EXTRACELLULAR PROTEINASES OF PATHOGENIC MICROORGANISMS

by

JONATHAN LLOYD MOON

(Under the Direction of James Travis)

ABSTRACT

Proteolytic enzymes are required by all organisms for nutrient acquisition. However, pathogenic microorganisms often secrete enzymes of this class that may aid in their virulence by other means, including tissue destruction and modulation of host defense responses. It is therefore important to investigate the production of these enzymes by known pathogens. With this in mind, we have studied the production of proteolytic enzymes by two human pathogens, *Penicillium marneffei* and *Staphylococcus epidermidis*.

P. marneffei is an AIDS related fungal pathogen, native to Southeast Asia. . Two proteinases secreted by *Penicillium marneffei* have been purified from liquid cultures. PMAP-1, a 24 kDa proteinase with optimal activity at acid pH, was purified from a culture grown at room temperature. PMNP, a 50 kDa serine protease with activity at neutral pH, was purified from a culture grown at 37°C. The cDNA for a third enzyme from *P. marneffei*, PMAP-2, was cloned. This putative proteinase has a predicted molecular weight of 22kDa and a pI of 4.41. Based on the amino terminal sequence and functionality of PMAP-1 and the full length sequence of the putative proteinase PMAP-2,

both of these enzymes have been assigned to the G1 family of proteinases, also known as the eqolisins.

S. epidermidis is a common nosocomial pathogen that is a frequent colonizer of indwelling medical devices. We have purified and characterized a proteinase secreted by this organism during culture at 37°C. This enzyme is a 25 kDa serine proteinase that has high homology to the V8 protease from *Staphylococcus aureus* and, like the latter enzyme, is highly specific for cleavage after glutamic acid residues.

These three enzymes from *P. marneffei* and the one from *S. epidermidis* may play roles in virulence.

INDEX WORDS: *Penicillium marneffei*, *Staphylococcus epidermidis*, AIDS, protease, proteinase, eqolisin, V8 protease

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DEDICATION

This dissertation is dedicated to my family and especially to my parents, Andy and Emily. Their constant love and support have lifted me through many difficult times during graduate school. Their steadfastness and advocacy have been invaluable to me, and I will be grateful to them forever.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Microbial Proteases as Virulence Factors

All living organisms require extracellular sources of nutrients to grow and survive. In microorganisms such as fungi and bacteria, carbon and nitrogen sources are often supplied via the enzymatic breakdown of extracellular materials such as carbohydrates (broken down into simpler sugars) and proteins (broken down into smaller peptides and free amino acids). The proteinases that are secreted by infectious organisms often act in a rampant and unchallenged manner, freely breaking down host proteins since host proteinase inhibitors cannot control these activities. Frequently, the function of the proteinases secreted by these pathogens is merely to provide a source of free amino acids. In some cases, however, these enzymes may play a more direct role in pathogenesis, including tissue destruction and invasion, inflammation, and modulation of the host immune system (Figure 1.1) (Travis et al., 1995).

To provide free amino acids and small peptides, many pathogens secrete proteinases with broad substrate specificities. In contrast, bacteria that colonize the gastrointestinal tract often do not secrete large quantities of proteinase as their environment is already rich with nutrients provided by host enzymes. Outside of this environment, however, some microorganisms can subvert the action of normal host proteinases as an alternative mechanism for providing nutrients (e.g., the use of enzymes from phagocytes, such as neutrophils and macrophages).

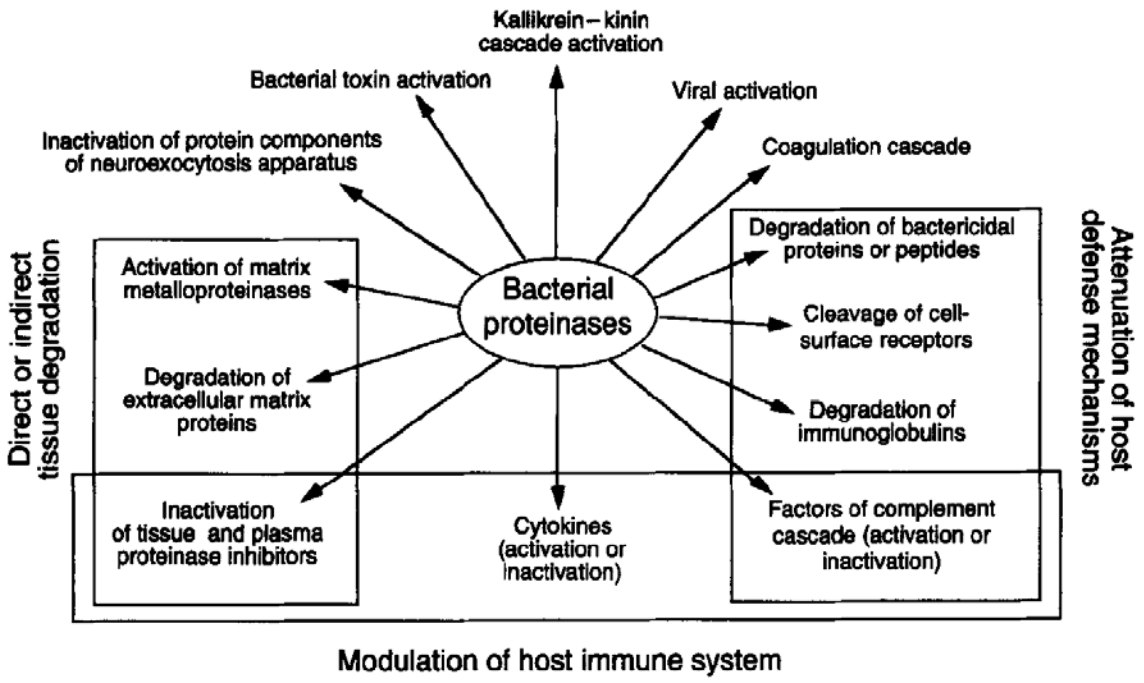

Figure 1.1 The roles of microbial proteinases in the progression of disease. While the center oval lists these as functions of bacterial proteinases, many apply to fungal enzymes as well. From Travis et al., 1995

The pain and swelling often found at the site of a microbial infection can sometimes be traced to the dysregulation of the kallikrein-kinin cascade by proteinases secreted by bacteria (Nilsson et al., 1985; Maeda and Molla, 1989). Other proteinase dependant systems that may be subverted or disrupted by microbial pathogens include the coagulation cascade, fibrinolysis, phagocytosis, and complement. While these systems are normally tightly controlled by an excess of proteinase inhibitors (Travis and Salvesen, 1983), the enzymes secreted by microbial pathogens are sometimes far more active against the substrates in these cascades. Moreover, host inhibitors often are not effective against these foreign enzymes but, instead, serve as substrates (Nilsson et al., 1985; Potempa et al., 1986; Maeda and Molla, 1989).

Induction of the kallikrein-kinin system by the proteinases of pathogenic bacteria increases vasopermeability which in turn results in an increased flow of nutrients to the site of infection (Nilsson et al., 1985; Maeda and Molla, 1989). Some pathogens lack sufficiently high or specific enzymatic activities to activate this system. Often, these organisms can benefit from the enzymes released by host phagocytic cells (especially neutrophils) during degranulation. These host proteinases degrade surrounding tissue and, thereby, provide an increased level of nutrients at the site of the infection.

Tissue destruction by microbial proteinases that have low substrate specificity is common in several species. *Porphyromonas gingivalis*, the causative organism in periodontitis (Sandholm, 1986), *Pseudomonas aeruginosa*, involved in both corneal keratitis and cystic fibrosis (Bejarano et al., 1989), and others including *Streptococcus*, *Clostridium*, *Staphylococcus*, and *Serratia* species (Molla et al., 1986) secrete potent enzymes, some of which can directly degrade tissue proteins such as elastin, fibronectin,

and collagen. Some fungal enzymes, for example the metallo- (Brouta et al., 2001) and serine (Mignon et al., 1998) proteinases from *Microsporum canis*, along with enzymes from *Trichophyton rubrum* (Apodaca and McKerrow, 1990) and *Trichophytyon mentagrophytes* (Tsuboi et al., 1989), produce keratinases that putatively aid fungi in invasion and tissue destruction. Although not found to be a virulence factor in animal studies (Monod et al., 2002), the *Aspergillus fumigatus* serine proteinase has been shown to degrade human lung elastin, fibronectin, and lung, type I, and type III collagens (Iadarola et al., 1998). Plant pathogens, such as *Sclerotinia sclerotiorum*, have also adopted this strategy, secreting proteinases that may aid in invasion and nutrient acquisition (Poussereau et al., 2001). In addition, proteinases produced by the infected host can play a significant role in the progression of disease. These enzymes are often the product of neutrophil degranulation but can also result from the activation of procollagenases from fibroblasts through cleavage by microbial proteinases.

A particularly good example of the subversion of host proteinases can be found in the case of periodontitis caused by *P. gingivalis*. In addition to activating the kallikrein-kinin system to increase nutrient flow to the site of infection (Imamura et al., 1994), *P. gingivalis* also subverts the complement system to produce a chemotactic protein for neutrophils (C5a) (Wingrove et al., 1992). This would appear to be counterproductive for the bacteria, but the neutrophils are often unable to phagocytose the infecting bacteria after arriving at the site of infection due to the destruction of complement proteins, their receptors, and of immunoglobulins by the pathogen's secreted proteinases (Cutler et al., 1993; Sundqvist, 1993). As these neutrophils die, they release their degradative enzymes

at the site of infection, breaking down components in the crevicular fluid and in the surrounding connective tissue (Weiss and Regiani, 1984).

In contrast to the above case of active recruitment of neutrophils to the site of infection by the invading bacteria, some organisms secrete enzymes which can disrupt the chemotactic response. Enzymes from both *Serratia marcescens* (Oda et al., 1990) and *Streptococcus pyogenes* (Cleary et al., 1992) specifically degrade C5a, a neutrophil chemotactic factor of the complement system. Additionally, enzymes from *S. marcescens* (Molla et al., 1986), *Staphylococcus aureus* (Prokesova et al., 1992), *Candida albicans* (Ruchel et al., 1982; Ruchel, 1986; Kaminishi et al., 1995), and many other pathogens can degrade immunoglobulins, preventing the immune response from reaching the site of infection (for a review of bacterial IgA degrading enzymes see Kilian et al., 1998). Other bacterial proteinases can kill immune cells after they are internalized as part of α_2-macroglobulin complexes. These complexes of the foreign proteinase with the host inhibitor are internalized and degraded in the lysosomes of macrophages and fibroblasts. However, the activity of these foreign enzymes is sometimes regenerated during partial degradation of the α_2-macroglobulin, allowing the bacterial proteinase to cleave intracellular proteins and cause cell death (Maeda et al., 1987). It has been theorized that several of the *C. albicans* secreted aspartyl proteinases (Sap4-Sap6) can function in a similar manner (Hube and Naglik, 2001; Monod et al., 2002). However, instead of proteinase-α_2-macroglobulin complexes, the entire fungal cell is phagocytosed. The Saps are then secreted, possibly degrading intracellular proteins and eventually causing cytolysis of the phagocyte.

Inhibitors of both microbial and host derived proteinases are emerging as therapeutic agents. Inhibitors of the HIV protease have been found to reduce the invasiveness of *C. albicans* which is mediated by Sap1-Sap3 (Borg-von Zepelin et al., 1999; Korting et al., 1999; Munro and Hube, 2002). Additionally, though not a secreted proteinase, ClpP, the core protein of a major bacterial protease complex, has recently emerged as the target for a new antibiotic (Brotz-Oesterhelt et al., 2005). The compound, an acyldepsipeptide, activates the normally tightly controlled complex, allowing it to act in an unregulated manner inside the cell, causing cell death.

These examples serve to illustrate both the diversity of function and the wide distribution across species of proteinases that aid in microbial virulence. Given the decreasing effectiveness of currently available antibiotic drugs, it is important to examine these enzymes as possible targets for new therapies. To that end, this thesis describes the isolation, characterization, and cloning of proteinases produced by two pathogenic microorganisms, *Penicillium marneffei* and *Staphylococcus epidermidis*. These types of studies are important for furthering our understanding of the pathologies of these species.

Penicillium marneffei

Penicillium marneffei is a dimorphic fungal pathogen native to Southeast Asia. First described in 1956 (Capponi et al., 1956), the first report of a natural infection in man was not made until 1973 (DiSalvo et al., 1973), though later articles report cases of penicilliosis marneffei in man as early as 1964 (Deng and Connor, 1985). Infection by this fungus is usually diagnosed in patients with some predisposing immunodeficiency such as HIV infection (Chariyalertsak et al., 1997), chemotherapy, or immunosuppressive

therapy following organ transplantation (Wang et al., 2003; Chan et al., 2004; Woo et al., 2005). Accordingly, the rate of diagnosis has risen dramatically in parallel with the increase in the incidence of AIDS in the native region of *P. marneffei* (Chierakul et al., 2004; Liyan et al., 2004). However, penicilliosis marneffei is often confused with other AIDS related illnesses, such as tuberculosis, histoplasmosis, and cryptococcosis, sometimes complicating its diagnosis (Cooper and McGinnis, 1997).

Cases of penicilliosis marneffei, with very rare exception, are restricted to those who either live in or have visited its endemic region, comprising Thailand, southern China, Vietnam, Laos, Malaysia, Cambodia, Indonesia (Cooper and Haycocks, 2000), Taiwan (Hung et al., 1998), and eastern India (Singh et al., 1999). In fact, penicilliosis marneffei is the third most common AIDS related disease in some areas (Supparatpinyo et al., 1994) and has been designated an AIDS defining illness in the region (Nittayananta, 1999). Unfortunately, relatively few studies of the biochemistry of this organism have been made despite its increased human infection rates over the last 20 years.

The most striking feature of *P. marneffei* is its dimorphism (Figure 1.2) (Andrianopoulos, 2002). No other member of the *Penicillium* genus has the ability to grow in this manner. At 25°C, *P. marneffei* grows as a mold having multiple conidia, or spores, which are released to form more mold colonies. Additionally, a distinctive pink pigment is secreted into the media during growth at this temperature. At 37°C, however, the fungus grows with a yeast-like morphology, or arthroconidia, which can grow and divide inside an infected host cell. This change in morphology can be reversed by reducing the growth temperature of cultures from 37°C to 25°C.

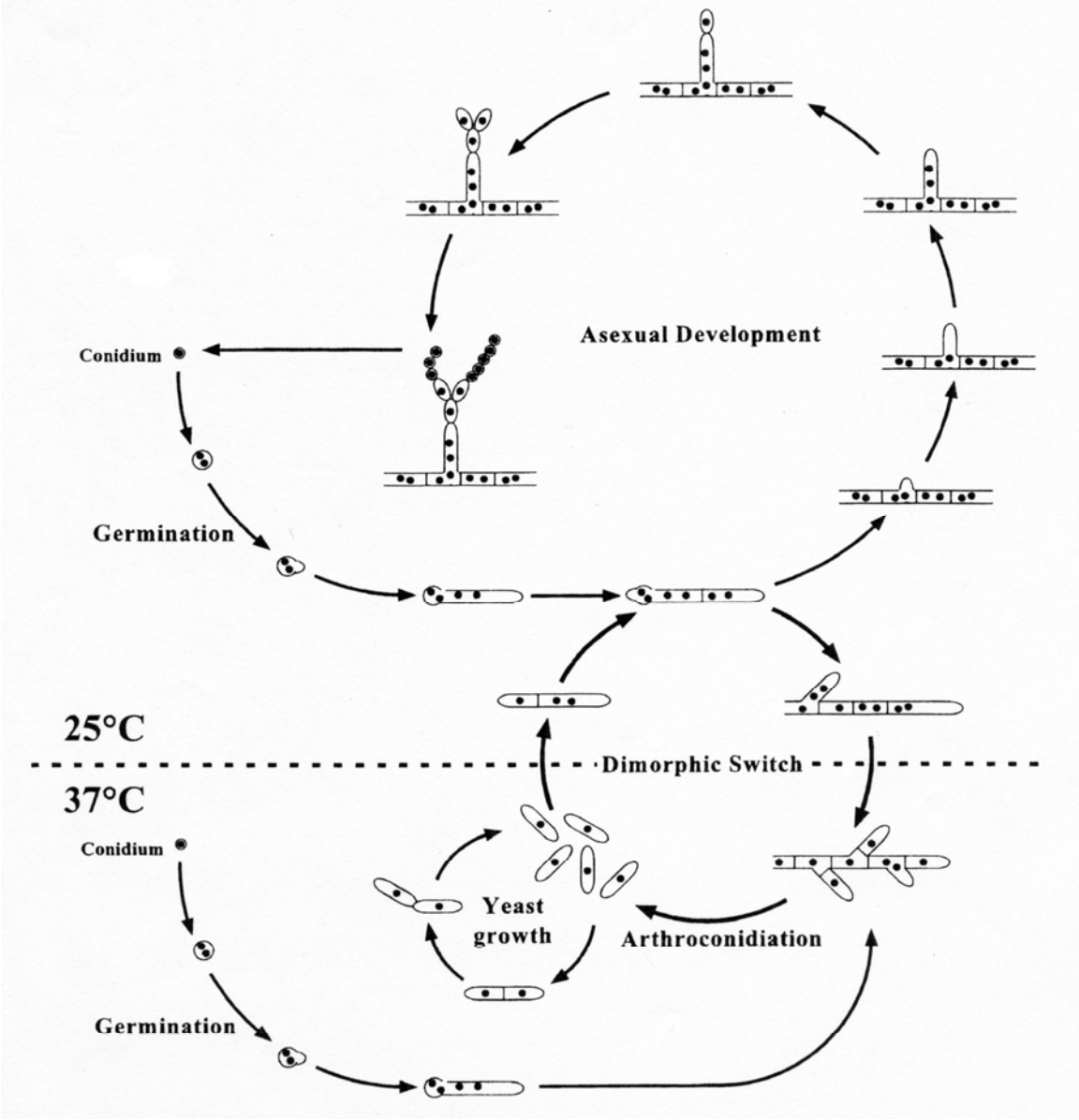

Figure 1.2 Dimorphic switching in *Penicillium marneffei.* From Andrianopoulos, 2002

The natural reservoir for infection by this organism remains unknown. Initially isolated from Bamboo rats during an unrelated study, the rate of infection in some species of these rats is as high as 90% (Deng et al., 1986; Ajello et al., 1995; Chariyalertsak et al., 1996). However, soil samples taken from their burrows in another study were almost always negative for *P. marneffei* (Vanittanakom et al., 1995). Further complicating matters, a comparison of AIDS patients with and without penicilliosis marneffei showed that a history of exposure to bamboo rats did not predispose patients to infection (Chariyalertsak et al., 1997). Although not yet proven, current theory holds that infection takes place by inhalation of conidia from the soil.

