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UNIVERSITY OF ALBERTA

Utilization of Peptide-Enzyme Linked Assay (PELA) for the Characterization of Adhesins

by

LIANE KIM

A THESIS

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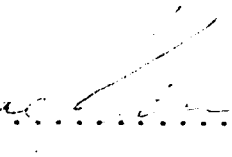

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Utilization of Peptide-Enzyme Linked Assay (PELA) for the Characterization of Adhesins submitted by Liane Kim in partial fulfillment of the requirements for the degree of Master of Science.

.....
Dr. Randall T. Irvin (Supervisor)

Glen Armstrong
Dr. Glen D. Armstrong

.....
Dr. Mark S. Peppler

H Anwar
.....
Dr. Hosmin Anwar
(External Examiner)

Date: April 21, 1993

To Mom and Dad, with love

Abstract

Infections caused by *Pseudomonas aeruginosa* are initiated by the adherence of the organism to the host mucosal surface. Of the various adhesins of *P. aeruginosa*, the proteinaceous pili are the most dominant. Previous studies on pili have led to the identification of the domain in the pilin monomer that makes contact with the host cell receptors: the epithelial cell-binding domain. Synthetic peptides of this domain have been synthesized and coupled to the reporter enzymes alkaline phosphatase and horse-radish peroxidase.

The present study employs the peptide-enzyme conjugates in a novel assay termed PELA (Peptide-Enzyme Linked Assay). Two peptides were used in this study: one encompasses 17 amino acids of the epithelial cell-binding domain (residues 128 to 144, containing a disulfide loop) and the other, containing 7 extra residues (residues 121 to 144). These peptides were used to evaluate PELA.

The peptide-enzyme conjugates were shown to bind to human buccal epithelial cells (BECs) in a manner similar to that of pure pili and bacterial whole cells, but with lower apparent Km values (therefore, higher affinities), and were also observed to be inhibited by free unconjugated peptides and pure pili. Thus, PELA is demonstrated to be an effective method for examining the interaction between peptide ligands and their receptors.

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List of Abbreviations

A_{405}	absorbance reading taken at 405 nm
A_{562}	absorbance reading taken at 562 nm
ABTS	2,2'-azido-di-(3-ethylbenzthiazoline sulfonic acid)
Ac	acetyl
AP	alkaline phosphatase
BECs	human buccal epithelial cell
BSA	bovine serum albumin
°C	degrees Celcius
cm	centimeter
D	Daltons
DME	Dulbecco's modified Eagle medium
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	fetal calf bovine serum
g	gram
g	gravity
h	hour
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICU	intensive care unit
IgG	immunoglobulin of the G class
K_d	equilibrium dissociation constant
kD	kiloDaltons
L	Liter
M	moles/liter or Molar

µ	micro-
µM	micromolar
Mab	monoclonal antibody
mg	milligram
mL	milliliter
mM	millimolar
min	minute
N	Normality
nm	nanometer
ox	oxidized
PAGE	polyacrylamide gel electrophoresis
PAK	*Pseudomonas aeruginosa* strain K
PAO	*Pseudomonas aeruginosa* strain O
PBS	phosphate buffered saline
PEG	polyethylene glycol
rpm	revolutions per minute
SDS	sodium dodecylsulfate
SSC	Standard Saline Citrate
TEC	tracheal epithelial cell
v	volume
wt	weight

1. Introduction

Pseudomonas aeruginosa is a major cause of descending pulmonary infections in individuals with cystic fibrosis (CF) (Rivera and Nicotra, 1982), burn victims (Sato *et al.* 1988), intensive care unit (ICU) patients (Garibaldi *et al.* 1981), and patients whose immune system is suppressed and compromised (Rivera *et al.* 1982). This bacterium is a gram-negative, rod-shaped organism that is commonly found in many environments, including that of a hospital. Thus, *Pseudomonas aeruginosa* is a significant nosocomial agent, along with other organisms such as *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (Cross, 1985). Because *P. aeruginosa* infections rarely occur in the normal, healthy population, it is also referred to as an opportunistic pathogen (Johanson *et al.* 1972).

This organism is responsible for 8 to 10% of all nosocomial infections (Bisbe *et al.* 1988). Of these, about 27% occur in the ICU (Costantini *et al.* 1987), where it is often endemic (Allen *et al.* 1987) and the mortality rate ranges from 15% for pneumonia (Craven *et al.* 1988) to 50% for bacteremia (Bisbe *et al.* 1988). In CF patients, pulmonary disease is a major cause of death. Forty to 90% of all CF patients acquire pulmonary disorders through infections of one or more of the following: *Aspergillus fumigatus*, *S. aureus*, and *P. aeruginosa* (Schonheyder *et al.* 1985; Sordelli *et al.* 1985; Rivera and Nicotra 1982). *Staphylococcus aureus*

and *Haemophilus influenzae* are the most prominent organisms in CF of childhood, but *P. aeruginosa* predominates in CF of older children and adults (Hoiby, 1991). The result is a chronic infection of the lower respiratory tract and almost all of the CF deaths due to respiratory diseases are associated with *P. aeruginosa* infections (Kulczycki *et al.* 1978).

In addition to the above, *P. aeruginosa* is also the causative agent of various other infections. One of these is bacteremia in burn victims where the mortality is high (up to 77%) (McManus *et al.* 1985). This organism has also been associated with urinary tract infections introduced by catheters (Kunin, 1979). Eye infections, such as bacterial keratitis can also be caused by *P. aeruginosa* (Stern *et al.* 1985). Keratitis is characterized by corneal ulceration and extensive destruction of the stroma which may lead to severe visual impairment and eventual loss of vision.

Thus, *P. aeruginosa* is an important opportunistic pathogen. Moreover, antibiotic therapy of *P. aeruginosa* is frequently ineffective due to innate antimicrobial resistance and the inherent plasmid-mediated resistance of this organism to many antibiotics. For these reasons, a greater understanding of its infection process is needed.

1.1 Virulence Factors of *Pseudomonas aeruginosa*

The success of *Pseudomonas aeruginosa* as a major pathogen can be attributed to its growth characteristics and its ability to produce a number of virulence factors, including several exoproducts, lipopolysaccharides, capsule and pili. These factors, along with the inherent resistance of the organism to many antibiotics contribute to the virulence of *P. aeruginosa*.

Some of the features that contribute to the pathogenicity of this organism are a.) its ability to grow over a wide temperature range, i.e. 20°C to 42°C (Cross, 1985), b.) its ability to grow with minimal nutrients (Costerton, 1984; Holder, 1977), and c.) its ability to adapt in response to environmental change. An example of the latter is the fact that *P. aeruginosa* is a nonfermentative aerobe that can also grow anaerobically if nitrates are supplied to act as the terminal electron acceptor instead of molecular oxygen (Vasil, 1986). It can also grow anaerobically if arginine is available; arginine is anaerobically converted to ornithine by the arginine dehydrolase enzyme pathway, thereby generating ATP by substrate level phosphorylation (Vasil, 1986). This may explain the ability of *P. aeruginosa* to survive in soil and to establish infections in parts of the body where the oxygen tension is low.

Although *P. aeruginosa* produces many virulence factors, they do not play prominent roles in all pseudomonad infections, i.e. the effects of each factor differ, depending on the strain, the site of isolation and the type of disease. For instance, elastase, exoenzyme S, alkaline protease, and exotoxin A are prominent in acute lung infections, whereas in chronic infections, such as those associated with CF, these exoproducts are of minor importance (Woods *et al.* 1986; Doring *et al.* 1987).

1.1.1 LPS.

The lipopolysaccharide, or LPS, of *P. aeruginosa* plays an important role in the pathogenesis of the organism and is unique to gram-negative bacteria. The LPS is also referred to as an endotoxin, although the *Pseudomonas* endotoxin is not as toxic as those found in other gram-negative bacteria (Sadoff, 1974; Liu, 1974; Wilkinson, 1983). Among the properties of the *P. aeruginosa* lipopolysaccharide are its ability to elicit the classical symptoms of endotoxic shock (Barasanin *et al.* 1978), and its mitogenicity for splenocytes (Barasanin *et al.* 1978; Pier *et al.* 1981).

The LPS is composed of three basic structures: the highly variable, O-specific polymeric side chain, a core oligosaccharide, and the hydrophobic lipid A (Wilkinson, 1983). The latter two are usually phosphorylated and conserved. Bacterial lipid A contains 1,6'-β-linked disaccharide of D-glucosamine carrying *O*- and *N*- fatty acyl

substituents, but is characterized in *P. aeruginosa* by its high content of acid labile phosphate residues (Drewry *et al.* 1972; Wilkinson *et al.* 1975). The core oligosaccharide, which connects the lipid A to the O-specific side chain, contains two residues of KDO (2-keto-3-deoxyoctonate) which facilitates identification of LPS. The core differs from that of the enterics in the composition of other sugars. For example, the outer region of the *Pseudomonas* core oligosaccharide consists of D-glucose, L-rhamnose, D-galactosamine and possibly the amino acid, L-alanine (Wilkinson, 1983).

The O-side chain consists of 15-20 repeating units of small sugar chains and is the basis of serotyping (Wilkinson, 1983). There are 17 serogroups of *P. aeruginosa* (Difco Manual, 1984) but evidence shows that strains associated with chronic infections are frequently nontypeable or polyagglutinable (Hancock *et al.* 1983). It is thought that this portion of the LPS, due to its high variability, is responsible for dodging the host's immune system. Studies with mutants lacking the O-side chain (rough mutants) have shown that these mutants are susceptible to complement action while those possessing the O-side chain (smooth phenotype) are not (Hancock *et al.* 1983). The O-side chain is rich in unique amino sugars. Sugars frequently present in this portion of the LPS are: 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, 2-amino-2,6-dideoxyglucose, 2-amino-2-deoxygalacturonic acid, 2,4-diamino-2,4,6-trideoxyglucose,

2,3-diamino-2,3-dideoxyhexuronic acid (with the D-gluco-, D-manno-, and L-gulo- configurations), and 2,3-(2-methyl-2-imidazolino-5,4)-2,3-dideoxymannuronic acid (Wilkinson, 1983).

Together with other components of the outer membrane, the LPS is an effective barrier against most antibiotics (Godfrey *et al.* 1984; Nikaido and Hancock, 1986). Another barrier to antibiotics is the lower number of pores found in the outer membrane of *P. aeruginosa*, as compared to those found in the enterics (Hancock *et al.* 1979). This may account for the innate resistance of this organism to antibiotics.

1.1.2 Exotoxin A.

Because the *Pseudomonas* endotoxin (LPS) was known to be less toxic than that of the *Enterobacteriaceae*, investigators searched for another, more toxic factor to account for the lethality of *P. aeruginosa* infections. Exotoxin A is produced by 80% to 90% of all clinical isolates of *P. aeruginosa* (Bjorn *et al.* 1977; Pollack *et al.* 1977). It is the most toxic product of this organism purified thus far, and is an extracellular enzyme with a reported molecular weight of 66,583 (Wick *et al.* 1990).

Like diphtheria toxin, the production of exotoxin A is regulated by the concentration of iron (Bjorn *et al.* 1979). Increasing amounts of iron results in a low yield of toxin production (Bjorn *et al.* 1978). Exotoxin A mutant

studies showed that toxin production is under the control of at least two chromosomal loci (Gray and Vasil, 1981) and at the transcriptional level (Wick *et al.* 1990). Recently, Storey *et al.* (1990) indicated that the regulation of exotoxin A production was from two promoters, P1 and P2. Expression from P1 was shown to be independent of the level of iron present while P2 was tightly controlled by iron.

Studies with infected animals have demonstrated the presence of enzymatically active toxin at infected burn sites and in the serum (Saelinger *et al.* 1977). Exotoxin A was also found to cause damage in eye infections in young adult mice (Snell *et al.* 1978). It was also shown that serum antibodies against exotoxin A confer protection, resulting in an increase in the chances of survival from a *Pseudomonas* bacteremia (Cross *et al.* 1980).

The toxin is a single-chain polypeptide, which is secreted as a proenzyme. This proenzyme toxin becomes activated by proteolytic cleavage or denaturation (Vasil *et al.* 1985). The current model for the structure of exotoxin A suggests three structural domains (Wick *et al.* 1990): the amino terminal (domain I) which is involved in the recognition of the eukaryotic target cells; the hydrophobic central region (domain II) which is involved in the secretion of the toxin into the periplasm and the translocation of the toxin once it has been internalized by the host cell; and the carboxy-terminal (domain III) which possesses the enzymatic

activity. This larger region was demonstrated to have a cleft, which is believed to be the active site of the toxin.

The mode of action of exotoxin A is similar to that of diphtheria toxin (Iglewski and Kabat, 1975). The toxin, once inside the cell, transfers the adenosine diphosphate (ADP) ribosyl moiety of nicotinamide-adenine dinucleotide (NAD) to elongation factor 2 (EF2). EF2 functions in the translocation of the ribosome along messenger RNA in the elongation of the polypeptide being synthesized. Exotoxin A inactivates EF2, resulting in the inhibition of protein synthesis (Iglewski and Kabat, 1975).

1.1.3 Proteases.

P. aeruginosa produces three extracellular proteases that are separable by ion-exchange chromatography and isoelectric focusing. These are protease I, protease II (elastase), and protease III (alkaline protease) (Morihara, 1964). These virulence factors are also referred to as "aggressins" (Vasil, 1986), and cause local tissue damage, as well as enhance virulence. Besides exerting their damaging effects, proteases can facilitate the spread of the bacteria to other tissues and to the bloodstream (Wretlind and Pavlovskis, 1983). In studies using the burned mouse model, it was observed that polymeric nutrients that were too large to enter through the bacterial cell envelope were modified by proteases into smaller molecules, where they were used by the organism, thereby contributing to the rapid establishment of

an infection (Cicmenec and Holder, 1979). The burned skin site allows for the initial colonization and proliferation of *P. aeruginosa*, and it was noted that as few as ten organisms caused 100 % lethality in the burned mice when inoculated subcutaneously; this, however, was not seen in normal skin (Stiertz and Holder, 1975).

Both elastase and alkaline protease cleave laminin associated with the basement membrane, although the two enzymes produce different cleavage products (Heck *et al.* 1986). The substrates of these proteases vary. Elastase is active against casein, elastin, hemoglobin and other proteins, but not against collagen, and has specificity for hydrophobic and bulky amino acids (Wretlind and Pavlovskis, 1983). It is thought that elastase and alkaline protease enhance *P. aeruginosa* invasiveness by inactivating C3b and C5a complement components and thereby inhibiting opsonization and the generation of chemotactic factors. It was also observed in patients with cystic fibrosis that these proteases inhibit the actions of IgA and IgG by degradation of these immunoglobulins (Doring *et al.* 1983). Proteases were also found to cause tissue damage in *Pseudomonas* pulmonary infections, bacteremias and burn infections. In pulmonary infections, elastase was found to be responsible for the disruption of epithelial cells from neighbouring cells and the basement membrane, whereas alkaline protease had no effect on the ciliated epithelium (Amitani *et al.* 1991).

Proteases play a destructive role in *Pseudomonas* eye infections. In studies where proteases were injected into the cornea, dissolution of collagen fibrils was seen, probably due to the loss of the proteoglycan ground substance of the cornea (Kreger and Griffin, 1974; Kessler *et al.* 1977; Snell *et al.* 1978). Passive or active immunization against the proteases conferred protection in experimental studies (Kawaharajo and Homma, 1976; Hirao and Homma, 1978). Elastase, when injected into the skin of animals, causes hemorrhagic lesions within minutes (Kawaharajo *et al.* 1975). Woods and Iglewski (1983) describe a study where injection of proteases causes lesions of the cornea, lungs and the intestinal tract. These lesions are thought to occur as a result of degradation of collagen, fibrin and other coagulation factors by elastase. Elastase also proteolytically inactivates human anti-proteinase, α_1-proteinase inhibitor and airway lysozyme (Morihara *et al.* 1979), thereby enhancing tissue damage.

