PBS and native elution buffer and a faint button of bacteria was seen following treatment with conditioned S2 media. In contrast, purified nBPIFA2, rBPIFA2 and saliva all showed clear agglutination of *S. aureus* (Figure 3-7).

As with *P. aeruginosa*, *E. coli* showed inconclusive results. Faint buttons of bacteria were seen following treatment with PBS and conditioned S2 media, however positive agglutination was seen with native elution buffer, purified nBPIFA2, rBPIFA2 and saliva.

P. gingivalis strains W50, E8, K1A and EK18 were incubated with only purified nBPIFA2 and PBS. *P. gingivalis* W50, E8 and K1A showed no button formation in PBS, while strain EK18 showed a tight button of bacteria, however in the presence of BPIFA2 this button became less pronounced and much smaller, suggesting that some agglutination was occurring.
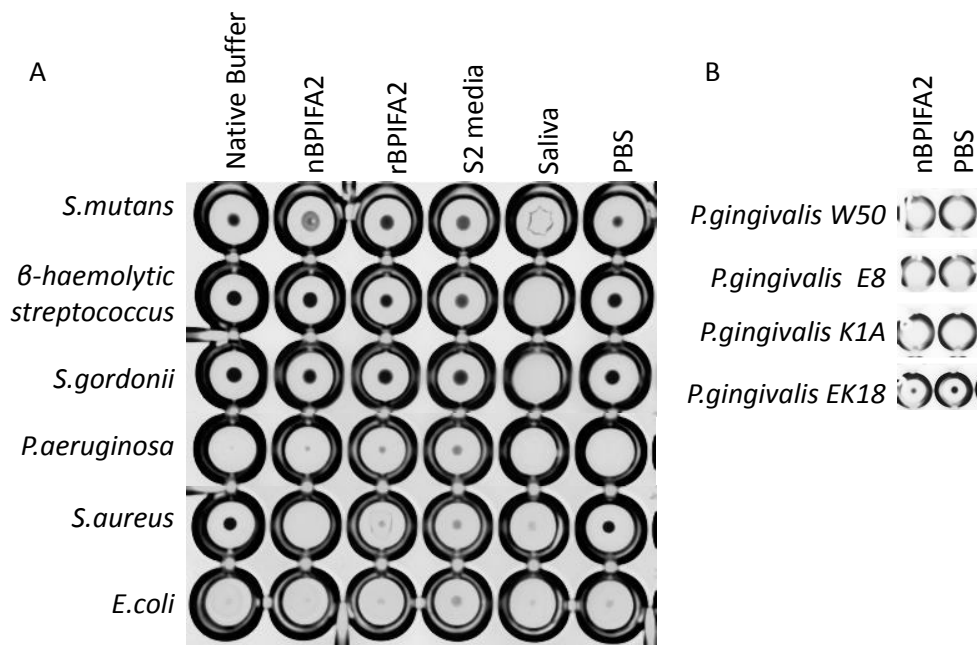
Figure 3-7: Agglutination of bacteria by purified nBPIFA2

$5x10^8$ ml^{-1} bacteria per treatment were labelled with FITC, following extensive washing to remove excess FITC, the bacteria were centrifuged (13,000rpm for 5 minutes) and resuspended in 100µl native elution buffer, purified nBPIFA2, rBPIFA2, conditioned S2 media saliva or PBS in a U-bottomed 96 well plate and incubated overnight at 4°C. Photographs were taken of each well using the Syngene G-box-imaging system for fluorescence.

The results seen with *P. aeruginosa* and *E. coli* were inconclusive. All of the other bacteria (*S. mutans,* β-haemolytic streptococcus*, S. gordonii* and *S. aureus*)(A) showed clear agglutination following incubation with saliva and none of the bacteria showed agglutination following incubation with conditioned S2 media or conditioned S2 media. *S. mutans* and *S. aureus* showed agglutination in the presence of purified nBPIFA2 and *S. aureus* also showed positive agglutination in the presence of rBPIFA2 to a slightly lesser degree. β-haemolytic streptococcus and *S. gordonii* showed no agglutination in the presence of purified nBPIFA2 or rBPIFA2.

In the presence of PBS and purified nBPIFA2, *P. gingivalis* (W50, E8 and K1A)(B) showed no dot of bacteria at the base of the well, however *P. gingivalis* EK18 shows a clear dot in the absence of BPIFA2 which becomes less distinct in the presence of purified nBPIFA2. Data is representative of 3 repeats.

3.4.6 Biofilm Disruption

It was interesting to observe the interaction between BPIFA2 and laboratory plastics during the purification stages of this project, which could suggest a possible functional significance. A biofilm assay would allow us to assess any functional activity associated with bound BPIFA2.

A key process in the survival of oral bacteria is their ability to adhere to surfaces and form biofilms. It has been proposed that the ability of *P. aeruginosa* to form biofilm can be inhibited in the presence of BPIFA2 peptides and rBPIFA1 (Gorr et al., 2008, Gakhar et al., 2010). The following results show that the presence of BPIFA2 reduced the ability of some bacteria to form biofilm. Development of the biofilm assay was carried out by a fellow student and she demonstrated that the control protein, bovine serum albumin, did not affect biofilm formation (unpublished data). The biofilm experiments for this study were performed alongside those of my colleague, and so control data was not collected, simply observed.

 In the presence of purified nBPIFA2, *T. forsythia* biofilm became thinner with less bacteria binding to the 96 well plates compared with the PBS control. The biofilm produced by *S. mutans* in the absence of BPIFA2 appears to be evenly spread over the surface of the 96 well plates, however in the presence of BPIFA2 this biofilm was less evenly spread and showed clear signs of clumps of bacteria. *P. aeruginosa* and *S. gordonii* biofilm was not reduced by the presence of BPIFA2 and in fact appeared to have increased in thickness (Figure 3-8). However this is a very subjective method and a more quantitative method is required.

PBS   **nBPIFA2**
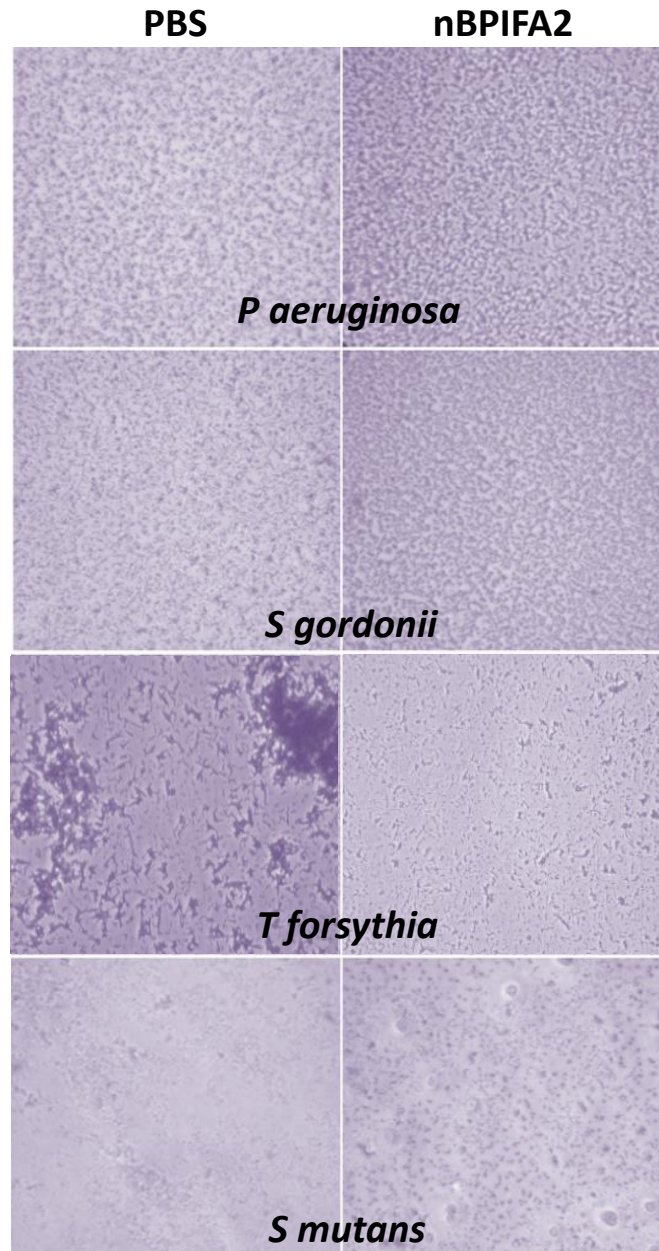
Figure 3-8: Biofilm disruption by BPIFA2

Polystyrene 96 well plates were pre-incubated with purified nBPIFA2 (right) or PBS (Left) before being washed to remove excess purified nBPIFA2. Bacteria were added at $OD_{(600)}$ of 0.05 and biofilms were allowed to form over 48-96 hours, these were washed and stained with crystal violet.

Biofilm formed by *P. aeruginosa* and *S. gordonii* showed no reduction in biofilm formation, the biofilm appears thicker in the presence of BPIFA2 when compared to the normal control. The biofilm produced by *T. forsythia* in the presence of BPIFA2 is much thinner, aggregation of the bacteria to each other looks to be greatly reduced when compared with the normal control.

The normal biofilm formed by *S. mutans* looks evenly distributed throughout the plate, the bacteria do not aggregate in big thick masses as with *T. forsythia*, however in the presence of BPIFA2 the biofilm looks much less uniform in nature, and it appears that the bacteria have formed small aggregated clumps rather than the 'blanket' of cells previously seen. Images are representative of 3 repeats.

3.4.7 Protein-Lipid Overlay Assay

Due to the predicted structural similarities between BPIF proteins and BPI, it is believed that BPIFA2 contains a hydrophobic cleft ideal for the binding of lipid-like molecules. Therefore, we assessed which lipids, if any, bind to BPIFA2. Commercial strips, dotted with 15 biologically active lipids present within cell membranes were used in a BPIFA2-lipid overlay assay. As expected, the conditioned media from untransfected S2 cells showed no binding to any of the lipids present on the membrane (Figure 3-9A), whilst the PI(4,5)P$_2$ Grip™ positive control did so (Figure 3-9B). Lipid binding by BPIFA2 appeared to be affected by the source of the protein. The enriched saliva (Figure 3-9C) showed clear binding to the PIP lipids (PtdIns(4)P, PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$) and Phosphatidic acid, whereas the S2 cell-expressed BPIFA2 only showed binding to PtdIns(4,5)P$_2$ and weak binding to PtdIns(3,4,5)P$_3$ (Figure 3-9D).
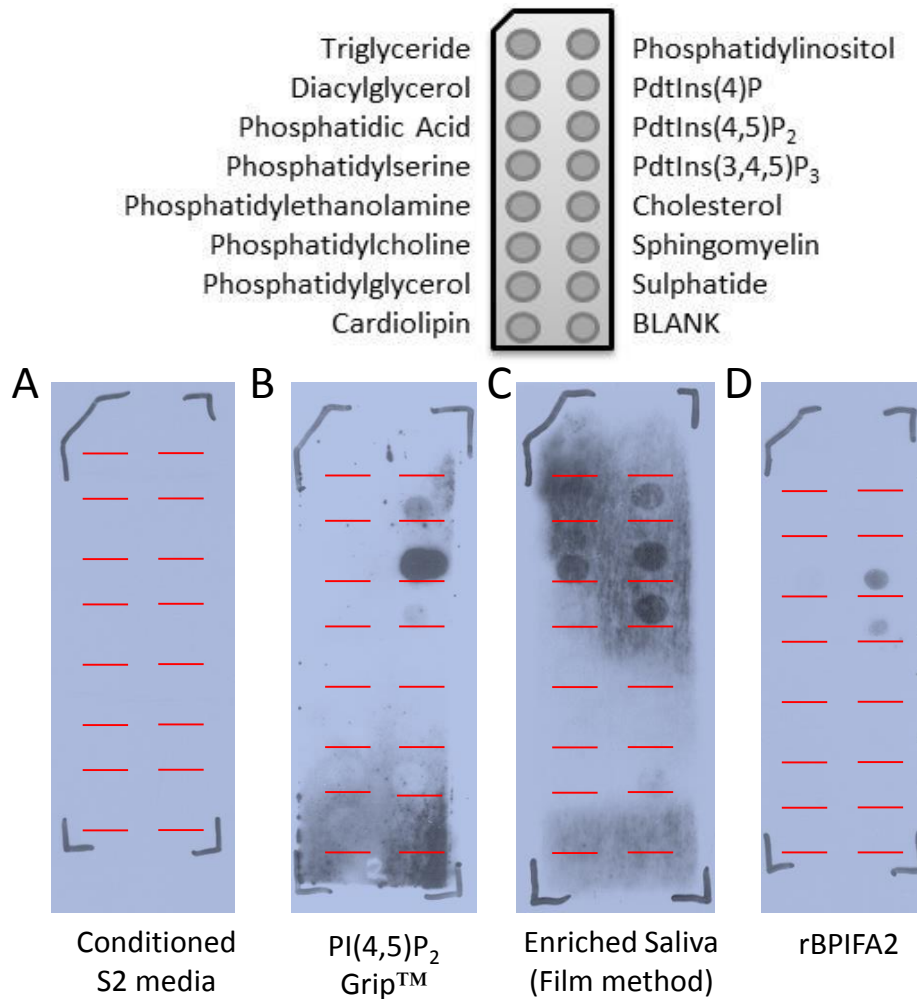
Figure 3-9: Interaction between BPIFA2 and membrane lipids.

Membrane Lipid Strips (Echelon, Salt lake city, UT) were incubated with Conditioned empty S2 cell media, Conditioned media from BPIFA2 expressing S2 cells, Enriched BPIFA2 from saliva or PI(4,5)P$_2$ (positive control) for 1 hour at room temperature before the bound BPIFA2 was detected by antibody detection.

As expected the untransfected S2 conditioned media (A) showed no binding and the PI(4,5)P^2 Grip™ positive control (B) strongly highlighted PI(4,5)P$_2$.

Incubation with enriched BPIFA2 from saliva (C) identified binding to the PtdIns and Phosphatidic Acid whereas rBPIFA2 (D) only identified binding of BPIFA2 to PtdIns (4,5)P$_2$ and PtdIns (3,4,5) P$_3$.

3.5   Discussion

The oral cavity contains over 700 species of bacteria, most of which have yet to be cultivated. The oral cavity contains gram positive *Streptococcus,* such as *S. mitis, S. oralis, S. sanguis* and *S. mutans*; gram-negative cocci and bacilli, such as *Neisseria* and *Fusobacterium* and gram-positive bacilli and filaments, such as *Actinomyces* and *Corynebacterium*. Oral pathogens may also be present and include *P. gingivalis, T. forsythia, Treponema denticola* and *Actinomyces* species*.* The oro-pharynx and pharynx contains its own microflora, predominantly consisting of alpha-haemolytic streptococci. Although it would be highly beneficial to examine all of the cultivable bacteria present in the oral cavity and upper airways, particularly for BPIFA2 binding, it was decided to focus on a select few bacteria. Thus two oral commensal species, *S. gordonii* and *S. mutans*; two oral pathogens, *P. gingivalis* and *T. forsythia;* one respiratory commensal species, *S. aureus*; one respiratory pathogen, β-haemolytic streptococci and two non-oral commensal species, *P. aeruginosa* and *E. coli* were used to examine the function of BPIFA2. Unusually, the literature has mainly focused on determining the function of various BPIFAs by studying effects on non-oral commensal bacteria, *E. coli* and *P. aeruginosa,* even those looking at the function of BPIFA2. As previously mentioned, BPIFA2 expression is isolated to the oral cavity and upper airways and if the function of BPIFA2 is in fact antimicrobial, based on the level of expression, it would be expected that BPIFA2 would act against bacteria that it is regularly exposed to otherwise the function would be redundant and expression senseless. Therefore, the study of purely non-oral bacteria does not appear to be the most rational choice. In contrast, here were

studied the effect of BPIFA2 on a variety of bacteria, including representative oral species, which showed some interesting contrasts.