Patients suffering from penicilliosis marneffei exhibit a diverse array of symptoms such as skin lesions (often the first sign of disseminated infection), fever, cough, pulmonary infiltrates, diarrhea, lymphadenopathy, hepatosplenomegaly, anemia, leukocytosis, weight loss, osteoarticular lesions, pericarditis, multiple subcutaneous abscesses, papule-like ulcers, and septicemia (Cooper and McGinnis, 1997). The organism tends to be found inside macrophages and in the yeast-like arthroconidia morphology. Frequent sites of isolation include the lymph nodes, lung, liver, kidney, bone, and bone marrow.

The most reliable method of diagnosis for penicilliosis marneffei is the microscopic observation of fungal cells in a biopsy of skin or bone marrow. Additionally, fungal cultures grown from samples of these and other locations such as blood, sputum, or bronchoalveolar lavage can be used to diagnose infection (Cooper and McGinnis, 1997). Treatment for penicilliosis marneffei involves the use of antifungal drugs such as itraconazole, ketoconazole, or, in severe cases, amphotericin B. Due to a

high relapse rate, prophylactic itraconazole has been recommended for the remainder of the patient's life (Supparatpinyo et al., 1998). However, use of HAART (Highly Active Anti Retroviral Therapy) and the discontinuance of prophylactic antifungal therapy has shown promising results (Hung et al., 2002).

In parallel with *Aspergillus fumigatus* (Tronchin et al., 1997), the conidia of *Penicillium marneffei* possess a surface protein that allows them to bind to fibronectin (Hamilton et al., 1999) or laminin (Hamilton et al., 1998), both of which are components of the extracellular matrix, possibly playing a role in the initial phase of infection in the lung. The phagocytosis of *P. marneffei* by macrophages appears to be based on a glycoprotein receptor or receptors that contain *N*-acetyl-β-D-glucosamine based on inhibition of internalization by wheat germ agglutinin (Rongrungruang and Levitz, 1999). Reports concerning the killing of internalized fungal cells are contradictory with respect to both the ability of conidia to activate macrophages and their susceptibility to nitric oxide (Cogliati et al., 1997; Kudeken et al., 1998). Kudeken and coworkers found, however, that arthroconidia (yeast cells) stimulated macrophages and were susceptible to both superoxide anion and nitric oxide (Kudeken et al., 1998; 1999). Polymorphonuclear leukocytes have also been found to be fungistatic against arthroconidia and fungicidal when stimulated by certain cytokines (Kudeken et al., 1999). Neutrophils specifically were examined, and it was found that heat-labile granular extracts were likely responsible for their fungicidal activity (Kudeken et al., 2000). Models of infection using both normal and athymic BALB/c mice indicate a role for $CD4^+$ T cells in the clearance of infection by *P. marneffei* whether the mice were infected via the lung (Kudeken et al., 1996) or intravenously (Cui et al., 1997). The only study concerning the production of

proteolytic activities by *P. marneffei* failed to find any positive results (Youngchim et al., 1999). However, that study only tested for the production of trypsin or chymotrypsin type activities, and the authors were careful to point out the possible existence of other proteases.

To address the lack of information concerning the proteinases of *P. marneffei*, we have purified and characterized two enzymes secreted by this organism during growth in liquid culture (Chapter 2). One of these enzymes (*P. marneffei* acid proteinase 1 (PMAP-1)), a glutamyl endopeptidase, was purified from cultures grown at room temperature and the other (*P. marneffei* neutral proteinase (PMNP)), a serine endopeptidase, from cultures grown at 37°C. Additionally, we have cloned a putative glutamyl endopeptidase (PMAP-2) from a cDNA library made from room temperature cultures, suggesting that this enzyme may be expressed along with PMAP-1 under these culture conditions. It is our hope that our research may lead to a better understanding of the roles that proteinases may play in the pathogenesis of *P. marneffei*.

Staphylococcus epidermidis

Staphylococcus epidermidis is a coagulase-negative staphylococcus that has long been regarded a harmless commensal organism. It is often found as a contaminant in blood samples and is the most common bacteria found on human skin (Patrick, 1990). However, *S. epidermidis* has emerged as a frequent opportunistic pathogen, often acquired nosocomially by patients who either have an indwelling device such as a shunt, catheter, or prosthesis or are in some way immunocompromised (Blum and Rodvold, 1987; Mayo, 1995; Costerton et al., 1999). Infection by *S. epidermidis* is in fact the

leading cause of infections having to do with implanted medical devices such as joint prostheses, intravascular catheters, cerebrospinal fluid shunts, and artificial heart valves (Rupp and Archer, 1994). While native valve endocarditis due to *S. epidermidis* is rare, intravenous heroin users are particularly susceptible to right-sided endocarditis due to this organism (von Eiff et al., 2002).

The ubiquity with which this organism is found as part of the normal flora often complicates diagnosis of a clinically significant infection. Treatment of nosocomial infections is complicated by several factors, including the high rate of resistance to methicillin, which indicates resistance to all beta-lactam antibiotics (Diekema et al., 2001). Often, these methicillin-resistant strains are also resistant to other antibiotic agents, further complicating treatment. Additionally, biofilm formation by *S. epidermidis* can provide a physical barrier to those antibiotic agents to which it is not resistant (Knobloch et al., 2002). While many studies have been undertaken to describe the biofilm formation and antibiotic resistance of this organism, less has been done with regard to its production and/or utilization of proteolytic enzymes.

Foreign body associated infections of *S. epidermidis* are quite common due largely to the ability of this organism to form a multilayered and complex biofilm (Figure 1.3) (von Eiff et al., 2002). Initially, small numbers of bacteria attach to the device prior to its implantation in the host. These cells may come from the patient's skin, contaminated instruments, or the skin of the hospital staff. After implantation, foreign devices are coated by a mixture of fibrin, fibrinogen, fibronectin, vitronectin, thrombospondin, and von Willebrand factor, conditioning the surface of the implant and providing a better surface for the attachment of *S. epidermidis* along with other

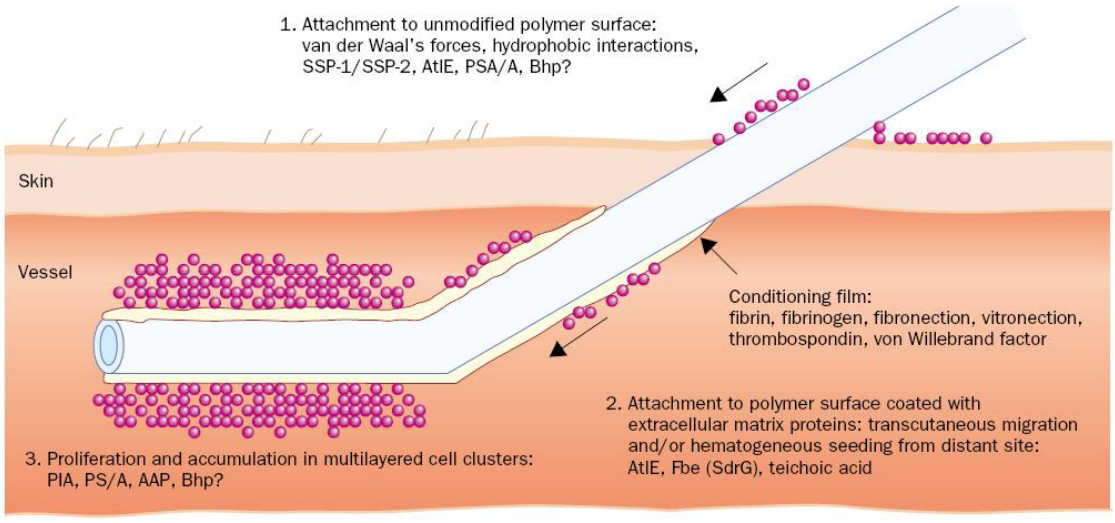

Figure 1.3 ***Staphylococcus epidermidis*** **biofilm formation on an indwelling device.** SSP-1/SSP-2: staphylococcal surface proteins; AtlE: autolysin; PSA/A: polysaccharide/adhesin; Bhp: biofilm-associated (Bap)-homologous protein; Fbe: fibrinogen-binding protein; SdrG: serine-aspartate-repeat-containing protein G; PIA: polysaccharide intercellular adhesin; AAP: accumulation-associated protein. From von Eiff et al. 2002

pathogenic bacteria such as *Staphylococcus aureus*. After adherence, *S. epidermidis* continues to reproduce, accumulating in cell clusters with multiple layers. The cells in these clusters adhere to each other via polysaccharide intercellular adhesin (PIA), of which there are two forms, a major polysaccharide I and minor polysaccharide II.

S. epidermidis is also known to produce many other virulence factors (von Eiff et al., 2002). Two proteinases with elastinolytic activity have been described, one by Teufel and Gotz, 1993 and one by Sloot et al., 1992 that also has activity against IgA, IgM, albumin, fibrinogen, and fibronectin. Given the substrates that are susceptible to cleavage by these enzymes, it is possible that proteinases from this organism function in dysregulation of the immune response as well as in tissue destruction in order to aid invasion. Lipases produced by *S. epidermidis* are thought to aid in skin colonization (Farrell et al., 1993; Simons et al., 1998). Despite the production of these and other virulence factors, *S. epidermidis* remains far less toxigenic than *S. aureus*.

The genome of *S. epidermidis* has been fully sequenced (Gill et al., 2005), allowing researchers to seek and study putative enzymes in addition to the ones already identified by biochemical means. These two sources of information have yielded a list of 78 known and putative peptidases and 13 non-peptidase homologues (Rawlings et al., 2004). These enzymes include representatives from all 4 major classes of proteases as well as both intracellular and secreted enzymes.

In an effort to further characterize the proteolytic activities produced by *S. epidermidis*, we have purified and characterized a serine endopeptidase produced by this organism (Chapter 3). This enzyme, a serine proteinase, has a high degree of sequence homology to the V8 proteinase produced by *S. aureus*. In addition, these two enzymes

share the same specificity for cleavage after glutamyl residues. It is possible that this enzyme plays a role in the pathogenesis of this organism in some of the same ways that V8 protease does for *S. aureus*.

Purpose of Research

This dissertation describes the studies of several proteolytic enzymes from the two pathogenic microorganisms introduced above, *Penicillium marneffei* and *Staphylococcus epidermidis*. Infection by these organisms is an increasing problem, and study of the proteinases produced by them is an important step in further understanding their mechanisms of virulence.

In Chapter 2, we describe the purification and characterization of two proteinases and the cloning of the cDNA for a third from *Penicillium marneffei*. The cloned cDNA and one of the purified enzymes share high sequence homology and are believed to be members of a recently identified family of proteinases, the eqolisins. The other purified enzyme is a serine proteinase that has the ability to cleave several physiologically relevant proteins. However, any of these proteinases may play a role in the virulence of this fungal pathogen.

In Chapter 3, we describe studies of another proteinase, a glutamyl endopeptidase purified from *Staphylococcus epidermidis*. This enzyme is highly homologous to the V8 protease from *Staphylococcus aureus*. It also possesses the ability to cleave certain physiological substrates, suggesting possible roles for it in the progression of disease caused by this organism.

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CHAPTER 2

ISOLATION AND PROPERTIES OF EXTRACELLULAR PROTEINASES OF *Penicillium marneffei*[1]

[1]Moon, J. L., Shaw, L. N., Mayo, J. A., Potempa, J., and Travis, J. 2005. Submitted for publication to Biological Chemistry

ABSTRACT

Penicillium marneffei is a dimorphic fungus native to Southeast Asia. Disease caused by this organism, until recently a very rare condition, has increased dramatically in parallel with the increase in the number of individuals in the region immunocompromised by AIDS and other conditions. While much research has been performed on the control of dimorphic switching in *P. marneffei*, there is a relative dearth of information regarding the proteinases secreted by this pathogen. Our laboratory has purified and characterized two proteinases produced by this organism in liquid culture and cloned a third. Both the cloned enzyme and one of those purified from culture supernatants have been identified as members of the eqolisin family, a group of pepstatin-insensitive acid proteinases. The other enzyme purified from a culture supernatant is a serine proteinase with activity in the neutral pH range. These enzymes appear to be differentially expressed depending on culture conditions.

Key Words

Penicillium marneffei, protease, eqolisin, AIDS

INTRODUCTION

Penicillium marneffei, a dimorphic pathogenic fungus native to Southeast Asia (Cooper and Haycocks, 2000), was first described in 1956 (Capponi et al., 1956). The first report of natural infection in humans was made in 1973 (DiSalvo et al., 1973), but a later publication (Deng and Connor, 1985) described a natural infection occurring in 1964. Although penicilliosis marneffei was initially seen as a rare condition, it has recently become considerably more common in parallel with an increase in the number of individuals living with compromised immune systems (Chierakul et al., 2004; Liyan et al., 2004). Such a rise in the number of cases reported can be clearly associated not only with the increasing number of AIDS patients (Chariyalertsak et al., 1997), but also with the increase in utilization of immunosuppressive therapy following organ transplantation or chemotherapy (Wang et al., 2003; Chan et al., 2004; Woo et al., 2005). Indeed, penicilliosis marneffei has been shown to be the third most common opportunistic infection in AIDS patients in one hospital in Northern Thailand (Supparatpinyo et al., 1994). As a result, this disease has been designated as an AIDS defining illness in that region (Nittayananta, 1999). Significantly, the rate of infection by this organism has grown rapidly over the past 20 years, yet few studies of its biology have been undertaken.

Most studies surrounding *P. marneffei* have focused on either its clinical pathology or the regulation of its dimorphic switching. This phase transition from sporulating mold form (25°C) to yeast like arthroconidia (37°C) is unique among the penicillia and is thought to contribute to the virulence of this fungus. Infection is thought to proceed by inhalation of conidia (spores) which are engulfed by phagocytosis. The

organism then undergoes phase transition to arthroconidia which can reproduce inside the lysosome. From there, it can spread to the lymph nodes, liver, kidney, bone, and bone marrow through the circulatory system. Patients with penicilliosis marneffei frequently present with intermittent fever, cough, lymphadenopathy, septicemia, hepatosplenomegaly, and skin lesions. Unfortunately, some of the symptoms associated with penicilliosis marneffei resemble other AIDS related diseases common in the region such as tuberculosis, histoplasmosis, and cryptococcosis, sometimes leading to misdiagnosis. When treated incorrectly, penicilliosis marneffei has a high mortality rate. For a review of these topics see Duong, 1996; Cooper and McGinnis, 1997; Cooper and Haycocks, 2000; Andrianopoulos, 2002.

Our research group has had a long interest in the extracellular proteinases produced by pathogenic microorganisms and the putative role(s) of these enzymes in the progression of disease (Potempa et al., 2000; Moon et al., 2001). Proteinases from such pathogens have been shown to play major roles in invasion, nutrient acquisition, host immune suppression, modulation of the clotting cascade, and other types of virulence. In this respect, another group of dimorphic fungi, the *Candida* species, are known to produce enzymes known as SAPs (secreted aspartic acid proteases) that have been shown to have roles in early fungal dissemination, invasion, and immune response modulation, all to the benefit of the invading organism (Naglik et al., 2003). Additionally, the fungal plant pathogen *Sclerotinia sclerotiorum* also secretes acid proteinases when cultured using sunflower leaves as the carbon and nitrogen sources (Poussereau et al., 2001). Other pathogenic fungi, including *Aspergillus niger* and *Aspergillus fumigatus*, release proteinases that are active at neutral or acidic pH (Monod et al., 2002), some of which

may aid in their virulence (Iadarola et al., 1998). Given the relative dearth of information concerning the biochemistry of *P. marneffei*, our laboratory has undertaken studies to determine what proteinases are secreted by this organism during its growth, both at room temperature and 37°C. Curiously, an earlier report indicated that *P. marneffei* produced no extracellular enzymes with trypsin or chymotrypsin type activities, but the authors pointed out that the possible production of other extracellular proteinases should not be overlooked (Youngchim et al., 1999).

We describe here the purification of two proteolytic enzymes from supernatants of cultures grown at 25°C and 37°C, respectively. These enzymes have been named *Penicillium marneffei* acid proteinase (PMAP-1) (25°C) and neutral proteinase (PMNP) (37°C). We have undertaken the characterization of these enzymes with respect to their physical properties such as pH optimum and cleavage specificity, as well as to their ability to cleave physiologically important substrates. In addition, we have cloned the gene for a third proteinase (*PMAP-2)* apparently synthesized during growth at 25°C. Subsequently, we have detected apparent production of PMAP-1 and PMAP-2 during culture at 37°C, in addition to that which occurs at 25°C.

RESULTS

Purification of PMAP-1

Early experiments focused on selection of growth conditions that would induce maximal proteinase production. The highest levels of proteolytic activity in cultures grown at

25°C were usually observed between 1 and 2 days after culture inoculation (during logarithmic growth) into Malt Extract Broth and were measured at pH 5.5 using EnzChek BODIPY-FL as the substrate. The presence of this activity was highly transient, returning to baseline levels during the late logarithmic and stationary phases of growth (Figure 2.1). Therefore, careful monitoring of the activity was necessary in order to obtain cultures with maximum activity levels for purification of the acid proteinase (PMAP-1).

After removal of the cells by centrifugation and filtration, the culture supernatant was concentrated using a 10 kDa filter to reduce both the volume and relative concentration of remaining media components as well as the pink pigment produced in room temperature cultures of *P. marneffei*. A DE-52 anion exchange column then allowed for the further removal of pigment and other components in the culture supernatant by exclusion as the acid proteinase activity was retained on the column and eluted with a gradient of NaCl in the buffer. After concentrating the activity containing fractions and making them 1 M in $(NH_4)_2SO_4$, a final step employing hydrophobic interaction (Phenyl Superose) was used to separate the remaining pigment and other components from PMAP-1 which was eluted in a gradient of buffer without $(NH_4)_2SO_4$ (Figure 2.2).