1.1.4 Phospholipase C.

Also known as heat-labile hemolysin, phospholipase C (PLC) is a virulence factor that contributes to the pathogenicity of *P. aeruginosa* in lung infections. PLC is a single polypeptide with molecular weight of 78,000 (Vasil *et al.* 1985). Together with rhamnolipid, PLC is regulated by the level of inorganic phosphate in the environment: low amounts of phosphate induces the synthesis of PLC and

rhamnolipid. The structural gene that encodes PLC, *plcS*, is regulated at the transcriptional level and its gene product is an 82,600 Dalton protein which is eventually cleaved into the mature PLC protein (Pritchard and Vasil, 1986). Two genes, designated as *plcR1* and *plcR2*, are located downstream of *plcS*. It is believed that the products of the *plcR* genes have a negative effect on the synthesis of PLC and other phosphate-regulated products in the presence of high levels of inorganic phosphate (Ostroff and Vasil, 1987).

PLC acts to hydrolyse certain phospholipids into diacylglycerol and phosphorylcholine. The phospholipid substrates of this enzyme are components of the eukaryotic cell membrane (Vasil, 1986). For example, PLC can act upon phosphatidylcholine, a major component of lung surfactant that is critical to lung physiology. Evidence that this enzyme is produced *in vivo* is seen in patients with cystic fibrosis, where antibodies against phospholipase C were detected (Granstrom *et al*. 1984). In addition to the hemolytic PLC, a non-hemolytic PLC was identified in 1987. This enzyme was capable of hydrolyzing phosphatidylcholine and a synthetic PLC substrate, p-nitrophenylphosphorylcholine (Ostroff and Vasil, 1987).

1.1.5 Rhamnolipid and heat-stable glycolipid.

The rhamnolipid is a heat-stable hemolysin (cytotoxin) excreted by *P. aeruginosa* during the stationary growth phase or during growth in media where phosphate is

limiting (Syldatk *et al.* 1985). The hemolysin, with a molecular weight of 660 (Sierra, 1960), is also known as 2-(2-α-L-rhamno-pyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoate and can exist without one of the rhamnoses. The rhamnolipid is an amphiphilic molecule that disrupts eukaryotic cell membranes by the insertion of its fatty acid chains (Dorian *et al.* 1987). Apparently, another function of rhamnolipids is to facilitate survival of *Pseudomonas* by ridding the environment of competing bacteria. Rhamnolipids are known to be bactericidal, mycoplasmacidal and antiviral (Leisinger and Margraff, 1979). In addition to this, it was found that rhamnolipids cause ciliastasis by altering the ultrastructure of the cilia (i.e. reduction of ciliary proteins) and reducing ATPase activity (Hastie *et al.* 1986), thereby facilitating bacterial colonization of the respiratory tract (Hingely *et al.* 1986). It is also suggested that the rhamnolipid stimulates chemotaxis and chemokinesis (enhanced random migration) of human neutrophils (Shryock *et al.* 1984).

The glycolipid of *P. aeruginosa* is also a heat-stable hemolysin consisting of a dimer of two major acidic glycolipids. One component of the dimer is composed of two moles of rhamnose and two moles of β-hydroxydecanoic acid. The other part is composed of one mole of rhamnose and two moles of β-hydroxydecanoic acid. The hemolysis-producing moiety resides in the dimer of the β-hydroxydecanoic acid in the glycolipid molecule (Fujita, 1987). The glycolipid may be responsible for some of the cytopathic effects observed in

the infected lung (Winkler *et al.* 1985). The glycolipid may also be involved in the induction and modulation of inflammatory mediators (such as histamines and leukotrienes) from various cells, as observed in burn patients (Bergmann *et al.* 1989).

1.1.6 Leucocidin.

Leucocidin is a 42,500 Dalton cytotoxin produced by most pathogenic strains of *P. aeruginosa* (Hirayama *et al.* 1983). Its significance in pathogenesis lies in the fact that it destroys granulocytes and leukocytes (Scharmann, 1976; Scharmann, 1976). It is suggested that the action of leucocidin is initiated by its binding to specific receptors on the surface of target cells (Godfrey and Putney, 1984; Scharmann, 1976). Hirayama *et al.* (1984) found that pseudomonal leukocidin bound to polymorphonuclear leucocytes from rabbit peripheral blood. It was also reported that the destruction of leucocytes by leucocidin was enhanced by an increase in the concentration of cytosolic calcium ions, resulting in the conversion of phosphatidylinositol to phosphatidic acid (Hirayama and Kato, 1983; Hirayama and Kato, 1984).

1.1.7 Exoenzyme S.

Exoenzyme S, like exotoxin A, is an ADP-ribosyl transferase. Its substrates include a small subset of cellular proteins (Woods and Que, 1987), primarily cell

membrane associated, with molecular weights ranging from 23,000 to 25,000, some of which are GTP-binding proteins (Coburn *et al.* 1989). Thus, it differs from exotoxin A in substrate specificity.

Exoenzyme S can be extracted in toxic or nontoxic form depending on the level of calcium ions present during purification. It binds to ganglioseries glycolipids and glycerolipid receptors and may have two interrelated binding sites (Lingwood *et al.* 1991). Recently, it was demonstrated that exoenzyme S can also function as an adhesin of *P. aeruginosa* (Baker *et al.* 1991).

Sokol *et al.* (1990) cloned the gene for exoenzyme S from *P. aeruginosa* strain DG1 into *Escherichia coli* DH1 and the resulting protein , which had a molecular weight of 68,000, reacted with antibodies to exoenzyme S. Although toxic, the protein failed to demonstrate ADP-ribosyl transferase activity. When the gene was cloned back into *P. aeruginosa*, a precursor form of exoenzyme S (64,000 Daltons) that was not seen in the *E. coli* strain, was detected along with the mature form, indicating that processing does not occur in *E. coli*. The toxin expressed in *P. aeruginosa* was observed to be enzymatically active. The promoter for exoenzyme S has not yet been located, but the gene can be expressed under the control of a suitable promoter in *E. coli*.

Exoenzyme S is involved in dissemination of the bacteria and not initial colonization because antibodies to

exoenzyme S inhibited dissemination but not colonization (Nicas *et al.* 1985; Nicas and Iglewski, 1985). Studies were carried out in which it was seen that strains producing exoenzyme S produced greater lung damage compared to those strains that did not produce the enzyme (Nicas *et al.* 1985). In a study with rat lungs, intratracheal administration of purified exoenzyme S produced damage within 2 hours, with necrosis of the respiratory epithelium and accumulation of polymorphonuclear leucocytes (PMNs); injury was also observed at the pulmonary arterioles and venules (Woods *et al.* 1988).

1.1.8 Capsule.

The majority of deaths of CF patients is due to chronic pulmonary infections caused by mucoid strains of *P. aeruginosa*. Chronic pulmonary infections usually involve endobronchial infections caused by mucoid strains that gradually lose most of the O-antigenic determinants of the LPS (Hoiby, 1991). The mucoid phenotype is characterized by the production of the capsule, which is composed of alginate. Alginate is an acetylated exopolysaccharide consisting of β-1,4-linked D-mannuronic acid and L-guluronic acid, similar to the alginate polysaccharide found in seaweed (Linker and Jones, 1966). Krieg *et al.* (1988) reported that mucoid strains are able to utilize phosphorylcholine for growth and for the gradual formation of a capsule. Phosphorylcholine is the product of the hydrolysis of phosphatidylcholine (a lung surfactant) by phospholipase C. Therefore, mucoid strains

have the ability to use lung surfactant as a nutrient source and for forming a capsule.

The alginate polysaccharide is produced by the bacteria in the exponential to the stationary phase of growth (Annison and Couperwhite, 1987) and is involved in various roles in the virulence of *P. aeruginosa*. Bacterial clearance is associated with a rise in the titre of antibodies against alginate, as seen in the rat lung model (Woods and Bryan, 1985). Alginate confers protection against antibiotics by promoting the formation of microcolonies (aggregates of mucoid bacteria) and by decreasing the activity of the antibiotics (Winkler *et al.* 1985; Baltimore *et al.* 1987). The involvement of alginate in the formation of microcolonies enables the bacteria to resist mucociliary clearance, opsonisation and phagocytosis, thereby impairing the host immune system (Oliver *et al.* 1985; Winkler *et al.* 1985; Simpson *et al.* 1988). In fact, exogenous alginate (including that which is found in seaweed) confers antibiotic resistance to non-mucoid *P. aeruginosa* (Baltimore *et al.* 1987) and inhibits their uptake and degradation by macrophages (Simpson *et al.* 1988). The alginate, by its ability to enhance microcolony formation, is also an adhesin of *P. aeruginosa*, although it is not as efficient an adhesin as the pilus (McEachran and Irvin, 1985). The binding of alginate to human buccal and tracheal epithelial cells was observed and characterized (Doig *et al.* 1987; 1989) as was its interaction with rat lung lectin (McArthur and Ceri, 1983).

Various studies indicate that different structural forms of alginate exist. Pugashetti *et al.* (1982) observed that mucoid strains from various clinical isolates had different chemical compositions; in 1985, Irvin and Ceri demonstrated that an alginate-specific monoclonal antibody, designated Ps 53, reacted with only some *P. aeruginosa* alginates. It was suggested that the degree of acetylation of D-mannuronic acid and the ratio of L-guluronic acid to D-mannuronic acid differed from strain to strain (Evans and Linker, 1983; McArthur and Ceri, 1983), accounting for the heterogeneity among *P. aeruginosa* alginates.

1.1.9 Pili.

The *Pseudomonas* bacteria possesses polar, chromosomally encoded appendages termed pili. These pili are distinct from the pili that are responsible for bacterial conjugation (Bradley and Pitt, 1974). The degree of piliation varies significantly from isolate to isolate. Pili function mainly in mediating the adhesion of the bacterium to various surfaces, including mammalian cells. Pili are also utilized as receptors by various pilus-specific bacteriophages (Bradley and Pitt, 1974) and pilus retraction and extension results in a form of movement called twitching motility (Bradley, 1980). Twitching motility is defined as a flagella-independent movement across a surface (Lautrop, 1961) and all species exhibiting this form of motility possess polar pili. Organisms capable of twitching motility

were observed to form spreading colonies on solid agar (Henrichsen *et al.* 1972). Therefore, this was different from flagella-mediated motility, which requires semi-solid or liquid medium. Bradley (1980) found that *P. aeruginosa* strains PAK and PAO possessed pili that were retractile and those strains that did not have pili or whose pili were non-retractile did not demonstrate twitching motility.

Pili of *Pseudomonas aeruginosa* strains K (PAK) and O (PAO) have been well characterized. The pilus of PAK is a flexible filament with a diameter of 5.2 nm and a length of approximately 2500 nm (Bradley, 1972; Folkhard *et al.* 1981). It is a polymer composed of a 15,000 D, 144 amino acid polypeptide subunit termed pilin (Paranchych *et al.* 1985). It is a type 4 pilus, comprised of the pilin monomer arranged in a helical array, with 5 pilin subunits per turn and a pitch of 4.1 nm (Paranchych *et al.* 1986).

The pilin protein has several interesting features. First of all, the N-terminal region of this polypeptide is highly conserved and begins with the residue N-methylphenylalanine, and thus, it is an N-MePhe pilus (Frost *et al.* 1978; Paranchych *et al.* 1979). This is a common feature in pilins from *Neisseria gonorrhoeae, Neisseria meningitidis, Dichelobacter (Bacteroides) nodosus, Moraxella bovis, Vibrio cholerae* and *Moraxella nonliquefaciens* (Froholm and Sletten, 1977; Paranchych *et al.* 1985) and suggests an important structural function for this domain. This region of the pilin is also extremely hydrophobic, suggesting that

this is the region responsible for subunit-subunit interactions (Watts *et al.* 1983).

Following the N-terminus is a highly conserved, hydrophobic region of about 30 amino acids, a hypervariable central region, and a semi-conserved C-terminal region (Paranchych *et al.* 1978). The latter region contains the domain that is responsible for binding to mammalian cell receptors (Sastry *et al.* 1985; Irvin *et al.* 1989). The amino acid sequence of the PAK pilin monomer is presented in Figure 1.

1.1.10 Motility.

P. aeruginosa is toxigenic due to its ability to produce numerous virulence factors (exoproducts), and invasive because it is motile. The latter is due to the flagella which, like the pili, is located at the poles of the bacterium. Using the burned mouse model, various studies were done to correlate bacterial motility with invasive infections (McManus *et al.* 1980; Craven and Montie, 1981; Montie *et al.* 1982; Luzar and Montie, 1985; Drake and Montie, 1988). In these studies, it was observed that non-flagellated bacteria were less virulent than those that were flagellated and non-motile bacteria were less virulent than those that were motile. Motility was seen to contribute to the invasiveness of the organism, although non-motile *P. aeruginosa* clinical isolates are also frequently obtained.

1.2 Role of Adherence in *Pseudomonas* Infections

Adherence is an important mechanism by which many bacteria establish infections. Several studies have suggested that adherence is the initial step in the establishment of *P. aeruginosa* respiratory infections (Johanson *et al.* 1972, 1979, 1980), and that adherence preceded bacterial colonization of mucosal surfaces (Johanson *et al.* 1972; Gibbons and van Houte, 1975). Johanson *et al.* (1972) observed that nosocomial respiratory infections developed in 23% of the patients who were colonized with gram-negative bacilli, compared to only 3.3% in noncolonized patients. In this study, underlying conditions such as previous respiratory disease, hypotension and tracheal intubation were associated with colonization. Todd *et al.* (1989) observed that increased adherence of bacteria to TECs of ICU patients was associated with gram-negative pneumonia and that adherence was not related to whether or not the patients were intubated.

In 1979, Johanson *et al.* demonstrated that *Pseudomonas aeruginosa* adhered to buccal epithelial cells in greater numbers in seriously ill patients as compared to healthy people. All of this suggests that the host cell surfaces undergo some sort of alteration that enhances adherence of the bacteria and facilitates the subsequent development of infections. More specifically, the affinity of *P. aeruginosa* to the epithelial cell surface changes with

the status of the patient (Irvin, 1991). Normal, healthy cells show no colonization of gram-negative bacilli, and it has been suggested that this is due to the unavailability of specific receptors on normal cells for the binding of these bacilli. Therefore, the persistence of infection is due to the ability of *P. aeruginosa* to adhere to the epithelial cell surfaces which are altered to allow the exposure of receptors and to escape mucociliary clearance (Woods *et al.* 1983).

Niederman *et al.* (1983) compared the binding of *P. aeruginosa* to ciliated tracheal, and nasal epithelial cells and squamous buccal epithelial cells. Their results indicate that ciliated cells bind bacteria better than the non-ciliated buccal cells and that factors such as IgA and elastase levels, pH, and nutritional status affect the binding of bacteria (Niederman *et al.* 1983, 1984, 1986).

Several adhesins mediate the attachment of *P. aeruginosa* to the host cell surface. Up until now, the major adhesins of *P. aeruginosa* that have been identified are exoenzyme S, alginate and pili. Each of these play a role in the adhesion process, but their specific contributions and mechanisms are not yet clearly understood.

1.3 *Pseudomonas* Adhesins

1.3.1 Exoenzyme S.

This virulence factor has only recently been identified as an adhesin (Baker *et al.* 1991). As mentioned

before, it is an ADP-ribosylating toxin, similar to exotoxin A, and is purified in toxic and nontoxic forms (Coburn *et al.* 1989; Woods and Que, 1987). Recently, exoenzyme S has been identified as an important adhesin of *P. aeruginosa*, binding to the possible receptors for *P. aeruginosa*: gangliotriosylceramide, gangliotetraosylceramide, and lactosylceramide (Baker *et al.* 1991). In this particular study, it was observed that exoenzyme S found on the outer surface of the bacteria functioned as an adhesin, and that the binding specificities for the receptors were the same as those of whole bacteria. Exoenzyme S was also found to have some homology to the C-terminal region of the pilin and reacted with monoclonal and polyclonal antibodies specific to the pilin C-terminus (unpublished observations, this laboratory).