Of the many bacteria tested, a number showed no significant response to BPIFA. For example, purified nBPIFA2 did not bind to *E. coli*, the initial investigation suggested that the growth was not affected in the presence of purified nBPIFA2 and no killing was observed. These results are in agreement with some of the previously published data relating to a number of BPIF family proteins. Ethanol purified native BPIFA1 from tracheobronchial secretions did not show any binding to *E. coli* LPS when competing with LBP (Campos et al., 2004). However as previously mentioned, exposure of protein to ethanol has led to denaturation and changes in structural conformation of other proteins such as human serum albumin (Lin et al., 2004) and this could influence binding capability. Furthermore, recombinant bovine BPIF proteins, BPIFA2A and BPIFA2B, also failed to show binding to *E. coli* LPS (Haigh et al., 2008). This recombinant protein was expressed in an *E. coli* expression system; it may be that the lipid binding sites of the purified recombinant protein already contain LPS, following the previous exposure to *E. coli*. These studies limit the binding assessment to *E. coli* LPS, and do not consider binding of alternative lipid like molecules such as lipoteichoic acid (LTA) of gram-positive bacteria, unlike in this study. Here a range of gram-positive and gram-negative bacteria were tested allowing for the assessment of the binding of BPIFA2 to a range of bacterial surface molecules. It must be assumed that the similarity between the BPIF proteins and BPI, which bind to gram-negative bacterial LPS, was the reason *E. coli* LPS was used in both the Haigh et al (2008) and Campos et al (2004) studies, however other

members of the BPI family, such as CETP and PLTP, bind cholesteryl esters, triglycerides and phospholipids but not bacterial LPS, demonstrating that not all members of this family bind the same lipid-like molecules. In contrast, Gahfouri et al (2003, 2004) believe that BPIFA1 does bind to *E. coli* LPS, however in both publications unpurified nasal lavage fluid was used as the source of BPIFA1. Nasal lavage fluid contains over 1000 proteins including a number of antibacterial proteins such as, lysozyme, lactoferrin and SLPI (Cole et al., 2002) and mucins (Ali et al., 2002), which could interfere with the binding of BPIFA1 to bacteria. The data shown here supports the hypothesis that BPIF protein binding from complex secretions may be through other accessory proteins. BPIFA2 in whole saliva bound to all of the bacteria tested. However, following purification of BPIFA2, binding was restricted to only two species, *S. mutans* and the β-haemolytic streptococcus, suggesting that the initial binding was via a second protein present in saliva, such as mucin. The absence of evidence demonstrating binding to *E. coli* or *E. coli* LPS may explain the small range of functional data currently available. Most of the previously published results have recorded that BPIFA2 does not have antibacterial activity towards *E. coli* (Khovidhunkit et al 2004; Bartlett et al 2008; Chu et al 2007). Our results are therefore in agreement with previously published studies.

Purified nBPIFA2 did not show any killing or agglutination activity against *S. gordonii* and the growth of *S. gordonii* biofilm was not affected in the presence of purified nBPIFA2. However the initial investigation into growth rate suggests that there may be a reduction in the doubling time of *S. gordonii* in the presence of purified nBPIFA2. Of the bacteria tested this was the largest reduction in growth rate seen

and resulted in a reduced final cell density, suggesting that some effect on cell membranes and/or metabolism is occurring. However, due to the absence of evidence supporting a direct interaction between BPIFA2 and *S. gordonii* (binding and agglutination) and our inability to demonstrate killing activity of purified nBPIFA2 against *S. gordonii,* further investigations are required determine if this reduction in growth is significant and, if so, to establish mechanism of action against this organism. Gorr et al (2008) demonstrated agglutination of this bacterium with an engineered BPIFA2 peptide GL13NH2, however removal of the amine group (NH_2) resulted in a 50% reduction of agglutination and addition of a second amine group resulted to an increase in agglutination. This demonstrates that the presence, and number of amine groups in these peptides appears to influence their binding function. For this reason, the peptide results must be interpreted with caution as the GL-13 peptides (GQIINLKASLDLL-) referred to were synthesised based on the functional region of BPI and LBP. This set of 13 amino acid peptides do not contain a large portion of the whole protein; there is no certainty therefore, that this domain in the natural protein has the opportunity to interact with bacteria as the rest of the BPIFA2 structure may sterically hinder it.

No binding of BPIFA2 to *P. aeruginosa* was observed, and the initial growth curve assay suggests there may be a very minimal increase in growth rate (1.41-fold). The agglutination assay gave inconclusive results, we are not able to confirm or otherwise this interpretation. It was expected that incubation of the bacteria with PBS and native elution buffer would provide a tight button of bacteria, however, in the case of *P. aeruginosa*, there was autoaggregation in the both buffers. The

agglutination method adopted here was chosen due to the small volume of pure BPIFA2 protein available, ideally it would have been beneficial to repeat the agglutination assays in solution with continuous optical density monitoring, however these assays would require much more pure BPIFA2, a volume which was not available at the time. In addition, no reduction or change in biofilm growth and/or quality was observed in our assays. Similar results have been reported previously (Haigh et al., 2008) using recombinant bovine BPIF proteins, BPIFA2A and BPIFA2B, which failed to show any direct binding though there was a significant suppression of *P. aeruginosa* growth. In that study no assessment of biofilm disruption or agglutination was made. As with *S. gordonii*, the BPIFA2 peptide GL13NH2 was shown to cause agglutination of *P. aeruginosa* in a dose dependent manner, however minimal inhibitory concentration assays showed no reduction in growth, similar to the results seen in this study. Abdolhosseini et al (2012) reported binding of BPIFA2 to *P. aeruginosa* with both human rBPIFA2 expressed in rat pituitary GH4C1 cells and ethanol purified nBPIFA2 from human saliva, no further functional studies have been published to date.

Binding of purified nBPIFA2 to β-haemolytic streptococcus was also noted, however no antibacterial activity against this organism was observed, including killing and agglutination.

The most interesting results from this part of the study were with *S. mutans*, a gram positive, oral commensal species which is an important player in the development of dental caries. Purified nBPIFA2 showed positive binding to this gram-positive bacterium, positive agglutination and an altered biofilm growth with the initial

growth curve assay suggesting that there may be a very slight suppression of growth (1.3-fold). It is possible that the agglutination of the bacteria by BPIFA2 is the cause of the slight reduction in growth rate (judged by change in optical density) and also the change in biofilm appearance. However, following incubation of *S. mutans* with purified nBPIFA2, surviving bacteria were counted on agar plates, it was noted that in the presence of purified nBPIFA2, the number of viable bacteria reduced, indicating that BPIFA2 does seem to have some antibacterial activity against *S. mutans*. Although, killing was not observed in the presence of BPIFA2 (both in the form of whole saliva and purified nBPIFA2) by diffusion through agar or by direct application of the agar surface. Consequently, the apparent reduction in viability of *S. mutans* in the presence of nBPIFA2 may be due to clumping of bacteria so reducing the number of colony forming units in the bacterial suspension and this would have little effect in the growth assay on agar. It should be noted that due to the limited volume of nBPIFA2 and the high volume required to complete the growth curve assays, these results are based on a single set of data and so have been included as a preliminary data set and should be viewed as such. Much more investigaton is required with this technique to establish any true relationship between purified nBPIFA2 and planktonic bacterial growth.

The binding results obtained from the assay using unpurified BPIFA2 (whole saliva) initially indicated that BPIFA2 was binding in a non-targeted manner, as the BPIFA2 bound to most of the bacteria tested, including *E. coli, S. gordonii, P. aeruginosa, S. mutans,* β-haemolytic streptococcus*, T. forsythia* and *P. gingivalis* (K1A), the only negative binding was seen with *P. gingivalis* (W50 and E8), however this was

probably due to destruction of the BPIFA2 by the lysine specific gingipain since it was recovered from the cells surface of a mutant that lacks this enzyme (K1A). Indeed it is possible that binding of BPIFA2 from saliva to all these bacteria actually reflects binding of a complex of proteins, amongst which one is BPIFA2. Support for this comes from our observation that purified nBPIFA2 did not bind to many species, only to *S. mutans* and β-haemolytic streptococcus. Previous studies have suggested that murine BPIFA2 and amylase expression is coordinated in the parotid gland, and this may further indicate that these proteins are naturally in association with each other. During the purification of nBPIFA2 we observed co-purification of BPIFA2 and amylase. The most well-known function of amylase is the digestion of starch; however amylase has also been shown to inhibit the growth of *Neisseria gonorrhoeae* and bind to oral streptococci including *S. gordonii, S. mitis,* and *S. anginosus* but not to *S. sanguis, S. oralis* or *S. mutans* and many gram-negative bacteria (Scannapieco et al., 1993). Amylase binding is known to be via two specific binding proteins (Gwynn and Douglas, 1994, Rogers et al., 1998). Amylase has also been found in the enamel pellicle indicating that amylase also binds to the tooth surface. It is believed that the binding of amylase to oral streptococci and the tooth surface aids in the formation of dental plaque. It may be interesting to compare the binding ability of BPIFA2 to the bacteria found to bind and not to bind amylase from saliva since it may be for some species their binding is synergistic.   In addition, the assessment of bacterial binding to the tooth surface in the presence and absence of BPIFA2 could be examined, using hydroxyapatite, to determine if BPIFA2 also increases the binding of bacteria to the tooth surface in a similar way to amylase. These binding assays were conducted early in the project, prior to the successful

production of rBPIFA2. Unfortunately due to the time constraints of the project rBPIFA2 was never assessed in this assay. This assay will be repeated in future studies with unpurified BRIFA2, purified nBPIFA2 and rBPIFA2.

As mentioned in the introduction, the predicted structural similarity of the BPIF proteins and the LBP-BPI-PLTP-CETP protein family has led to the belief that BPIF proteins share functional attributes with BPI and LBP proteins, which interact with gram negative bacteria in the innate immune pathway. It is interesting that our data suggests that BPIFA2 may selectively bind to gram-positive bacteria and not to gram-negative bacteria; however no binding was observed between BPIFA2 and the gram-positive respiratory commensal, *S. aureus* or gram-positive oral commensal *S. gordonii*. Thus a simple distinction cannot be the case. The β-haemolytic streptococcus and *S. mutans* are both considered opportunistic pathogens of the upper respiratory tract and oral cavity: *S. mutans,* as mentioned previously, is believed to be an important coloniser of the tooth surface and under the correct conditions, may contribute to dental caries while β-haemolytic streptococci, such as *Streptococcus pyogenes*, is a common cause of pharyngitis and tonsillitis (Kreth et al., 2005). Infections are rare with the bacteria which did not bind to BPIFA2: *E. coli, S. aureus* and *S. gordonii*. *P. aeruginosa*, which also did not bind, has been shown to colonise the upper airways, particularly the nasopharynx and oropharynx of cystic fibrosis patients (Taylor et al., 1992) but infections of uncompromised upper airway tissues are rare. This preliminary data suggests that bacteria, known to colonise the oral cavity and upper respiratory tract are targeted by BPIFA2, with the exception of *P. aeruginosa.*

Interestingly, demonstration of the binding of purified nBPIFA2 to *P. gingivalis* was dependent upon the removal of the lysine specific gingipain. *P. gingivalis* expresses three gingipains which are trypsin-like cysteine proteinases. RgpA and RgpB act to degrade the Arg-Xaa peptide bond. The third gingipain, Kgp, targets the Lys-Xaa peptide bond. BPIFA2 is a protein which contains a large number of lysine residues and only 1 arginine. The degradation of the peptide bonds between the lysine residues causes total degradation of the protein, leading to the loss of function, therefore explaining why BPIFA binding to W50 (wild type) and E8 strains of *P. gingivalis* could not be demonstrated (they still express the lysine specific gingipain). BPIFA2 only contains 1 arginine residue at position 221. Degradation of the Arg(221)-Ile(222) peptide bond by the arginine specific gingipain would lead to the removal of the terminal region of BPIFA2 (27 amino acids), resulting in a much less severe degradation than seen with the lysine-specific gingipain. Also, since BPIFA2 clearly bound to the kgp mutant, *P. gingivalis* K1A, the arg(221)-Ile(222) peptide bond within BPIFA2 does not appear to affect the binding ability of the protein to this organism, and possibly others.

Biofilm formation is important in the initiation of periodontal disease, as formation of a bacterial biofilm leads to an innate immune inflammatory response. Although this can lead to the recruitment of immune cells to prevent the invasion of bacteria into the system, it can also lead to the weakening of the gingiva supporting the teeth (Kinane et al., 2008). Therefore, it may be beneficial to the host, particularly in cases of chronic inflammation, to elicit a system which reduces the bacterial load before inflammation occurs. It is therefore conceivable that some salivary proteins,

such as BPIFA2, act upon bacterial biofilms to disrupt and prevent an inflammatory infiltrate, reducing the effects of chronic inflammation on the supporting structures of the teeth. No previous data has been published relating to the important periodontopathogens, although there were clear effects of BPFIA2 on *T. forsythia* biofilm as well as on *S. mutans* biofilms. Others have reported on disruption of a *P. aeruginosa* biofilm by BPIFA2 peptides (Gorr et al., 2008); which contradicts the results seen in this study, although, as previously mentioned, this was done with a peptide, rather than the intact protein. As biofilms are made up of a combination of bacteria, it would be interesting to see how the presence of BPIFA2 affects biofilms containing both *T. forsythia* and *S. mutans*.

Future studies will require purification of rBPIFA2 without the use of imidazole or after the imidazole has been successfully removed. The current study found that the low concentrations of imidazole used to elute recombinant protein from the nickel resin, resulted in bacterial killing, and thus any rBPIFA2 preparations containing imidazole would not be suitable for assays investigating growth, killing and biofilm growth. As agglutination of bacteria was not reliant upon live bacteria, possible contaminating antibacterial agents present did not affect the results and so "non-purified" rBPIFA2 protein could be used in this assay without concern.

There is very little information available regarding the interaction between BPIFA2 and lipids. This study highlighted the binding of BPIFA2 to a number of different lipids depending upon the source of the BPIFA2. BPIFA2, enriched using saliva film method, suggested BPIFA2 binds to three PIP lipids, PtdIns(4)P, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and phosphatidic acid, whilst recombinant BPIFA2 from S2 cells

showed binding to PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$. As the native BPIFA2 was not pure and the preparation contained a number of other salivary proteins, it is possible that some of the lipid binding observed was due to the binding of BPIFA2 in conjunction with salivary proteins such as mucin or amylase. It is also possible that the differences in binding may be due to differential glycosylation of the native BPIFA2 and the rBPIFA2. Although the rBPIFA2 was also not pure, the other proteins present would originate from the drosophila, not human, and so may not interact with the lipids explaining the difference in binding seen in this assay. Both recombinant and purified native BPIFA2 bound to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$), located in plasma membranes. It has been suggested that binding to these membrane lipids may be involved in the localisation of BPIFA2 protein into granules of the acinar cells for release into the salivary ducts (Venkatesh et al., 2011). If this is the case, the functional significance of this binding is minimal. However, it is possible that some interaction with bacterial lipids occurs but further investigation of the functional basis of lipid binding is clearly warranted.