Purification of PMNP

For cultures grown at 37 °C, maximum proteolytic activity was observed during growth in media containing yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ but

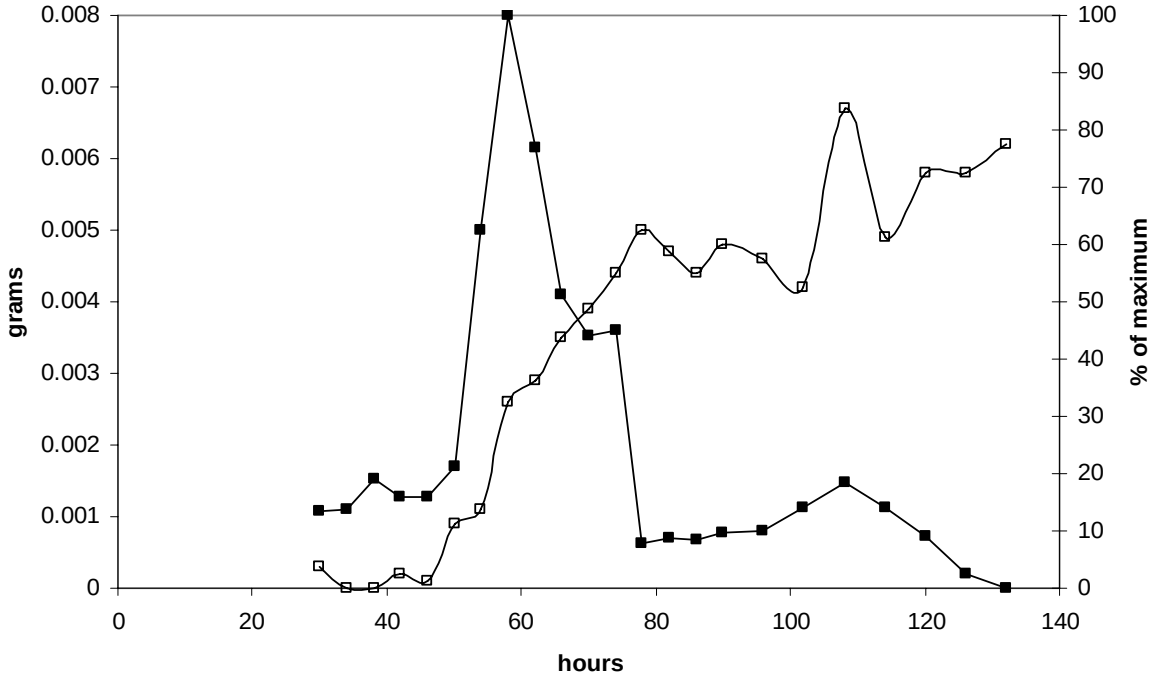


Figure 2.1. **Growth and production of acid pH proteolytic activity of *P. marneffei*.** Dry weight of 1 ml of culture (*open squares*) and activity against EnzChek BODIPY-FL at pH 5.5 (*filled squares*) of a liquid culture of *P. marneffei* grown at 25°C were measured and plotted against time.

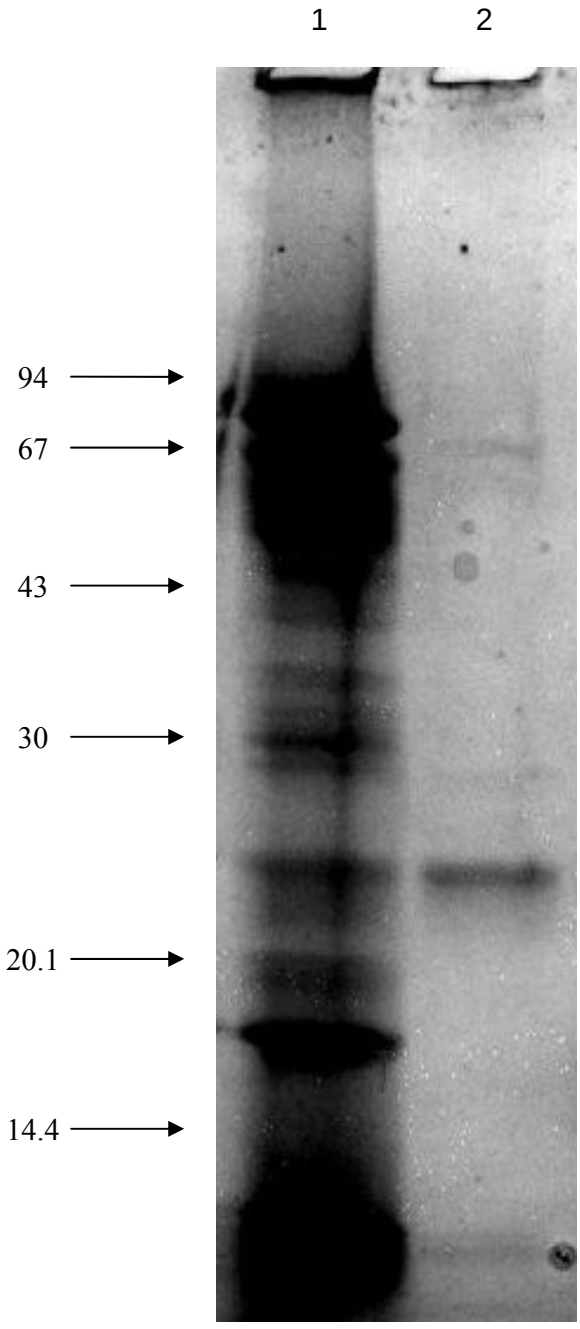


Figure 2.2. **Purification of PMAP-1.** Pooled, concentrated, activity containing fractions from a DE-52 column (*lane 1*) and 1 fraction from a Phenyl Superose column (*lane 2*) containing purified PMAP-1 were subjected to SDS-PAGE. The positions of molecular weight markers are indicated on the left. The gel was stained with Coomassie Brilliant Blue.

supplemented with 1% dextrose and 0.5% BSA. This activity was generally monitored using EnzChek BODIPY-FL substrate at pH 8.0 and normally reached maximum levels after 5-7 days of growth.

Again, the cells were separated from the supernatant using centrifugation followed by filtration. An initial anion exchange step as utilized earlier for PMAP-1 purification gave unexpected results, as the proteolytic activity was not retained on the column. This step, however, was still of value, as much of the black pigment produced during growth along with other components were bound. The flow through was concentrated and its buffer changed to 50 mM Tris-HCl, pH 8.0 by diafiltration. The next step employed a stronger anion exchange medium (Source 15Q), but most of the proteolytic activity remained in the flow through volume. Nevertheless, this step removed additional contaminants. Again, concentration and diafiltration were used to reduce the volume and exchange the buffer to 50 mM Tris-HCl, pH 7.5 containing 1 M $(NH_4)_2SO_4$. The sample was then applied to a Phenyl Superose hydrophobic interaction column. Elution with a gradient of buffer without $(NH_4)_2SO_4$ yielded purified PMNP (Figure 2.3).

Initial characterization of PMAP-1 and PMNP

SDS-PAGE analysis showed that PMAP-1 migrated as a single band of approximately 24 kDa (Figure 2.2). Activity against EnzChek BODIPY-FL was observed at pH values from 2.0 to 5.5 with the peak activity at pH 3.0 (Figure 2.4A). Storage of the enzyme at

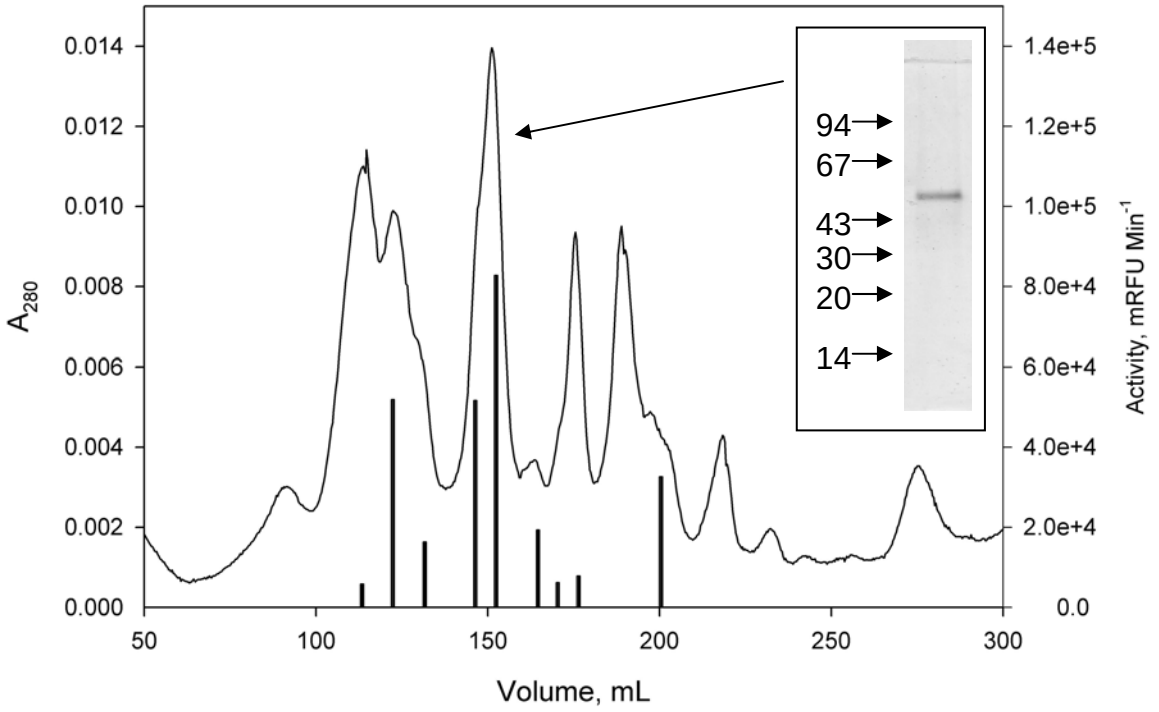


Figure 2.3. **Purification of PMNP.** One fraction from the indicated peak from a Phenyl Superose column was subjected to SDS-PAGE (inset). The positions of molecular weight markers are indicated on the left. The bars under the A280 trace indicate the activity of individual fractions against EnzChek BODIPY-FL. The gel was stained with Coomassie Brilliant Blue.

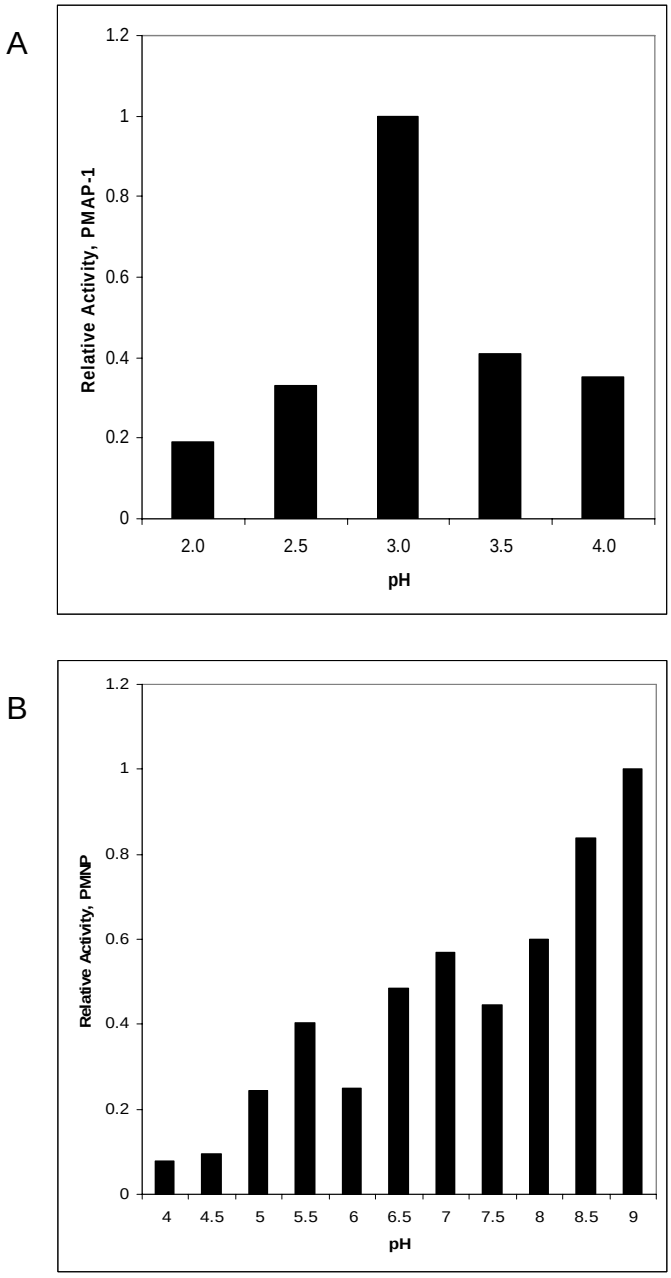


Figure 2.4. **pH optima for PMAP-1 and PMNP.** PMAP-1 (*A*) and PMNP (*B*) were assayed at varying pH levels with the EnzChek BODIPY-FL substrate.

4°C for extended periods seemed to have little effect on its activity. PMNP migrated as a single band of approximately 50 kDa on SDS-PAGE (Figure 2.3) and showed activity over a wide range of pH values (pH 4.0 to 9.0) (Figure 2.4B). Storage of the enzyme at 4°C for extended periods did not seem to adversely affect the activity of the proteinase.

Cleavage specificities of PMAP-1 and PMNP

The B chain of insulin was used to assess the peptide bond cleavage specificity of both PMAP-1 and PMNP (data not shown). In both instances, the products of the cleavage experiments were analyzed by LC-MS to determine the identity of the fragments generated. In the case of PMAP-1, the products showed a wide variety of cleavage sites, suggesting the enzyme has little preference for specific residues at the P1 or P1′ sites. However, products generated by cleavage after either glutamic acid or phenylalanine residues were more prevalent than others. PMNP also cleaved the insulin B chain in several locations, with fragments frequently being found that had been generated from B chain cleavage after leucine residues.

Substrates with small reporter groups in their P1′ positions were also tested for susceptibility to cleavage by PMAP-1 and PMNP. While PMAP-1 had no detectable activity against this type of substrate, PMNP showed some activity against substrates with leucine residues in the P1 position such as N-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide and p-Glu-Phe-Leu-*p*-nitroanilide (data not shown).

Additionally, PMNP was tested for activity against fibrinogen and fibronectin, with both of these proteins being cleaved during incubation at 37°C (Figure 2.5). PMNP

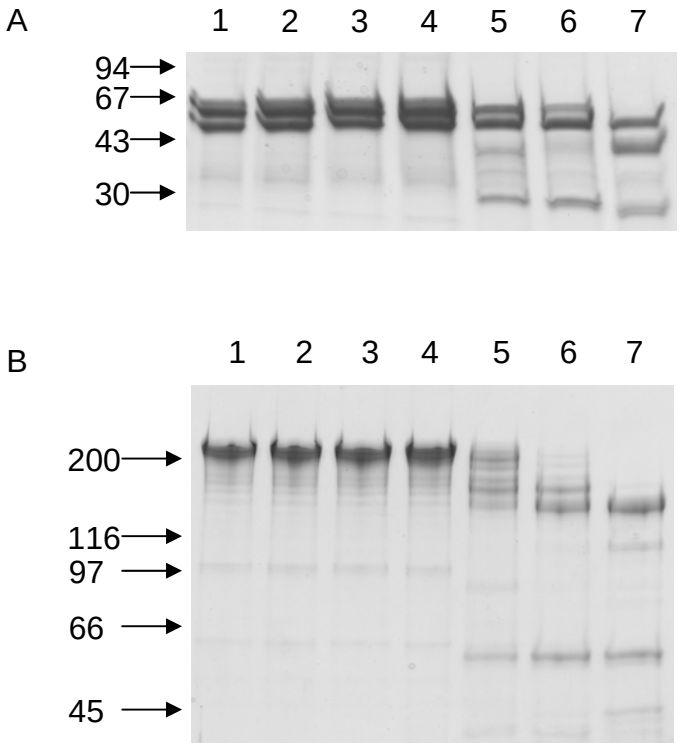


Figure 2.5. **Degradation of fibrinogen and fibronectin by PMNP.** SDS-PAGE was performed on samples of fibrinogen (*A*) and fibronectin (*B*) after incubation with PMNP for varying times at 37°C. For each gel, lanes 1-4 contain only the substrate protein incubated for 0, 1, 4, and 24 hours. Lanes 5-7 contain the substrate protein along with PMNP incubated for 1, 4, and 24 hours. The positions of molecular weight markers are indicated on the left.

also showed moderate activity against elastin-rhodamine and native elastin (data not shown). PMAP-1 was not assayed for cleavage of these substrates as they are only present above its functional pH range *in vivo*. As mentioned previously, both PMNP and PMAP-1 showed activity against EnzChek BODIPY-FL (fluorophore substituted casein).

Identification of the mechanistic classes of PMAP-1 and PMNP

Studies were performed with various catalytic class specific inhibitors in order to further characterize both PMAP-1 and PMNP (data not shown). In the case of PMAP-1, none of the inhibitors tested (including pepstatin, normally an inhibitor of acid proteinases), had any significant effect on its cleavage of the EnzChek BODIPY-FL substrate. The lack of inhibition required reliance on other data to identify the catalytic class of this enzyme. PMNP however was strongly inhibited by three different serine proteinase inhibitors, di-isopropylfluorophosphate (DFP), phenylmethylsulphonyl fluoride (PMSF), and 3,4 – dichloroisocoumarin (DCI), with inhibitors of proteinases from other classes having little effect. These data allow for the classification of PMNP as a serine proteinase.