1.3.2 Alginate.

Mucoid strains of *P. aeruginosa* are associated with cystic fibrosis (CF), as stated earlier. The mucoid phenotype is due to the production of the alginate polysaccharide, which is composed of polymers of D-mannuronic acid and L-guluronic acid (Linker and Jones, 1966). Alginate has been identified as an adhesin for mucoid strains (Ramphal and Pier, 1985). In fact, McArthur and Ceri (1983) observed that rat lung lectin interacted with exopolysaccharide from CF isolates and ruled out the role of LPS in adherence. Initial adherence to the host is mediated

by nonmucoid strains which change to the mucoid phenotype during the course of the infection (Pier, 1985). These form microcolonies (Costerton *et al.* 1983). Doig *et al.* (1989) investigated the effect of microcolony formation on the adherence of *P. aeruginosa* to BECs and found that it increases the number of bacteria binding to epithelial cells and promotes bacterial proliferation.

The alginate remains associated with the cell envelope of *P. aeruginosa*, forming a slime layer (glycocalyx) (Sutherland, 1977), or a capsule (Costerton, 1981; Costerton *et al.* 1981.). Irvin and Ceri (1985) demonstrated that alginic acid or L-guluronic acid is associated with the outer membrane where a monoclonal antibody (Ps 53) specific for *P. aeruginosa* alginate reacted with L-guluronic acid residues of alginic acid and the outer membrane. Among other roles, the antiphagocytic activity of alginate allows the bacteria to persist in the respiratory tract.

Although alginate plays a role in the adhesion of *P. aeruginosa* to human buccal epithelial cells (BECs), the pilus is a more efficient adhesin (McEachran and Irvin, 1985). Doig *et al.* (1987) kinetically analysed the binding characteristics of purified alginate from *P. aeruginosa* strain 492c to BECs and tracheal epithelial cells (TECs). The purified alginate adhered to both cell types in a concentration-dependent and specific manner. It was found to be multivalent, capable of agglutinating both TECs and BECs. It was also observed that there is a structural diversity

among alginates produced by *P. aeruginosa*, i.e., some strains of *P. aeruginosa* produce alginate that bind to BECs and TECs while some strains do not. Irvin and Ceri (1985) demonstrated the heterogeneity in alginates by noting that the previously mentioned monoclonal antibody Ps 53 did not react with L-guluronic acid from all alginates and that the degree of acetylation in the alginate may be responsible for this heterogeneity.

1.3.3 Pili.

Pili have been identified as the adhesins that mediate the attachment of nonmucoid strains of *P. aeruginosa* to human BECs (Woods *et al.* 1980). Ramphal *et al.* (1984) extended this further by observing that pili also mediated the adherence of nonmucoid strains to TECs. It was concluded from this study that pili mediated the adherence of nonmucoid strains but not mucoid strains: nonmucoid variants derived from mucoid strains also used pili for attachment to the cell surface. Previous studies indicated that the pilus is probably responsible for the initial adherence of the bacteria to the host cell surface, as opposed to the alginate, due to the higher efficacy with which it binds (Doig *et al.* 1987, 1988; McEachran and Irvin, 1985, 1986). These studies show that adhesion mediated by pili reaches saturation, occupying all of the available binding sites on the epithelial cell surfaces. While there are pilus-associated adhesins in other pili types (*e.g.* P pili), there

is no evidence of such proteins in the *P. aeruginosa* pilus (Irvin, 1991).

Experiments with naturally cleaved and synthetic peptide fragments of the *Pseudomonas* pilin subunit demonstrate that the epithelial cell-binding domain resides in the C-terminal region of the pilin structural protein (Doig *et al.* 1988). Naturally cleaved fragments of *P. aeruginosa* strain K (PAK) pilin were derived by arginine-specific cleavage with trypsin, generating fragments termed, cT-I, cT-II, cT-III, and cT-IV, corresponding to the N-terminal to C-terminal regions, respectively (Watts *et al.* 1983). Synthetic peptides with amino acid sequences identical to the C-terminal region of the PAK pilin were also generated and bound specifically to BECs and TECs (Irvin *et al.* 1989). These synthetic peptides were also found to compete with purified pili for the binding sites on BECs. The creation of the epithelial cell-binding domain in the C-terminal region was supported by two additional studies. The first used monoclonal antibodies and their Fab fragments specific to various regions of the pilin (to inhibit binding) (Doig *et al.* 1990). The second showed that anti-peptide Fab fragments directed against the C-terminal region inhibited the binding of PAK pili to buccal epithelial cells (Lee *et al.* 1989). It was also found that a disulfide bridge existing between amino acid residues Cys-129 and Cys-142 in the C-terminus was important in the maintenance of the cell-binding function of the pilin, giving the adhesin a higher

affinity for the receptors (Doig *et al.* 1988; Irvin *et al.* 1989).

Monoclonal antibodies have been raised against various regions of the pilin polypeptide (Doig *et al.* 1990). The monoclonal antibody that is specific to the C-terminal region, PK99H, has been shown to inhibit the function of the pilus adhesin. In fact, in a study using colloidal gold, PK99H bound to the tip of the pilus, the site of interaction with the host cell surface (Irvin, 1990). The epitopes for other monoclonal antibodies, PK41C and PK34C, were localized at the N-terminal region and the C-terminal region, respectively, the latter being dependent on the disulfide bridge. The epitope for monoclonal antibody PK3B is suspected to be dependent on the three-dimensional structure of the pilin protein (Doig *et al.* 1990).

1.4 Receptors for *Pseudomonas aeruginosa*

Until recently, human receptors for *P. aeruginosa* pili have not been clearly identified. Woods *et al.* (1980) observed that a decrease in bacterial binding was associated with a decreased level of fibronectin on the surface of respiratory epithelial cells. Surface glycoproteins were indeed found to bind PAK pili, as well as certain sugar moieties (Doig *et al.* 1989). There have been several findings indicating that carbohydrate moieties play an important role in *Pseudomonas* adherence to epithelial cells.

The latter group of investigators identified L-fucose and N-acetylneuraminic acid as necessary components of the receptor activity for *P. aeruginosa* PAK pili. It has also been reported that mucins and sialic acid are components of receptors for *P. aeruginosa* in the lower respiratory tract (Ramphal and Pyle, 1983).

Asialo-GM1 and asialo-GM2 have been implicated as possible receptors for *Pseudomonas* (Krivan *et al.* 1988a). *P. aeruginosa* and *P. cepacia*, two common isolates found in cystic fibrosis patients, were seen to bind specifically to asialo-GM1 and asialo-GM2. β-N-acetylgalactosamine appeared to be necessary for binding. Asialo-GM1 and asialo-GM2. are also recognized by other pulmonary pathogens, (such as *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*), in addition to fucosylasialo-GM1 (Krivan *et al.* 1988b). Within these glycolipids, the sequence GalNAcβ1-4Gal was found to be essential. It was also demonstrated in this study that asialo-GM1 occurred frequently in human lung tissue. However, in these studies, it was not revealed whether the adhesin mediating attachment was pili, alginate, or exoenzyme S. The latter was also observed to bind to asialo-GM1 (Baker *et al.* 1991).

1.5 Ligand-Receptor Interactions

The development of reliable ligand binding assays is of considerable importance due to the need for a better

understanding of interactions between drugs, hormones, toxins and adhesins and their respective receptors present on the cell membrane. Even small peptides have been observed to interact with their receptors *in vivo*. For example, vasoactive intestinal polypeptide (VIP), a 28-amino acid peptide hormone that was first isolated from the small intestine of a hog, is known to be responsible for several physiological conditions such as systemic vasodilation, hypotension and hyperglycemia (Said and Mutt, 1970). Andersson *et al.* (1991) observed that natural VIP displaced ^{125}I-VIP in a dose-dependent manner from pig liver membrane, further demonstrating that a small peptide can bind to a receptor. Other examples of small peptides that can bind specifically to their receptors include glucagon (29 amino acids; Rodbell *et al.* 1971), neuropeptide Y, or NPY (36 amino acids; Kazuhiko *et al.* 1982), and the heat-stable toxin of enterotoxigenic *Escherichia coli* (18 amino acids; Thompson, 1987).

Previous assays have employed radiolabeled ligands to quantitate precise measurements of binding. Although these assays have many advantages (such as sensitivity and the ability of the labeled protein to retain biological activity), there are various limitations and considerations. First, it is necessary to know that the specific radioactivity of the ligand is enough to detect binding to the expected number of receptors. Also, a differentiation has to be made between specific binding to receptors and

nonspecific binding to components of the membrane other than the receptor (Hollenberg and Nexo, 1981). Another limitation to ligand binding assays is the degradation of receptors and ligands by proteases, resulting on the underestimation of binding affinities and capacities. Thus, a rapid and more efficient method of analysing binding kinetics is essential.

Irvin *et al.* (1989) utilized monoclonal antibodies and a second labeled antibody to detect the binding of synthetic peptide ligands to epithelial cell receptors, thereby eliminating the use of radioactivity. However, the limitations of this assay included time-consuming wash steps and incubation periods. A modification of this assay led to the development of a more rapid and efficient assay termed PELA (peptide-enzyme linked assay), to be used as a tool for li d binding assays (see below).

1.6 Research Objectives

The purpose of this project was to kinetically characterize the epithelial cell-binding domains of PAK pilin and other *P.aeruginosa* pilins, using a new method PELA (peptide-enzyme linked assay). Characterization of this assay is of importance, due to its potential as the basis for a diagnostic kit and as a tool for studying subunit-subunit interactions and interactions between a bacterial adhesin and the host cell surface. Because of the importance of adherence, and therefore adhesins, in the pathogenesis of *P.*

aeruginosa, it is necessary to look at the interaction between the epithelial cell-binding domain of the pilin and the epithelial cell. Previous studies demonstrated that acetylated synthetic peptides of the epithelial cell-binding domain (including the previously mentioned intrachain disulfide bridge between Cys-129 and Cys 142) bound to and competed with PAK pili for receptor sites on the surface of human respiratory epithelial cell (Irvin *et al.* 1989). The previous study involved the use of monoclonal antibodies and a labeled secondary antibody. In PELA, the synthetic peptides are conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase to facilitate the detection of adherence, thereby shortening the procedure.

2. Materials and Methods

2.1 Bacterial strain, culture conditions and pilus purification.

The conditions used for the growth of *Pseudomonas aeruginosa* strain K (PAK) have been previously described (Bradley and Pitt, 1974; Paranchych *et al.* 1979). Bacteria were grown on Oxoid Nutrient Broth (2% (wt/v)) agar plates) for 24 h at 37°C. Pili from PAK were purified as previously described by Paranchych *et al.* (1979) and obtained from Dr. W. Paranchych. The following procedure was used: PAK grown on solid media was harvested by scraping the surface of the agar and suspending the cells in 1 L of SSC Buffer (110 g wet wt/1 L buffer). The suspension was stirred with a magnetic stirrer at 5°C for 2 h. Large pieces of agar were removed by passage through a sieve. Pili were sheared from the cells by blending 200 mL portions for 2 min at 2,000 rpm with a Sorvall Omnimixer and bacteria were removed by centrifugation at 10000 x *g* for 15 min. The sodium chloride concentration of the supernatant was adjusted to 0.5 M. Polyethylene glycol (PEG) 6000 was then added to a final concentration of 1% (wt/v). Following this, the solution was stored for 18 h at 4°C while the flagella and pili precipitated out. The precipitates were collected by centrifugation at 7,000 x *g* for 20 min. The resulting PEG pellet was resuspended in 10 % (wt/v) ammonium sulfate solution, pH 4.0, and allowed to incubate at 4°C for 2 h. During this time, pili were

precipitated while the majority of flagella remained in solution. This step was repeated once more to remove any remaining flagella from the pili. Finally, the pellet was resuspended in water and dialysed to remove ammonium sulfate. The solution was then subjected to CsCl density gradient centrifugation which involved layering 20 mL of the pili solution onto 16 mL of a preformed step gradient in which the density of CsCl ranged from 1.1 to 1.5 g/ml. The centrifugation lasted 20 h (20,000 rpm in an SW27 rotor using a Beckman L2-65B ultracentrifuge). The pilus band was removed, subjected to another CsCl density gradient, and dialysed against distilled water to remove the CsCl (the buoyant density of pili is approximately 1.3 g/ml). The pili were suspended in distilled water, washed several times by centrifugation (2 h at 50,000 rpm in a 60-Ti fixed angle rotor), and the purity was verified by SDS-PAGE (100 µg per sample).

2.2 Peptide synthesis and conjugation to enzymes.

Synthetic peptides AcPAK(121-144)ox and AcPAK(128-144)ox (corresponding to residues 121 to 144 and 128 to 144 of the PAK pilin, respectively, with a disulfide bond between Cys-129 and Cys-142), as well as the P1 and PAO peptides, were synthesized by the solid phase method previously described (Parker and Hodges, 1984; Merrifield, 1986; Doig *et al.* 1988; Lee *et al.* 1989). Briefly, the peptides were synthesized using the Merrifield solid-phase procedure on a

Beckman 990 peptide synthesizer. The insoluble solid support was a styrene-divinylbenzene polymer. The C-terminal of the first amino acid served as the attachment point to the resin support. Each subsequent amino acid to be added was protected at its N^{α}-amino group by tertiary butyloxycarbonyl, which was later removed by acid treatment with hydrogen fluoride. The peptides were then purified by reversed-phase HPLC.

Conjugation of the synthetic peptides to bovine alkaline phosphatase (AP) (Sigma) or horseradish peroxidase (HRP) (Accurate Chemicals) were as follows: At the N-terminal end of the peptide, the photoreactive moiety benzoylbenzoic acid (BBA) (Aldrich, Milwaukee, U.S.A.) had been attached using the solid phase synthesis coupling method described earlier. One mg of the peptide and 4 mg of the enzyme were dissolved in a minimal volume of 0.01 M PBS, pH 7.4 in a 10 x 75 mm glass tube and irradiated at 350 nm with a RPR 208 preparative reactor (Rayonet, Southern New England Ultraviolet Company) for 30 min as described by Parker and Hodges (1985). The conjugates were then brought up to 1 mL of PBS and dialysed (to remove unconjugated peptides) against PBS at $4^{o}C$ (several changes), divided into aliquots, and stored at $-20^{o}C$.

2.3 Amino acid analysis.

Amino acid analysis was carried out by freeze-drying 50 µL of the peptide-enzyme conjugates. Five hundred µL of 6

N HCl was added and the hydrolysis was allowed to continue overnight in a 110°C oven. The analysis was carried out in the Department of Biochemistry, University of Alberta, Edmonton, Canada, on a Durram D-500 amino acid analyzer (Dionex, Palo Alto, CA, U.S.A.).

2.4 Purification of IgG.

IgGs were obtained as described by Irvin and Ceri (1985) and Doig *et al.* (1990). Separation of the ascites fluid from hybridoma cells was carried out by centrifugation (5,000 x g for 20 min at 4°C). IgGs were precipitated out of solution with 50 % (v/v) saturated ammonium sulfate. The precipitated IgG was dissolved in 10 mM PBS, pH 7.2, and then dialysed against PBS (several changes) and purified by HPLC (Waters 501 pump) (100 µL of crude IgG per injection), using a HiPac protein G affinity column (ChromatoChem). The antibodies were eluted in glycine buffer, pH 2.75, while the flowthrough was in PBS, pH 7.2. The IgGs were concentrated to approximately 2 mL using PEG 15000 (Sigma), divided into aliquots, and frozen at -20°C until further use. Th ability of the IgGs to bind to PAK pili was evaluated by ELISA and the protein concentration was determined by the mini Folin-Lowry protein assay (Lowry *et al.* 1951).