It has been demonstrated here that BPIFA2 binds non-specifically to a number of oral commensal and oral non-commensal bacteria in the presence of other salivary proteins, however in their absence BPIFA2 shows specific binding to the gram-positive bacteria *S. mutans* and β–haemoltic streptococcus. It has been hypothesised that BPIFA2 shares functional similarities with BPI and/or LBP and thus would target gram-negative bacterium this is, therefore a novel finding. In

addition to the binding of *S. mutans* and β–haemoltic streptococcus it has been demonstrated, using agar and planktonic killing assays, that BPIFA2 shows no bactericidal activity against a number of bacteria including *E. coli, S. aureus, P. aeruginosa, S. gordonii* and β-haemolytic streptococcus, however, preliminary studies suggest that the planktonic growth of *S. mutans* is reduced in the presence of BPIFA2. As BPIFA2 caused agglutination of *S. mutans* it is possible that the reduced planktonic viability is due to agglutination rather than a reduction in viability. This has not yet been supported by similar experiments with rBPIFA2. An altered *S. mutans* biofilm in the presence of BPIFA2 was also demonstrated, in that an evenly distributed biofilm changed to a more uneven, aggregated biofilm. Again activity against gram-positive bacterium have, to date, not been reported in the literature and so these results suggest a new focus for the function of BPIF proteins, particularly BPIFA2.

Chapter 4.   Molecular Mimicry

4.1 Introduction

It is almost 100 years since the house dust mite was proposed as a causative agent in allergic reactions. This suggestion was, however, largely ignored and it was not until 1964 that it was truly considered (Voorhorst et al., 1964). Over the past few decades it has been conclusively shown that both deceased and living house dust mites contribute to the development of respiratory allergies, such as extrinsic asthma and allergic rhinitis. The proteolytic nature of dust mite allergens is reported to damage the lung epithelium, which in addition to an exaggerated immune response to LPS in the presence of dust mite allergens leads to inflammation and the classical symptoms of asthma (Nadchatram, 2005).

The families of mites known to induce an allergic response include *Acaridae, Pyroglyphidae* and *Glycophagidae* and of these, the *Pyroglyphidae* family is believed to be the most allergenic, particularly the *Dermatophagoides* species (Thomas and Hales, 2007). *Dermatophagoides pterontssinus* allergens 1 and 2 (Der p 1 and Der p 2) are believed to be the most important allergens accounting for 80% of allergic cases. These allergens are believed to be present at high concentrations in dust mite extract (20-100μg ml^{-1}). Although Group 3 (Der p 3), 5 (Der p 5), 7 (Der p 7), 10 (Der p 10), 11 (Der p 11) and 14 (Der p 14) allergens are believed to be present at low concentrations in comparison to group 1 and 2 allergens, they are believed to be very potent, inducing a high allergic response. The concentrations of other dust mite allergens are unknown. Dust mite allergens have a wide variety of biochemical functions which characterise them, for example, group 1 allergens are all cysteine

proteases, group 9 allergens are collagenolytic serine proteases and group 13

allergens are fatty acid binding proteins (Table 4.1). Functions of all groups have not

been defined though, including groups 5 and 7 (Table 4.1) (Thomas et al., 2004). A

number of research groups have hypothesised that the interaction of bacterial LPS

and some dust mite allergens can either initiate or exacerbate allergic reactions

(Mueller et al., 2010).

Table 4.1: House Dust Mite Allergens and their biochemical functions

Group	Biochemical Function
1	cysteine protease
2	(Niemann Pick C3 homologue)
3	trypsin
4	α-amylase
5	unknown
6	chymotrypsin
7	unknown
8	glutathione-S-transferase
9	collagenolytic serine protease
10	tropomyosin
11	paramyosin
12	unknown
13	fatty acid binding protein
14	vitellogenin/apolipophorin-like
15	98K chitinase
16	gelsolin
17	Ca binding EF protein
18	chitinase
19	anti-microbial peptide

(Thomas et al., 2004)

Der p 7 from Dermatophagoides pteronyssinus is a protein of 198 amino acids that

can be differentially glycosylated to give 3 proteins of between 22 and 31 kDa.

Structural analysis has identified an elongated protein containing two 4-stranded β

sheets in a head to toe orientation wrapped around a C-terminal helix. The

structure is described as having a cleft at the N-terminal helix between adjacent β sheets. Recent analysis has determined that juvenile hormone binding protein, Takeout proteins and BPI are significantly structurally similar to Der p 7 (Mueller et al., 2010). In particular, the 'super roll', present in the N-terminal domain of BPI, has been described as having a significant match to Der p 7 with an alignment RMSD of 3.5 Å over 174 Cα atoms. This similarity has led to the hypothesis that Der p 7 may have a similar mechanism of action to BPI whereby bacterial LPS leads to the initiation of an immune reaction (Mueller et al., 2010).

BPI is found in the cytoplasmic granules of polymorphonuclear leukocytes and, as previously mentioned, has strong antimicrobial properties towards LPS of gram-negative bacteria (Weiss et al., 1992). It has also been shown that BPI and N-terminal BPI proteins within the blood stream act to bind and neutralise gram-negative bacteria, reducing their potency and inhibiting the action of the immune response (Weiss et al., 1984, Beamer et al., 1998). In contrast to this action LBP, expressed by the hepatocytes of the liver and released into the blood stream, causes an enhancement of the immune system (Beamer et al., 1998). LBP is structurally (45% amino acid identity) and functionally related to BPI and both have been identified as gram-negative bacterial LPS-binding proteins (Weiss et al., 1984, Lei and Morrison, 1988, Mannion et al., 1989, Halling et al., 1992).

As previously mentioned, although the structure of the BPIF family of proteins has yet to be established, it has been predicted that all of the BPIFs contain a fold similar to that seen in BPI with a 95% confidence value based on the primary amino acid structure (Bingle and Craven, 2002).

The oral cavity and respiratory tract is one of the first areas exposed to extrinsic pathogens via inhalation and ingestion. Recognition and clearance of these pathogens in a rapid manner is important to survival. The innate immune system provides this rapid protection, allowing time for the adaptive immune system to react in a slower but more specific way.

The tight intercellular junctions of the oral and respiratory epithelial cells provide a barrier against the invasion of bacteria (Davies et al., 1998). As previously mentioned, the production and secretion of saliva into the oral cavity repeatedly washes away planktonic bacteria and exposes bacteria to a number of antimicrobial proteins, peptides and mucins, which work in concert to minimise adhesion, colonisation and invasion of the oral tissues (Staines et al., 1993). This physical and mechanical defence does not require any form of recognition; it is non-specific and non-selective over organic and inorganic molecules.

The immune system also recognises pathogens by their expression of conserved antigens known as pathogen-associated molecular patterns (PAMPs) not found on host cells (Akira et al., 2006). PAMPs include gram-negative bacterial LPS, lipoproteins of eubacteria, LTA of gram-positive bacteria, CpG, peptidoglycan and Lipoarabinomannan of mycobacteria, N-formyl-Met of prokaryotes, heat shock proteins of both prokaryotes and eukaryotes and mannans, mannoproteins and cell walls of yeast (Aderem and Ulevitch, 2000).

PAMPs are detected by preformed, non-specific, germline-encoded pattern-recognition receptors (PRRs), which are constitutively expressed by cells of the

innate immune system (Akira et al., 2006). PRRs include cytoplasmic receptors, such as retinoic acid inducible gene-I (RIG-I)-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors and membrane bound and DNA receptors, such as TLRs. The founding member of the TLR protein family was initially identified in *Drosophila* fruit fly in 1985 when the toll gene was identified as being necessary for the development of the dorsoventral polarity. Later it was proposed that not only did the toll gene control embryogenesis but it also played a vital role in the immunity of the fruit fly. It was shown that through binding of toll to a ventralisation inducer protein, spatzle, the NF-kB pathway was activated (Lemaitre et al., 1996) and that a lack of Toll in the fruit fly left it susceptible to fungal infections (Takeda et al., 2003).

The human homologue of toll was reported in 1997 (Medzhitov et al., 1997) and it is believed that mammals have 10-15 TLR genes (Iwasaki and Medzhitov, 2004) with TLR-4 being the founding member (Takeda et al., 2003). TLRs are integral proteins containing an extracellular domain made up of leucine-rich-repeat motifs of varying lengths, a transmembrane segment and an intracellular signalling domain named the toll/IL-1 receptor homology (TIR) domain (Akira et al., 2006). The overexpression of TLRs has been shown to induce a number of inflammatory cytokine and co-stimulatory molecule genes. TLRs recognise conserved elements common to a number of pathogens for example: TLR-2 recognises lipoproteins and LTA of gram-positive bacteria; TLR-5, flagellin; TLR-9, CpG DNA; TLR-3, dsRNA and TLR-7, ss viral RNA (Iwasaki and Medzhitov, 2004). TLR-4 has been shown to recognise the LPS portion of gram-negative bacteria (Takeda et al., 2003) with the

help of innate immune component, LBP. Only a small concentration of LPS is required to activate TLR-4 (Takeda and Akira, 2005). As mentioned previously, the recognition of LPS by TLR-4 leads to an increased affinity of the cellular receptor CD14. This complex then interacts with both TLR-4 and MD-2. This complex activates the intracellular domain of TLR-4 resulting in a cytoplasmic signalling cascade leading to the activation of NF-kB, and increased expression of inflammatory cytokines such as IL-8 (Figure 4-1).
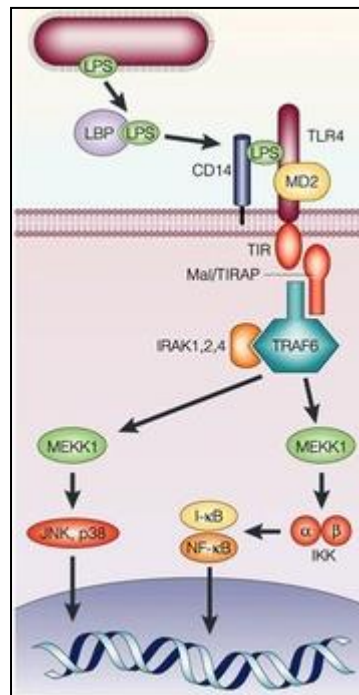
Finlay and Hancock (2004)

Figure 4-1: Activation of NF-kB in response to gram-negative bacerial LPS

Bacterial LPS associated with LBP is transported to CD14, leading to the formation of a complex consisting of LPS, CD14, TLR-4 and MD-2. The association of each component of this complex leads to the interaction of the intracellular toll-interleukin receptor domain of TLR-4 with an intracellular protein, MyD88 and following a cytoplasmic signalling cascade, NF-κB is activated which up-regulates the expression of various inflammatory genes including the IL-8 gene.

4.2 Hypothesis and Aim

The recognition of bacterial LPS by TLR-4 requires a number of accessory molecules. LPS is collected by LBP, which delivers it to membrane bound CD14 (Wright et al., 1990). This complex then transfers the LPS to MD-2 and TLR-4 which results in a series of reactions leading to the activation of NF-κB (Takeuchi et al., 1999). NF-κB is a family of transcription factors that, when activated, lead to immune and inflammatory responses including the increased expression of the chemoattractant IL-8. Molecular mimicry has previously been demonstrated by house dust mite allergens in that Der p 2 can hijack the TLR4/MD-2 interaction. The related protein, Der p 7, has structural similarity to BPI and LBP and thus could mimic LBP and either instigate an innate immune response against gram-negative bacterial LPS or prevent LBP from presenting LPS to CD14 resulting in a reduction in the innate immune response.

Hypothesis

As BPIFA2 is predicted to share structural homology with the N-terminal domain of BPI (and so also with LBP and Der p 7), we hypothesised that the mimicry of LBP by Der p 7, could suggest a possible mode of action for BPIFA2.

Aim

The aim of this chapter was to identify the effect Der p 7 has on the innate immune pathway in the presence and absence of LBP and to use the same assay system to determine whether BPIFA2 has a similar functional role.

4.3   Materials and Methods

4.3.1   Cell lines

THP-1 cells, an acute monocytic leukaemia cell line, were kindly provided by Dr Craig Murdoch (University of Sheffield Dental School, Sheffield, UK), and were cultured in a humidified atmosphere at 37°C and 5% CO_2 in RPMI-1640 with L glutamine (Sigma, UK) supplemented with 10% tissue culture grade (low endotoxin ≤10EU/mL) FCS (Sigma, UK), 100U ml^{-1} penicillin and 100µg ml^{-1} Streptomycin (Sigma, UK). The cells were centrifuged every 3 to 4 days at 1000rpm for 5 minutes and resuspended in fresh media. The THP-1 cells were maintained at 6-8 $x10^5$ cells ml^{-1}.

HEK 293 cells were kindly supplied by Dr Clare Byrant (University of Cambridge, Cambridge, UK) and were cultured in a humidified atmosphere at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS (Sigma, UK), 2mM L-glutamine (Sigma, UK) 100U ml^{-1} penicillin and 100µg ml^{-1} Streptomycin (Sigma, UK). Media was changed every 2-3 days and cells were passaged when 70-80% confluent. Cells were washed in PBS and then incubated with 0.05% trypsin-EDTA (Sigma, UK) for 5 minutes. Cells were pelleted by centrifugation, 1000rpm, 5 minutes, and resuspended in 1 ml complete media before re-seeding 1:10 in a T-75 flask.

All cells were regularly tested for mycoplasma infection.

Frozen stocks of both cell lines were stored in 50% complete media, 40% FCS and 10% dimethylsulphoxide (DMSO) (Sigma, UK).

4.3.2   Bacterial LPS Extraction

Escherichia coli (U125643)*, Pseudomonas aeruginosa* (clinical strain)*, Porphyromonas gingivalis* (W50) and *Haemophilus Influenzae* (clinical strain) were cultured as previously described (3.3.2) and the LPS was extracted using an LPS extraction kit (Intron Biotechnologies), according to the manufacturer's instructions. The LPS was analysed using an LPS polyacrylamide gel with LPS silverstain and developed with silverstain plus (BIORAD).

4.3.3   LPS SDS-Polyacrylamide gel electrophoresis and LPS silverstain

Bacterial LPS was separated on an SDS-Polyacrylamide gel containing 0.8M UREA. The gel was then fixed in 0.7% periodic acid, 40% ethanol and 5% Acetic Acid. Following a 2 hour wash under running water the gel was soaked in staining reagent containing Ammonia (2mls), 20% Silver nitrate (5mls), distilled water (115ml) and 0.1M NaOH (drop wise until solution becomes clear). After three 15 minute washes the gel was developed with silverstain plus developer (BIORAD) until bands reached the desired intensity. The reaction was stopped in 5% acetic acid and the gel scanned with Lab Scanner Image Master (Amershem).

4.3.4   THP-1 Cell Culture

For all assays, THP-1 cells were seeded at a density of 6×10^5 viable cells ml^{-1} in 200µl volumes (1.2×10^5 cells per well) and incubated overnight in the presence and absence of FCS. For FCS free samples, the cells were centrifuged (1000rpm for 5 minutes) and resuspended in FCS free medium 2 times, to remove any residual FCS, before being added to the plate.

4.3.5 IL-8 Enzyme-linked immunosorbent assay

To detect changes in inflammatory response, via the TLR 4 pathway, expression of IL-8 was measured following treatment of the THP-1 cells using an Enzyme-linked immunosorbent assay (ELISA). Due to large variations in the level of responses between replicate experiments all results were normalised to the culture media negative control and expressed as fold change.