Amino terminal sequencing of PMAP-1

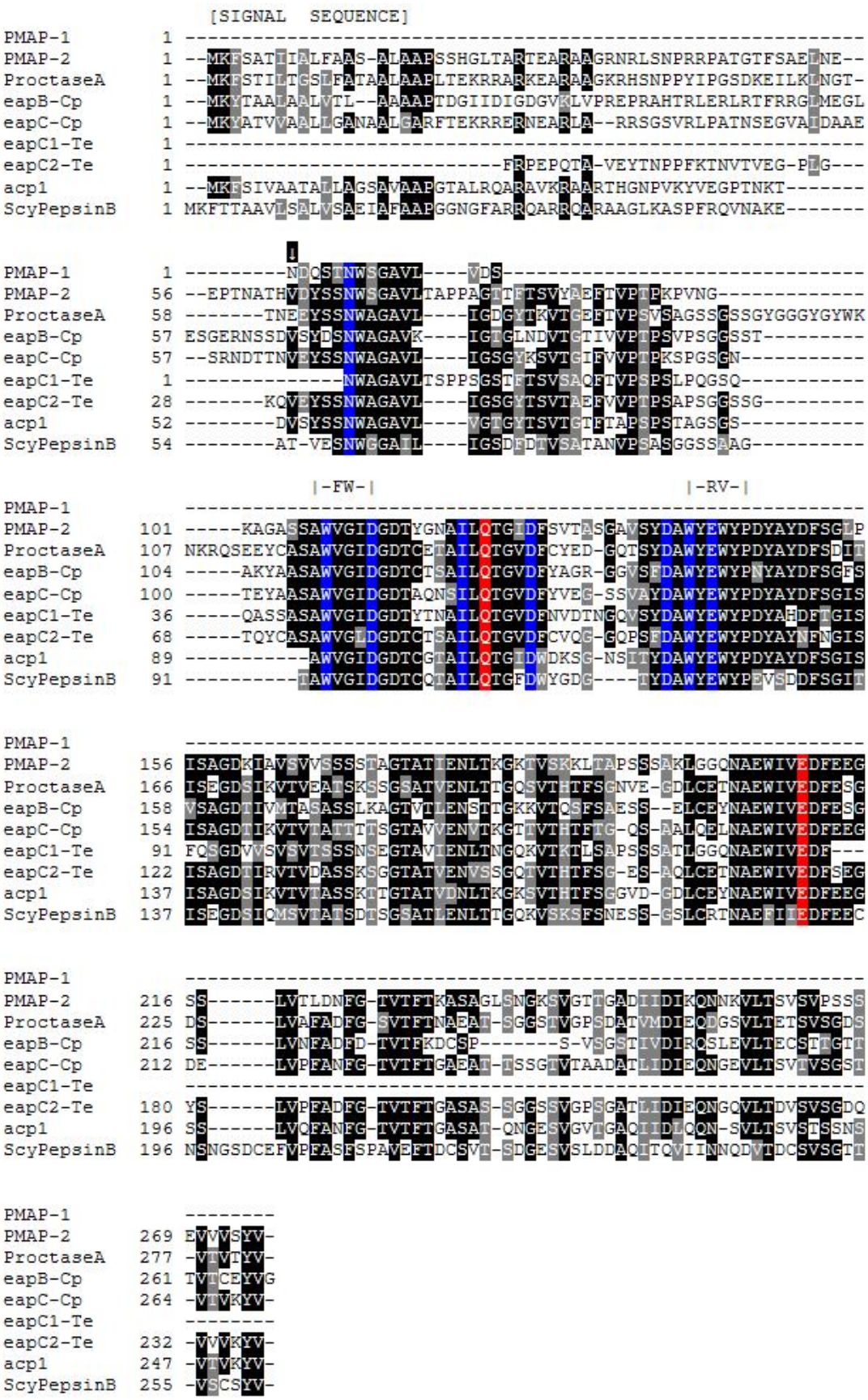
After SDS-PAGE (as described in Laemmli, 1970) followed by blotting to a PVDF membrane, the amino terminal sequence of PMAP-1 was determined to be NDQSTNXSGAVLVDV by Edman degradation. Subsequent *de novo* sequencing of tryptic digests of PMAP-1 by MS/MS yielded multiple sequence fragments, one of which

overlapped with the sequence obtained from Edman degradation. The amino terminus of PMAP-1 was thus determined to have the sequence NDQSTNWSGAVLVD. This sequence was found to possess high homology to members of the G1 sub-class of acid proteinases. These proteinases differ from other acid proteinases in several ways including their insensitivity to pepstatin and other proteinase inhibitors, explaining the results of the earlier inhibition studies as well as the failure of the protein to be retained on a pepstatin agarose column (data not shown).

Cloning of *PMAP-2*

As we were in possession of the amino terminal sequence of PMAP-1, it allowed us to identify this enzyme as a member of the G1 protease family. Based on a ClustalW (Chenna et al., 2003) alignment of this family it became apparent that there were certain regions of these proteins having high degrees of identity and that these regions might enable us to clone the PMAP-1 gene as a result of this sequence conservation. A *P. marneffei* cDNA library was generated and used as the template for degenerate PCR reactions in an attempt to isolate the DNA sequence encoding PMAP-1. Degenerate primers were designed on the basis of a homology alignment with members of the G1 protease family (see Figure 2.6). Experiments with one set of primers (designed from the regions indicated in Figure 2.6) generated a 116bp fragment that was TOPO TA cloned (Invitrogen) and subjected to DNA sequencing. This revealed a sequence of:

Figure 2.6. **Sequence alignment of PMAP-1, PMAP-2, and several other members of the G1 family.** The amino terminus of PMAP-1 and the putative gene product of *PMAP-2* are aligned with several other members of the G1 family of proteinases: Proctase A is from *Aspergillus niger* and is also known as Aspergillopepsin II; eapC-Cp and eapB-Cp are from *Cryphonectria parasitica*; EapC1-Te and EapC2-Te are from *Talaromyces emersonii*; acp1 is an EapC homolog from *Sclerotinia sclerotiorum*; ScyPepsinB is scytalidopepsin B from *Scytalidium lignicolum*, now known as scytalidoglutamic peptidase. The putative signal sequence for PMAP-2 is indicated, as well as the regions from which successful primers were designed (FW and RV), and the putative mature amino terminal residue (↓). Some important conserved residues are shown in red and the active site residues in blue.


[SIGNAL SEQUENCE]
PMAP-1
PMAP-2
ProctaseA
eapB-Cp
eapC-Cp
eapC1-Te
eapC2-Te
acp1
ScyPepsinB
|-FW-|
|-RV-|

GCGTGGGTGGGAATTGACGGTGACACCTACGGCAATGCCATTCTCCAAACTG
GTATTGACTTCAGTGTCACTGCCAGCGGCGCCGTTTCCTACGATGCCTGGTAC
GAATGGTATCC

When subjected to *in silico* translation analysis, the sequence derived proved an excellent match when included into the G1 family homology alignment (see Figure 2.6). Thus in order to identify the entire coding region of this protein we designed two further, gene specific primers based on the DNA sequence above in order to sequence out the remainder of the open reading frame. These primers were based on a 24bp internal fragment from the identified DNA (underlined sequence above) but were divergent to each other so that they would amplify out into the unknown sequence. These primers were used separately in conjunction with primers based either side of the multiple cloning site of the cDNA host vector pSL1180. Thus using this technique we were able to generate a 343bp fragment corresponding to the 5′ sequence of the gene and a 464bp fragment corresponding to the 3′ end of the gene. These fragments were then TOPO TA cloned and sequenced in order to reassemble, *in silico*, the coding sequence for PMAP-1. However, after further analysis it was found that the resulting sequence varied significantly in the amino terminal region from that obtained for PMAP-1:

PMAP-1: NDQSTNWSGAVLVD

PMAP-2: VDYSSNWSGAVLTA

Therefore, the full sequence was denoted *PMAP-2* (the cDNA sequence of *PMAP-2* was deposited under GenBank Accession Number DQ166816) and is aligned in Figure 2.6 with other members of the G1 class with which it shares a high degree of identity.

Assuming the protein to have a mature structure beginning at the Val residue indicated by ⇓ in Figure 2.6, the gene product of *PMAP-2* should possess a molecular mass of 21,914 Da and a theoretical pI of 4.41. Residues 1-17 make up a putative signal sequence also indicated in Figure 2.6. Southern blotting suggested only one copy of the gene to be present in the DNA of *P. marneffei*. Despite repeated attempts to identify the DNA sequence encoding PMAP-1, we were unable to generate this result.

Western blot analysis of the production of PMAP-1 and -2

Liquid cultures of *P. marneffei* were grown at 25°C and 37°C using various media for two weeks. The cultures were sampled every 8 hours and assayed for proteolytic activity at pH 4.3 and 7.4 using EnzChek BODIPY-FL. Samples with high levels of low pH activity were concentrated using Centricon YM-3 (Amicon) concentrators and subjected to Western Blot analysis using serum from rabbits immunized with the recombinant putative proteinase PMAP-2 (Figure 2.7). For cultures grown at 25°C (Figure 2.7A), two bands (24 and 22 kDa) were detected from high activity samples early in the time course, while high activity samples taken later in the course of *P. marneffei* growth generally exhibited only one band (24 kDa) that was much fainter (not shown). In cultures grown at 37°C, at least two bands could be detected in early, high activity fractions, but no activity was observed in any of the cultures past 3 days of growth. For cultures grown on 1% Peptone (Difco, Figure 2.7B), three bands were visible (24, 22, and 20 kDa), and for those grown on MEB (Figure 2.7C), as many as four bands (24, 20, 18, and 17 kDa) could be seen in some samples. All of the samples with low pH activity had a band at

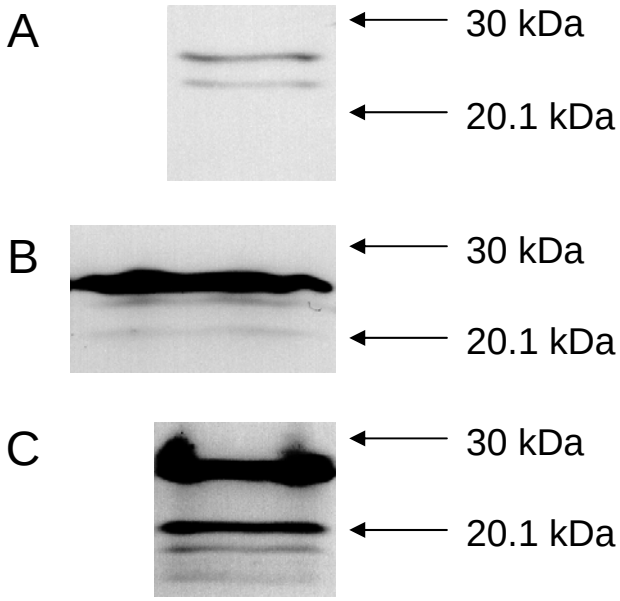


Figure 2.7. **Western blot of concentrated culture supernatants of *P. marneffei*.** Culture supernatants (500 µl) were concentrated using Centricon YM-3 concentrators and separated via SDS-PAGE on a 4-20% gradient gel. The gel was then subjected to Western blotting using the sera from two rabbits immunized with the expressed *PMAP-2* gene product for the primary antibody step. (*A*) Room temperature cultures exhibited two bands (24 and 22 kDa) with high activity when grown on MEB, while cultures grown at 37°C on 1% Peptone (*B*) and MEB (*C*) show 3 (24, 22, and 20 kDa) and 4 (24, 20, 18, and 17 kDa) bands respectively.

approximately 24 kDa on the Western blots, probably corresponding to PMAP-1. Interestingly, the intensity of these bands seemed to have little correspondence with the overall level of proteolytic activity (data not shown).

DISCUSSION

As a dimorphic fungus, *P. marneffei* expresses different proteins depending on growth temperature (Andrianopoulos, 2002; Borneman et al., 2002; Zuber et al., 2002; Boyce et al., 2003; Todd et al., 2003; Zuber et al., 2003; Boyce et al., 2005). We have found that in its mold form (room temperature growth) in liquid culture, *P. marneffei* secretes at least one acid proteinase (PMAP-1) into the growth media. The enzyme was determined to have a molecular mass of approximately 24 kDa and was not inhibited by any of the proteinase inhibitors used. The lack of susceptibility to inhibitors combined with its amino terminal sequence and broad specificity place this enzyme in the G1 family of proteinases. It can also be surmised that a protein corresponding to the sequence of PMAP-2 is produced during room temperature growth, as it was originally cloned using a cDNA library from a room temperature culture. The putative protein encoded by this sequence would have a molecular mass of 21,914 Da and a pI of 4.41. Its translated DNA sequence also shows a high degree of identity to the primary structures of the G1 proteinase family, but it clearly differs from PMAP-1 based on amino terminal sequence comparisons. Our difficulties in obtaining both the purified proteinase and its corresponding gene may be in parallel with the results of Poussereau et al., 2001, in which production of another eqolisin, ACP1 from *Sclerotinia sclerotiorum*, was tracked

by molecular techniques with no information presented concerning the purification and properties of the actual ACP1 gene product.

Expressed recombinant PMAP-2 found in inclusion bodies was used to immunize rabbits to obtain specific antisera for use in Western blot analysis of *P. marneffei* culture supernatants. Initial Western blots showed that the serum cross-reacted not only with the expressed protein used as the antigen, but also with purified PMAP-1 (data not shown). Experiments on culture supernatants from *P. marneffei* grown at 25°C showed the presence of two bands of molecular masses corresponding to those of PMAP-1 and the putative PMAP-2 proteinase, indicating expression of both enzymes (Figure 2.7A).

Subsequent experiments performed on supernatants from cultures grown at 37°C on both MEB and 1% peptone media also showed proteolytic activity at low pH (data not shown). Western blot experiments (Figure 2.7) using the serum developed against recombinant PMAP-2 showed multiple bands in activity containing samples. Among them, a band at 24 kDa was consistently found, possibly indicating the presence of PMAP-1. An activity containing supernatant from a culture grown on 1% peptone also exhibited a band at 22 kDa, suggesting the presence of PMAP-2. However, in both 1% peptone and MEB cultures, several bands were present on the Western blot that could not be assigned to any previously purified or cloned proteinase. It is possible that these bands are the result of proteolytic degradation of PMAP-1 and/or -2 or that *P. marneffei* produces other enzymes with sufficient similarity to PMAP-2 to cross react with immune serum raised against PMAP-2. Moreover, there was little correlation between the activity of the samples against EnzChek BODIPY-FL and the intensity of the bands on the Western blot. This could be due to any number of factors, including the variations in

growth temperature and media components. It is also possible that the antibody bound to an immature and inactive form of one or more of the PMAP proteinases.

Another enzyme, PMNP was secreted into the growth media during liquid culture at 37°C but has not been detected during growth at room temperature. This serine proteinase was observed to have a molecular mass of 50 kDa and had the ability to degrade fibrinogen, fibronectin, and, to a lesser extent, elastin. As these substrates are not found in an acid pH environment *in vivo*, PMAP-1 was not assayed for activity against them. It may be speculated that PMAP-1 is produced *in vivo* during growth in the acidic environment of the phagosome after phagocytosis of *P. marneffei* by macrophages. These observations of differential patterns of proteinase expression based on temperature are similar to those seen in other fungal species including *Candida albicans* (for review see Naglik et al., 2003).

Both PMAP-1 and the putative proteinase PMAP-2 belong to the G1 family of proteinases ((Rawlings et al., 2004); http://merops.sanger.ac.uk/), for which the common name eqolisins has been suggested (Fujinaga et al., 2004). The secretion of a G1 proteinase by *Sclerotinia sclerotiorum* has been shown to increase suddenly during the necrosis phase of sunflower cotyledon infection, suggesting a role for this enzyme in the progression of disease (Poussereau et al., 2001). Unfortunately, very little is known about the biological role of the other members of the eqolisin family. As is evident from the ClustalW (Chenna et al., 2003) alignment (Figure 2.6), a high degree of identity exists between members of this family, especially around the active site residues Q53 and E136 (numbering from Fujinaga et al., 2004) indicated in blue on the diagram. Several other

residues (indicated in red) identified as critical to the active site of SCP-B are also conserved in PMAP-2.

Microbial pathogens, including fungi (notably the *Candida* species (Naglik et al., 2003)), often secrete proteinases that have been demonstrated to contribute considerably to virulence. Indeed members of the secreted aspartic acid protease (SAP) family have been implicated in *Candida* dissemination, invasion of the intestinal wall, and degradation of the extracellular matrix and other host proteins. While the eqolisins and the SAPs are clearly members of different proteinase families, their similar pH optima may allow them to act on similar substrates and microenvironments, and thus act in similar ways with respect to virulence. Both candidiasis and penicilliosis marneffei proceed to disseminated infection if untreated and therefore require proteinase production during this phase.

Many microbial proteinases have been shown to act by inactivating host proteinase inhibitors, degrading connective tissue components, interfering with the immune system, deregulating the clotting cascade, and interrupting host cell communication (Travis et al., 1995). The degradation of elastin and fibronectin (connective tissue components) and of fibrinogen (a clotting system component) by PMNP *in vitro* suggests that this enzyme may play a significant role in the virulence of *P. marneffei*, especially considering its proposed route of infection (inhalation). Indeed, the break down of the connective tissue proteins may both enhance fungal invasion as well as aid in the acquisition of small peptides and amino acids for use by the fungus. However, roles for PMAP-1 and the putative enzyme PMAP-2 remain to be established. Our current efforts are centered on the cloning of PMAP-1 and PMNP. Additionally, studies

comparing the virulence of wild-type and PMAP-1, -2, and PMNP knockout *P. marneffei* should be made. It is our belief that this information will lead to a better understanding of their role in the pathogenesis of this organism.

MATERIALS AND METHODS

Organism and growth conditions

Penicillium marneffei (strain UTMB 4354, a generous gift from Prof. Michael McGinnis) was cultured at 25°C in Malt Extract Broth (ME; Difco), or on ME supplemented with 2% agar for solid phase growth. Cultures grown at 37°C utilized Yeast Nitrogen Base, without either amino acids or Ammonium Sulfate, (Difco) and supplemented with 1% dextrose and 0.5% bovine serum albumin (BSA) (Sigma A-9647) unless otherwise noted. All broth cultures were grown with shaking at either 120 RPM or 180 RPM.

Proteinase assays

Proteolytic activity during culture growth and enzyme purification was monitored using general proteinase substrates. Azocasein degradation assays were conducted by incubating the sample of interest with substrate (Fluka, 1% final concentration) and various buffers for specific time periods. Trichloroacetic acid was then added to precipitate undegraded protein and the absorbance of the supernatant measured at 360 nm. Activity against BSA was followed in a parallel manner, with the absorbance being

measured at 280 nm. Additionally, activity was measured using the EnzChek BODIPY-FL substrate (Molecular Probes) where casein has been conjugated to 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY-FL) fluorophore groups. Activity was quantified by an increase in fluorescence (excitation at 485 nm, emission at 514 nm, cutoff at 495 nm) using a Gemini EM microplate fluorometer (Molecular Devices).

Protein determination

Protein concentrations were determined using a bicinchoninic acid (BCA) reagent kit according to the manufacturer's instructions (Pierce). Briefly, BCA solution was mixed in a 20:1 ratio with 4% $CuSO_4$ solution to make a working solution. The sample of interest (10 µl) along with a set of standards containing varying concentrations of BSA were then mixed with 200 µl working solution in a microtiter plate and incubated at 37°C for 30 minutes. The plate was then allowed to cool for 10 minutes and the absorbance measured at 562 nm. After subjecting the standards to linear regression analysis, the protein concentration of the sample of interest was calculated.