2.5 Preparation of Fab fragments.

Briefly, antibody was dialysed against 20 mM cysteine-HCl, 10 mM EDTA in 20 mM sodium phosphate buffer, pH

6.2. One mg of antibody was added to 150 µL of immobilized papain (Pierce) and incubated at 37°C with shaking at 250 rpm. The reaction was stopped at various intervals by centrifugation to remove immobilized papain and the samples were put through a protein G column to remove Fc fragments and uncleaved antibodies. The Fab fragments were collected in the flowthrough (PBS, pH 7.2), while the Fc fragments and uncleaved antibodies were eluted with 0.1 M glycine buffer, pH 2.75. Activity of the different time fractions was determined by ELISA. The active fractions were then concentrated with PEG (Doig *et al.* 1990), dialysed against PBS and activity determined again by ELISA. The concentrations of the Fab fragments were determined by the mini Folin-Lowry assay with bovine serum albumin (BSA) (Boeringer Manheim) as a standard. The cleavage products were analysed by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using Rainbow Markers (Amersham) (14,300 - 200,000 D) (Laemmli and Favre, 1973).

2.6 ELISA.

Ten µg of PAK pili in 100 µL of 0.1 M sodium carbonate buffer, pH 9.5, was added to each well of a microtitre ELISA plate (Linbro) and incubated overnight at 4°C. The remaining protein binding sites were saturated by adding 200 µL of 5% (wt/v) BSA in PBS, pH 7.4. After incubating the plate for 2 h at room temperature, the wells

were washed 5 times with 250 µL of PBS, pH 7.4, containing 0.05 % (wt/v) BSA. Ten-fold serial dilutions of the Fab fragments were added to the wells in triplicate (100 µL/well) and incubated at 37°C for 2 h. The wells were then washed 5 times with 250 µL of 0.05% (wt/v) BSA by aspiration. Following this, 100 µL of the second antibody, goat anti-mouse IgG (heavy plus light chains [H+L]) immunoglobulin-horseradish peroxidase conjugate (Jackson Laboratory), was added to each well, and the plate was incubated at 37°C for 2 h. The wells were washed again 5 times with 0.05 % (wt/v) BSA in PBS, pH 7.4, and 125 µL of the substrate solution [1 mM 2,2'-azido-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), 0.03% (v/v) hydrogen peroxide in 10 mM sodium citrate buffer, pH 4.2] was added to each well. The plate was incubated for 45 min at room temperature and the reaction was stopped by the addition of 125 µL/well of 4 mM sodium azide. Absorbance at 405 nm was determined by using an ELISA plate reader (Titertek Multiskan Plus).

2.7 Mini-Folin Lowry Protein Assay.

Using BSA as a standard, the concentration of a sample of protein was determined. 40 µL of standard and sample were added to the wells of an ELISA plate in triplicate. Dilutions of the sample used were 1:2, 1:5, and 1:10. The concentrations of the BSA standards ranged from 300 to 25 µg/mL. 160 µL of Reagent A (see below) was added to each well and the plate was allowed to incubate for 10 min

at room temperature with gentle shaking. Following this, 50 µL of 0.4 N Folin phenol reagent was added to each well and after careful mixing, the plate was incubated for 10 min at room temperature with shaking. Absorbance was read at 562 nm (Lowry *et al.* 1951) using a microplate reader (BioTek Instruments).

Reagent A was prepared as follows: Reagent A1 consisted of 1 g of NaK tartrate dissolved in 100 mL of water. Reagent A2 was prepared by dissolving 0.5 g $CuSO_4 \cdot 5H_2O$ in 100 mL of water and Reagent A3 consisted of 2 g of Na_2CO_3 in 100 mL of 0.1 N NaOH. Working Reagent A was prepared by the addition of 1.25 mL of Reagent A1, and then 1.25 mL of Reagent A2 to 100 mL of Reagent A3.

2.8 SDS-PAGE.

Antibodies and Fab fragments were analyzed using 12 % acrylamide discontinuous mini-gels by the procedure described by Laemmli and Favre (1973). Electrophoresis was performed using a voltage of 200 for approximately 45 min in a Mini-Protean II cell (Bio-Rad).

2.9 Buccal epithelial cells (BECs).

Human buccal epithelial cells were collected with wooden applicator sticks from healthy male non-smoking volunteers (n=10). The cells were removed from the sticks by gentle agitation in PBS, pH 7.2 and washed three times with PBS (2 000 x g for 10 min at 4° C). The BECs were then

passed through a piece of nylon mesh twice (pore size 70 μm) and counted using a hemocytometer. Cell concentration was adjusted to 2.0×10^5 cells/mL.

2.10 Binding of horseradish peroxidase (HRP) and alkaline phosphatase (AP) to BECs.

Aliquots of 1 mL of BECs (at a concentration of 2.0×10^5 cells/mL) were placed in 1.5 mL polypropylene Eppendorf vials (Fisher). 0.1 mL of 5 % BSA was added to each vial (to minimize nonspecific binding), along with 0.1 mL of the enzyme (HRP or AP), with concentrations of the enzymes ranging from 0 to 0.025 μM. The vials were incubated for 1 h at 37°C with rotation on a labquake shaker (Labindustries) and subsequently washed 7 times with 0.1 mM PBS, pH 7.2 (12,000 x *g* for 1 min at room temperature). After washing, the cells were transferred to new Eppendorf vials. Six hundred μL of the substrate solution was added to each vial and incubated for 30 min at 37°C. The substrate solution for HRP was 1 mM ABTS, 0.03% (v/v) hydrogen peroxide in 10 mM sodium citrate buffer, pH 4.2 while the substrate solution for AP was 1.0 mg/mL p-nitrophenyl phosphate disodium in diethanolamine buffer, pH 9.8. The reaction was stopped by the addition of 125 μL of 4 mM sodium azide (for HRP) or 2 N sodium hydroxide (for AP). BECs were then collected by centrifugation (12,000 x *g* for 1 min) and A_{405} of the supernatant was determined using an ELISA plate reader

(Titertek Multiskan Plus). BECs were resuspended in 1.0 mL of PBS, and counted using a hemocytometer.

2.11 Use of BSA to reduce nonspecific enzyme-BEC interactions.

BECs were placed in Eppendorf vials as previously described above (section 2.10), along with 0.1 mL of 0.18 µM of the conjugate PAK(121-144)-AP. 0.1 mL of BSA (final concentrations ranging from 0 to 1.9 % (wt/v)) was added to each vial and incubated for 1 h at 37°C with shaking. The cells were then washed 7 times as described above, transferred to new vials, and incubated with 600 µL of the AP substrate solution for 30 min at 37°C. The reaction was stopped using 125 µL of 2 N sodium hydroxide. BECs were then collected by centrifugation and A_{405} of the supernatant was determined. BECs were resuspended in 1.0 mL of PBS, and counted using a hemocytometer.

2.12 Binding of synthetic peptide-enzyme conjugates to BECs.

Aliquots of BECs (1.0 mL at 2.0 x 10^5 cells/mL) were placed in 1.5 mL polypropylene Eppendorf vials and incubated with 0.1 mL of 5 % (final concentration: 1.9 %) BSA and 0.1 mL of the peptide-enzyme conjugates (concentrations ranging from 0 to 250 nM) at 37°C with rotation on a labquake shaker (Labindustries). After 1 h the cells were washed 7 times with PBS (12,000 x *g* for 1 min at room temperature) and

transferred to new Eppendorf vials. The appropriate substrate solution (600 µL) was added to each vial and incubated at 37°C. The reaction was stopped using 125 µL of 4 mM sodium azide in the case of the horseradish peroxidase conjugate or 2 N sodium hydroxide in the case of the alkaline phosphatase conjugate. BECs were then collected by centrifugation and A_{405} of the supernatant was determined as described above (section 2.10). BECs were resuspended in 1.0 mL of PBS, and counted using a hemocytometer.

This assay was also performed on a Manifold™ filtration apparatus (Hoefer Scientific Instruments) using 12 µm polycarbonate filters (Nuclepore). In this version of the assay, 1 mL of BECs at 1 x 10^5 BECs/mL were added to the filters that had previously been incubated with 5 % BSA (1 h at room temperature). 0.1 mL of the conjugates and 0.1 mL of 1 % BSA was added to the cells and incubated for 1.5 h at room temperature. Following incubation, the filters were washed seven times with 2 mL of PBS and each filter was incubated in 1 mL of substrate buffer in scintillation vials. The colourimetric reaction was stopped with 125 µL of 2 N NaOH (in the case of alkaline phosphatase conjugates) or 4 mM sodium azide (in the case of horseradish peroxidase conjugates). The solution was centrifuged to remove any cellular debris. The results were recorded using an ELISA plate reader and A_{405} was determined.

2.13 Time course assay.

The time course of binding was determined. BECs (1.0 mL at 2.0×10^5 cells/mL) in Eppendorf vials were incubated with 0.1 mL of 5% (wt/vol) BSA and 0.1 mL of 0.18 µM PAK(121-144)-AP as described earlier. The binding reaction was stopped at 10 minute intervals over a period of 1 h. The cells were washed as described above, transferred to new vials, and incubated with substrate solution. The reaction was stopped by the addition of 125 µL of 2 N sodium hydroxide. BECs were then collected by centrifugation and A_{405} of the supernatant was determined. BECs were resuspended in 1.0 mL of PBS, and counted using a hemocytometer.

2.14 Dissociation of PAK(128-144)-AP from BECs.

The 17-amino acid peptide-alkaline phosphatase conjugate PAK(128-144)-AP (final concentration of 0.015 µM) was added to 1 mL of BECs at 2.0×10^5 BECs/mL, along with 0.1 mL of 5 % BSA in 1.5 mL polypropylene Eppendorf vials. The mixture was incubated for 1 h at 37°C and then washed seven times with PBS, pH 7.2. The alkaline phosphatase substrate buffer (600 µL per tube) was added after allowing the cells to sit in the PBS buffer for 0, 20, 30, 40 and 60 minutes. Incubation of the cells with substrate was maintained for approximately 45 min at room temperature. The reaction was stopped with 2 N NaOH and A_{405} was determined.

BECs were collected as described earlier and counted using a hemocytometer.

2.15 Inhibition of conjugate binding by free peptides.

BECs (1.0 mL at 2.0 x 10^5 cells/mL) were incubated with 0.1 mL of 5% (wt/vol) BSA, 0.1 mL of 0.178 µM PAK(128-144)-AP and 0.1 mL of varying concentrations of free peptides (ranging from 0 to 5 µM) in Eppendorf vials. Incubation was at 37°C for 1 h. The cells were collected and washed as described above and incubated with the phosphatase substrate solution. The reaction was stopped, A_{405} was determined, and the final cell concentration was determined. The assay was also performed on the Manifold™ filtration apparatus.

2.16 Inhibition of PELA by PAK pili.

A competition assay between the conjugates and PAK pili was performed by the addition 0.1 mL of 1 % (wt/v) BSA, 0.1 mL of PAK pili and 0.1 mL of 2.8 µM PAK(128-144)-AP to BECs immobilized onto 12 µm polycarbonate filter. The filters had been incubated with BSA as described above (section 2.12). The concentration of the pili ranged from 0 to 80 µg/mL. Incubation with BECs was carried out on the Manifold™ filtration apparatus at room temperature. After 1.5 h, the filters were washed 7 times as described above (section 2.12) and incubated with substrate buffer in scintillation vials for 45 min. The colourimetric reaction

was stopped with 125 µL of 2 N sodium hydroxide. The solution was centrifuged to remove any cellular debris. The results were recorded using an ELISA plate reader and A_{405} was determined.

2.17 Effect of Fab fragments on the binding of conjugates to BECs.

Three different dilutions of each of PK99H, PK3B, commercial normal mouse (Jackson Laboratory) Fab fragments were pre-incubated with an equal volume of conjugate at 37°C for 1 h. 0.1 mL of the Fab and conjugate solution was added to 1.0 mL of BECs (2.0×10^5 cells/mL) along with 0.1 mL of 5 % (wt/vol) BSA in 1.5 mL Eppendorf vials. The incubation was carried out at 37°C for 1 h. The cells were washed as described above (section 2.12) and incubated with the appropriate substrate solution. The reaction was stopped after 45 min. BECs were then collected by centrifugation and A_{405} of the supernatant was determined. BECs were resuspended in 1.0 mL of PBS, and counted using a hemocytometer.

2.18 Binding of PAK(128-144)-HRP to monoclonal antibodies.

5 µg/mL of PK99H, PK3B, PK129A monoclonal antibodies, and normal mouse IgGs were coated onto an ELISA plate (Linbro) and nonspecific protein binding sites were blocked with 5 % BSA (200 µL per well) by incubation overnight at

4°C. Dilutions of the 17-amino acid residue peptide conjugates were added to the IgGs (100 µL per well) and incubated at 37°C for 2 h with shaking at 100 rpm using a Gyrotory™ shaker (model G2) (New Brunswick). The wells were then washed five times with 0.05 % BSA in PBS, pH 7.4 and incubated with 125 µL of the appropriate substrate buffer. After 1 h, the reaction was stopped and the A_{405} was determined.

2.19 Competitive binding of free AcPAK(128-144)ox and PAK(128-144)-HRP to PK99H IgG.

An ELISA plate was coated with 10 µM of PK99H IgG and blocked with 5 % BSA as described above (section 2.18). Equal volumes of a constant amount of conjugate and varying concentrations of free peptide were mixed and added to the coated wells. Incubation was for 1 h at 37°C with shaking at 100 rpm. The wells were then washed 5 times as described above and incubated with 125 µL of the horseradish peroxidase substrate. The reaction was stopped, and A_{405} was then determined.

2.20 Binding of the peptide-enzyme conjugates to PAK pili.

10 µg/mL of PAK pili was coated onto ELISA plates (100 µL per well). The remaining protein binding sites were saturated by adding 200 µL of 5 % BSA and incubating the plate overnight at 4°C. Dilutions of the peptide-enzyme

conjugates were added to the pili-coated wells (100 µL/well) and incubated at 37°C for 2 h. The wells were then washed 5 times with 0.05 % BSA and incubated with the appropriate substrate buffer. After 45 min, the reaction was stopped and A_{405} was determined.

3. Results

3.1 Binding of alkaline phosphatase (AP) and horseradish peroxidase (HRP) to BECs.

The binding of AP and HRP to BECs in 0.01 M PBS buffer, pH 7.2, was performed to assess the amount of background absorbance present when synthetic peptides conjugated to these enzymes are bound to BECs. AP was observed to bind weakly to BECs in a concentration-dependent manner, reaching a maximum $A_{405}/10^5$ BECs reading of 0.16 at a concentration of 0.025 μM (Figure 3A) Similarily, HRP also bound to BECs in a concentration-dependent manner, approaching an $A_{405}/10^5$ BECs reading of 0.25 at a concentration of 0.025 μM (Figure 3B). Assay conditions were set to maximize specific binding while minimizing nonspecific interactions.

3.2 Use of BSA to reduce nonspecific enzyme-BEC interactions.

Various concentrations of BSA (ranging from 0 to 1.9 % (wt/v)) were incubated with BECs and the conjugate PAK(121-144)-AP to assess the ability of BSA to reduce nonspecific binding sites on BECs while retaining the ability of the peptide to interact with its receptor. As the concentration of BSA increased, the amount of conjugate binding to BECs decreased (Figure 4). Binding of the conjugates was highest when no BSA was present, indicating the adherence of the

conjugates to nonspecific sites as well as specific receptor sites.