ELISA analysis was performed using the BD OptEIA™ Human IL-8 ELISA kit (Minimum detection limit - 0.8pg/ml, BD Biosciences). The ELISA plate (NUNC, Thermo Scientific, UK) was coated with IL-8 antibody overnight at 4°C. The plate was then blocked with PBS containing 10% FCS for 1 hour at room temperature. Conditioned media or prepared standards (Figure 4-2) were then added to the plate to allow the IL-8 to complex with the coating antibody for 2 hours at room temperature. Samples with high concentrations of IL-8 and those which exceeded the standard curve limit were diluted appropriately and all of the samples re-tested to ensure accurate concentrations were collected. IL-8 was then detected with IL-8 antibody conjugated with streptavidin-HRP for 1 hour at room temperature and the final addition of substrate solution. The reaction was stopped with 2N H_2SO_4 and the absorbance was read at 570nm and 450nm to allow for wavelength correction. Concentrations were calculated using Delta Soft Microplate Analysis Software (Delta Soft Inc).
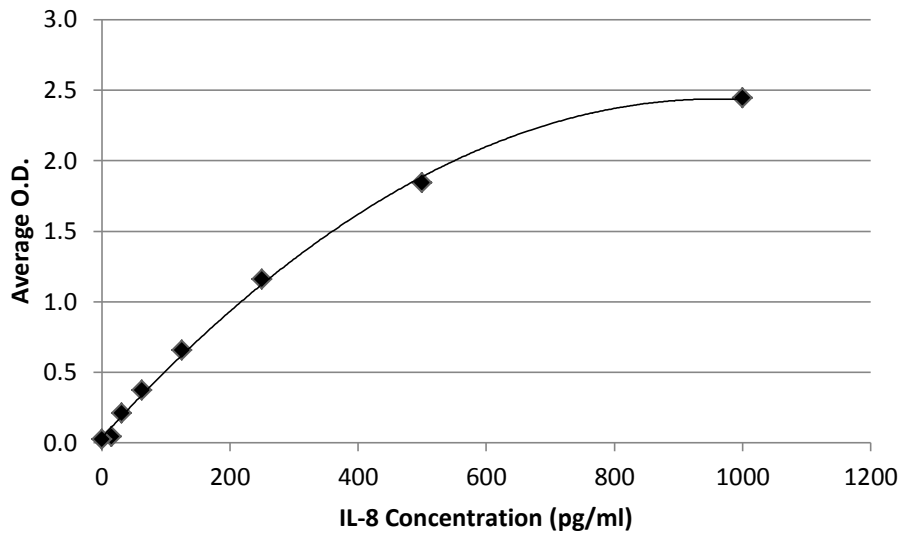
Figure 4-2: Representitive IL-8 standard curvedemonstarting the range of the assay

The concentration of IL-8 was determined using the BD OptEIA™ Human IL-8 ELISA kit. A standard curve was determined alongside the samples. This standard curve is representative of the standard curves throughout the project. The sensitivity of the ELISA is reported to be 0.8pg/ml, the minimum concentration detected from the negative controls exceeded 8pg/ml, well within this minimum detection limit.

4.3.6 Effect of Bacterial LPS on the inflammatory response via the TLR4/MD-2/CD14 pathway in THP-1 cells

To determine which bacterial LPS THP-1 cells responded to, the cells were treated with *E. coli, P. aeruginosa, P. gingivalis* or *H. influenzae* LPS and TNF-α (40ng ml^{-1}) and incubated at 37°C for 1.5, 3.0, 4.5, 6.0, 7.5 and 9.0 hours. The samples were centrifuged at 1000rpm for 5 minutes to remove the THP-1 cells and the conditioned media stored at -20°C before analysis by ELISA.

4.3.7 LPS optimisation

THP-1 cells were treated with *E. coli* (0111:B4) LPS in the presence and absence of FCS. THP-1 cells were treated with increasing concentrations of *E. coli* (0111:B4) LPS and incubated for 6 hours at 37°C. The samples were centrifuged (1000rpm, 5

minutes) to remove the THP-1 cells and the conditioned media was stored at -20°C until analysed by ELISA.

4.3.8 rDer p 7 optimisation

THP-1 cells were treated with rDer p 7 (Indoor Biotechnologies, Charlottesville, VA) in the presence and absence of FCS. THP-1 cells were treated with increasing concentrations of rDer p 7 and incubated for 6 hours at 37°C. The samples were centrifuged (1000rpm, 5 minutes) to remove the THP-1 cells and the conditioned media was stored at -20°C until analysed by ELISA.

4.3.9 Mimicry of LBP by Der p 7

THP-1 cells were treated with low concentrations of *E. coli* LPS (3.125ng ml^{-1}, 6.25ng ml^{-1} and 12.5ng ml^{-1}) in the presence and absence of rDer p 7 (2µg ml^{-1}, 1µg ml^{-1}, 0.5µg ml^{-1}, 0.25µg ml^{-1}) and in the presence and absence of FCS. Cells were treated for 6 hours, centrifuged (1000rpm, 5 minutes) to remove the THP-1 cells and the conditioned media was stored at -20°C before analysis by ELISA.

4.3.10 Data and Stastistical Analysis

IL-8 assay data was normalised to the negative control and is presented as fold changed due to a large amount of variability seen in the THP-1 cells during the determination of suitable timescales assays (Appendix 6.7).

Where possible all data is presented as means + SEM. Statistical analysis was performed using the student's t-test for comparisons between two groups. All data was analysed compared to the corresponding negative control to determine any

significant increase in IL-8 activation. Values were considered significant if *p* values were <0.05.

4.3.11 TLR4/MD-2/CD14 Transient Transfection

HEK 294 cells were seeded into a flat bottomed 96 well plate at 1.5×10^5 cells ml^{-1}. Following a 48 hour incubation, cells were transfected using jetPEI transfection reagent (polyplus transfection) with 10ng pcDNA5-frt-V5-His-Topo/BPIFA1, pcDNA5-frt-V5-His-Topo/BPIFA2, pcDNA5-frt-V5-His-Topo/BPIFB1 or pcDNA3 along with TLR-4 pathway specific DNA, pcDNA3/TLR-4, pEFIRES/MD2, pcDNA3/CD14 and luciferase (pNF-κβ-luc) and renilla (hRG-TK) as internal controls for transfection efficiency (Table 4.2) (Lee Hopkins, University of Cambridge, Cambridge UK) to a total concentration of 100ng/well.

Following 48 hours incubation the media was removed and LPS was added at 0.05ng ml^{-1} or 0.5ng ml^{-1} in FCS free medium. Following a 6 hour incubation at 37°C the medium was removed and the HEK-293 cells were washed with PBS before lysis with 1x passive lysis buffer (Promega). Plates were stored at -80°C until analysis. Luciferase activity was quantified using the Dual Luciferase kit (Promega) according to the manufacturer's instructions and the GloMax®-96 Microplate luminometer (Promega), which assessed the NF-κB response. Data was normalised to the equivalent 0ng ml^{-1} treatment for analysis.

Table 4.2. Transient transfection set up

(Stock Concentration (ng/µl))	(volume µl per 10 wells)			
	Control	BPIFA1	BPIFA2	BPIFB1
pcDNA3/TLR-4 (100)	1	1	1	1
pEFIRES/MD-2 (10)	1	1	1	1
pcDNA3/CD14 (100)	1	1	1	1
pNF-κβ-luc (10)	10	10	10	10
hRG-TK (10)	5	5	5	5
pcDNA3 (100)	6.4	6.3	6.3	6.3
pcDNA5-frt-V5-His-Topo /BPIFA1 (10)	-	1	-	-
pcDNA5-frt-V5-His-Topo /BPIFA2 (10)	-	-	1	-
pcDNA5-frt-V5-His-Topo /BPIFB1 (10)	-	-	-	1
10x TE	2.44	2.44	2.44	2.44
150mM NaCl	23.16	22.26	22.26	22.26
Total Volume (µl)	**50**	**50**	**50**	**50**

4.4 Results

4.4.1 Initial Experiments

4.4.1.1 Determination of suitable timescales for IL-8 assays

LPS from *E. coli, P. aeruginosa, P. gingivalis* and *H. influenzae* were extracted and analysed by SDS-polyacrylamide gel electrophoresis to ensure that LPS preparations were representative and that LPS was present in each of the samples. LPS is composed of varying length carbohydrate chains in association with Lipid A which allows them to be anchored to the outer membrane of gram-negative bacteria. Migration through the gel is based on the length of the carbohydrate chain; Lipid A with smaller carbohydrate chains travels further than Lipid A with larger chains. Lipid A molecules with the same length of carbohydrate chains will migrate together, producing a denser band in the LPS gel. The pattern seen from this migration is unique to each bacterium and these 'profiles' can vary considerably. If no LPS were purified, no pattern would be seen. Clear carbohydrate patterns can be seen following LPS Polyacrylamide gel electrophoresis (Figure 4-3) for each bacterium indicating that LPS was successfully isolated.

THP-1 cells were treated with each bacterial LPS to determine their response via the TLR-4/MD-2/CD14 pathway resulting in the immune response of increased IL-8 expression.
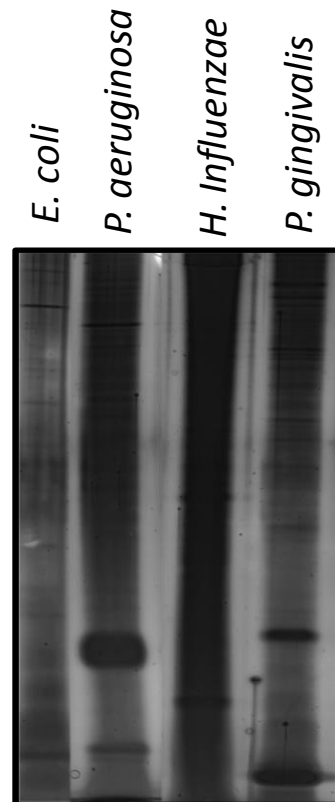
Figure 4-3: SDS-PAGE analysis of extracted bacterial LPS

LPS from *E. coli, P. aeruginosa, H. influenzae* and *P. gingivalis* were extracted using the LPS Extraction Kit (Intron Biotechnologies). LPS presence was confirmed by SDS polyacrylamide gel electrophoresis and LPS silverstain and comparison with previously extracted LPS from the same species of bacteria, using the same method.

No increased IL-8 expression, over that of the negative control, was seen following treatment of the THP-1 cells with 1µl ml^{-1} of extracted *P. gingivalis* LPS for 1.5 hours and 3.0 hours (0.9 fold and 0.6 fold respectively). IL-8 expression increased slightly following 4.5 hours treatment (1.3 fold), but this diminished after 6.0 hours and 7.5 hours (1.0 fold and 0.8 fold respectively) (Figure 4-4A). Treating the THP-1 cells for extended time periods failed to increase the expression of IL-8.

No increase in IL-8 expression was observed at any time point in response to treatment with 1µl ml^{-1} of extracted *P. aeruginosa* LPS (Figure 4-4B).
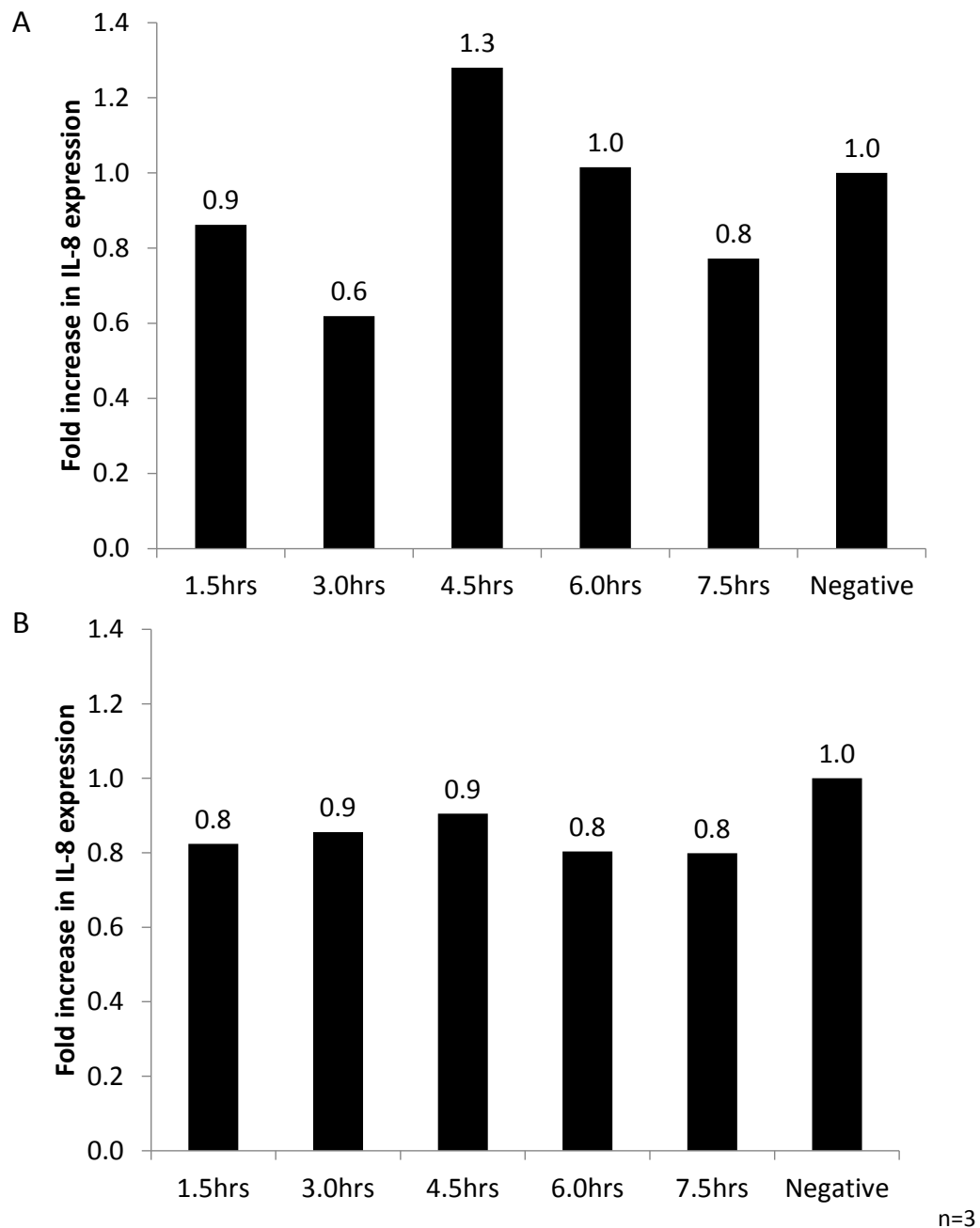
Figure 4-4: Change in IL-8 expression following incubation of the monocytic cell line, THP-1, with *P. gingivalis* (A) and *P. aeruginosa* (B) for various periods of time

THP-1 cells were treated with extracted LPS from *P. gingivalis* or *P. aeruginosa*; samples were taken at varying time points to assess the levels of IL-8 expression. *P. gingivalis showed* a slight increase in IL-8 expression at 4.5 hours with a 1.3 fold increase and no increase at any of the other time points tested. *P. aeruginosa* failed to cause any increase in IL-8 expression following 7.5 hours of treatment. The negative control was culture media.

In contrast, treatment with *H. influenzae* LPS (Figure 4-5A) led to a significant IL-8 response, with almost a 20 fold increase in expression following treatment for 3 hours. The response reduced gradually after 4.5, 6.0 and 7.5 hours to 13.6, 7.5 and 7.4 fold respectively.

The largest response was seen with *E. coli* LPS (Figure 4-5B) as after just 1.5hours the IL-8 expression increased by almost 15 fold compared to the untreated control, and this gradually increased to 19.3 fold after 3.0 hours and finally peaked after 4.5 hours to a 48 fold increase in IL-8 expression. The IL-8 expression diminished to 24.4 and 17.7 fold that of the negative control following treatment for 6.0 and 7.5 hours.