Purification of PMAP-1

To purify PMAP-1, *P. marneffei* was grown in a liquid culture at room temperature for 1-2 days until proteolytic activity was evident by one of the proteinase detection assays described above. During culturing, a pink pigment, characteristic of room temperature

growth in *P. marneffei*, was secreted into the culture medium. The culture was then centrifuged and filtered to remove any cells. The supernatant was retained and concentrated through a YM10 10 kDa cutoff membrane (Millipore). The retentate was applied to a DE-52 (Whatman) anion exchange column (1.5 by 60 cm, 100 ml) equilibrated with 0.1 M sodium acetate pH 5.5 (Buffer A). The column was developed with a 0-100% gradient of the same buffer containing 1 M NaCl (Buffer B). Proteinase activity was present in several adjacent salt eluted fractions. These were pooled, dialyzed against Buffer A, and concentrated using a Centriprep 10 kDa cutoff concentrator (Millipore). The concentrate was then made 1 M in ammonium sulfate and applied to a Phenyl Superose 10/10 column (Amersham Pharmacia Biotech, fast protein liquid chromatography system) equilibrated with Buffer A containing 1 M ammonium sulfate. The column was washed with the same buffer and eluted with a linear decreasing gradient of ammonium sulfate from 1 M to 0 M. One protein peak was found to contain proteolytic activity. A fraction from that peak was visible as a single band after 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Tris-HCl/Tricine system according to Schagger and von Jagow (Schagger and von Jagow, 1987). All fractions containing the purified proteinase (PMAP-1) were pooled, concentrated, and used in further experiments.

Purification of PMNP

PMNP was purified from the supernatant of a 37°C culture. Unlike growth at room temperature, at 37°C *P. marneffei* secretes a melanin like pigment that has recently been

characterized (Youngchim et al., 2005). After centrifugation and filtration on a 0.22 μm filter (Corning) to remove all cells, the culture supernatant was again applied to a DE-52 anion exchange column (1.5 by 60 cm, 100 mL) equilibrated and eluted as described above. In this case, the flow through from the column was found to contain most of the activity. After concentration and pH adjustment to 8.0 with 50 mM Tris-HCl, the flow through was loaded onto a 1.6 by 10 cm (20 ml) Source Q 15 anion exchange column (Amersham Pharmacia, fast protein liquid chromatography system) equilibrated with 50 mM Tris-HCl pH 8.0 and eluted with a 0-100% gradient of the same buffer made 1 M in NaCl. Again, the majority of the of the proteolytic activity was found in the flow through fractions which were pooled and concentrated with a simultaneous change of buffer to 50mM Tris-HCl pH 7.5 with 1 M ammonium sulfate, followed by application to a Phenyl Superose column (Amersham Pharmacia) equilibrated with the same buffer. The column was developed with a decreasing linear gradient of ammonium sulfate from 1 M to 0 M.

pH optima

The optimal pH for the activities of PMAP-1 and PMNP were determined using the EnzChek protease substrate with buffers ranging from pH 1 to 9 as follows: pH 1-2, 0.1 M KCl-HCl; pH 2.5-3, 0.1 M Glycine-HCl; pH 3.6-5.6, 0.1 M sodium acetate; pH 6-7, 0.1 M Bis-Tris-HCl; pH 7.5-9, 0.1 M Tris-HCl. Buffer (95 μl) was mixed in a black plastic 96-well plate (Costar) with 10 μl of purified PMAP-1 (2.2 μM) or PMNP (0.27 μM) and warmed to 37°C. EnzChek BODIPY-FL substrate (10 μl containing 1 μg of

substrate) was then added and the rate of increase of fluorescence measured as described in the proteinase assay section.

Inhibitor studies

To determine the catalytic class of each enzyme, PMAP-1 (2.2 μM) or PMNP (38 nM final concentration) was mixed with either 50 mM Sodium Acetate pH 3.6 or 50 mM Tris-HCl pH 9.0 respectively, together with various inhibitors in a total volume of 80 μl, and allowed to incubate for 15 minutes at 37°C. EnzChek BODIPY-FL substrate (1 μg in 20 μl dissolved in the same buffer as the enzyme) was then added and the increase in fluorescence measured.

Cleavage specificity

The cleavage specificities of PMAP-1 and PMNP were determined using the oxidized B chain of insulin as a substrate and analyzing the resulting peptide fragments using a liquid chromatography system coupled with an electrospray mass spectrometer. PMAP-1 and PMNP were incubated, separately, with the insulin B chain (1:100 or 1:5000 molar ratio respectively) in either 50 mM sodium acetate buffer pH 3.6 or 50 mM Tris-HCl pH 9.0, respectively, overnight at 37°C. The reaction mixture was then treated with 6% TCA and centrifuged at 16,000 x g to halt the reaction and precipitate the proteinase, leaving leftover intact insulin B chain as well as the products of cleavage in solution. The supernatant was applied to an Applied Biosystems syringe pump HPLC system with a

C18 column interfaced with a Perkin Elmer Sciex API I Plus Quadrupole Mass Spectrometer (LC-MS) at the University of Georgia Chemical and Biological Sciences Mass Spectrometry Facility, according to the manufacturer's instructions. The masses corresponding to peaks from the LC-MS were then compared to the masses of peptides that could theoretically be generated by cleavage of the insulin B chain and cleavage sites assigned.

Protein sequence analysis

Amino terminal sequence analysis was performed after electrophoresis followed by electrotransfer to polyvinylidene difluoride membranes (PVDF) (BioRad) (Matsudaira, 1987). These membranes were then stained with 0.1% Coomassie Brilliant Blue in 50% methanol and subjected to sequence analysis at the Emory University Microchemical Facility using a PE-Biosystems 494 cLC Pulsed-Liquid Sequencer on-line with PE-Biosystems Capillary PTH Analyzer according to the manufacturer's instructions.

To obtain the amino acid sequences of internal regions of PMNP and PMAP-1, the purified enzymes were again subjected to electrophoresis and the band corresponding to the proteinase of interest was then excised. The excised protein band was subjected to *in situ* tryptic digestion followed by tandem mass spectrometry at the University of Georgia Integrated Biotechnology Laboratories (IBL).

Proteolysis of natural substrates

PMNP was mixed with fibrinogen or fibronectin (1:500 or 1:2000 molar ratios respectively), incubated in 50mM Tris-HCl, pH 7.0 for varying time periods at 37°C, and the resulting solutions then analyzed on either 10% or 7.5% SDS-PAGE gels. To control for any proteolytic impurities, fibrinogen and fibronectin controls without PMNP were utilized at each time point.

PMNP was also assayed for activity against elastin using both native elastin and elastin-rhodamine conjugates (Molecular Probes). These were each incubated in 50 mM Tris-HCl, pH 7.0 over several time points at 37°C. The reaction mixtures were then centrifuged to pellet all remaining insoluble elastin, and the concentration of peptide fragments in the supernatant measured either by absorbance at 280 nm (native elastin) or fluorescence (excitation at 498 nm, emission at 521 nm) to determine the extent of cleavage. Porcine elastase (Molecular Probes) was used as a positive control in these assays.

DNA manipulations

All DNA manipulations and cloning techniques were conducted according to manufacturers' protocols, and performed as described previously (Sambrook et al., 1989).

Generation of a *P. marneffei* cDNA library

A 10 ml aliquot of a 3-day 100 ml starter culture of *P. marneffei* (grown at 27°C) was used to inoculate 900 ml of fresh media. Activity assays (pH 3.0, EnzChek BODIPY-FL substrate) were performed at 22, 25, and 27 hours, and 300 ml aliquots were removed, filtered on Miracloth (Calbiochem), and frozen at -80°C. The three samples were combined and ground in a freezer mill under liquid nitrogen. Trizol (Invitrogen) was then used to isolate total RNA from the samples followed by Dynal oligo-dT beads to purify the mRNA, both according to their respective manufacturer's instructions. Generation of a cDNA library was accomplished using a cDNA Synthesis Kit (Stratagene), substituting Superscript III (Invitrogen) in the first strand synthesis reaction. cDNAs were size selected by agarose gel electrophoresis followed by gel purification using a Qiagen gel purification kit. Purified cDNA was ligated into pSL1180 and transformed into *E. coli* DH10B (Invitrogen). Qiagen Mini-Preps of these libraries were then used as templates for *Taq* PCR experiments.

Expression of PMAP-2

The cDNA coding for PMAP-2 was amplified by PCR using AccuPrime *Pfx* DNA polymerase (Invitrogen) and primers corresponding to its putative mature first amino acid (based on sequence homology) and one corresponding to its carboxy terminal. The PMAP-2 coding region was cloned into pGEX5-T using restriction endonuclease sites encoded in the 5' ends of the PCR primers, producing pJON1. pJON1 was then

transformed into *E. coli* expression host BL-21 cells for purification studies. BL-21 containing pJON1 was grown overnight at 37°C (250rpm) in a 5ml LB starter culture containing ampicillin (0.1 mg/ml). This was then used to inoculate a 1 l culture of LB containing ampicillin (0.1mg/ml) to an initial OD_{600} of 0.075. This 1 l culture was allowed to grow at 37°C (250rpm) until the OD_{600} reached 0.6. At this point, isopropyl-ß -D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM and the growth temperature reduced to 30°C. After 4 hours, the cells were harvested, centrifuged at 3000 x g for 10 minutes, and the supernatant discarded. The cellular proteins and the inclusion bodies were then isolated using the B-PER reagent (Pierce) according to the manufacturer's instructions. The inclusion bodies were then dissolved in 8 M Urea. Both the inclusion bodies and the soluble proteins were analyzed by SDS-PAGE, and the inclusion bodies were found to contain the vast majority of the expressed protein.

Generation of polyclonal antibodies to the PMAP proteinases

Several attempts were made to purify the over-expressed protein from the inclusion body fraction, but the protein consistently degraded when non-denaturing conditions were used. Therefore, the entire inclusion body fraction was utilized as the antigen by the University of Georgia Animal Resources Facility to generate polyclonal antibodies. Briefly, pre-immune serum samples were taken from two New Zealand White rabbits. The rabbits were subsequently injected intradermally with a 1:1 emulsion of inclusion bodies and Complete Freund's Adjuvant (CFA). After 3 weeks, a secondary

immunization was performed in the same way except using Incomplete Freund's Adjuvant and performing the injections subcutaneously. After 2 additional weeks, a small blood sample was collected and the serum retained for analysis. This serum was used in subsequent experiments.

Western blots were performed in order to screen the serum produced by the rabbits immunized with the expressed PMAP-2 protein. Briefly, the sample of interest was separated by electrophoresis under the system described by Laemmli (Laemmli, 1970) using a 10% gel (Bio-Rad). The proteins were then electro-transferred to nitrocellulose, stained with Ponceau S, blocked with 5% nonfat dry milk, incubated in immune or pre-immune serum (diluted up to 10,000 fold), and, finally, in anti-rabbit immunoglobulin conjugated to alkaline phosphatase (diluted 15,000 fold) according to the method of Towbin et al.(Towbin et al., 1979). An alkaline phosphatase color development reagent (Bio-Rad) was used to develop the blot.

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CHAPTER 3

ISOLATION AND CHARACTERIZATION OF A HIGHLY SPECIFIC SERINE ENDOPEPTIDASE FROM AN ORAL STRAIN OF *Staphylococcus epidermidis*[1]

[1]Moon, J. L., Banbula, A., Oleksy, A., Mayo, J.A., and Travis, J. 2001. Biological Chemistry, 382(7): 1095-1099

ABSTRACT

Infection by *Staphylococcus epidermidis*, an opportunistic pathogen, has become a major problem due to the increased use of implanted medical devices and the growing number of patients who are therapeutically or infectiously immunosuppressed. These infections appear to proceed via modulation of the coagulation and complement systems. In this communication we describe the purification and characterization of a novel extracellular proteinase from an oral strain of *S. epidermidis* that can degrade fibrinogen, complement protein C5, and several other proteins. This proteinase has a strong preference for cleavage after glutamic acid residues, but not after aspartic acid. The *S. epidermidis* enzyme may be a multifunctional protein which not only provides this organism with both the ability to evade the complement defense system and to dysregulate the coagulation cascade, but also supplies nutrients for its growth through the degradation of Glu-rich proteins.

KEY WORDS: coagulation/complement/glutamic/protease/proteinase/serine/

Staphylococcus epidermidis is a Gram-positive bacterium that is a component of the normal microflora of human skin where it apparently functions to degrade components of the epidermis of this organ. It is, in fact, the most common bacterium found on skin and, as a result, often present as a contaminant in blood samples (Patrick, 1990). However, *S. epidermidis* has also been recognized as a pathogen, infecting those individuals who are either immunocompromised or who have indwelling foreign devices such as catheters, shunts, or implants (Blum and Rodvold, 1987; Mayo, 1995; Costerton *et al.*, 1999). These infections often proceed, albeit at a slow rate, to the sepsis stage with resultant fever and, ultimately, disseminated intravascular coagulation. The organism is also a suspected oral pathogen, as it is frequently isolated from individuals with apical periodontitis or severe marginal periodontitis (Johnson *et al.*, 1999). The main recognized virulence factor of *S. epidermidis* appears to be a slime which is produced to provide the organism with protection from host defense (Bayston and Rodgers, 1990), although how this occurs is not precisely understood. Significantly, other possible virulence factors which allow *S. epidermidis* to be an opportunistic pathogen have not yet been characterized.

While the initial host defense against *S. epidermidis* infection has been shown to occur mainly through the alternative complement pathway, with the classical pathway only being involved in the more advanced stages of infection, this pathogen has shown an ability to reduce complement-associated phagocytosis; however, how this occurs has not yet been elucidated (Dobrin *et al.*, 1975; Fleer *et al.*, 1985; Kawasaki *et al.*, 1987; Riber *et al.*, 1990; Wakabayashi *et al.*, 1991; Giese *et al.*, 1994; de Fijter *et al.*, 1996). In

addition, *S. epidermidis* can deregulate the clotting cascade, again by unknown mechanisms (Bykowska *et al.*, 1985; Leibowitz and Ramakrishnan, 1995; Sapatnekar *et al.*, 1995).

In this communication we report the purification of an extracellular proteinase, released by *S. epidermidis* during its growth. This enzyme shows high homology to the amino-terminal sequence of the well-characterized V8 proteinase from *Staphylococcus aureus* (Drapeau *et al.*, 1972; Hua *et al.*, 1992). The two proteinases also have partial similarity in terms of biochemical properties and peptide bond specificity. However, while the V8 proteinase cleaves substrates with glutamic acid or aspartic acid residues in the P1 position, our initial studies seem to indicate that the proteinase purified from *S. epidermidis* cleaves exclusively after glutamic acid.

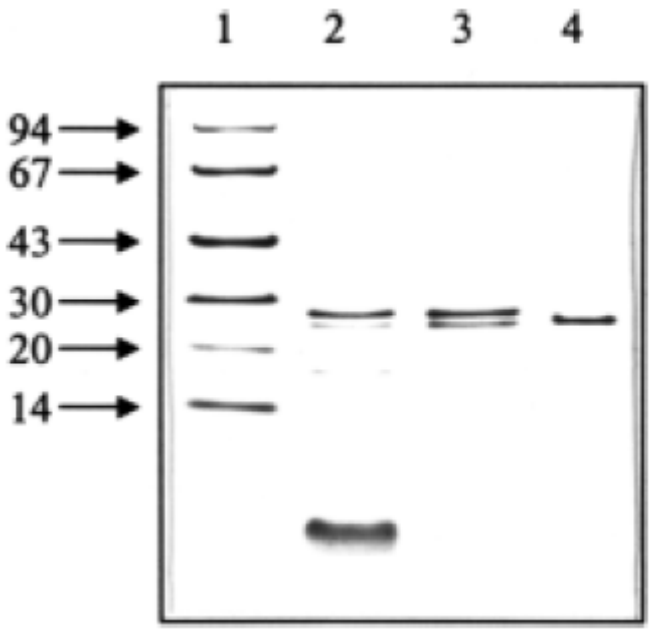
The proteinase was purified from *S. epidermidis* culture supernatants using standard protein purification techniques and azocasein digestion to track activity. At each step, homogeneity was examined by SDS-PAGE analysis (Figure 3.1), with the purified enzyme migrating as a single band with a molecular weight of about 25 kDa. In confirmation of enzyme purity, N-terminal sequence analysis indicated a unique sequence VILPNNNRHQIFNTTQGHYDAVSFIYIPID. When this sequence was entered into a blastp search (http://www.ncbi.nlm.nih.gov/BLAST/), the most significant match was shown to be with that of the *Staphylococcus aureus* V8 protease as shown in Figure 3.2.

Figure 3.1 Purification of *Staphylococcus epidermidis* proteinase as monitored by Tris-Tricine SDS-PAGE

Lane 1, molecular mass markers (rabbit muscle phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa; soybean trypsin inhibitor 20 kDa; bovine milk α-lactalbumin, 14 kDa). *Lane 2,* dialyzed, centrifuged, 80% (NH4)2SO4 precipitate of culture supernatant; *lane 3,* flow through from DE-52 anion exchange column; *lane 4,* pure fraction from Resource Q column.

Bacterial Growth – Staphylococcus epidermidis (strain BJ0018) was isolated from a periodontal patient and identified according to Johnson *et al.* (1999). The organism was cultured in a liquid medium and grown to stationary phase (24h), according to the method used for the growth of *Staphylococcus aureus* (Drapeau, 1972).