3.3 Binding of synthetic peptide-enzyme conjugates to BECs.

Both the 17-amino acid peptide and the 24-amino acid peptide (PAK(128-144)ox and PAK(121-144)ox, respectively), conjugated to alkaline phosphatase or horseradish peroxidase, bound to human BECs in a time-dependent (Figure 5) and concentration-dependent (Figure 6) manner, reaching equilibrium in approximately 30 minutes, at a concentration of 0.18 μM. The data was subsequently replotted as double reciprocal plots (Figure 7) and the apparent K_d values of the peptide-enzyme conjugates binding to BECs were determined. The average calculated K_d values of the conjugates, along with that of the peptide analogous to the epithelial cell-binding domain of the P1 strain of *Pseudomonas aeruginosa* are presented in Table 1. The K_d values represent the apparent equilibrium dissociation constants of the conjugates to their receptors on BECs. According to these results, it appears that the PAK peptides have a higher affinity for the BEC receptors than the P1 conjugate. However, the fact that the P1 conjugate binds to BECs in a concentration-dependent manner (Figure 8) indicates that the epithelial cell-binding domain resides in the region of the pilin represented by the the synthetic peptide (amino acid residues 126-148) (Figure

2). The data for the binding of the P1 conjugate to BECs was replotted as a double reciprocal plot (Figure 9).

The binding assay was also carried out using the Manifold™ filtration apparatus and the results were comparable to those reactions carried out in polypropylene Eppendorf vials. Statistical analysis was carried out on the K_d values of the PAK conjugates using the (non-parametric) Wilcoxon Rank Sum Test. In comparisons between each of the conjugates, the p values indicate that there was no statistically significant difference between AP or HRP conjugates or utilizing AcPAK(121-144)ox or AcPAK(128-144)ox for the conjugation.

3.4 Dissociation of PAK(128-144)-AP from BECs.

The dissociation of bound peptide-enzyme conjugate PAK(128-144)-AP was examined to determine if the observed binding of the conjugates to BECs was reversible (*i.e.* in equilibrium). The amount of the peptide-enzyme conjugate bound to BECs was observed to decrease with the length of time the conjugates were left in solution with the BECs (Figure 10). After the incubation time and washing, the conjugates that were bound to the BECs were left in PBS for various lengths of time, and then incubated with substrate buffer. The fractions that were left the longest in PBS prior to the addition of the substrate buffer showed the lowest A_{405} readings, indicating that the binding of the peptide-enzyme conjugates to BECs was reversible.

3.5 Competitive binding of free peptide and conjugate to BECs.

A competition study employing free acetylated peptide as a competitor indicated that there was a competition for the same binding sites on the BECs (Figure 11). The binding of the conjugates decreased as the concentration of free peptide increased. This indicates that the binding of the conjugates to the BECs is specific. The IC_{50} (the concentration at which there is 50 % inhibition) was extrapolated from the graph to be 0.04 μM.

3.6 Inhibition of PELA by PAK pili.

A competition assay was performed using PAK pili and the PAK synthetic peptide-enzyme conjugate PAK(128-144)-AP as competitors (Figure 12). The binding of the peptide-enzyme conjugates to BECs was inhibited by the addition of PAK pili and the inhibition was dependent on the concentration of the pili. The A_{405} decreased as the concentration of the pili was increased, indicating the specificity of the binding and a competition for the same receptor sites on the BECs. The IC_{50} was determined to be 5.6 μg/mL.

3.7 Effect of PK99H IgGs and Fab fragments on the binding of the peptide-enzyme conjugates to BECs.

The effect of various Fab fragments on the binding of the peptide-enzyme conjugates to BECs was studied to confirm that the specificity of the monoclonal antibody PK99H was

directed to the C-terminal region of the pilin monomer. Intact IgGs were found to increase A_{405} readings, which represented increased binding of the conjugates to BECs. Therefore, monovalent Fab fragments were used.

In an experiment where a single dilution of Fab fragments was employed and compared to the fraction containing no Fab fragments, there was relative inhibition of binding with the fraction containing the PK99H Fab fragments (Table 2). According to the results presented in Table 2, PK3B Fab fragments also appear to inhibit conjugate binding, which is the opposite of what was expected. This could be due to nonspecific hydrophobic peptide-protein-antibody-BEC interactions. Incubation with the polyclonal normal mouse IgG Fab fragments (used as controls) displayed higher A_{405} readings, indicating little or no inhibition, but rather, enhancement of binding.

3.8 Binding of PAK(128-144)-HRP to monoclonal antibodies.

Three different concentrations of the 17-amino acid long peptide-horseradish peroxidase conjugate were added to immobilized monoclonal IgGs in a binding assay to confirm the specificity of the monoclonal antibody for that region of the pilin. The binding was demonstrated to occur in a concentration-dependent manner to PK99H IgGs (Figure 13). The conjugates did not bind significantly to PK3B IgG nor to

PK129A IgG, confirming that the AcPAK(128-144)ox peptide was specific for PK99H.

3.9 Competitive binding of free AcPAK(128-144)ox and PAK(128-144)-HRP to PK99H IgG.

A competition experiment between free AcPAK(128-144)ox and its HRP conjugate was performed to assess the affinity of the conjugate for the PK99H antibody. When a constant amount of the peptide-enzyme conjugate and varying concentrations of the free peptides were added to immobilized PK99H IgG, there was a reduction in the A_{405} readings. This indicates that the binding of the peptide to PK99H is specific (Figure 14).

3.10 Binding of the peptide-enzyme conjugates to PAK pili.

The interaction between PAK(121-144)-AP and PAK pili was examined in the earlier studies of Doig *et al.* (1988). These authors showed that the 24 amino acid peptide could bind to PAK pili. The 24-amino acid peptide-enzyme conjugated to AP bound to immobilized PAK pili in a concentration-dependent manner (Figure 15). However, the same assay performed with the 17-amino acid conjugate indicated no binding to the pili, suggesting that the extra seven amino acids in the longer peptide play a role in pilin-pilin subunit interactions.

4. Discussion

Pseudomonas aeruginosa is a major nosocomial pathogen of humans, responsible for morbidity and mortality among patients with burns (Sato *et al.* 1988) cystic fibrosis (Rivera and Nicotra, 1982), and those who are immunocompromised or immunosuppressed (Rivera *et al.* 1982). Infections caused by *P. aeruginosa* are thought to be initiated by the adherence of the organism to the respiratory mucosa (Johanson *et al.* 1972, 1979, 1980; Niederman *et al.* 1983, 1984, 1986) and the organism accomplishes this by means of a number of adhesins. The major adhesins identified thus far are the capsule, exoenzyme S and pili (Woods *et al.* 1980; Ramphal and Pier, 1985; Baker *et al.* 1991). Of these, the dominant adhesin is considered to be the pilus, due to the efficacy with which it adheres to epithelial surfaces (Doig *et al.* 1987; McEachran and Irvin, 1985, 1986).

The pilus, made up of pilin subunits, has previously been extensively characterized, both biochemically (Bradley, 1972; Folkhard *et al.* 1985; Paranchych *et al.* 1978; 1985, 1986; Watts *et al.* 1983) and functionally as an adhesin (Woods *et al.* 1980; Ramphal *et al.* 1984; Doig *et al.* 1988; Irvin *et al.* 1989). The domain of the pilin which makes contact with the epithelial cell surface (the epithelial cell-binding domain) has been localized in the C-terminal of the polypeptide (Doig *et al.* 1988). In this research project, a 24- and 17- amino acid synthetic peptide

encompassing the epithelial cell-binding domain of the pilin of *P. aeruginosa* strain K (PAK) has been used to study the effectiveness of an assay called PELA (Peptide-Enzyme Linked Assay). This assay can be used to facilitate the study of interactions between subunits within a protein, and can also be used as the basis for a diagnostic kit that will detect patients who are susceptible to ventilator-associated pneumonia. The previously mentioned synthetic peptides were conjugated to enzymes such as alkaline phosphatase (AP) and horseradish peroxidase (HRP) to measure their adherence to the epithelial cell surface.

Alkaline phosphatase and horseradish peroxidase have molecular weights of 140,000 and 40,000, respectively. After conjugation of the enzymes with the peptides, the resulting peptide:enzyme ratio ranged from 3:1 to 7:1. When the peptide:enzyme ratios of the conjugates was lower than 7:1, there was little difference in the binding characteristics of the conjugates. However, at higher coupling ratios (*e.g.* 17:1), the experiments were difficult to carry out. This was due to the cross-linking of the BECs. The conjugates caused agglutination of the BECs, making the counting of the cells difficult. Under the light microscope, the cells were seen as three-dimensional clumps (data not shown). Agglutination of the cells serves as further evidence of the interaction between the C-terminal region of the pilin and the BEC surface.

The binding of the peptide-enzyme conjugates occurred in a specific, concentration-dependent manner, very much like the binding characteristics of *P. aeruginosa* whole cells (McEachran and Irvin, 1985), consistent with the epithelial cell-binding domain being localized within that region. These experiments were performed in polypropylene Eppendorf vials and on the Manifold™ filtration apparatus. In the former method, BECs were washed seven times after the incubation step to remove unbound conjugates. This was a time consuming step, and due to the possibility of cell loss during the removal of the supernatant, final BEC concentration had to be determined. Using the latter method proved to be rapid, because the final stage of counting BECs (to account for those lost in the washing steps) was eliminated, since the BECs were immobilized on the filters. However, both methods were reproducible and results were comparable.

The affinities of the peptide-enzyme conjugates are expressed as equilibrium dissociation constants (K_d) (Table 1). In a comparison between the binding of the 24-amino acid peptide and the 17-amino acid peptide, one can see that the average K_d value of the 17-amino acid residue peptide is slightly lower. This, therefore, would indicate a higher affinity of the shorter conjugates for the receptors present on the BEC surface. However, these differences are not statistically significant, according to the Wilcoxon Rank Sum Test. It was originally anticipated that the extra seven

amino acids present in AcPAK(121-144)ox functioned to stabilize the peptide-enzyme conjugate and to provide a longer linker arm to remove the peptide off the surface of the enzyme.

The results presented in Table 1 suggest that there is no significant role played by these extra residues. The results also demonstrate that the type of reporter enzyme used in the conjugates has little effect on the binding of the conjugates to the BECs. The only difference lay in the fact that when horseradish peroxidase was used as the reporter enzyme, colour development in the substrate buffer was faster and the signal was higher. Therefore, it would seem that horseradish peroxidase is the enzyme of choice in practical situations.

The apparent K_d value of the 17-amino acid peptide that was determined by Irvin *et al.* (1989) using monoclonal antibodies as a means of detecting adherence is reported as 6.40 μM, which is significantly higher than the values calculated for the peptides used in PELA (all of which were less than 1 μM). Therefore, by using PELA, the conjugates were shown to have higher affinities for the BECs than demonstrated by the previous method. This discrepancy could be due to the loss of peptides from BECs due to shifts in equilibrium of binding during the washing steps that were used by Irvin *et al.* (1989). In PELA, several of these wash steps are eliminated, so there is only one wash step after

the incubation with the peptides. In this way, efficiency is increased and error is reduced.

For the sake of comparison, a binding assay was performed with a peptide representing the epithelial cell-binding domain of the pilin of the clinical isolate *P. aeruginosa* strain P1. *P. aeruginosa* P1 was originally isolated from a CF patient in Minnesota, U.S.A. The pilin subunit of the P1 pilus contains a large C-terminal disulfide loop which consists of 17 residues (Figure 2) (Pasloske *et al.* 1988). The K_d value of the P1 peptide (3.707 μM) is higher than that of all the PAK peptide-enzyme conjugates, indicating that the binding affinity of the P1 peptide is less than that of the PAK peptides. However, the fact that the P1 peptide does adhere to BECs confirms that the epithelial cell-binding domain is within the disulfide loop, which is considered functionally important in maintaining receptor binding activity, possibly due to the three-dimensional structure it provides.

Specificity of conjugate binding to BECs was demonstrated by competition with free, unconjugated peptides (Figure 11). There is a discrepency between the IC_{50} of PAK(128-144)-AP (0.04 μM) and its K_d value of 0.293 μM. One possible explanation for this is the fact that these experiments were performed on different days. Another reason for this difference can be explained by the difference in binding affinities between the conjugate and free peptide. According to the results, competition did not reach

completion. High background and insufficient amounts of free peptide are possible explanations for this observation. The binding of HRP and AP to BECs was performed to demonstrate the level of background present in the assays (Figure 3) and can be seen in other figures.

PAK pili were observed to inhibit the binding of PAK(128-144)-AP in a concentration-dependent manner (Figure 12), indicating a competition for the same receptor sites on the host cell epithelial cell surface. Doig *et al.* (1988) observed the same result when they competed whole bacterial cells with purified pili. This again confirms that the *P. aeruginosa* pilin has its epithelial cell-binding domain localized within the region represented by the synthetic peptides. Inhibition of conjugate binding by PAK pili and free unconjugated peptides also demonstrates the specificity of binding. The IC_{50} of PAK pili was relatively high (5.6 µg/mL), supporting the high affinity (low K_d values) of the conjugates.

Experiments were performed using Fab fragments of monoclonal antibodies directed against various regions of the PAK pilin. PK99H is specific to the C-terminal region and therefore, is postulated to inhibit adherence. It had been previously demonstrated to inhibit whole cell and pilus binding to BECs (Doig *et al.* 1990). The epitope for PK3B is thought to be dependent on the three-dimensional structure of the pilin. The negative control was the normal mouse Fab fragments (Pierce).

According to the results presented in Table 2, competitive binding with PK99H Fab fragments inhibits adherence of the conjugates to BECs. At first, the experiment was carried out using IgGs. This did not work because of the bivalency of the antibodies. The bivalency caused cross-linking of the conjugates, resulting in increased conjugate binding to the BECs. Therefore, the A_{405} readings were higher, the opposite of what was expected.

In most circumstances, the assay with Fab fragments was difficult to carry out. The assay was carried out with different dilutions of the Fab fragments and a constant amount of the conjugate PAK(128-144)-AP. The results did not demonstrate inhibition of conjugate binding to BECs by the appropriate Fab fragments (*i.e.* PK99H Fab fragments were expected to inhibit binding). This could be explained by nonspecific protein-protein interactions, considering that there are many proteins present in the system (Fab fragments, enzymes, peptides, BSA, and especially the high molecular weight enzymes). In previously published inhibition studies with Fab fragments, increasing the amount of Fab fragments of PK99H IgGs inhibited the binding of PAK whole cells (Doig *et al.* 1988; 1990), and pili (Doig *et al.* 1990) to BECs. The methods employed in these assays do not utilize the high molecular weight enzymes (horseradish peroxidase and alkaline phosphatase), reducing any nonspecific interactions and accounting for specific interactions between Fab fragments and the antigens.

In order to better investigate the specificity of binding of the monoclonal antibodies to the synthetic peptides, assays were performed using immobilized antibodies on ELISA plates. PK99H, PK3B and normal mouse IgGs were coated onto plates and reacted with the 17-amino acid synthetic peptide conjugates. The conjugates bound well to PK99H, somewhat less efficiently to PK3B and not at all to the normal mouse antibody. This further shows that PK99H, which is specific to the epithelial cell-binding domain of the pilin, is also specific to the synthetic peptide of that domain, confirming Doig *et al.* (1990). Also, in this assay, competitive binding between free and conjugated peptides was demonstrated. This data also indicates that both AcPAK(121-144)ox and AcPAK(128-144)ox are freely accessible to PK99H when they are coupled to either AP or HRP.

Nonspecific interactions are suspected to be caused by the hydrophobic nature of the peptides. Hydrophobicity is considered to be controlled by surface charges and solvent and hydration energetics (Irvin, 1990). In addition to the use of BSA to minimize nonspecific interactions, one can manipulate the system by using other proteins, such as gelatin or ovalbumin, increasing the ionic strength of the solvent, or decreasing the surface tension of the aqueous solution. However, there are limits as to what extent one can manipulate the reaction system, since it is necessary to mimic as close as possible, host physiological conditions.

The very fact that the peptide-enzyme conjugates bind effectively to human BECs in the same manner as bacterial cells makes PELA an effective candidate for a diagnostic kit. BECs would be obtained from patients from the ICU and binding assays would be performed in order to identify individuals who would need to undergo prophylactic treatment, whether by antibiotic therapy or vaccination.