A

B
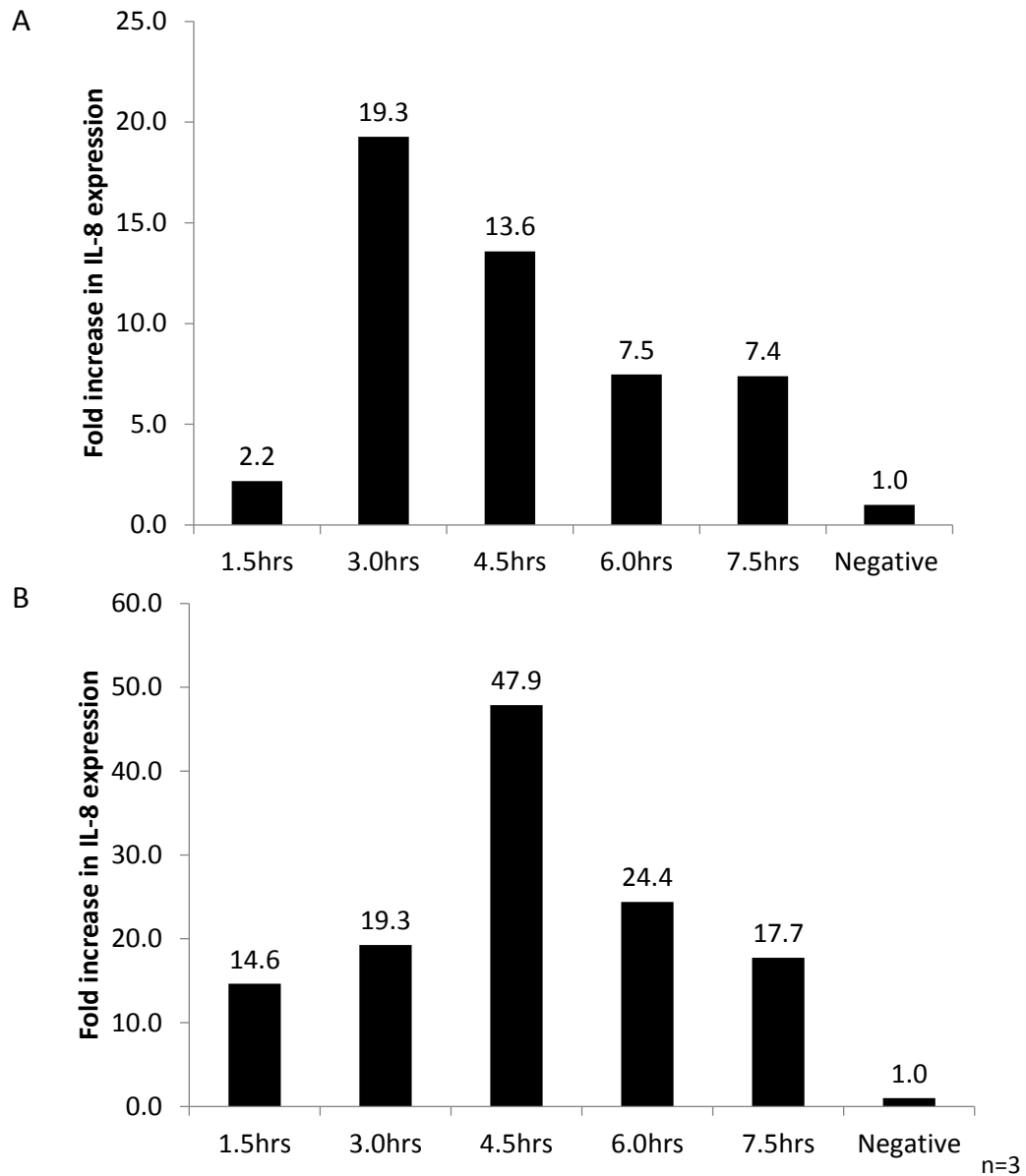
Figure 4-5: Change in IL-8 expression following incubation of the monocytic cell line, THP-1, with *H. influenzae* (A) and *E. coli* (B) for various periods of time.

THP-1 cells were treated with LPS extracted from *H. influenzae* or *E. coli*; samples were taken at varying time points to assess the levels of IL-8 expression. *H. influenzae* caused a rapid increase in IL-8 expression from 2.2 fold increase at 1.5 hours to almost a 20 fold increase at 3 hours. *E. coli* showed a more dramatic effect on the expression of IL-8 with almost a 15 fold increase after 1.5 hours peaking at almost 50 fold after 4.5 hours. IL-8 expression reduced at 6 hours and then again at 7.5hours. The negative control was culture media.

4.4.1.2   Dose response of LPS concentration on IL-8 production

To ensure the results were consistent and reliable, commercial *E. coli* (0111:B4) LPS was used for further assays. THP-1 cells were treated with increasing concentrations of *E. coli* (0111:B4) LPS ranging from 0.3ng ml^{-1} to 20ng ml^{-1} in the presence of FCS, and thus LBP, to establish basal levels of the IL-8 response. IL-8 expression levels increased in a dose dependent manner with increasing LPS concentrations ranging from a 2-fold increase with 0.3ng ml^{-1} to 10-fold with 20ng ml^{-1} (Figure 4-6 - black).

THP-1 cells were also treated with similar concentrations of LPS in the absence of FCS, and thus absence of LBP. THP-1 cells failed to respond at these concentrations of *E. coli* (0111:B4) LPS (Figure 4-6 - grey).

Data is displayed as fold change compared to the negative control. IL-8 concentrations (pg/ml) can be seen in appendix 6.8.

IL-8 concentration following treatment with E.coli LPS or Der p 7 at increasing concentrations in the presence and absence of Serum.

**, P<0.01
*, p<0.05

n=3
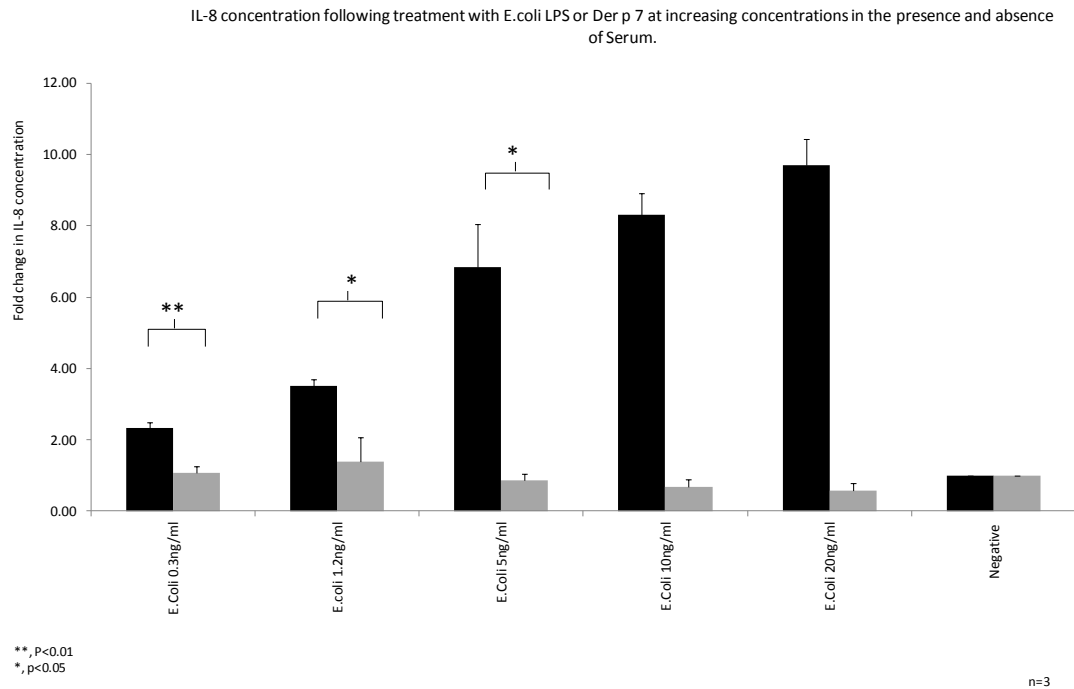
Figure 4-6: IL-8 concentration following treatment with increasing doses of *E. coli* (0111:B4) LPS in the presence of FCS (black) and absence of FCS (grey) to establish a normal response.

In the presence of FCS the IL-8 expression increased in a dose dependent manner showing a 10 fold increase in expression after treatment of 20ng ml^{-1} *E. coli* (0111:B4) LPS for 6 hours at 37°C. In contrast in the absence of FCS the IL-8 expression fails to increase at low concentrations of *E. coli* LPS. The negative control was culture media. Values are means of 3 experiments in triplicate. Error bars are standard error of the mean. Statistical analysis was performed using the student's t-test. Comparisons are made between samples with FCS and the associated sample without FCS. Significance is based on expression of IL-8 compared to the sample with/without FCS (n=3).

4.4.1.3 Dose response of rDep p7 on IL-8 expression

To determine if the rDer p 7 allergen had the potential to induce an immune response via the TLR4/MD2/CD14 pathway in the absence of LPS, THP-1 cells were treated with 0.25 to 2µg ml^{-1} rDer p 7 in the presence (Figure 4-7: black) and absence of FCS (Figure 4-7: grey), and so the presence and absence of LBP. With the exception of a slight increase (1.4 fold) observed following the addition of 0.25µg ml^{-1} rDer p 7 no increase in IL-8 expression was detected.
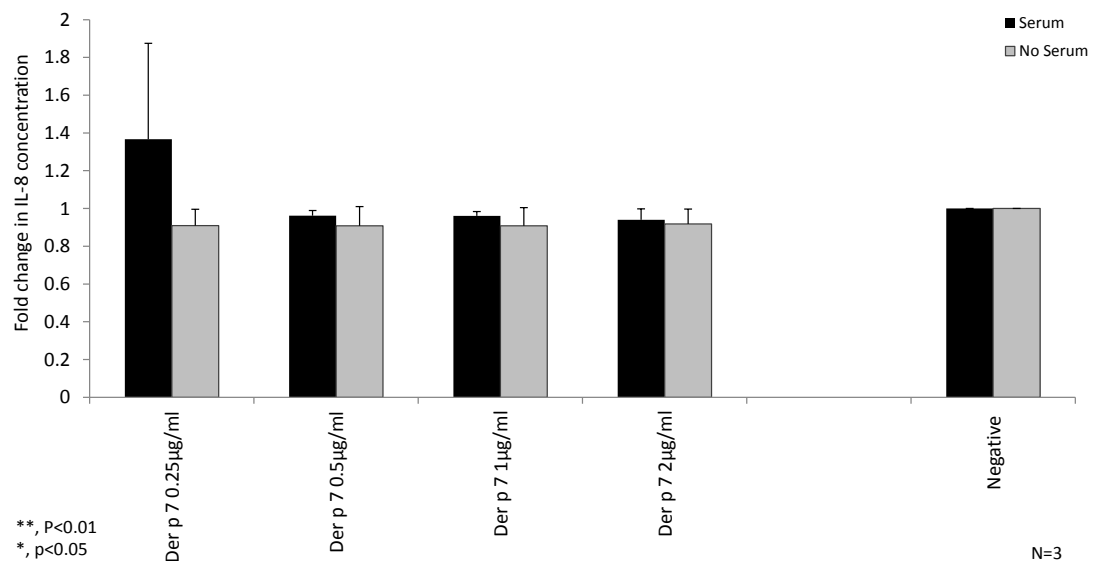
Figure 4-7: IL-8 concentration following treatment with increasing doses of rDer p 7 in the presence of FCS (black) and absence of FCS (grey) to establish a normal response.

Treating THP-1 cells for 6 hours with rDer p 7 did not induce expression of IL-8. With the exception of 0.25µg ml^{-1} (1.4 fold), all treatments showed expression in line with the negative control containing no rDer p 7. The negative control was culture media. Values are means of 3 experiments in triplicate. Error bars are standard error of the mean. Statistical analysis was performed using the student's t-test. Comparisons are made between samples with FCS and the associated sample without FCS. Significance is based on expression compared to the sample with/without FCS (n=3).

4.4.2 Induction of IL-8 by bacterial LPS in the absence of LBP and the presence of rDer p 7.

To establish if rDer p 7 has the ability to mimic the action of LBP the THP-1 cells were treated with low concentrations of *E. coli* LPS and Der p 7 in the absence of FCS.

THP-1 cells were treated with 12.5ng ml^{-1}, 6.25ng ml^{-1}, 3.125ng ml^{-1} or 0ng ml^{-1} *E. coli* (0111:B4) LPS at the same time as being treated with rDer p 7 at concentrations of 2µg ml^{-1}, 1µg ml^{-1}, 0.5µg ml^{-1}, 0.25µg ml^{-1} or 0µg ml^{-1}. THP-1 cells were treated with 100ng ml^{-1} *E. coli* LPS as a positive control.

In the absence of LBP and rDer p 7, *E. coli* LPS, as seen in preliminary assays, did not increase IL-8 expression at any concentration (12.5ng ml^{-1}, 6.25ng ml^{-1}. 3.125ng ml^{-1}). Treatment of the THP-1 cells with 12.5ng ml^{-1} *E. coli* LPS and 0.25µg ml^{-1} rDer p 7 increased IL-8 expression in excess of that produced by the 100ng ml^{-1} positive control. The expression of IL-8 increased in a dose dependent manner as the concentration of *E. coli* was increased (Figure 4-8A). A similar pattern was observed with 0.5µg ml^{-1}, 1µg ml^{-1} and 2µg ml^{-1} rDer p 7 (results not shown) but interestingly, increasing the rDer p 7 concentration further did not lead to any more increase in IL-8 expression, showing that the response had reached a plateau at 0.25µg ml^{-1} rDer p 7.

It was considered that suitable statistical analysis of this data would be a two-way ANOVA with post-hoc tests, however due to the simple structure of the Der p 7 treatment, i.e. present or absent, it was subsequently decided that a Levene's Test of Equality of Error Variancies followed by an Analysis of Co-varience (ANCOVA)

would be more beneficial. This analysis identified a non-parallel relationship between LPS treatments with and without Der p 7 (Figure 4.8B) indicating a clear difference between the response in the presence and absence of Der p 7. This is further supported by the ANCOVA analysis (Appendix 6.7) which indicates that Der p 7 affects the IL-8 response only through its interaction with LPS. However, the Levene's Test has highlighted concerns regarding the homogeneity of varience assumption and the quality of fit. Further investigation wil be required to determine significance.
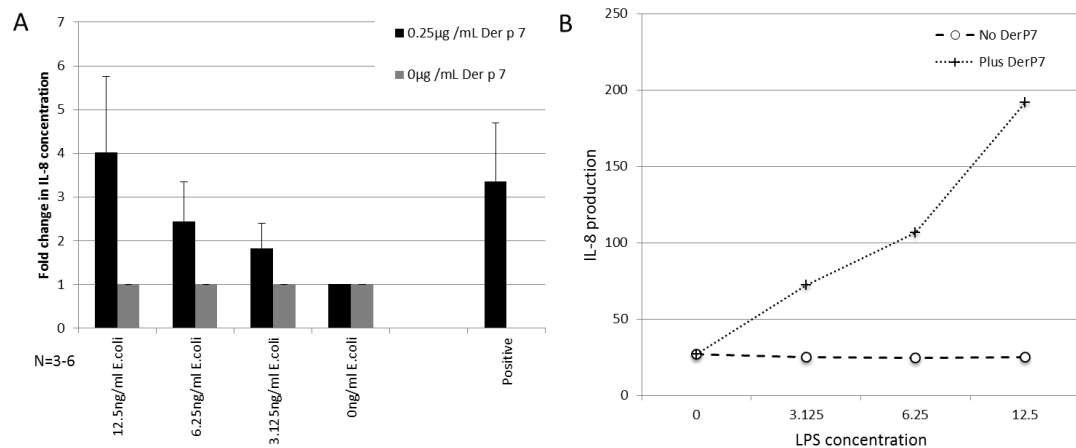
Figure 4-8. IL-8 concentration following treatment with *E. coli* LPS and rDer p 7 in the absence of FCS.

In the absence of *E. coli* LPS the presence of the rDer p 7 allergen fails to cause an increase in IL-8 expression, and similarly the presence of *E. coli* LPS at low concentrations in the absence of rDer p 7 fails to induce an IL-8 response. However, with the addition of as little as 0.25µg ml^{-1} rDer p 7 allergen the THP-1 cells can be induced to express IL-8 at a higher level compared to the positive control of 100ng ml^{-1} *E. coli* LPS. ANCOVA analysis has identified a non-zero intercept and a non-parallel relationship between treatments with and without Der p 7, indicating that Der p 7 affects the IL-8 response only through its interaction with LPS. The negative control was culture media. Values are means of 3-6 experiments in triplicate. Error bars are standard error of the mean. Statistical analysis was performed using the Levene's Test and Analysis of Covarience (n=3-6).