Initial Purification – The culture was centrifuged at 4000 x g to pellet the cells and the supernatant removed, brought to 80% saturation with (NH4)2SO4 in order to precipitate most of the proteins from solution and re-centrifuged at 10000 x g for 30 minutes. The precipitate was re-suspended in 50mM Tris-HCl buffer pH 8.0 (Buffer A) and dialyzed against several changes of the same buffer using a 3.5 kDa molecular mass cut off membrane. The retentate was then applied to a DE-52 anion exchange column equilibrated with the same buffer, washed to remove unbound protein and then treated with 50mM Tris-HCl, pH 8.0, 1M NaCl (Buffer B). The flow through and the salt eluted fractions were checked for azocasein hydrolysis with the flow through being found to possess the majority of the activity. This fraction was concentrated (YM-10 membrane), applied to a Resource Q column equilibrated with Buffer A, and eluted with Buffer A as the washing buffer and a 0-100% gradient of Buffer B. Several peaks were obtained, with all activity being located within the first peak eluted from this column after gradient salt elution had begun. While this procedure was usually satisfactory for the preparation of homogeneous protein, an additional step was sometimes necessary. In that case protein fractions containing proteinase activity were first dialyzed against 25mM Bis-Tris-HCl, pH 7.1, applied to a Mono P column equilibrated with the same buffer and the purified enzyme eluted with Polybuffer 74 pH 5.0.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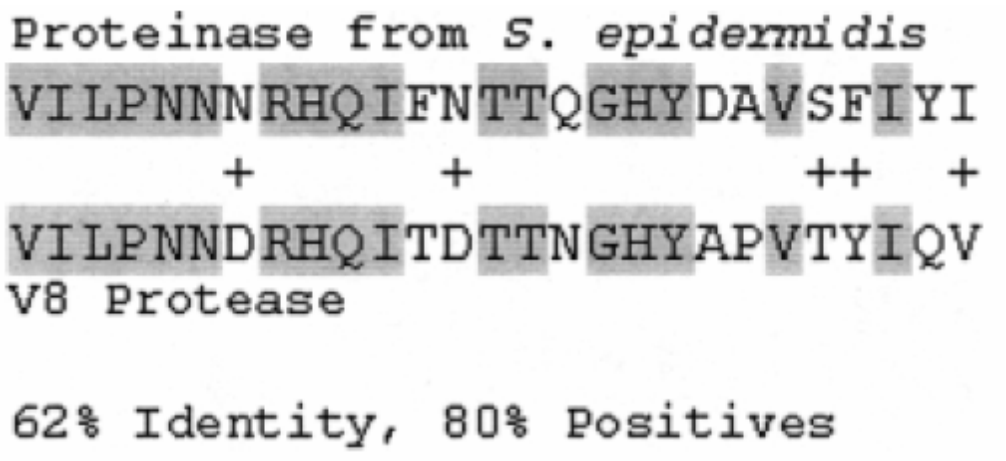


Figure 3.2 Sequence comparison (blastp) for amino terminal fragment of *S. epidermidis* proteinase with *S. aureus* V8 proteinase.
Sequencing was performed by both the Molecular Genetics Information Facility, University of Georgia, USA, and by the Emory University Microchemical Facility. Sequence homology information was obtained from a blastp search at http://www.ncbi.nlm.nih.gov/BLAST

After it was determined that the synthetic substrate Suc-Ala-Ala-Pro-Glu-pNA was readily cleaved by this enzyme, it was used in further experiments to study the biochemical properties of this proteinase. The enzyme was found to have a pH optimum between 7 and 8, with activity seen at pH values from 5.5 to 9 (data not shown). Inhibition studies showed that the enzyme was completely inactivated by DFP and partially by Pefabloc, indicating that the proteinase is a member of the serine class of endopeptidases (Table 3.1).

While the initial purification was carried out using azocasein as a substrate to mark the activity in each step, cleavage experiments were also performed in detail using various other substrates to determine peptide bond specificity. Tests with chromogenic substrates with glutamic acid as the P1 residue showed a preference for longer peptide substrates, with Ala-Ala-Pro-Glu-pNA giving the highest turnover (Km ≅ 5mM). Preliminary studies on non-chromogenic substrates showed cleavage after glutamic acid residues in substrates such as insulin beta chain (FVNQHLCGSHLV**E**ALYLVCG**E**RGFFYTPKA) but not after aspartic acid in such peptides as GSDGQWGLWRDHTQC. It should be noted that these experiments were performed at 37º C in 50 mM Tris pH 8.0, and that varying buffering conditions could alter the substrate specificity as has been suggested with the V8 proteinase (Houmard and Drapeau, 1972; Drapeau, 1977). Experiments were also conducted incubating varying molar ratios of the *S. epidermidis* protease with the serpin α_1-PI. In stark contrast to the action of *Staphylococcus aureus* V-8 proteinase (Potempa *et al.*, 1986), the proteinase from *S. epidermidis* only slowly degraded α_1-PI, and only at high concentrations of the proteinase. While the publication from Potempa *et*

Table 3.1 Inhibitor Studies
Inhibitor assays were performed using inhibitor concentrations specified by (Beynon and Bond, 1989) and using Suc-Ala-Ala-Pro-Glu-pNA (1 mM) for the substrate. Proteinase concentration in each case was 1 μM. The inhibitors used and their relative concentrations were DFP (1:75 enzyme:inhibitor molar ratio), Pefabloc (1:3000), TLCK (1:75), TPCK (1:75), PMSF (1:750), EDTA (1:7500), E64 (1:7.5), 1,10-phenanthroline (1:7500), and β-mercaptoethanol (1:750).

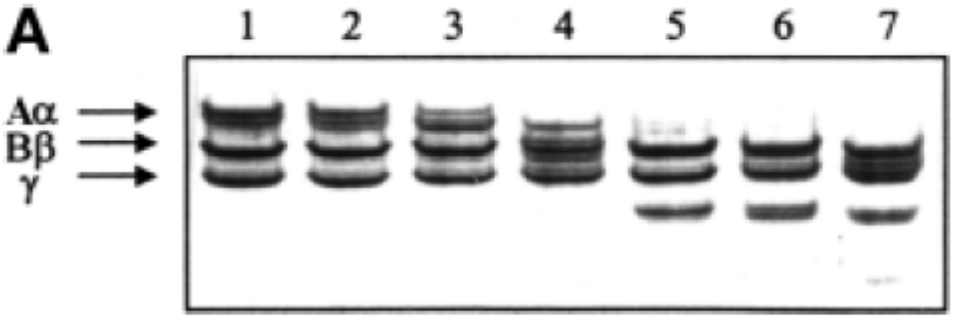
Inhibitor	Inhibitor concentration (mM)	V8 Proteinase residual activity	*S. epidermidis* proteinase residual activity
DFP	0.1	3%	2%
Pefabloc	1.0	50%	55%
TLCK	0.1	115%	123%
TPCK	0.1	124%	136%
PMSF	1.0	133%	118%
EDTA	10	103%	101%
E64	0.01	116%	116%
1,10-Phenanthroline	10	115%	106%
beta-Mercaptoethanol	4	101%	110%

al. (1986) showed greater than 50% inactivation of α_1-PI in one hour using a 500:1 excess of α_1-PI, our data showed that the proteinase from *S. epidermidis* required a ratio of 1:1 to cleave α_1-PI to the same extent (data not shown). This implies that although there are strong similarities in both N-terminal sequence and substrate specificity, there are structural differences between the proteinase from *S. epidermidis* and the V8 proteinase.

To further characterize the possible role of this enzyme in the virulence of *S. epidermidis*, experiments were performed to examine its activity against certain physiological substrates. In the cases of fibrinogen, complement protein C5, and denatured keratin, enzyme and substrate were mixed in known molar ratios, incubated for varying times at 37º C, and the extent of cleavage measured by SDS-PAGE. The Aα chain of fibrinogen (Figure 3.3A), the α chain of C5 (Figure 3.3B), and the major bands in the keratin sample (Figure 3.3C) were degraded by the proteinase. Hydrolysis of the other subunits of these target proteins was found to occur at a slower rate.

Mechanisms utilized in the host defense against *Staphylococcus epidermidis* have been shown to occur mainly through activation of the alternative complement pathway during early stages of infection and through the classical pathway during advanced bacteremia and sepsis. (Dobrin *et al*., 1975; Fleer *et al*., 1985; Kawasaki *et al*., 1987; Riber *et al*., 1990; Wakabayashi *et al*., 1991; Giese *et al*., 1994; de Fijter *et al*., 1996). Thus, cleavage of C5 by bacterial pathogens can cause one of two effects (Joiner, 1988), either transient activation to release the potent neutrophil chemotactic factor C5a, as has been shown for

Figure 3.3: Degradation of protein substrates.
Hydrolysis of protein substrates was measured as follows: human complement protein C5, bovine fibrinogen, and denatured keratin were each mixed with enzyme and incubated overnight at 37º C. The total digests were then run out on Tris-Tricine gels in the presence of SDS. For specific analysis of protein degradation patterns, fibrinogen **(A)** and complement protein C5 **(B)** were incubated at enzyme:substrate ratios of 1:30 and 1:70 (proteinase concentration 39 nM), respectively, at 37º C for varying lengths of time. For experiments with denatured keratin **(C)**, 30 μg of keratin was mixed with 39 μg (1.5 pmol) of the *S. epidermidis* protease. The digests obtained were then separated as above and the banding patterns at increasing time intervals compared with the zero time controls.
(A) Degradation of fibrinogen by *S. epidermidis* proteinase.
Notation on the left designates the various chains of fibrinogen. Lane 1, native fibrinogen; lanes 2, 3, and 4, time points 1, 4, and 24 hours respectively for fibrinogen alone incubated at 37º C; lanes 5, 6, and 7, time points 1, 4, and 24 hours respectively for fibrinogen incubated with *S. epidermidis* proteinase. Lane 4 clearly shows the effects of residual plasmin activity on the fibrinogen samples.
(B) Degradation of complement protein C5 by *S. epidermidis* proteinase.
Notation on the left designates the two chains of C5. Lane 1, native C5; lanes 2, 3, and 4, time points 1, 4, and 24 hours respectively for C5 incubated with *S. epidermidis* proteinase.
(C) Degradation of keratin by *S. epidermidis* protease.
Lanes 1 and 2, keratin alone incubated for 0 and 24 hrs respectively. Lanes 3-6, keratin incubated with *S. epidermidis* proteinase for 1, 3, 12, and 24 hours.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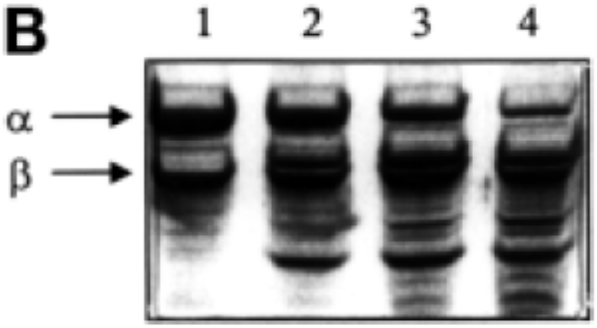
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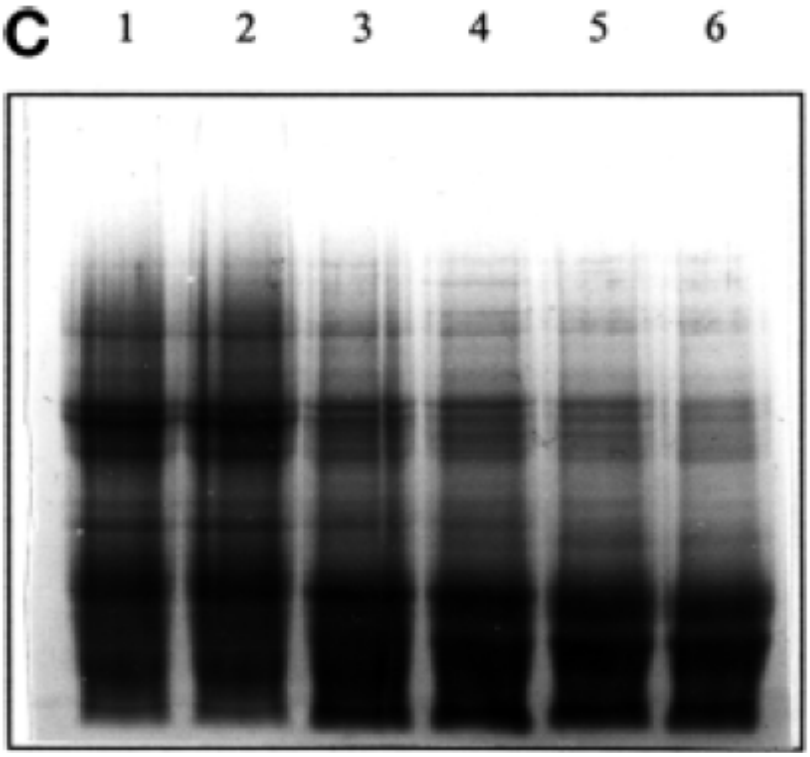
C
1 2 3 4 5 6

Porphyromonas gingivalis, the major pathogen associated with periodontal disease (Wingrove *et al.*, 1992), or C5 inactivation to permanently cripple the complement pathway. Studies to determine whether this occurs with the *S. epidermidis* enzyme are now in progress. However it should be pointed out that there are multiple glutamic acid residues near the normal cleavage site, the closest being 22 residues before and 13 residues after the C5a releasing cleavage site in C5, which might result in either destruction (internal cleavage) or activation (proximal cleavage to give an elongated C5a analogue. In addition, C5a is generated from cleavage of the α chain of C5, which is also the chain most rapidly degraded by the *S. epidermidis* glutamyl endopeptidase (Figure 3.3B). There is a report suggesting the possibility of generation of a chemotactic peptide from C5 alpha without production of full length C5a anaphylatoxin (Gerard and Gerard, 1994). Thus, a fragment produced by *S. epidermidis* proteinase may be sufficiently similar to C5a anaphylatoxin to induce neutrophil chemotaxis to the site of the infection. This could benefit the bacteria by allowing the action of neutrophil proteinases on skin proteins, thus aiding *S. epidermidis* in the production of amino acids as a nutrient source. Studies are underway to investigate such possibilities.

The rapid cleavage of the Aα chain of fibrinogen is intriguing. Experimental and clinical data suggest that *Staphylococcus epidermidis* inhibits the coagulation process (Bykowska *et al*., 1985) (except in cases of subacute bacterial endocarditis (Kessler *et al.*, 1987)). Since our results show degradation of the Aα chain of fibrinogen (Figure 3.3A), we hypothesize that the proteinase acts on this coagulation factor to inhibit its clot forming ability. To further investigate this possibility, thrombin dependent clot formation from a

fibrinogen solution was measured after treatment with varying concentrations of the *S. epidermidis* proteinase (Table 2). Clotting took longer than the 60 second maximum time measured by the Coag-A-Mate instrument at enzyme to substrate molar ratios as low as 1:300 and clotting time increased compared to controls at ratios as low as 1:1000. These results clearly demonstrate the potential for this enzyme to dysregulate the clotting cascade through the degradation of fibrinogen.

Despite the evidence showing that slime production is a virulence factor in various strains of *Staphylococcus epidermidis* (Bayston and Rodgers, 1990; Costerton *et al*., 1999), proteinases are obviously still required for nutrient acquisition and host defense evasion. In particular, as a major organism on skin, it is almost certain that *S. epidermidis* uses proteins from this organ as a nitrogen source. Indeed, keratin contains numerous repeating sequences of glutamic acid residues and would make an extremely suitable substrate for the enzyme described in this report under normal conditions. Considering the fact that the *S. epidermidis* proteinase is one of the major proteins secreted into the culture medium during growth of this organism, it is possible that this is one of its major functions.

The ability of this novel proteinase to degrade keratin, as well as the major physiological substrates complement C5 and fibrinogen, positions it as a highly versatile enzyme which may be involved in both normal and pathological functions. Certainly, it remains to be proven whether such activities are physiologically relevant. Nevertheless, the fact that

Table 3.2: Clotting Time Dependence on Pretreatment with *S. epidermidis* Proteinase

Fibrinogen (3 mg/mL) was mixed with decreasing amounts of proteinase from *S. epidermidis* and incubated for 4 hours at 37º C. The solutions were then spun down and the supernatant removed. Aliquots of the supernatant (200 μL) were placed in the sample wells of a Coag-A-Mate clotting time analyzer. The thrombin dependent clotting time was then measured using a solution of Human Thrombin (200 μL of 3.3 U/mL for each well).

Ratio of Enzyme:Fibrinogen	Clotting Time (s)
1:100000	8.7
1:30000	8.7
1:10000	8.7
1:3000	8.8
1:1000	14.9
1:300	>60
1:100	>60
1:30	>60

the enzyme is produced in copious quantities during culturing of *S. epidermidis* clearly indicates a major role not only in its survival but also in its growth.

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CHAPTER 4

DISCUSSION

The proteinases produced by pathogenic microorganisms often play an important part in their virulence. Tissue destruction by these foreign enzymes can aid in both invasion and nutrient acquisition. Disruption of the host response to infection is often another consequence of the secretion of these enzymes. Paradoxically, it is sometimes to the benefit of the pathogen if their secreted proteinases can activate host defenses as is the case with *Porphyromonas gingivalis* (Wingrove et al., 1992). Sometimes, all that is required is the inactivation of host proteinase inhibitors by these pathogens. This allows the proteinases that are part of the normal defenses to run amok, cleaving at inappropriate sites and at inappropriate times, all to the benefit of the pathogen. The study of the proteinases produced by invading bacteria and fungi and their roles in infection can lead to a better understanding of disease and aid in the development of new treatments.

The first step in understanding the roles of the proteinases produced by pathogenic microorganisms is either the isolation and characterization of these enzymes or the cloning of the genes responsible for their production. It is difficult if not impossible to perform experiments characterizing these enzymes without first purifying them from culture medium and other microbial components. To that end, our laboratory has undertaken the study of the proteolytic enzymes produced by these pathogens and their place in disease progression.

In Chapter 2, we describe the purification and characterization of two proteinases produced by *Penicillium marneffei*. The first enzyme purified (PMAP-1), isolated from liquid cultures grown at room temperature, was found to be a member of the eqolisins, a relatively new group of proteinases and the only members of the G1 family of enzymes classified in the MEROPS protease database http://merops.sanger.ac.uk/ (Rawlings et al., 2004). Like other members of the eqolisin family, this enzyme is optimally active at very low pH and is not inhibited by pepstatin, an inhibitor of almost all other acid acting proteinases. The second enzyme purified (PMNP) is a serine proteinase with activity at neutral pH. This enzyme can degrade fibrinogen, fibronectin, and, to a lesser extent, elastin *in vitro*, suggesting several possible roles for it in penicilliosis marneffei.

Also in Chapter 2, we describe the cloning of a putative proteinase (PMAP-2), the sequence of which is highly homologous to that of PMAP-1 and the other eqolisins. As this putative enzyme was cloned from a cDNA library made from a room temperature culture, we propose that the protein corresponding to this sequence is made during this growth condition.

In Chapter 3, the purification and characterization of a protease from *Staphylococcus epidermidis* is described. This enzyme, which possesses both sequence and functional similarity to the V8 protease of *Staphylococcus aureus*, is highly specific for cleavage after glutamyl residues. It also demonstrated the ability to degrade the C5 protein of the complement system, fibrinogen, and denatured keratin.