Another application for PELA would be its role as a tool for studying subunit-subunit interactions within a protein molecule. The 24-amino acid peptide conjugated to alkaline phosphatase bound to PAK pili (which were immobilized on to ELISA plates) in a concentration dependent manner and in competition with its respective unconjugated form (data not shown). The binding experiment was also carried out using the 17-amino acid peptide-enzyme conjugate but it failed to bind to the pili (data not shown). It is therefore speculated that the extra amino acids present in the 24-amino acid peptide are involved in the interaction between the pilin monomers, interacting in some way with the N-terminal region of the pilin, possibly by folding mechanisms.

Summary.

PELA (peptide-enzyme linked assay) was demonstrated to be a simple and effective method in examining the kinetics of ligand-receptor interactions. The epithelial cell-binding domain of the *Pseudomonas aeruginosa* strain K pilin was

represented by the synthetic peptides used in this project and the binding characteristics of the peptides were similar to those of pure pili and the *P. aeruginosa* bacteria.

Table 1. Apparent average K_d values of synthetic peptide-enzyme conjugate binding to human BECs

	PAK(121-144)-HRP	PAK(121-144)-AP	PAK(128-144)-HRP	PAK(128-144)-AP	P1-HRP
Apparent Average Km (μM)	0.670	0.272	0.191	0.293	3.707
Standard deviation	0.832	0.277	0.054	0.136	3.644

Statistical analysis on the PAK peptide conjugates using the Wilcoxon Rank Sum Test indicate that the differences among the K_d values are not statistically significant and that they are comparable. For each of the conjugates, average K_d values were derived from N=4 values.

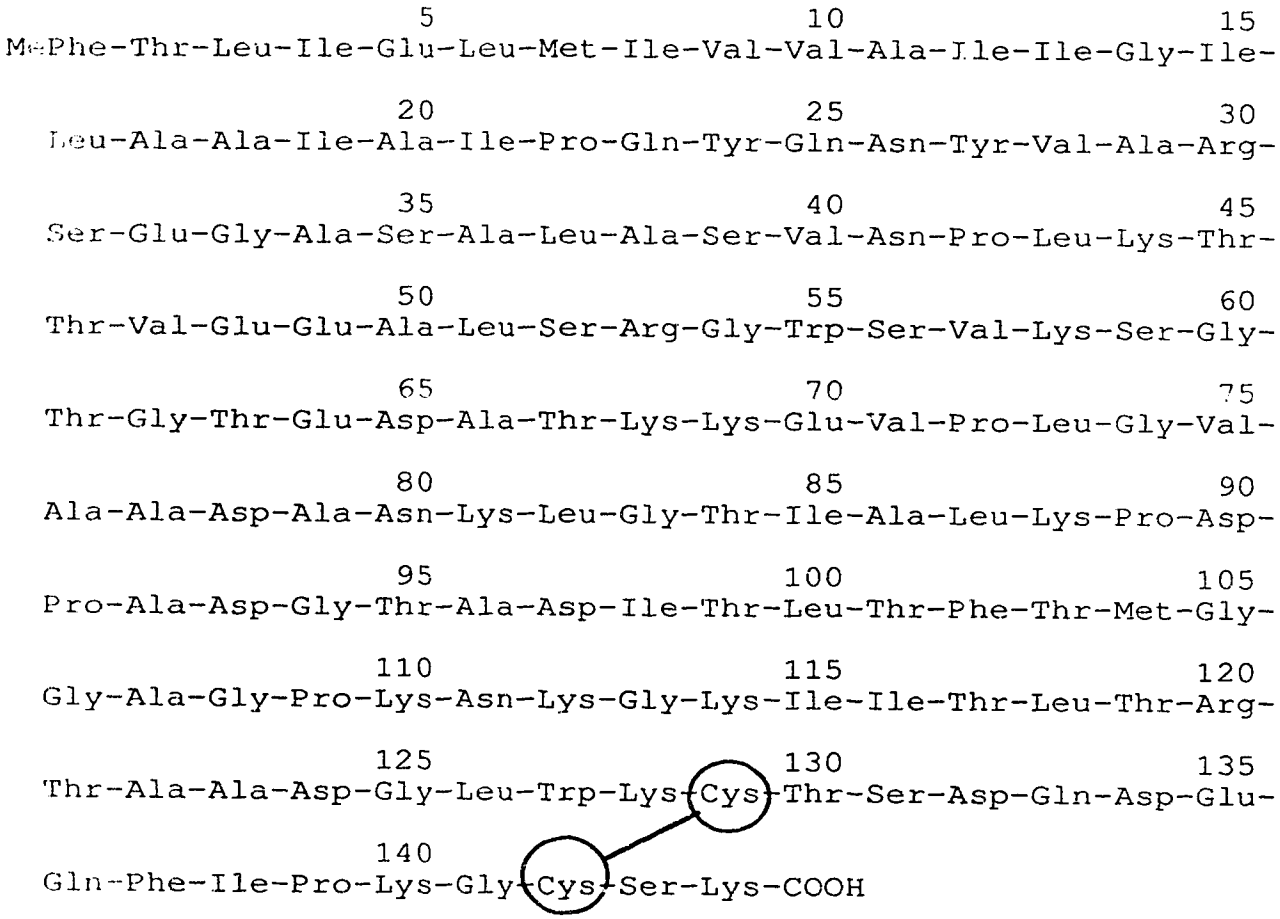
Table 2. Effect of monoclonal antibody Fab fragments on the binding of PAK(128-144)-AP to human BECs

Fab Fragment	Mean $A_{405}/10^5$ BECs	Standard Deviation
no Fab fragment	1.975	0.071
normal mouse Fab	1.340	0.082
PK3B	0.5980	0.103
PK99H	0.4065	0.056

N=2

A_{405} values represent the amount of the peptide-enzyme conjugate PAK(128-144)-AP bound to BECs. The PK99H Fab fragments were shown to inhibit conjugate binding to BECs when compared to the normal mouse Fab fragments and when no Fab fragments are present.

Figure 1. The amino acid sequence of the PAK pilin monomer. Cysteine residues 129 and 142 are linked by a disulfide bridge in the native protein and in the synthetic peptide-enzyme conjugates used (Paranchych *et al.* 1986).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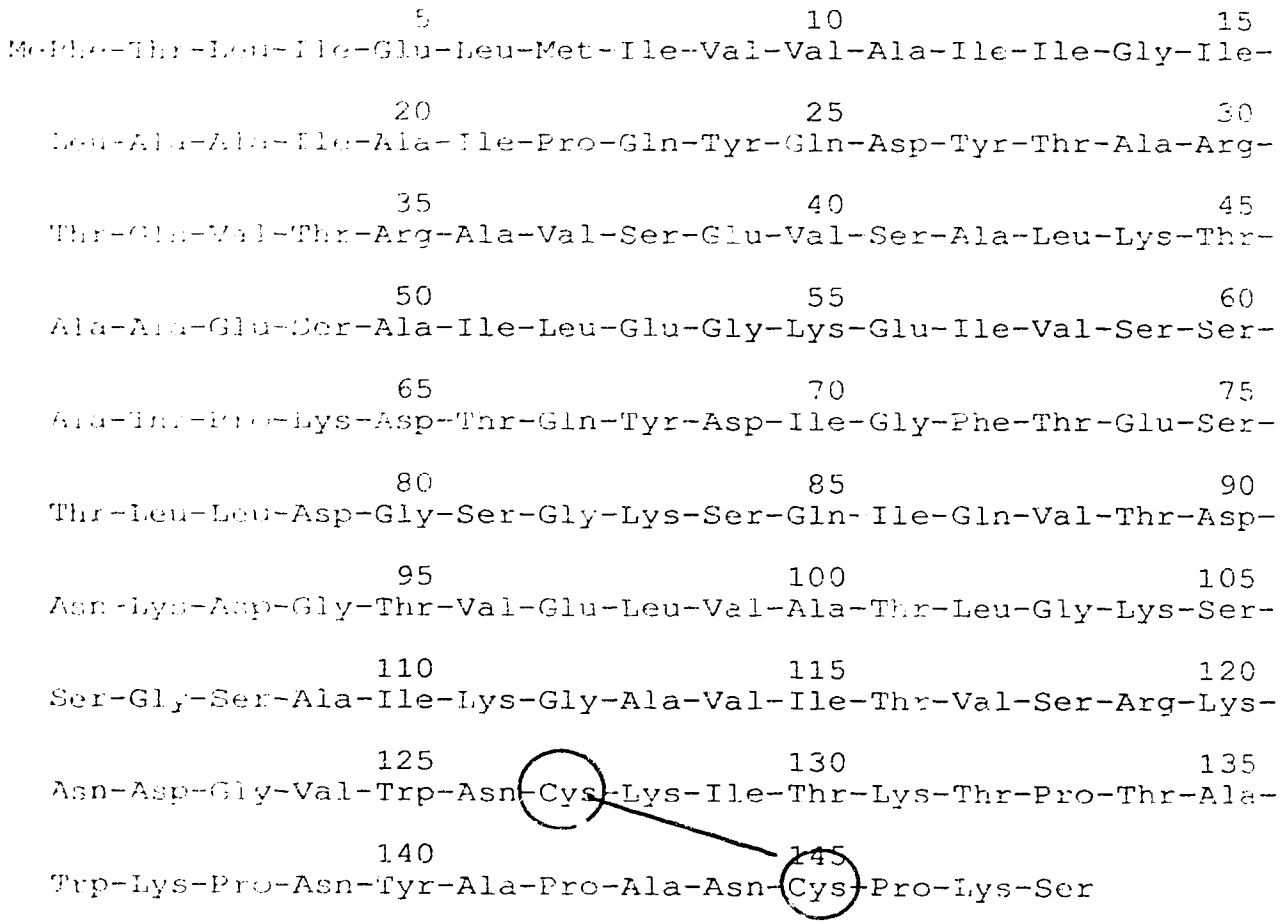
MePhe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Val-Ala-Ile-Ile-Gly-Ile-
20 25 30
Leu-Ala-Ala-Ile-Ala-Ile-Pro-Gln-Tyr-Gln-Asn-Tyr-Val-Ala-Arg-
35 40 45
Ser-Glu-Gly-Ala-Ser-Ala-Leu-Ala-Ser-Val-Asn-Pro-Leu-Lys-Thr-
50 55 60
Thr-Val-Glu-Glu-Ala-Leu-Ser-Arg-Gly-Trp-Ser-Val-Lys-Ser-Gly-
65 70 75
Thr-Gly-Thr-Glu-Asp-Ala-Thr-Lys-Lys-Glu-Val-Pro-Leu-Gly-Val-
80 85 90
Ala-Ala-Asp-Ala-Asn-Lys-Leu-Gly-Thr-Ile-Ala-Leu-Lys-Pro-Asp-
95 100 105
Pro-Ala-Asp-Gly-Thr-Ala-Asp-Ile-Thr-Leu-Thr-Phe-Thr-Met-Gly-
110 115 120
Gly-Ala-Gly-Pro-Lys-Asn-Lys-Gly-Lys-Ile-Ile-Thr-Leu-Thr-Arg-
125 130 135
Thr-Ala-Ala-Asp-Gly-Leu-Trp-Lys-Cys-Thr-Ser-Asp-Gln-Asp-Glu-
140
Gln-Phe-Ile-Pro-Lys-Gly-Cys-Ser-Lys-COOH

Figure 2. The amino acid sequence of the P1 pilin monomer.A disulfide bridge exists between cysteine residues 127 and 145 (Pasloske *et al.* 1988). The synthetic peptide used in the experiments include amino acid residues 126-148.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20 25 30
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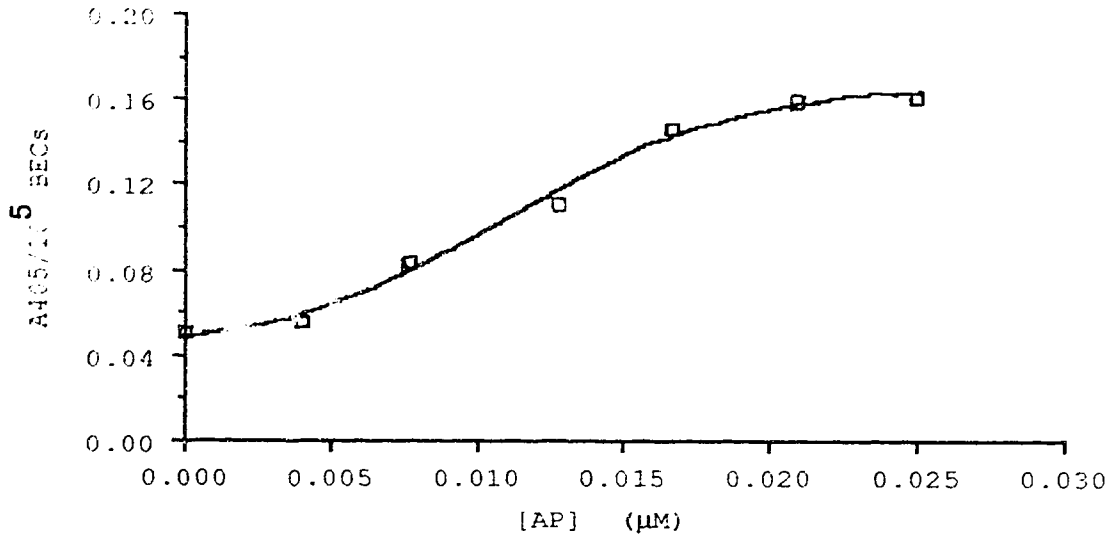
35 40 45
Thr-Gln-Val-Thr-Arg-Ala-Val-Ser-Glu-Val-Ser-Ala-Leu-Lys-Thr-
50 55 60
Ala-Ala-Glu-Ser-Ala-Ile-Leu-Glu-Gly-Lys-Glu-Ile-Val-Ser-Ser-
65 70 75
Ala-Thr-Pro-Lys-Asp-Thr-Gln-Tyr-Asp-Ile-Gly-Phe-Thr-Glu-Ser-
80 85 90
Thr-Leu-Leu-Asp-Gly-Ser-Gly-Lys-Ser-Gln-Ile-Gln-Val-Thr-Asp-
95 100 105
Asn-Lys-Asp-Gly-Thr-Val-Glu-Leu-Val-Ala-Thr-Leu-Gly-Lys-Ser-
110 115 120
Ser-Gly-Ser-Ala-Ile-Lys-Gly-Ala-Val-Ile-Thr-Val-Ser-Arg-Lys-
125 130 135
Asn-Asp-Gly-Val-Trp-Asn-Cys-Lys-Ile-Thr-Lys-Thr-Pro-Thr-Ala-
140 145
Trp-Lys-Pro-Asn-Tyr-Ala-Pro-Ala-Asn-Cys-Pro-Lys-Ser

Figure 3. A.) Binding of alkaline phosphatase (AP) was observed to occur in a concentration-dependent manner, reaching a maximum A_{405} reading of 0.16 at a concentration of 0.025 μM. 0.16 A_{405} accounts for the background absorbance observed in subsequent figures involving AP conjugates. B.) Binding of horseradish peroxidase (HRP) to BECs approached saturation at an A_{405} reading of 0.25 at a concentration of 0.025 μM.

A.


0.20
0.16
0.12
0.08
0.04
0.00
0.000
0.005
0.010
0.015
0.020
0.025
0.030
[AP] (μM)

B.