4.4.3   Transfection of HEK-293 cell line with *TLR4/MD-2/CD14* and *BPIF* genes

HEK-293 cells were transiently transfected with TLR-4, CD-14, MD-2 and either pcDNA5-frt-V5-His-Topo/BPIFA1, pcDNA5-frt-V5-His-Topo/BPIFA2, pcDNA5-frt-V5-His-Topo/BPIFB1 or empty pcDNA3 and treated with low concentrations of *E. coli* (0111:B4) LPS (0.05 and 0.5ng mL^{-1}). Expression of the BPIF protein in this vector have previously described using in vitro transcription/translation reactions and western blot analysis (Bingle et al 2009).

As a transfection control, a LPS dose response was performed with HEK-293 cells transfected with TLR-4, CD14, MD2, pcDNA3, Luciferase and Renilla (internal control for transfection efficiency). Although a dose response to the LPS was observed, indicating the success of the transfection, a maximum response (with 50ng ml^{-1} *E. coli* (0111:B4) LPS) of only a 2-fold increase in NF-κB activity was seen compared to that in the absence of *E. coli* (0111:B4) LPS (Figure 4-9). Initial observations indicate that no clear difference in NF-κB activity occurs in the cells co-transfected with any of the *BPIF* genes (Figure 4-10); however a trend of increased activation can be observed with BPIFA1, which requires further investigation.
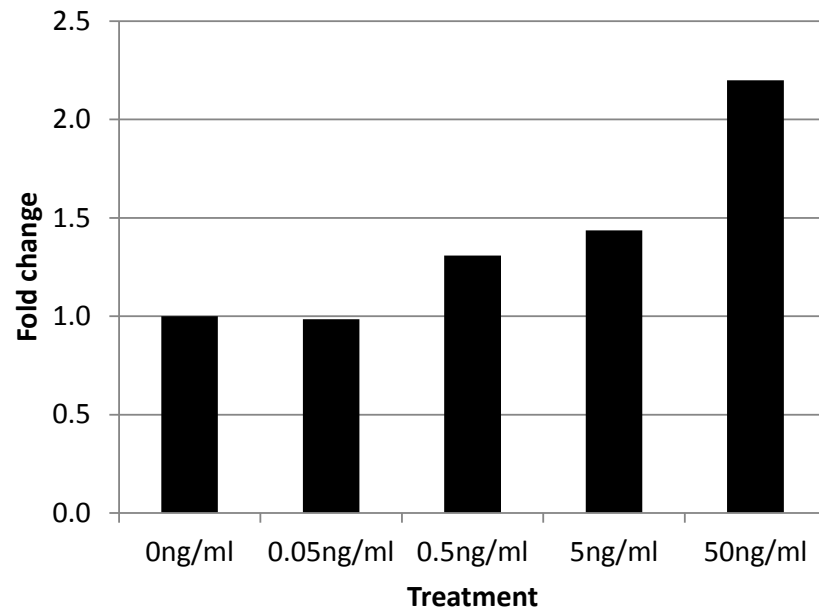
Figure 4-9: Dose response in NF-κB following treatment of TLR-4/MD-2/CD14 transfected HEK293 cells with _E. coli_ LPS

HEK-293 cells were transiently transfected with TLR-4, MD-2 and CD14. The cells were treated with increasing concentrations of _E. coli_ (0111:B4) LPS to establish levels of activation. The transfected HEK-293 cells showed dose dependent response to the LPS; however 50ng ml^{-1} of LPS only showed a 2-fold increase in NF-κB activation.
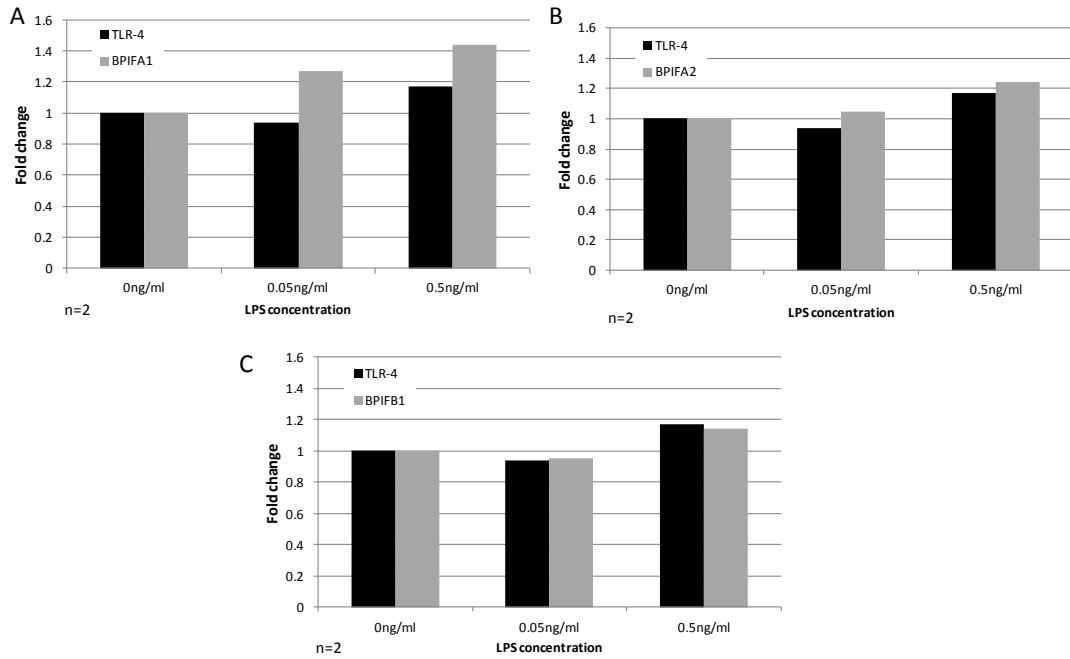
Figure 4-10: Effect of BPIF proteins on the activation of NF-κB

HEK-293 cells were transiently transfected with TLR-4, CD14, MD2, Renilla and Luciferase (as a transfection control) and either BPIFA1, BPIFA2 or BPIFB1 before being treated with either 0.05ng ml^{-1} or 0.5ng ml^{-1} *E. coli* (O111:B4) LPS. Data was normalised to the equivalent 0ng ml^{-1} treatment to assess any changes. Transfection of HEK-293 cells with BPIFA1 (A), BPIFA2 (B) or BPIFB1 (C) did not appear to affect the NF-κB pathway. Treatment with 0.05ng ml^{-1} and 0.5ng ml^{-1} resulted in no significant difference in NF-κB activation compared to the negative controls, however these preliminary studies have identified a trend of increased activation can be seen with BPIFA1. Values are means of duplicate experiments in triplicate (n=2).

4.5   Discussion

TLR-4 recognises gram-negative bacterial LPS, and results in an immune response; however this requires a number of accessory proteins. A complex, containing LPS, CD14, MD-2 and TLR-4, has the ability to elicit an immune response but the system requires LBP to deliver the LPS to CD14 in order to maximise this response. It has previously been demonstrated that dust mite allergen, Der p 2, shares structural homology to the accessory protein MD-2, and that this structural similarity allows functional mimicry by Der p 2 restoring the LPS-driven TLR-4 signalling in the absence of the essential MD-2 molecule (Trompette et al., 2009). Recently, dust mite allergen Der p 7 has been reported to share structural homology with the N-terminal region of BPI, both sharing the characteristic "super-roll" architecture (Mueller et al., 2010). BPIFA2 is also predicted to share close structural homology with the N-terminal domain of BPI, though this has yet to be determined experimentally. Due to the method adopted for the purification of nBPIFA2 it was presumed that the purified nBPIFA2 would be contaminated with LPS as the use of LPS free materials was not possible for the whole process. As a result of this presumed contamination, any studies involving the activation of the TLR-4 pathway would not be reproducible or valid as the concentration and type of LPS in the nBPIFA2 sample is unknown. Quantification of this LPS would be possible using assays such as the limulus amoebocyte lysate (LAL) assay; however this was not performed as this would only indicate the concentration of LPS in the sample and not the type of LPS nor its origin, which could affect the result of the immunological assays. Removal of this presumed contaminant LPS was attempted using the Pierce

endotoxin removal resin (Thermo Scientific, UK), unfortunately resulting in a loss of nBPIFA2. Therefore, due to the predicted structural similarity between Der p 7 and BPIFA2, endotoxin free rDer p 7 was used to investigate the potential functional role of BPIFA2 in the TLR-4 pathway. Following the mimicry of Der p 2 to MD-2, it was hypothesised that Der p 7 could also mimic the action of either BPI or the structurally similar protein LBP in the TLR-4 complex. Furthermore, it seemed reasonable to consider that BPIFA2 could mimic BPI and /or LPS in the oral cavity and upper airways in a similar manner to Der p 7.

The initial experiments in this study demonstrated activation of the TLR-4 complex, in the presence of LBP supplied by FCS, by some bacterial LPS but not others. *P. gingivalis* and *P. aeruginosa* did not produce a response in the THP-1 cell line, whilst *H. influenzae* and *E. coli* did. It has previously been reported that *P. gingivalis* LPS leads to a significant rise in IL-8 expression by THP-1 cells after only 4 hours of stimulation. This increase continued for 7 days following the addition of LPS (Baqui et al., 1999). A similar increase in IL-8 expression was thus expected in this study. Similarly an increase in IL-8 expression was expected with *P. aeruginosa* based on previously published data (Cigana et al., 2009). Although LPS was successfully extracted from these species it could not be accurately quantified. The inability to standardise the LPS dosage is likely to be a major cause of the differences in IL-8 expression between the previously published data and the results in this study.

Incubation of THP-1 cells with *E. coli* for 4.5 hours caused a significant increase in IL-8 expression, which gradually declined over the following 6 hours of incubation. Due to the extremely high response at 4.5 hours it was decided that the 6 hour

incubation period would allow more accurate detection and quantification in future assays. In addition, the decision as to whether *H. influenzae* or *E. coli* should be used was made solely on the availability and ease of access to relevant material and *E. coli* LPS (0111:B4) was commercially available. This also overcame the problem of quantification so that we could be sure a consistent amount of LPS was being added to the assay system.

As expected, commercial *E. coli* (0111:B4) LPS resulted in an increase in IL-8 production by THP-1 cells in a dose dependent manner in the presence of LBP provided by FCS in the culture medium. Removal of LBP from the assay, through the use of FCS free media, prevented this increase in IL-8 expression, until higher doses of *E. coli* LPS (25-100ng ml^{-1}) were used and these resulted in IL-8 expression levels similar to those seen with *E. coli* concentrations of less than 5ng ml^{-1} in the presence of LBP.

Activation of the TLR-4 complex involves the interaction between LBP and LPS; followed by an interaction between CD14, MD-2 and TLR-4, ultimately leading to the initiation of an inflammatory response. To verify rDer p 7 was able to mimic LBP and thus only act on the TLR-4 pathway through this mimicry, the levels of IL-8 expression were assessed both in the presence and absence of LBP. Although previous studies have described an increase in inflammatory cytokines, including IL-8, by the direct actions of house dust mite extracts (Hongjia et al., 2010) and Der p 2 (Osterlund et al., 2009), no increase in IL-8 was observed by Der p 7 in the presence of LBP (without LPS). Although this is in contrast to the literature, the house dust mite extract used by Hongjia et al (2010) was shown to contain 44ng of

LPS per 100µg protein and the Der p 2 utilised by Osterlund et al (2009) contained up to 632ng LPS/mg protein. Osterlund et al (2009) demonstrated a response with 10µg Der p 2, which would have contained 6.3ng LPS. This study has demonstrated that LPS concentrations of 20ng can elicit a 10-fold increase in IL-8 expression in the presence of FCS and concentrations of as low as 0.3ng ml^{-1} increased the IL-8 response above the background levels. Also we are confident that the rDer p 7 allergen used in our study was not contaminated with LPS and consequently no increase in IL-8 expression was detected. The increase in cytokine levels in the previous studies are therefore probably due to the presence of contaminant LPS, not the presence of the dust mite extract or Der p 2.

Replacing the LBP in FCS free media with rDer p 7 restored TLR-4 activity, suggesting that Der p 7 may be able to mimic the role of LBP by completing the TLR-4-MD-2-CD14 complex. This supported our hypothesis that BPIFA2 may play a similar role in TLR-4 activation in the oral cavity. FCS contains a number of molecules involved in the TLR-4 pathway including LBP and soluble CD14. Although not absolutely proven our results suggest that mimicry of LBP is occurring, based on the structural similarity between Der p 7 and LBP, rather than that of an alternative molecule such as CD14. Further studies to determine the accuracy of this suggestion are required, including the differentiation of the THP-1 cells into monocytes, and the use of primary monocytes. In addition, although an IL-8 response was seen after just 6 hours of treatment during the initial investigation, resulting in this timepoint being chosen for later assays, it would also be beneficial

for future studies to extend the treatment times to 12, 24 and 48 hours to determine if the responses seen are comparable over a longer timeframe.

It would be ideal to compare these results with other activators; however this assay was specifically designed to assess the ability of Der p 7 to mimic LBP and result in an IL-8 output as a measure of induction of the inflammatory response, not as a comparable method to other activators used in a similar system. Therefore, comparisons with other activators in similar systems would not be beneficial or appropriate.

Removal of LPS from the purified nBPIFA2 protein was attempted, using the Pierce endotoxin removal resin (Thermo Scientific, UK), and was repeatedly unsuccessful and thus a different assessment method was attempted. HEK-293 cells, which do not express TLR-4 and MD-2, were co-transfected with TLR-4, MD-2, CD14, renilla (internal control for transfection efficiency), luciferase and a number of *BPIF* genes to assess activation of NF-κB following LPS stimulation. In these assays the LPS dose response only showed a 2 fold increase in NF-κB following stimulation with 50ng/ml *E.coli* LPS. Initially it was thought that this response was low, however compared with work recently published by Herre et al (2013), who demonstrated that stimulation with 100ng/ml *E.coli* LPS induces an increase in NF-κB activation of just 4-fold, these values are considered reasonable. No difference in NF-κB activation was observed following transfection of any of the *BPIF* genes. Herre et al (2013) performed a similar investigation, using the cat allergen Fel D 1 in place of our BPIF proteins. They demonstrated a significant increase in NF-κB activation of approximately 15-fold. This further suggests that, as transfection of BPIF genes into

this systemshowed no real increase in NF-κB, unlike Der p 7, the BPIF proteins have no role in the stimulation of the TLR-4 pathway and the activation of NF-κB. However, it is possible that the BPIF proteins influence an alternative pathway, such as TLR-2, or bind bacterial molecules other than gram-negative bacterial LPS, such as gram-positive bacterial lipoteichoic acids (LTA), which have previously been shown to stimulate an increase in TNF-α, IL-1β, IL-6 and IL-10 but not IL-8 (Hermann et al., 2002). LTA is a surface associated molecule found in gram-positive bacteria such as streptococci, pneumococci and staphylococci (Ginsburg, 2002). *Streptococcus mutans* LTA has been shown to induce periodontal lesions and is considered a major cause of caries development (Bab et al., 1979). LTA induces the activation of NF-κB via TLR-2 positive macrophages and it has been suggested that LTA may be neutralised by proteins resembling BPI (Ginsburg, 2002). Thus LTA is a potential target for BPIFA2 in the oral cavity and warrants further investigation.