Physiological Role of the *Penicillium marneffei* Proteinases

Not much is known about the biological role of the eqolisins. However, in the plant pathogen *Sclerotinia sclerotiorum*, an eqolisin appears to play a role in infection (Poussereau et al., 2001). The protease, encoded by the *acp1* gene, is expressed at low levels during the initial stages of infection followed by a rapid increase in production as the fungus switches into a necrotizing phase. Expression of *acp1* was found to be repressed by both glucose and nitrogen as well as by high pH. During infection, *S. sclerotiorum* secretes oxalic acid, lowering the pH of the surrounding area, allowing for expression of *acp1*. Unfortunately, while *acp1* expression and general protease activity were followed closely in this study, the proteinase corresponding to this gene was never purified or characterized. However, it was proposed that this enzyme acts in nutrient acquisition and invasiveness by degrading the proteinaceous components of the plant cell wall.

In *P. marneffei*, it is possible that the eqolisins PMAP-1 and PMAP-2 function in much the same way, breaking down tissues surrounding the site of infection after acidification by the organism. However, it is unknown if *P. marneffei* possesses the ability to modulate its surrounding pH *in vivo* and whether the eqolisins are produced in this environment. It would seem more likely that PMNP, the neutral proteinase produced by *P. marneffei*, plays such a role. PMNP has the ability to cleave (in addition to fibrinogen) fibronectin and elastin, both connective tissue proteins, possibly allowing the fungus to be more invasive and to enhance tissue destruction.

A more likely role for the eqolisins in penicilliosis marneffei would seem to be after the phagocytosis of the fungal cells by macrophages. The low pH environment of

the lysosome would appear to be ideal for the activity of PMAP-1 and PMAP-2. These enzymes could aid in the survival of the fungal cells by degrading the host enzymes inside the phagosome. It is even possible that PMAP-1 and -2 could cause or aid in cytolysis of the macrophage as has been proposed for Sap4-Sap6 of *Candida albicans* (Hube and Naglik, 2001; Monod et al., 2002).

While both PMAP-1 and PMNP show some preference for cleavage at certain residues, they maintain (in insulin B chain degradation studies) the ability to cleave at several other locations. These activities may, therefore, function in the generation of peptides and amino acids for uptake by the fungus.

Experiments to further elucidate the roles of these proteinases should be performed. After determining the structure of the genes coding for PMAP-1 and PMNP, all three of the proteins described should be knocked out (individually and in combinations) and the knockout strains compared with the wild-type with respect to virulence. Additionally, antibodies made against these enzymes could be used in immunofluorescence studies to localize them in models of infection.

The G1 Family of Peptidases

PMAP-1 and -2 are members of the G1 family of proteinases classified in the MEROPS protease database http://merops.sanger.ac.uk/ (Rawlings et al., 2004). The proteinases of this family were formerly classed in a group of enzymes known as the "pepstatin-insensitive carboxyl proteinases". Prior to their discovery, it was widely believed that pepstatin, a pentapeptide, acted as an inhibitor of all proteolytic enzymes active at acid pH and, therefore, that all of these enzymes shared a common mechanism.

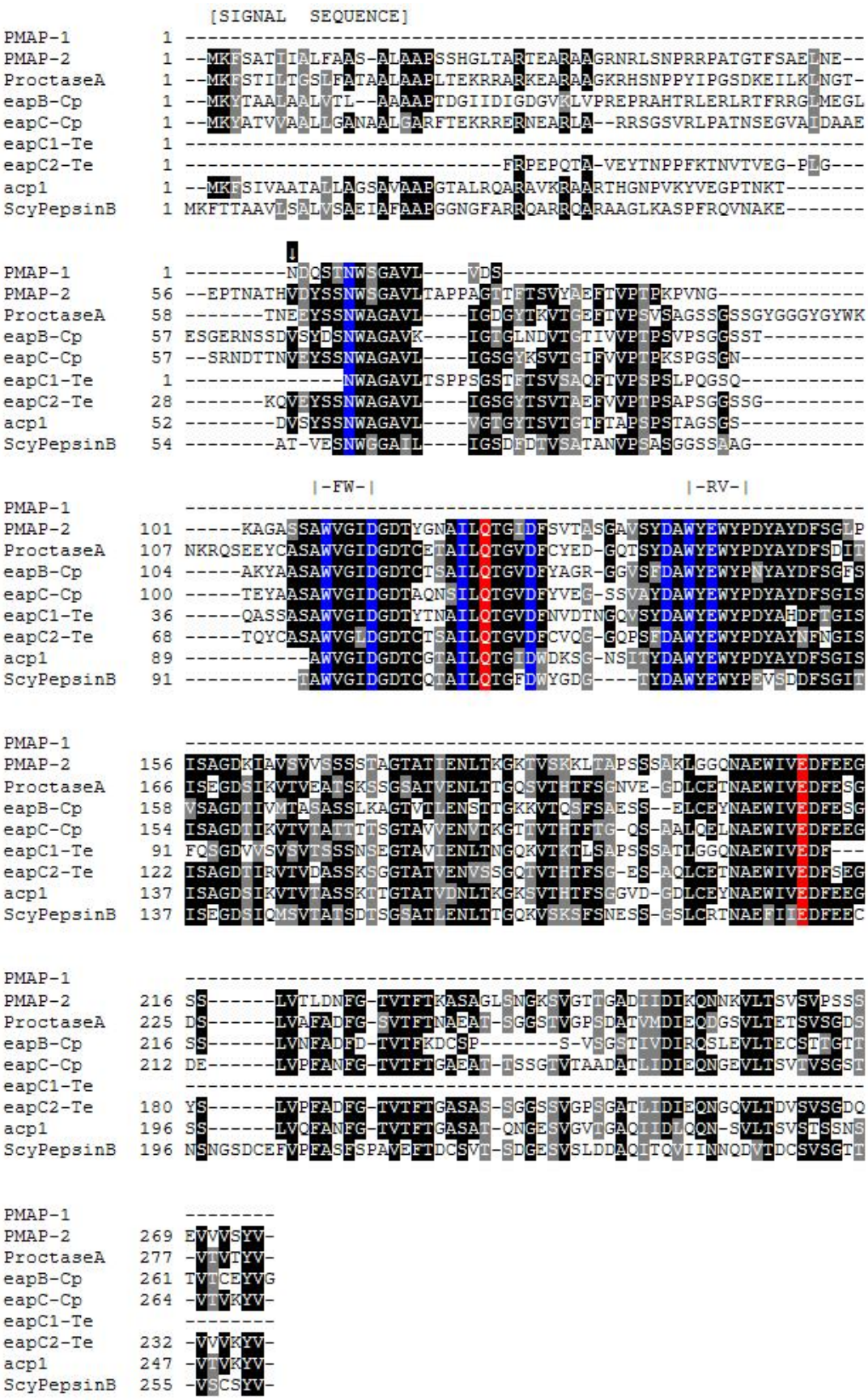
However, by 1985, several bacterial and fungal enzymes had been identified that acted at acid pH and were unaffected by the presence of pepstatin. While the bacterial enzymes were eventually identified as acid acting serine proteinases (Family S53), the fungal enzymes were placed in the new (2004) G1 family. These enzymes differ in a number of ways from other acid proteinases, notably in their active site, which contains one glutamate (E) and one glutamine (Q), prompting researchers to name the G1 family the 'eqolisins'.

The G1 family of proteinases (eqolisins) is made up of fungal enzymes, all of which have low pH optima. These enzymes possess high homology to each other as shown in Figure 4.1. The crystal structure of one of these enzymes, scytalidoglutamic peptidase, has been determined and has a beta-sandwich tertiary structure with each half of the 'sandwich' having seven anti-parallel beta strands (Fujinaga et al., 2004). Enzymes of the G1 family have been found in *Aspergillus niger* (Iio and Yamasaki, 1976), *Cryphonectria parasitica* (Jara et al., 1996), *Talaromyces emersonii* (SUBMITTED DIRECTLY TO GENBANK), *Sclerotinia sclerotiorum* (Poussereau et al., 2001), *Scytalidium lignicolum* (Oda and Murao, 1974), and now *Penicillium marneffei*.

Mechanism of the G1 Family

Proteinases are grouped into families based on sequence homology and mechanistic class. For example, the G1 family of enzymes is the 1st family in the glutamic mechanism class, whereas omptin, a protease produced by *Escherichia coli*, is in the A26 family, the 26th family in the aspartic mechanism class. The mechanistic class

Figure 4.1 Sequence alignment of PMAP-1, PMAP-2, and several other members of the G1 family. The amino terminus of PMAP-1 and the putative gene product of *PMAP-2* are aligned with several other members of the G1 family of proteinases: Proctase A is from *Aspergillus niger* and is also known as Aspergillopepsin II; eapC-Cp and eapB-Cp are from *Cryphonectria parasitica*; EapC1-Te and EapC2-Te are from *Talaromyces emersonii*; acp1 is an EapC homolog from *Sclerotinia sclerotiorum*; ScyPepsinB is scytalidopepsin B from *Scytalidium lignicolum*. The putative signal sequence for *PMAP-2* is indicated, as well as the regions from which successful primers were designed (FW and RV), and the putative mature amino terminal residue (↓). Some important conserved residues are shown in red and the active site residues in blue


[SIGNAL SEQUENCE]
PMAP-1
PMAP-2
ProctaseA
eapB-Cp
eapC-Cp
eapC1-Te
eapC2-Te
acp1
ScyPepsinB
|-FW-|
|-RV-|

of an enzyme describes how it performs its catalytic reaction and is basically the same for all the members of a family. Often, inhibitors of one member of a mechanistic class work for all of its members. It is, therefore, especially important to examine the differences in mechanism between the glutamic proteases (of which the G1 family is the only member) and the aspartic proteases from which they were recently, definitively differentiated (Fujinaga et al., 2004).

The proposed catalytic mechanism of the members of the G1 family (Figure 4.2) (Fujinaga et al., 2004) is somewhat similar to that of the acid (aspartic) proteinases (Davies, 1990) (Figure 4.3)**.** Aspartic proteinases catalyze the cleavage of peptide bonds without nucleophilic attack by the enzyme itself. Instead, these enzymes use a water molecule as the nucleophile. No covalent bond is formed between the enzyme and the substrate in either the aspartic proteinase mechanism or in the proposed eqolisin mechanism. A hydroxyl group is transferred from a water molecule to the carbonyl of the peptide bond to be cleaved in both mechanisms, stabilized by Asp-32 and Asp-215 in the aspartic proteinase mechanism and by both Glu-136 and Gln-53 in the eqolisin mechanism. This intermediate is stabilized by hydrogen bonding. In the case of the aspartic proteinases, the oxygen of the peptide bond is hydrogen bonded to the hydrogen covalently bound to Asp-32.

In the proposed eqolisin mechanism, however, this hydrogen bonding occurs with the hydrogen covalently bound to the side chain amide nitrogen of Gln-53. These hydrogen bonds function in both the formation of the tetrahedral intermediate and the stabilization of the resulting oxyanion. Additionally, the oxygen of the Gln-53 side chain functions in hydrogen bonding to the hydroxyl just added to the carbonyl of the substrate. No such

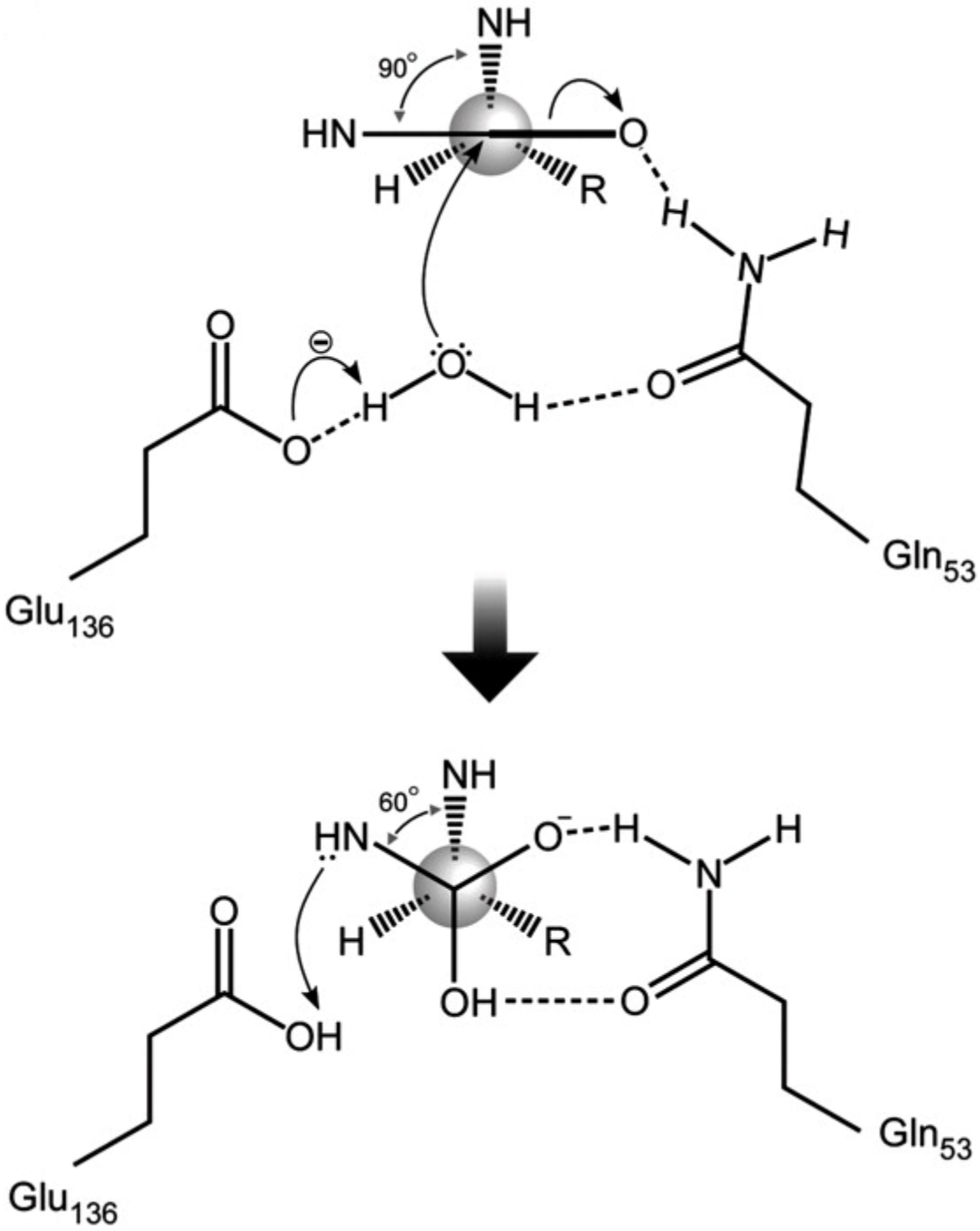
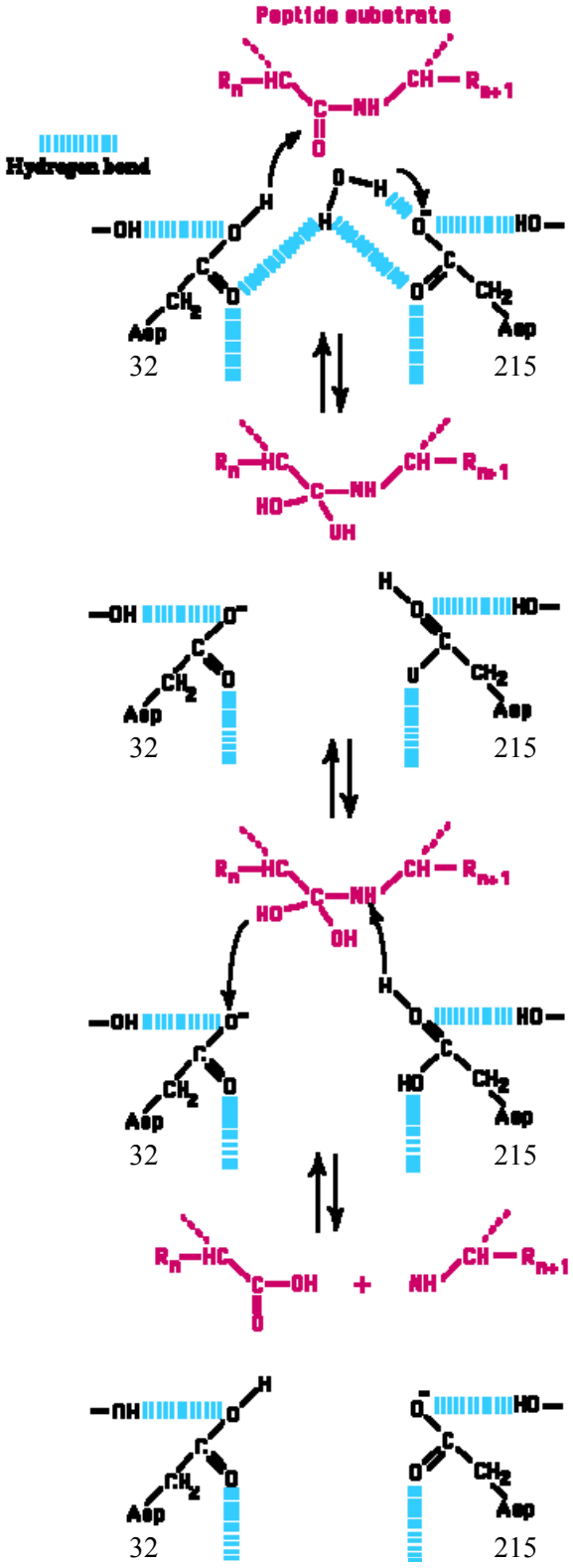


Figure 4.2 Proposed mechanism for the eqolisins. Modified from Fujinaga et al., 2004

Figure 4.3 Mechanism of aspartyl proteinases. Modified from Moreau. T. 1996-2005. Prolysis: a protease and protease inhibitor Web server. [Online.] http://delphi.phys.univ-tours.fr/Prolysis/introprotease.html Accessed 31 October 2005


Peptide substrate
Hydrogen bond
Asp
32
215

stabilization occurs in the mechanism of aspartic proteinases. Furthermore, the original nucleophilic attack by water on the peptide bond carbon occurs on the *si* face of the substrate in the eqolisin mechanism and not the *re* face as in aspartic proteinases. A more complete discussion of the mechanism of aspartic proteinases can be found in Dunn, 2002 (Dunn, 2002).

In the case of PMAP-2, the both the stabilizing residues (blue) and the active site residues (red) common to the other eqolisins are present (Figure 4.1), suggesting that this enzyme too utilizes the proposed eqolisin mechanism. Until the full primary structure of PMAP-1 is know, this cannot be confirmed for that enzyme.