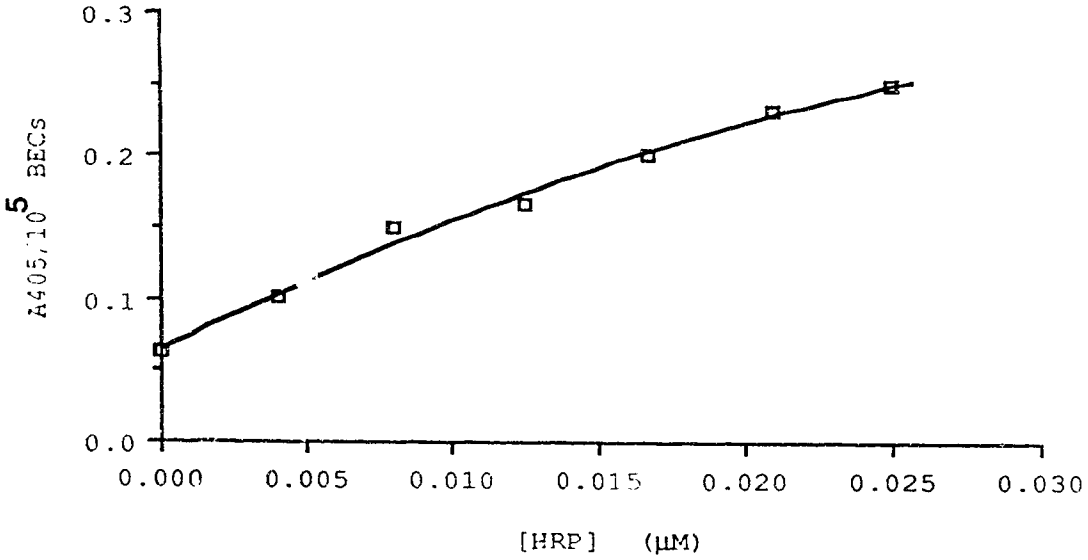

0.3
0.2
0.1
0.0
0.000
0.005
0.010
0.015
0.020
0.025
0.030
[HRP] (μM)

Figure 4. Binding of the conjugate PAK(121-144)-AP [illegible] in the presence of increasing amounts of BSA was evaluated to assess the efficiency of BSA in minimizing nonspecific binding. Increasing concentrations of BSA resulted in decreased nonspecific binding of the conjugate to BECs.

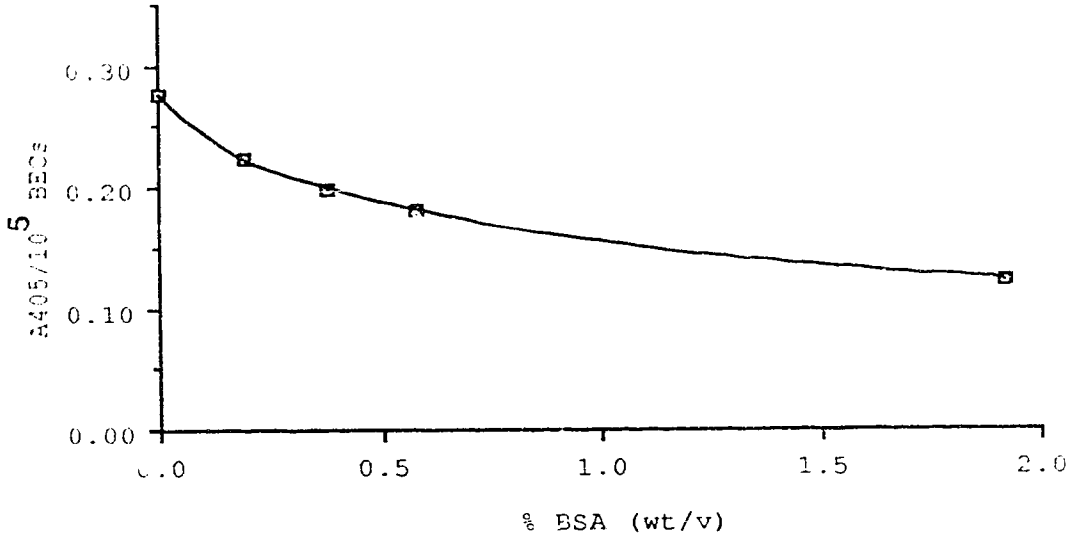

0.30
0.20
0.10
0.00
A405/10 BEC5
0.0
0.5
1.0
1.5
2.0
% BSA (wt/v)

Figure 5. Time course: binding of the peptide-enzyme conjugate PAK(121-144)-AP to BECs reached equilibrium after 30 minutes of incubation. The concentration of the conjug was 0.18 μM. The error bars represent standard deviation (N=3). The background A_{405} reading was at 0.04.

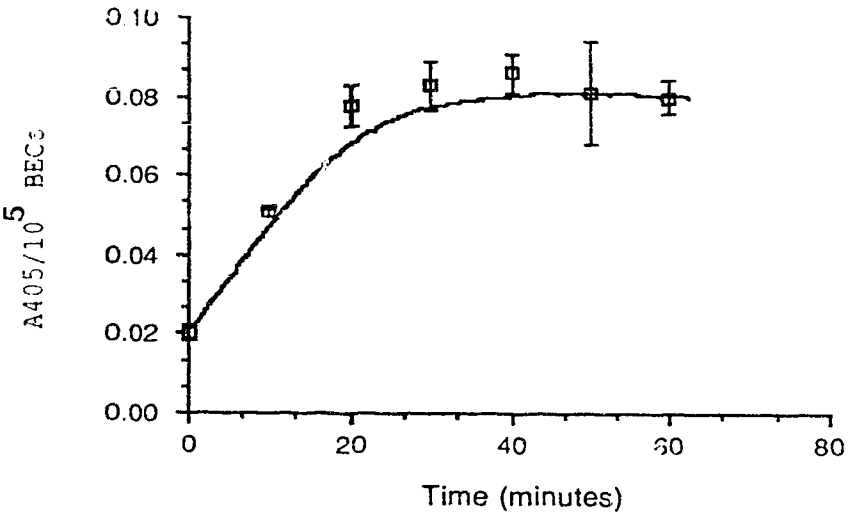

0.10
0.08
0.06
0.04
0.02
0.00
A405/10^5 BEC
0
20
40
60
80
Time (minutes)

Figure 6. Binding of the peptide-enzyme conjugate PAK(128-141)-AP to BECs. The molar coupling ratio was 3 peptides:1 enzyme. Binding was observed to occur in a concentration-dependent manner, approaching saturation of receptor sites, after 1 h of incubation. Error bars were not included due to variations occurring from day to day (*i.e.* physiological conditions of BECs).

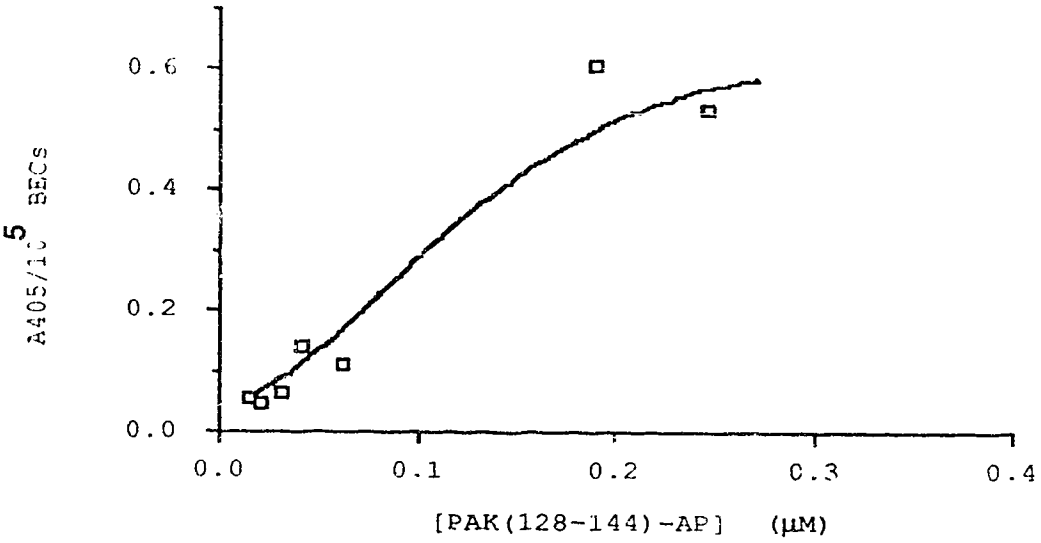

A405/10^5 BECs
0.6
0.4
0.2
0.0
0.0
0.1
0.2
0.3
0.4
[PAK(128-144)-AP] (μM)

Figure 7. Double reciprocal plots of the binding data represented in Figure 6 for the determination of K_d values for the binding of the PAK peptide-enzyme conjugates to BECs. A.) Binding of PAK(121-144)-AP to BECs. Linear regression coefficient=0.926. B.) Binding of PAK(121-144)-HRP to BECs. Linear regression coefficient=0.957. C.) Binding of PAK(128-144)-AP to BECs. Linear regression coefficient=0.919. D.) Binding of PAK(128-144)-HRP to BECs. Linear regression coefficient=0.934. Average K_d values for the conjugates are listed in Table 1.

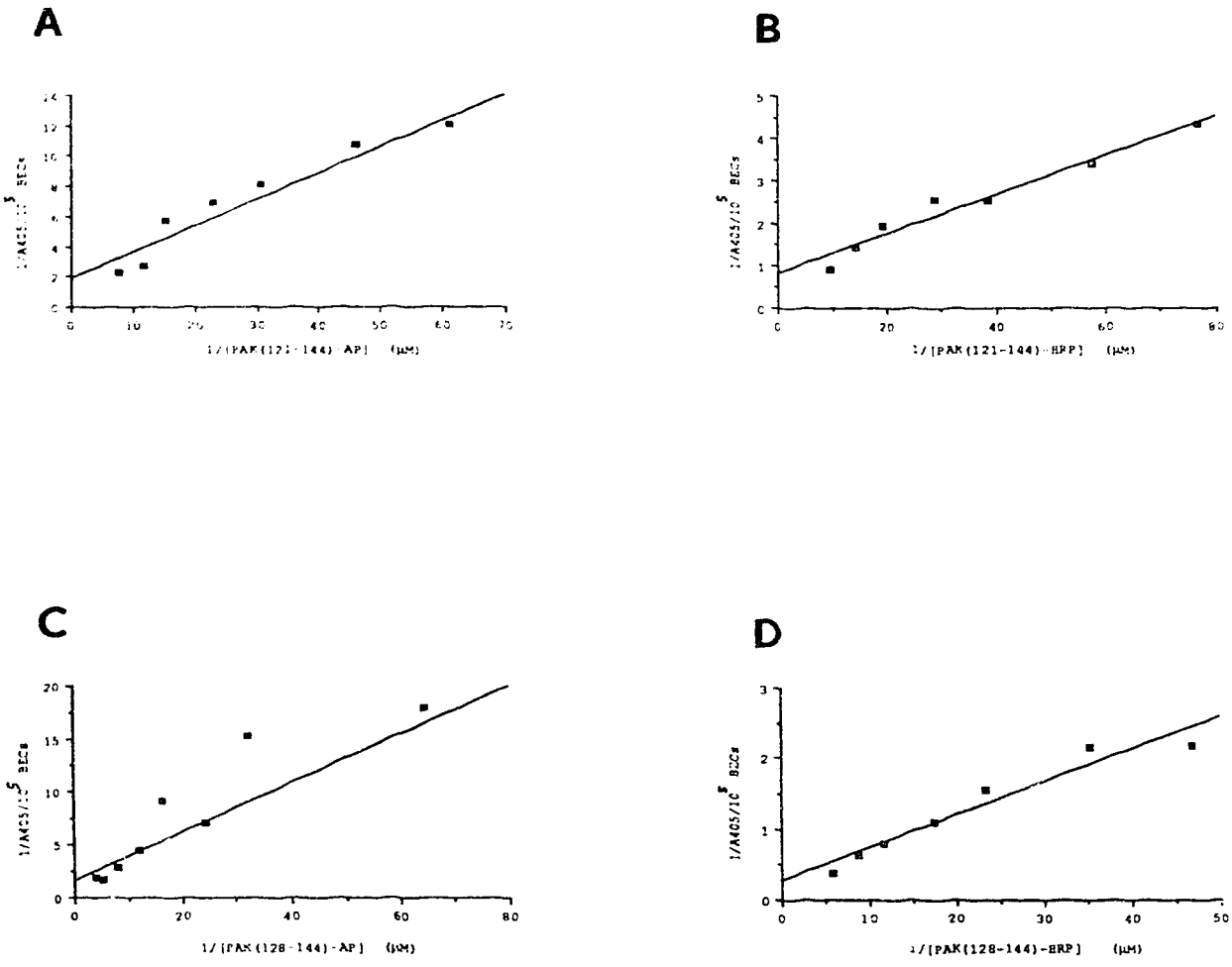

A
B
C
D
1/[PAK(121-144)-AP] (μM)
1/[PAK(121-144)-BRP] (μM)
1/[PAK(128-144)-AP] (μM)
1/[PAK(128-144)-BRP] (μM)

Figure 8. Binding of P1 peptide-HRP to BECs. Binding is shown to [illegible] in a concentration-dependent manner, identical to the b[illegible]g curve demonstrated for the PAK peptides. The molar coupling ratio was 3 peptides:1 HRP enzyme.

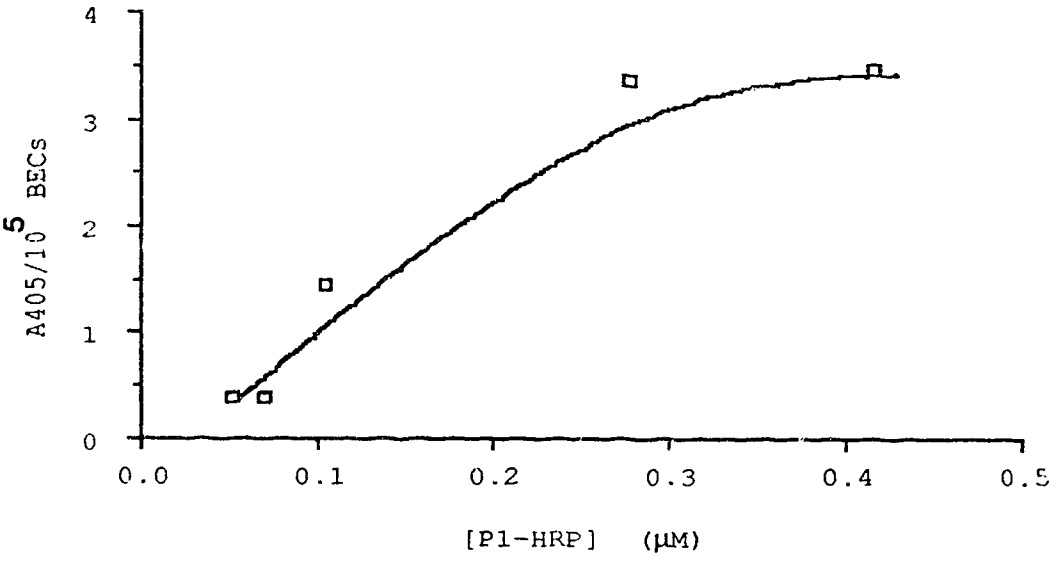

4
3
2
1
0
A405/10^5 BECs
0.0
0.1
0.2
0.3
0.4
0.5
[P1-HRP] (μM)

Figure 9. Determination of K_d for the binding of the P1 peptide-HRP conjugate to BECs. The apparent K_d is calculated to be 3.478 μM. The linear regression coefficient is 0.83.