The investigation of Der p 7, as an indicator for the role of BPIFA2, in the TLR-4 pathway has demonstrated that upon removal of FCS (as a source of LBP), the addition of Der p 7 can increase the expression of IL-8 in excess of the levels seen with high (100ng ml^{-1}) *E.coli* LPS stimulation. The predicted structural similarity between Der p 7 and BPIFA2 and the proven function of LBP as an important part of the TLR-4 pathway therefore lead to the suggestion that BPIFA2 may also posses the ability to influence the activation of this pathway. In order to further investigate this purified, LPS-free BPIFA2 needs to be collected and quantified, a key focus in the progression of this project. The activation of the TLR-4 pathway with the

allergen Der p 7 has not yet been reported in the literature, and so these findings

may provide a basis for further investigation into the connection between dust mite

allergy,   the   innate   immune   response   and   allergy.

Chapter 5.   Final Discussion

The oral cavity is a complex environment and in order to ensure that it remains healthy, it is supplied with approximately 1500mL of saliva per day (Zalewska et al., 2000), which provides moisture, digestive enzymes, buffering agents and immune defence molecules. It has been hypothesised that BPIFA2, a protein expressed in major and minor salivary glands and secreted in saliva, plays a role in immune regulation. This hypothesis is based on predicted structural similarities to acknowledged innate immune molecules, BPI and LBP. The aim of this study was to establish a method that would allow purification of native BPIFA2 from human saliva in which the pure protein would retain its heavily glycosylated state and to use this protein to determine its true function. A number of purification methods were attempted, based on the current literature, and we were able to successfully purify native, glycosylated protein. The activity of purified BPIFA2 was examined against a range of bacteria using a variety of assays including bacterial binding, growth, killing and agglutination, with recombinant protein being used for comparison. Interesting results were observed against gram-positive, oral commensal bacteria, *S. mutans*. We also investigated the TLR4/MD-2/CD14 pathway to demonstrate the potential for BPIFA2 to mimic the action of BPI or LBP.

The optimal method for purification of native, active BPIFA2 from human saliva with the highest probability of the correct glycosylation was through electro-elution following native gel electrophoresis with the final product being stabilised in a buffer compatible with functional assays by dialysis. A number of methods previously described in the literature for purification of similar proteins and family

members of BPIFA2 were examined; however a number of problems were highlighted. Precipitation with both ammonium sulphate and ethanol/acetone have been used to purify the founding family member, BPIFA1 (Campos et al, 2004) but in the experiments conducted here, ammonium sulphate fractionation proved to be the most ineffective method with no separation of BPIFA2 from other salivary proteins. Our initial experiments suggested that ethanol precipitation alone would provide a relatively simple but successful method for purification of BPIFA2, however, further analysis demonstrated the presence of significant amounts of contaminant proteins between 10-15kDa. Specifically, N-terminal sequencing identified cystatin, a known antibacterial protein, as a contaminant protein. Size exclusion chromatography was used to remove contaminating proteins; however, this led to a dramatic decrease in the yield of BPIFA2 protein, which appeared to adhere non-reversibly to the column. Similar problems were encountered using ion exchange chromatography implying that impractically large volumes of saliva would be required in order to purify detectable and useable concentrations of BPIFA2 protein. In addition to the problems seen with concentrations, additional concerns were raised regarding the quality of the purified protein as the method involved relatively high concentrations of ethanol (75%) and the many ethanol purification steps needed were carried out at temperatures greater than 4°C which have previously been shown to result in protein deformation and denaturation (Zellner et al., 2005, Lin et al., 2004).

Data presented by McGillivary and Bakaletz (2010) provided the basis for the electro-elution method from native polyacrylamide gels. The published method

included electrophoresis, detergent removal, denaturation, renaturation, dialysis lyophilisation and resuspension, which, unlike the optimal method we developed, provides numerous opportunities for the incorrect folding of the protein. The method finally developed, using a native gel, allows for the separation of native protein from other salivary proteins, with a final dialysis step being necessary as the salts present in the elution buffer were not compatible with the downstream functional assays. The optimised method uses fewer steps than that of McGillivary and Bakaletz (2010) thus limiting the risk of deformation and denaturation.

As the project progressed it became evident that a significant proportion of protein was being lost. As described in Chapter 2 (2.5) this has posed a major problem due to the tendency of BPIFA2 to adhere firmly to many surfaces, including laboratory plastics, This was highlighted as a particular problem when attempting column chromatography, as the sample was fed through numerous plastic tubes, and also in the many centrifuge steps required for ethanol precipitation. The interaction with plastic surfaces was less of a problem with the native gel elution method as there are many fewer steps in the process and throughout the protein rarely comes into contact with plastic surfaces; protein losses are thus minimised. The reason for this interaction is unknown, however it could be suggested that the predicted hydrophobic cleft in the BPIFA2 protein may be involved due to the aqueous nature of the buffer. However, if this were the case it would be expected that BPIFA2 protein would interact with all surfaces and not just laboratory plastics as observed during this study. This interaction may indicate some functional significance, as BPIFA2 could potentially coat hard surfaces of the oral cavity, such as the teeth, and

protect from colonisation by pathogenic bacteria. To understand this interaction fully, more information is required regarding the structure of BPIF2. It would also be beneficial to investigate these interactions further to assess if BPIFA2 shows specific affinity to the oral structures. This is something that will be studied further in the future of the project.

Although purification of BPIFA2 protein from a native source is the most accurate for functional studies, a level of variation has previously been seen between saliva donors (data not shown) and so recombinant protein would provide a more consistent supply of BPIFA2 protein and would give the project more independence without the reliance of saliva donors. It is unclear why the expression of BPIFA2 was so unsuccessful in CHO cells; however expression using the insect cell (S2) system was very successful. Purification of this rBPIFA2, however, proved to be quite a problem. Successful purification was achieved using imidazole buffer, however the presence of this buffer resulted in the killing of bacteria compromising further functional analysis of the BPIFA2 protein. In order to conduct functional analysis on the rBPIFA2 protein, unpurified protein was used in subsequent experiments, using conditioned media from untransfected S2 cells as a control. The inability to purify the rBPIFA2 prevented true comparisons between the purified nBPIFA2 however the use of the empty S2 cell media as a control strengthened the results seen with the rBPIFA2. As with nBPIFA2, no killing was observed with any of the bacteria but recombinant protein did cause agglutination of *S. aureus* in a similar manner to the native protein. Unfortunately, the activity of the two protein forms was very different with regard to agglutination of *S. mutans*; nBPIFA2 presence resulted in

clear agglutination of *S. mutans* whilst rBPIFA2 did not. This difference could be due to a number of reasons. Indications from our previous investigations (data not shown), that BPIFA2 is a glycosylated protein and although insect cell line glycosylation is reported to be similar to that of mammalian cells, there still could be significant differences in the level of glycosylation of rBPIFA2 expressed in the insect cell line compared with the nBPIFA2 and this could affect the function. However, if this were true, it might have been expected that rBPIFA2 would also have been less effective against *S. aureus*, it could also suggest that the binding of BPIFA2 to *S. mutans* is different from that to *S. aureus*, either via a different binding site or method. An alternative reason for the different results could be that although bacterial numbers and protein concentration were consistent between each bacterium, more protein may be required to agglutinate certain bacteria than others. Alternatively, the arrangement of the bacteria may be important; for example *S. aureus* grows in clusters, while *S. mutans* grows in pairs and chains. Thus as BPIFA2 agglutinates two or more *S. aureus* it could be agglutinating preformed clusters and this may appear to be more efficient than cross-linking chains of cells. It may be then that we would see similar amounts of agglutination of the *S. mutans* if the concentration of BPIFA2 is higher.

Unfortunately, a successful quantification method has yet to be discovered for the purified BPIFA2 protein and the problems encountered in the purification of recombinant protein, again, prevented successful quantification. However if quantification was possible equal quantities of recombinant and native protein could have been added to the assays allowing for more direct and accurate

comparisons to be made. The differences observed between nBPIFA2 and rBPIFA2 in this study, therefore, may be attributable to differences in concentration of rBPIFA2 and nBPIFA2.

No previous studies have examined the activity of BPIFA2 on such an extensive range of bacterial species as used in this study. The results presented are thus more overarching, giving a wider picture of the true function of this novel protein.

The results reported here demonstrate that in its native environment (whole saliva) BPIFA2 binds to a number of bacterial species, however, following purification the protein only bound to two of the eight species tested, *S. mutans* and β-haemolytic streptococcus, suggesting the binding to the other 6 bacteria was via, alternative or intermediary (i.e. complexed) salivary constituents. An interesting observation made, in relation to bacterial binding, was the ability of BPIFA2 to bind to *P. gingivalis* following the removal of the lysine-specific gingipain enzyme.

In addition to the binding of BPIFA2 to *S. mutans*, further assays supported the hypothesis that BPIFA2 plays a vital role in the immune response to this bacterium. A small inhibition of growth; a reduction in viable bacteria following incubation; positive agglutination and an altered biofilm growth all implicate BPIFA2 in the innate immune response against this bacterium. The predicted structural similarities between LBP, BPI and BPIFA2 initially suggested that binding might selectively occur between BPIFA2 and gram-negative bacterial LPS; however *S. mutans* is a gram-positive bacterium. This was a novel discovery in the field and indicates that BPIFA2 may bind LPS-like molecules found on gram-positive bacteria, i.e. lipoteichoic acid (Stashenko et al., 1986). The project may have yielded many

more interesting results if a wider range of gram-positive bacteria were tested, however the hypothesis was raised based on the structural similarities to BPI and LBP, both of which bind and respond to the presence of gram-negative bacterial LPS. In order to extend the project further, a larger range of bacteria need to be included in each of the assays, particularly gram-positive bacteria; it would also be interesting to see how BPIFA2 affects different strains of the same species.

Purified nBPIFA2 positively bound to two species, *S. mutans* and β-haemolytic streptococcus, and it is interesting to note that each of these poses a threat to the health of the oral cavity and upper airway: *S. mutans* is an important cariogenic organism (Bab et al., 1979) and β-haemolytic streptococcus is a common cause of pharyngitis and tonsillitis (Kreth et al., 2005). Although binding alone may not pose a great threat to the bacteria, with the exception of agglutination, the fact that binding occurs opens the possibility that other defence molecules complexed with BPIFA2 would be brought close to the bacterial surface and so potentiate their action.

Technical difficulties were encountered in removing all LPS from the purified sample of BPIFA2 and this would have interfered with the assay system used for our TLR4/MD2/CD14 studies. For this reason we initially carried out experiments with a dust mite allergen, Der p 7, which like BPIFA2, is also predicted to share structural homology with the N-terminal domain of BPI. It has previously been shown that dust mite allergen, Der p 2, mimics the action of MD-2 in the TLR-4 pathway (Trompette et al., 2009) and so we hypothesised that this structurally similar protein could mimic the action of LBP in the same pathway. The development of

this assay, using Der p 7, would also provide a suitable method to examine whether BPIFA2 has the ability to mimic LBP in the TLR-4 pathway, following successful removal of contaminant LPS. Successful activation of the TLR-4 pathway was achieved using *E. coli* O111:B4 in the presence of LBP, inactivation was observed when the LBP was removed and activation was restored in the presence of Der p 7, implying that Der p 7 mimics the action of LBP in activating an immune response in the presence of gram-negative bacterial LPS. Using Der p 7 in our initial experiments allowed us to develop the assay system but as we were never able to completely remove all LPS from our purified nBPIFA2 without sacrificing too much of the protein we could not use this assay to fully determine the potential for BPIFA2 to mimic the activity of LBP. In order to elucidate any functional role for BPIFA2 in the TLR pathway a different assay system was used in which HEK293 cells were transiently transfected with BPIFA2 and the TLR-4 machinery prior to an *E. coli* LPS challenge. We hypothesised that in an LBP-deprived assay, the presence of BPIFA2 would increase TLR-4 activation; however no such activation was observed.

The most interesting advance made throughout these studies into BPIFA2 function was the positive interaction and antibacterial actions occurring between BPIFA2 and gram-positive bacteria, particularly *S. mutans.* These included direct binding to the bacterial surface, agglutination, slight growth inhibition and an altered biofilm. The binding and action of BPIFA2 to gram-positive bacteria rather than gram-negative bacteria could provide an explanation to the absence of a change in response through the TLR-4 pathway. The absence of LPS on gram-positive bacteria leads to the belief that the binding of BPIFA2 to *S. mutans,* β-haemolytic streptococcus and potentially *S. aureus* may be through lipoteichoic acid present. If

this is the case, activation of NF-kB, via the TLR path way would not occur via the TLR-4 receptor but via the TLR-2 receptor and pathway, explaining the negative activation of NF-κB via the TLR-4 pathway described in chapter 3. It is interesting to speculate then that subject-to-subject variation in the levels and glycosylation type of BPIFA2 may correlate with oral colonisation by *S. mutans* and other species.

These findings suggest that further work is required to examine this new hypothesis and so it would be beneficial to repeat this assay using TLR-2 and LTA from gram-positive bacteria, including *S. mutans*, to identify any change in NF-kB activation through this pathway in the presence of the purified nBPIFA2.

In addition, although glycosylation is not exclusive to immune proteins and the glycosylation could simply function to stabilise the protein, glycosylation of immune proteins allows for host cell-protein interaction via lectins. Identification of specific interactions between BPIFA2 protein and host cells, particularly immune cells could provide further evidence that the protein has some immune function.

Chapter 6.   Appendices

6.1   SDS-Polyacrylamide Gel Electrophoresis

<u>2x SDS PAGE sample buffer</u>

0.5M Tris-HCl pH6.8	-	1.25ml
10% SDS	-	2ml
Glycerol	-	2ml
1M DTT	-	1ml
Protease Inhibitor tablets (roche)	-	1ml
Bromophenol Blue	-	0.05g
Distilled water	-	3.55ml

<u>SDS-PAGE Running buffer</u>

10x Native Tank Buffer	-	100mls
10% SDS	-	10ml
dH2O	-	890ml

<u>12% Denaturing Polyacrylamide Gel</u>

1.5M Tris HCl with SDS, pH 8.8

Tris Base	-	54.5g
SDS	-	30g
Distilled Water	-	150mls

Adjust pH to 8.8 with 1M HCl and top up to 300ml with distilled water.

0.5M Tris-HCl with SDS, pH 6.8

Tris Base	-	6g
SDS	-	10g
Distilled Water	-	60mls

Adjust pH to 6.8 with 1M HCl and top up to 100ml with distilled water.

12% Polyacrylamide Gel Composition

	Acrylamide (40%)(ml)	1.5M Tris HCl with SDS (pH8.8) (ml)	0.5MTris HCl with SDS (pH6.8) (ml)	dH2O (ml)	TEMED (µl)	APS (10%) (µl)
Resolving	3.0	2.5	-	4.3	5	350
Stacking	0.975	-	2.1	4.725	17	1000

6.2   Bio-Rad Silver Stain Procedure

Fixative Step: 20 minutes

Methanol	-	200mL
Acetic Acid	-	40mL
Fixative Enhancer Concentrate	-	40mL
dH_2O	-	120mL

Rinse Step: 20 minutes

3 x 10 minute washes with dH_2O

Staining/Development Step: 20 minutes

dH_2O	-	35mL
Silver Complex Solution	-	5mL
Reduction Moderator Solution	-	5mL
Image Development Reagent	-	5mL
Development Accelerator Solution	-	50mL

Stop Step: 15 minutes

5% Acetic acid solution

6.3   Western Blot

Transfer Buffer

Methanol	-	20mls per gel
NuPAGE (10x) Transfer Buffer (Invitrogen)	-	10ml
Distilled Water	-	Up to 200mls

Tris-buffered Saline (TBS)

Tris pH 7.5	-	50nM
NaCl	-	0.15M
dH2O	-	Up to 1L

Tris-buffered Saline with Tween (TBST)

Tris pH 7.5	-	50nM
NaCl	-	0.15M
Tween 20	-	500µl
dH_2O	-	Up to 1L

Transfer Buffer

Methanol	-	20mls per gel
NuPAGE (10x) Transfer Buffer (Invitrogen)	-	10ml
Distilled Water	-	Up to 200mls

6.4   Transfection of *Drosophila* S2 Cells

BPIFA2/pMT plasmids were purified from Top10 cells using the Qiagen mini prep kit according to manufacturer's instructions and sequenced confirmed using the pMTfor   5'd[CATCTCAGTGCAACTAAA]3'   and   BGHRev   BGH   Rev 5´d[TAGAAGGCACAGTCGAGG]3´ primers. High quality DNA suitable for transfection of Drosophila Schneider S2 cells were purified using the Qiagen midiprep kits.

For stable transfections, BPIFA2/pMT vectors (19 µg plasmid per transfection) were co-transfected with 1µg pCoBlast plasmid (Invitrogen). Following transfections, cells were incubated for 24h at 20°C and then washed twice before incubation with selection media (complete media supplemented with 25µg/ml blasticidin) to select for resistance to blasticidin and generate stable cell lines. Stable cells were passaged and maintained in selection media. Protein expression was induced as by the addition of 500 µM CuSO4 and confirmed by Western Blotting with the BPIFA2 and an anti-His antibody

6.5   Protein Purification

Phosphate Buffered Saline (PBS)

NaCl	-	8g
KCl	-	0.2g
Na_2HPO_4	-	1.44g
KH_2PO_4	-	0.24g
Distilled Water	-	1L

2x Native loading buffer

0.5M Tris-HCl pH6.8	-	1.0mls
Glycerol	-	2.0mls
Distilled Water	-	6.55mls
Bromophenol Blue	-	0.05g

<u>10x Native running buffer and Native Elution Buffer</u>

Trizma Base	-	15.15g
Glycine	-	72g
dH_2O	-	500mls

<u>12% Native Polyacrylamide Gels</u>

1.5M Tris HCl, pH 8.8

Tris Base	-	54.5g
Distilled Water	-	150mls

Adjust pH to 8.8 with 1M HCl and top up to 300ml with distilled water.

0.5M Tris-HCl, pH 6.8

Tris Base	-	6g
Distilled Water	-	60mls

Adjust pH to 6.8 with 1M HCl and top up to 100ml with distilled water.

Gel composition

	Acrylamide (40%)(ml)	1.5M Tris HCl (pH8.8) (ml)	0.5M Tris HCl (pH6.8) (ml)	dH2O (ml)	TEMED (µl)	APS (10%) (µl)
Resolving	24	15	-	21	30	2100
Stacking	2.6	-	5	12.4	68	200

6.6   IMAC Buffer

<u>Buffer base</u>

NaCl	-	300mM
NaH_2PO_4	-	50mM

Add imidazole to required concentration:
20mM, 25mM, 50mM, 150mM, 250mM, 500mM

## 6.7   Determination of suitable timescales for IL-8 assay Raw Data.

E. coli LPS	n 1	n 2	n 3	Average
1.5 hours	312.962	2737.661	1920.522	1657.048
3.0 hours	851.59	4107.454	1190.11	2049.718
4.5 hours	1082.27	4495.586	1346.05	2307.969
6.0 hours	1043.329	3724.014	2097.284	2288.209
7.5 hours	761.991	4186.86	1372.189	2107.013
9.0 hours	192.375	3137.935	1008.888	1446.399
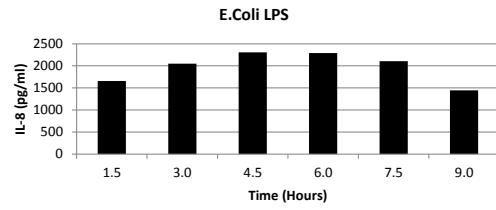
H. Influenzae LPS	n 1	n 2	n 3	Average
1.5 hours	19.781	908.087	636.616	**521.495**
3.0 hours	110.197	3326.469	1104.307	**1513.658**
4.5 hours	247.741	3888.303	1280.168	**1805.404**
6.0 hours	180.222	4149.720	1471.107	**1933.683**
7.5 hours	175.718	4078.586	1739.738	**1998.014**
9.0 hours	724.276	3951.170	1766.055	**2147.167**
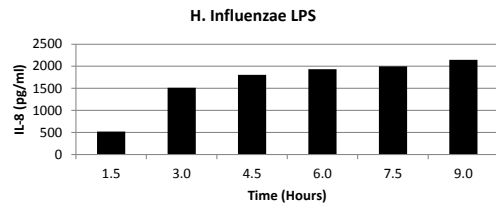
P. gingivalis LPS	n 1	n 2	n 3	Average
1.5 hours	11.328	161.463	325.871	**166.221**
3.0 hours	10.864	208.779	221.256	**146.966**
4.5 hours	19.903	204.242	267.630	**163.925**
6.0 hours	33.340	231.324	213.928	**159.531**
7.5 hours	16.357	301.571	256.485	**191.471**
9.0 hours	23.293	321.362	306.122	**216.926**
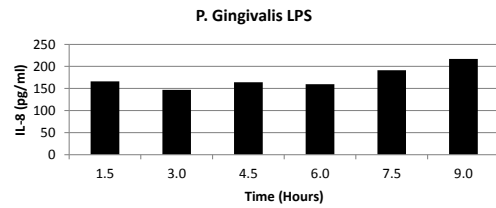
P. aeruginosa LPS	n 1	n 2	n 3	Average
1.5	11.772	173.194	266.286	**150.417**
3.0	20.118	211.115	280.801	**170.678**
4.5	12.594	231.324	204.799	**149.572**
6.0	19.628	257.025	243.583	**173.412**
7.5	20.457	218.271	248.912	**162.547**
9.0	15.753	2978.240	249.644	**1081.212**
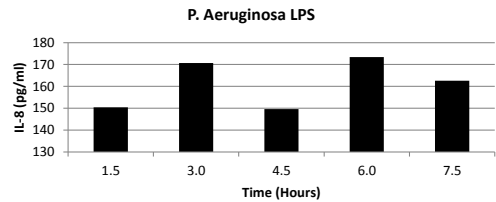
No LPS	n 1	n 2	n 3	Average
1.5	10.481	345.081	314.386	**223.316**
3.0	19.232	452.809	266.286	**246.109**
4.5	8.203	768.211	233.169	**336.528**
6.0	17.526	599.279	282.774	**299.860**
7.5	18.120	708.655	259.444	**328.740**
9.0	19.293	252.457	299.000	**190.250**
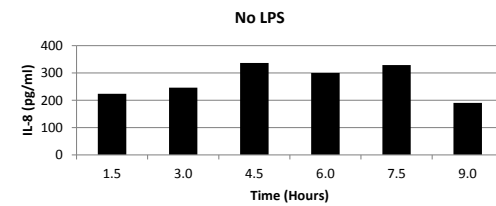
6.8 IL-8 data displayed at pg/ml
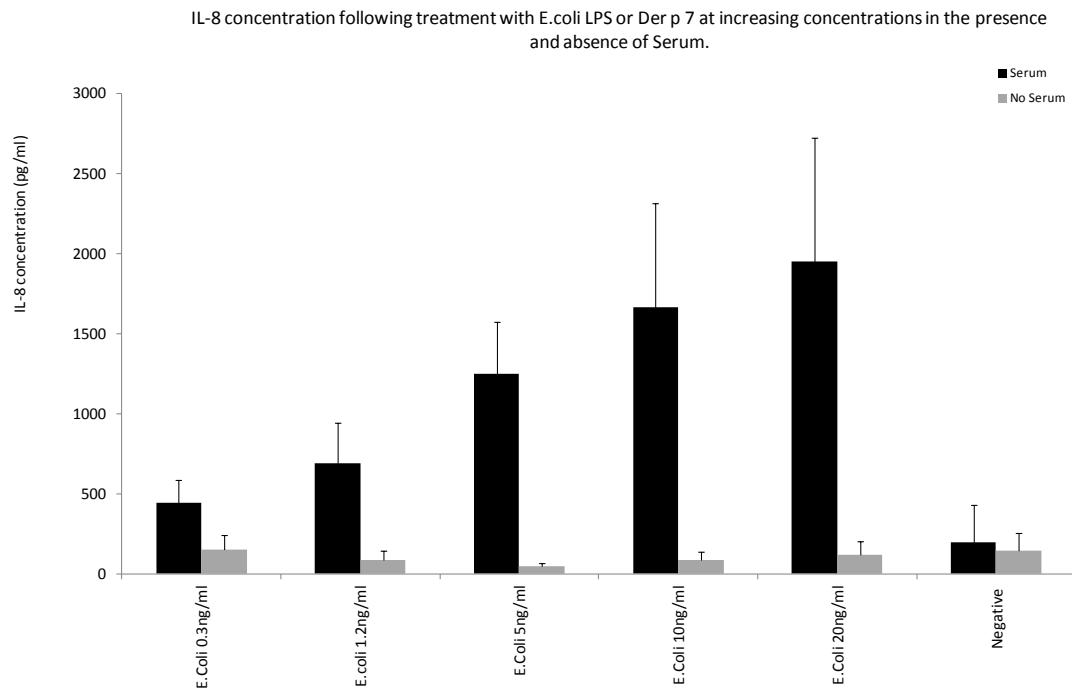
IL-8 concentration following treatment with E.coli LPS or Der p 7 at increasing concentrations in the presence and absence of Serum.

IL-8 concentration following treatment with increasing doses of *E. coli* (0111:B4) LPS in the presence of FCS (black) and absence of FCS (grey) to establish a normal response.

In the presence of FCS the IL-8 expression increased in a dose dependent manner following incubation of *E. coli* (0111:B4) LPS for 6 hours at 37°C. In contrast in the absence of FCS the IL-8 expression fails to increase at low concentrations of *E. coli* LPS. The negative control was culture media. Values are means of 3 experiments in triplicate. Error bars are standard error of the mean.

IL-8 concentration following treatment with E.coli LPS or Der p 7 at increasing concentrations in the presence of Serum.

n=3
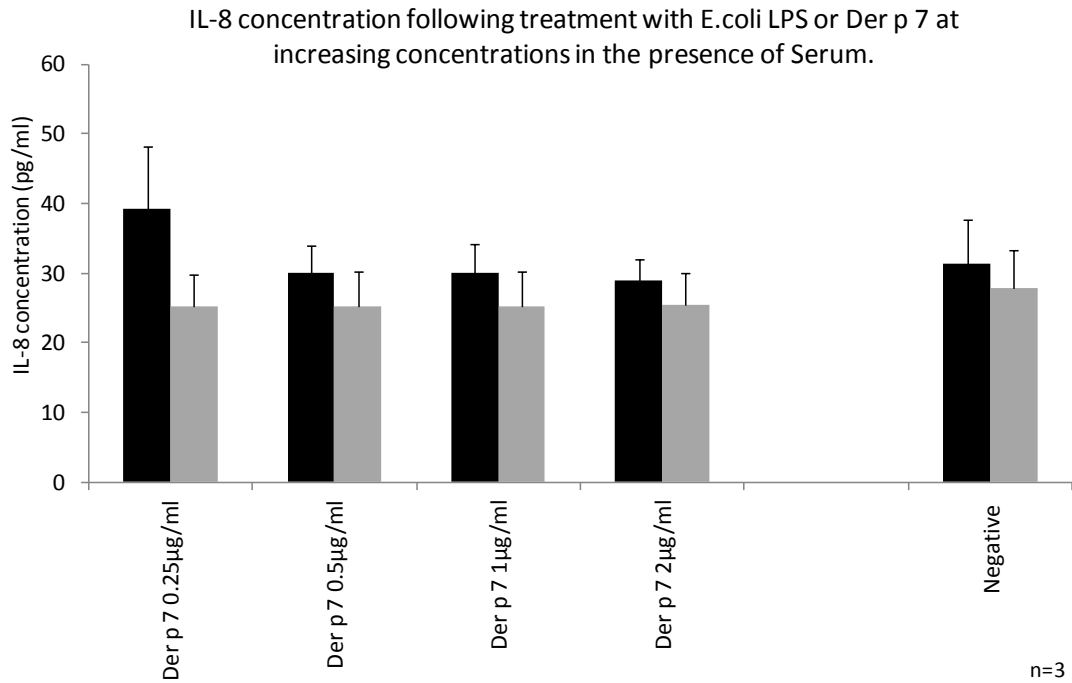
IL-8 concentration following treatment with increasing doses of rDer p 7 in the presence of FCS (black) and absence of FCS (grey) to establish a normal response.

Treating THP-1 cells for 6 hours with rDer p 7 did not induce expression of IL-8. With the exception of 0.25μg ml^{-1} (from 25pg/ml to 39pg/ml), all treatments showed expression in line with the negative control containing no rDer p 7. The negative control was culture media. Values are means of 3 experiments in triplicate. Error bars are standard error of the mean.

6.9   ANCOVA analysis

Tests of Between-Subjects Effects

Dependent Variable: IL8

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	229633.770[a]	3	76544.590	26.428	.000	.538
Intercept	21927.476	1	21927.476	7.571	.008	.100
DerP7	27.342	1	27.342	.009	.923	.000
LPS	64136.277	1	64136.277	22.144	.000	.246
DerP7 * LPS	66836.745	1	66836.745	23.076	.000	.253
Error	196949.574	68	2896.317			
Total	706644.603	72				
Corrected Total	426583.344	71				

a. R Squared = .538 (Adjusted R Squared = .518)

N.B. Significance identifies that there is a non-zero intercept, indicating that Der p 7 affects the IL-8 response only through interaction with LPS.
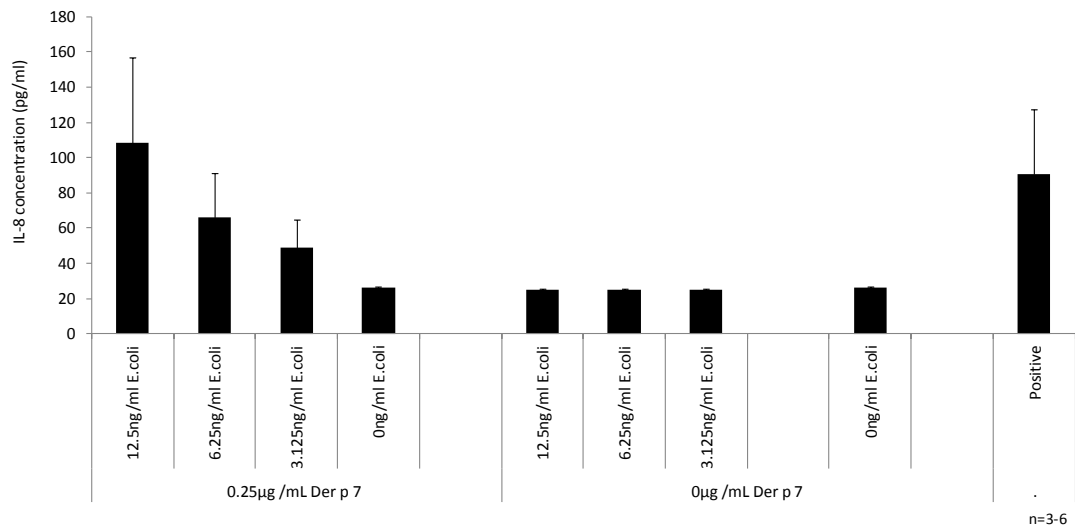
IL-8 concentration following treatment with *E. coli* LPS and rDer p 7 in the absence of FCS.

In the absence of *E. coli* LPS the presence of the rDer p 7 allergen fails to cause an increase in IL-8 expression, and similarly the presence of *E. coli* LPS at low concentrations in the absence of rDer p 7 fails to induce an IL-8 response. However, with the addition of as little as 0.25µg ml^{-1} rDer p 7 allergen the THP-1 cells can be induced to express IL-8 at a higher level compared to the positive control of 100ng ml^{-1} *E. coli* LPS. The negative control was culture media. Values are means of 3-6 experiments in triplicate. Error bars are standard error of the mean.

Chapter 7.   References

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