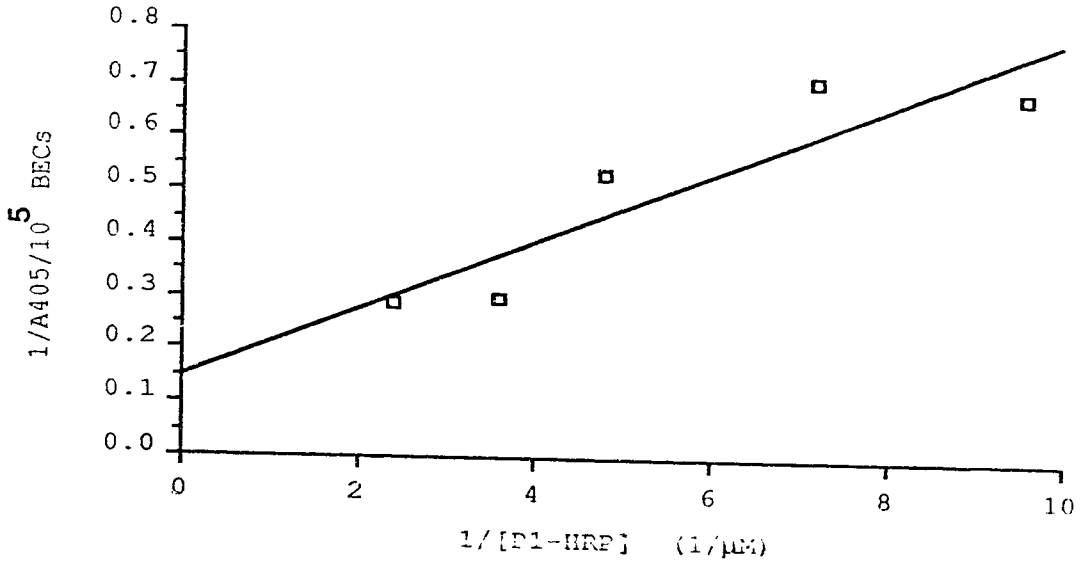

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1/A405/10^5 BECs
0
2
4
6
8
10
1/[P1-HRP] (1/μM)

Figure 10. Dissociation of bound PAK(128-144)-AP from BECs. After incubating the conjugates with the BECs, the bound peptide-enzyme conjugates were allowed to sit in 1 mL of 0.1 M PBS, pH 7.2, for varying lengths of time. The amount of bound conjugates appeared to decrease with increased time in the buffer, indicating that another equilibrium had been reached. The result presented is a single representative experiment, due to limitations in the number of BECs that are obtainable for a given experiment. Also, the number of cells required for each data point precluded utilizing multiple replicates. Thus, error bars are not included.

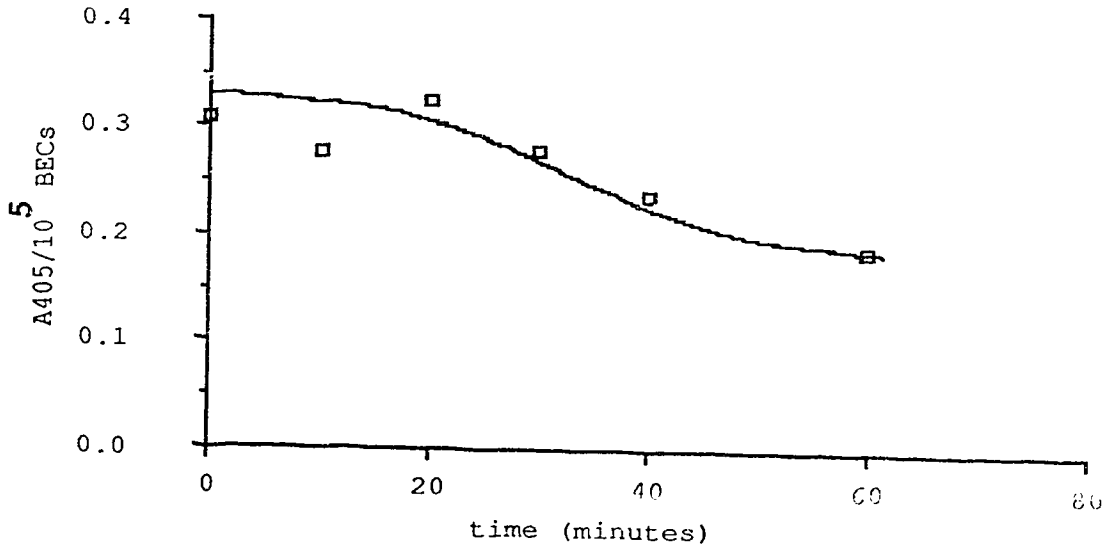

0.4
0.3
0.2
0.1
0.0
A405/10^5 BECs
0
20
40
60
80
time (minutes)

Figure 11. Binding of free AcPAK(128-144)ox in competition with 0.178 μM PAK(128-144)-AP to BECs. Binding of the peptide-enzyme conjugates decreased with increasing addition of free peptides. Concentration of the free peptides ranged from 0 to 1 μM. The IC_{50} was extrapolated from the graph to be 0.04 μM. Error bars are not included (see legend to Figure 10).

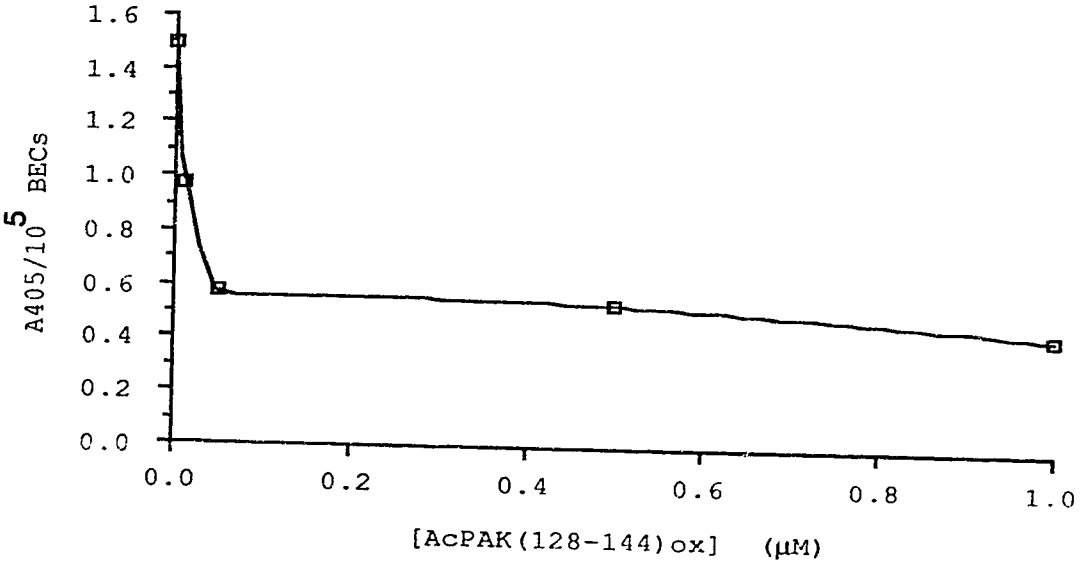

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A405/10^5 BECs
0.0
0.2
0.4
0.6
0.8
1.0
[AcPAK(128-144)ox] (μM)

Figure 12. Inhibition of PELA by PAK pili. 2.8 μM of the conjugate PAK(128-144)-AP was added to BECs along with varying concentrations of PAK pili (0 to 80 μg/mL) and incubated at room temperature for 1.5 h. The binding of the conjugates decreased with increased amounts of pili. The IC_{50} was extrapolated from the graph to be 5.6 μg/mL.

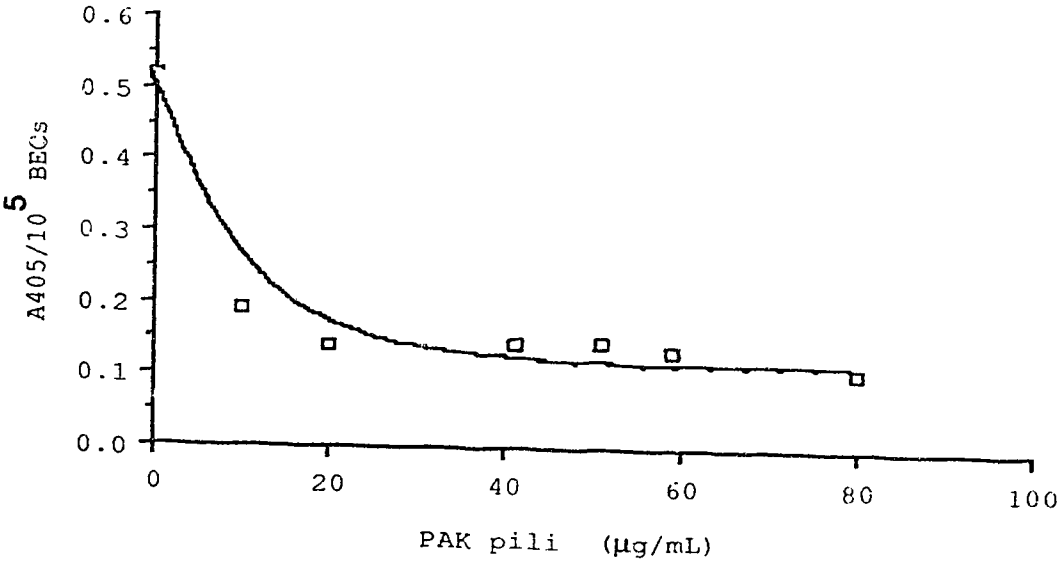

0.6
0.5
0.4
0.3
0.2
0.1
0.0
A405/10^5 BECs
0
20
40
60
80
100
PAK pili (μg/mL)

Figure 13. Binding of PAK(128-144)-HRP to monoclonal antibodies PK99H, PK3B and PK129A bound to the wells of a microtitre plate. The conjugates bound to PK99H in a concentration-dependent manner in an ELISA assay. Binding to PK3B and PK129A was not significant. 5 µg/mL of each antibody was coated on to ELISA plates.

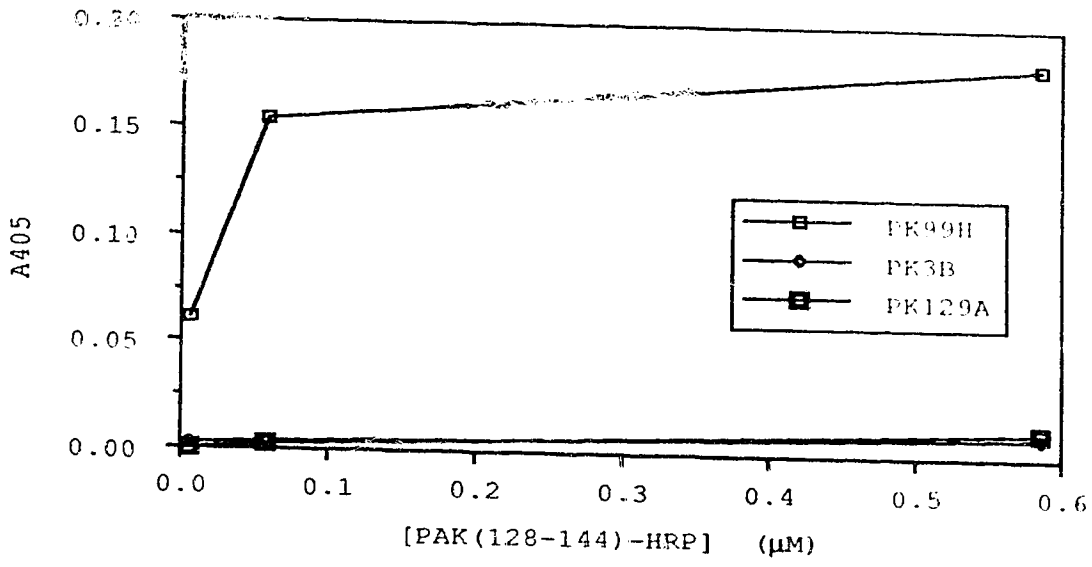

A405
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
[PAK(128-144)-HRP] (μM)
PK99H
PK3B
PK129A

Figure 14. Binding of PAK(128-144)-HRP to monoclonal antibody PK99H in competition with free AcPAK(128-144)ox. Equal volumes of free conjugate and peptides were added to 5 µg/mL of immobilized IgG in an ELISA assay. Increasing the free peptide concentration resulted in a decrease of the conjugated peptide binding to the antibody.

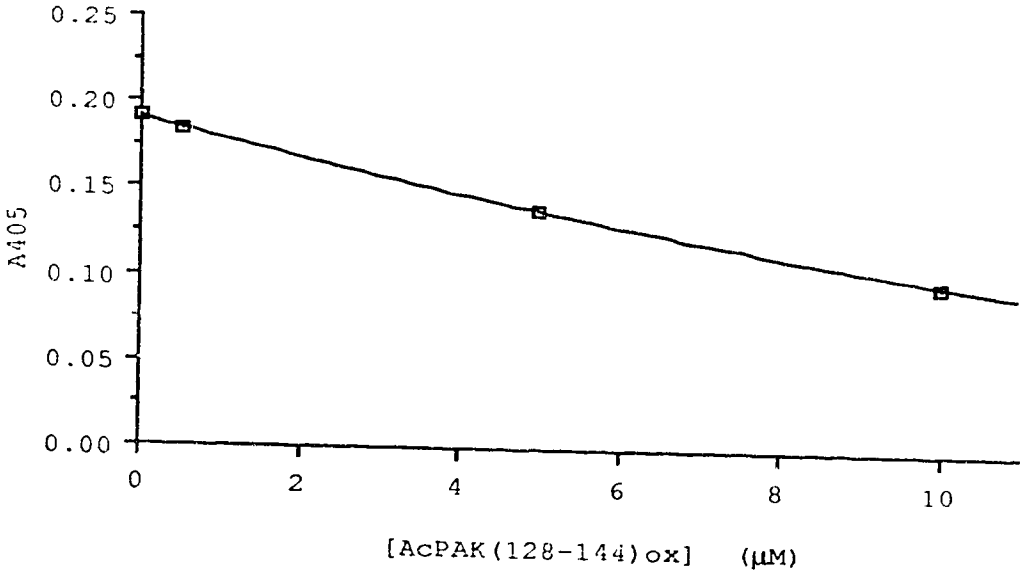
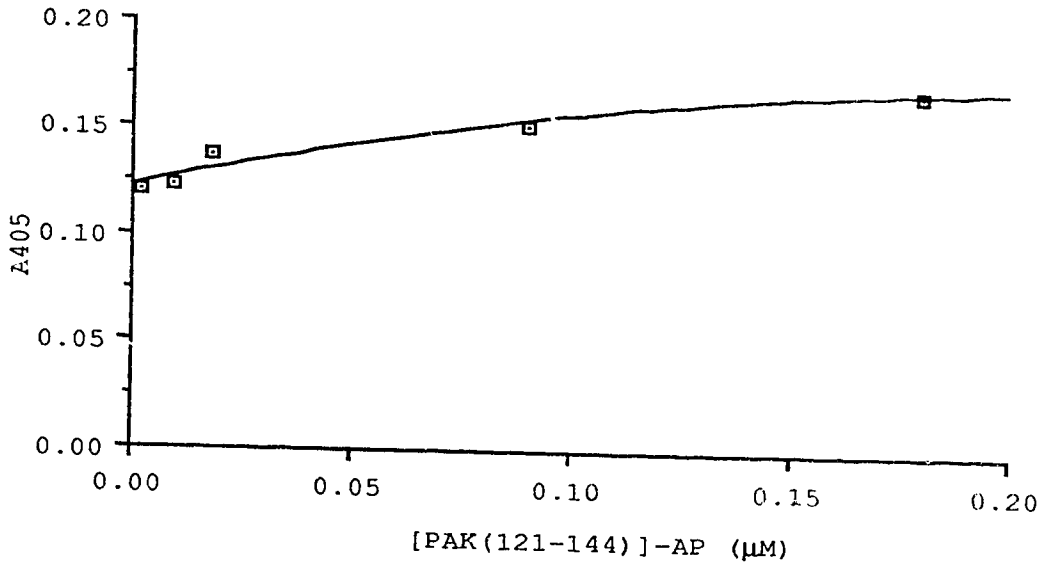

0.25
0.20
0.15
0.10
0.05
0.00
A405
0
2
4
6
8
10
[AcPAK(128-144)ox] (μM)

Figure 15. Binding of the 24-amino acid peptide-enzyme conjugate PAK(121-144)-AP to PAK pili was concentration-dependent. The same assay performed with the 17-amino acid conjugate did not demonstrate adherence.


0.20
0.15
0.10
0.05
0.00
A405
0.00
0.05
0.10
0.15
0.20
[PAK(121-144)]-AP (μM)

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