Staphylococcal Proteinases and Their Roles in Virulence

The glutamyl endopeptidase from *Staphylococcus epidermidis* bears high homology to the V8 proteinase of *Staphylococcus aureus* originally discovered by Drapeau (Drapeau, 1976). The most striking feature of these enzymes is their specificity for cleavage only after glutamic acid residues. Indeed, the specificity of these enzymes is so strong that they have been used for studies on other proteins that require cleavage at glutamyl residues. Homologues of this proteinase have been found in four other staphylococci, namely *S. warneri* (Yokoi et al., 2001), *S. sp AJ* (Rawlings et al., 2004), *S. saprophyticus* (Kuroda et al., 2005), and *S. haemolyticus* (Takeuchi et al., 2005). All four of these enzymes have been placed in the S1B family in the MEROPS database (http://merops.sanger.ac.uk/) (Rawlings et al., 2004).

In *S. aureus,* the V8 protease (also known as SspA) is theorized to be activated by cleavage by Aur, a metalloprotease (Figure 4.4) (Drapeau, 1978; Shaw et al., 2004).

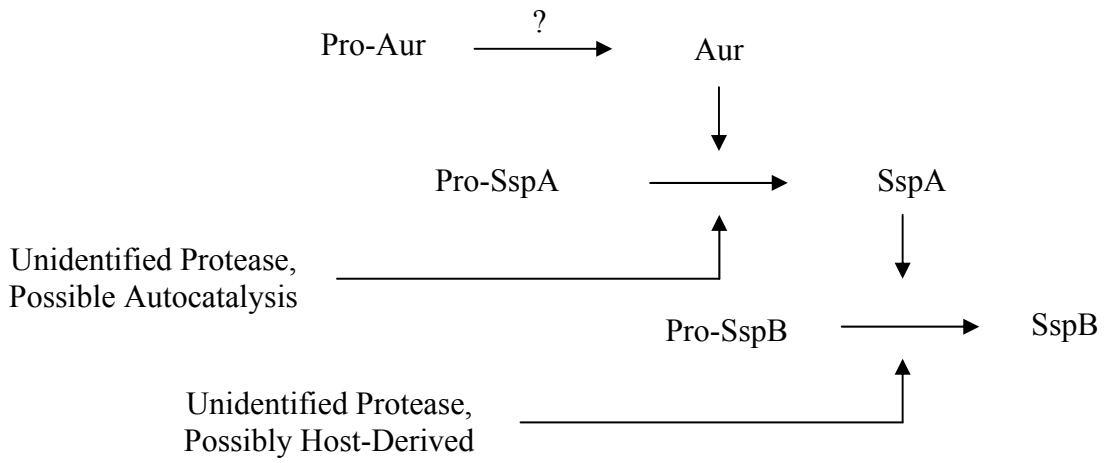


Figure 4.4 Post-translational activation of *Staphylococcus aureus* proteases. Adapted from Shaw et al., 2004

However, in Aur mutants, some residual SspA activity remains, indicating that Aur cleavage is not the sole path for activation of SspA (Shaw et al., 2004). It is possible that this activation occurs via autocatalysis as with the glutamyl endopeptidases found in *Bacillus* and *Streptomyces* species (Birktoft and Breddam, 1994). SspA has been shown to activate SspB, a cysteine protease for which the gene is directly downstream from that of SspA (Rice et al., 2001). SspB, though not present in *S. epidermidis*, has been shown to be an important virulence determinant for *S. aureus* (Rice et al., 2001; Shaw et al., 2004). In the case of *S. epidermidis*, there is an Aur homolog that may function in activation of the glutamyl endopeptidase described in Chapter 3. However, as there is no SspB homolog in *S. epidermidis*, this enzyme must have some other function in this organism.

Mutation experiments on the gene for SspA (*SspA*) have shown conflicting results. Two publications have indicated that a mutation in *SspA* results in markedly reduced virulence of *S. aureus* (Coulter et al., 1998; Shaw et al., 2004). However, due to the fact that the gene for SspB (*SspB*) resides directly downstream from *SspA*, it is likely that the mutations made in *SspA* affect the downstream *SspB* as well. Indeed, another strain containing a mutant in *SspA* that did not affect *SspB* showed no reduction in virulence when compared to wild-type *S. aureus* (Rice et al., 2001). This suggests that there is some other method of activation for SspB, possibly by host proteases.

Several other putative roles exist for the V8 protease (SspA). In *S. aureus*, SspA can cleave fibronectin-binding protein (for which there are homologues in *S. epidermidis*) (McGavin et al., 1997; Karlsson et al., 2001), allowing the cells to detach from their locations and become more invasive. While this finding has been disputed(Rice et al.,

2001), SspA can also cleave surface protein A (Karlsson et al., 2001), reducing the clumping of the bacterial cells. While surface protein A is not present in *S. epidermidis*, other surface proteins with homology to surface protein A are present. Remodeling the bacterial cell surface to increase invasiveness is consistent with the currently accepted model of *S. aureus* disseminated infection (Lowy, 1998).

Additionally, SspA, along with other staphylococcal secreted proteases, has been shown to degrade α_1-proteinase inhibitor along with other human proteinase inhibitors (Potempa et al., 1986; Rapala-Kozik et al., 1999). Cleavage of host inhibitors allows host proteinases to act in an unregulated fashion, providing nutrients for the invading bacteria through host tissue destruction. SspA has also been shown to cleave the heavy chain portions of human immunoglobulins (Prokesova et al., 1992).

The glutamyl endopeptidase produced by *S. epidermidis*, the purification of which is described in Chapter 3, has several possible roles. Its cleavage of denatured keratin, a glutamic acid rich protein, suggests a role for the enzyme in invasion and tissue destruction. Cleavage of fibrinogen suggests a possible link between production of this enzyme and the ability of *S. epidermidis* to dysregulate the clotting cascade (Bykowska et al., 1985; Leibowitz and Ramakrishnan, 1995; Sapatnekar et al., 1995). In a similar way, the degradation of the C5 complement protein by the glutamyl endopeptidase may be the way in which it avoids complement-associated phagocytosis (Dobrin et al., 1975; Fleer et al., 1985; Kawasaki et al., 1987; Riber et al., 1990; Wakabayashi et al., 1991; Giese et al., 1994; de Fijter et al., 1996).

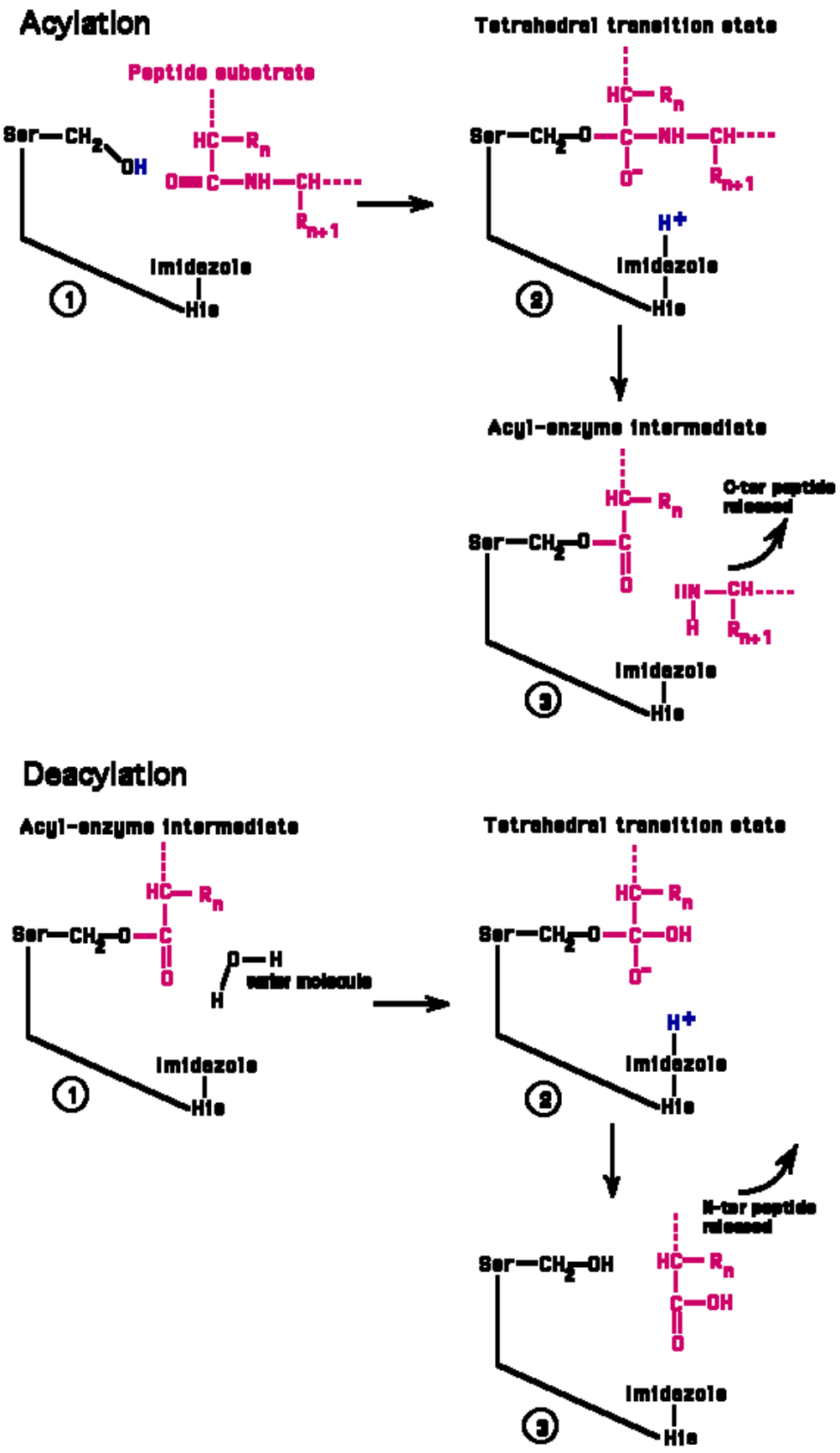
Unlike the V8 protease from *S. aureus*, the *S. epidermidis* enzyme only degrades α_1-proteinase inhibitor very slowly, suggesting that this is not the primary role for this

enzyme in pathogenesis. Also, as *S. epidermidis* does not possess the homolog of *S. aureus* SspB, the function of its SspA homolog (the purified glutamyl endopeptidase) cannot be for its activation. However, several of the other functions of the *S. aureus* V8 protease listed above have not been examined with respect to the purified enzyme from *S. epidermidis*. It is possible that this enzyme plays a role in the modulation of bacterial attachment through bacterial surface remodeling, cleavage of host proteinase inhibitors other than α_1-proteinase inhibitor, or cleavage of immunoglobulins. These possibilities should be investigated further. Furthermore, since the writing of Chapter 3, the gene sequence for the purified proteinase has been determined (Dubin et al., 2001). A strain with this gene knocked out should be constructed and compared with the wild-type strain with respect to virulence.

Structure and Mechanism of V8 Protease

A crystal structure of the V8 protease from *S. aureus* was recently published (Prasad et al., 2004). It was found to possess the standard Ser-His-Asp catalytic triad and shares the basic mechanism common to all serine proteinases (Figure 4.5). Interestingly, though V8 protease shares very little sequence identity with other serine proteinases, the tertiary structure is quite similar to such enzymes as epidermolytic toxin A and B (also from *S. aureus*) and to bovine beta trypsin. The authors also constructed a model of the active site of the enzyme in complex with a glutamic acid containing substrate, showing how the enzyme maintains its specificity (Figure 4.6).

Figure 4.5 Mechanism of serine proteinases. Modified from Moreau. T. 1996-2005. Prolysis: a protease and protease inhibitor Web server. [Online.] http://delphi.phys.univ-tours.fr/Prolysis/introprotease.html Accessed 31 October 2005


Acylation
Peptide substrate
Tetrahedral transition state
Ser—CH2—OH
HC—Rn
O=C—NH—CH----
Rn+1
Imidazole
His
1
Ser—CH2—O—C—NH—CH----
O−
H+
2
Acyl-enzyme intermediate
C-ter peptide released
IIN—CH----
H
3
Deacylation
Acyl-enzyme intermediate
Tetrahedral transition state
Ser—CH2—O—C
O—H
water molecule
Ser—CH2—O—C—OH
N-ter peptide released
Ser—CH2—OH
C—OH

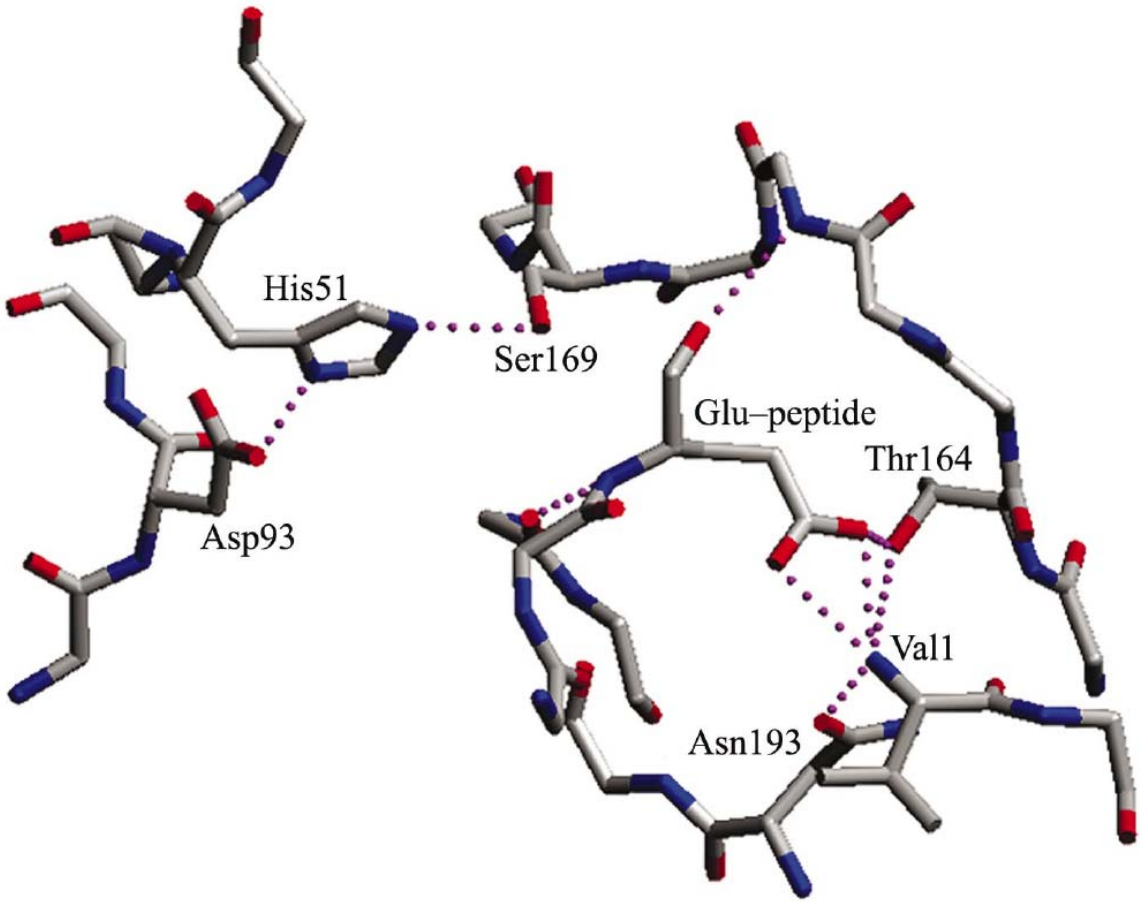


Figure 4.6 Active site of *Staphylococcus aureus* V8 protease. The S1 site confers specificity for glutamyl residues in the P1 of the substrate. Adapted from Prasad et al., 2004

Summary and Conclusions

In this dissertation, three proteinases have been purified and one cloned from two pathogenic microorganisms. In the case of *Penicillium marneffei*, an AIDS related fungal pathogen native to Southeast Asia, two enzymes, PMAP-1 (an acid acting glutamic proteinase) and PMNP (a serine proteinase with a neutral pH optimum) were purified and the gene for another putative glutamic proteinase (PMAP-2) cloned (Chapter 2). In Chapter 3, a V8-like serine proteinase was purified from *Staphylococcus epidermidis*. We propose that these enzymes may be involved in various manners in the virulence of these organisms in human infection. Through nutrient acquisition, invasion, tissue destruction, dysregulation of the clotting cascade, interruption of the complement response, or in other as yet untested means, these proteinases may aid the organisms that produce them in their survival and propagation in the human host.

In conclusion, it has been determined that:

A) PMAP-1 is a 24 kDa proteinase secreted by *Penicillium marneffei* that belongs to the G1 family of enzymes, the eqolisins. Like the other eqolisins, this enzyme catalyses peptide bond cleavage only at acid pH.

B) PMNP is a 50 kDa serine proteinase that is also secreted by *P. marneffei*. It has the ability to cleave physiologically relevant substrates such as fibrinogen, fibronectin, and elastin. This enzyme operates at neutral pH and cleaves mainly after leucine residues.

C) *P. marneffei* also possesses the gene for another putative G1 proteinase (PMAP-2). This putative enzyme has a molecular weight of 22 kDa and shares high sequence homology with PMAP-1 and the other members of the eqolisin family.

D) *Staphylococcus epidermidis* secretes a 25 kDa serine proteinase with high specificity for cleavage after glutamic acid. This enzyme is highly homologous to the V8 protease from *Staphylococcus aureus*.

Future Research

A) *Cloning of PMAP-1 and PMNP.* Obtaining the primary sequence information about these enzymes would aid in the definitive determination of their families, especially with respect to PMNP, about which little is known.

B) *Reverse genetics of all the enzymes described in this dissertation.* Performing knockout studies of PMAP-1, -2, PMNP, and the V8-like proteinase from *S. epidermidis* would clarify any putative roles in the pathogenesis of their respective organisms.

C) *Immunofluorescence studies*. After making antibodies to these proteinases, immunofluorescence studies should be performed to try and localize the sites of production of these enzymes in mouse models of infection, hopefully yielding more information concerning their physiological roles.

D) *Crystallization studies.* None of the above enzymes studied in this dissertation has been subject to X-ray crystallographic study. This information would be

valuable to further understand their function and specificity and help to differentiate them from their homologues in other species.

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APPENDIX

The following abstracts are from publications of which the author of this dissertation was a co-author during his graduate studies. The work described in these publications has not been discussed elsewhere in this dissertation.

Goldstein, J.M., Kordula, T., Moon, J.L., Mayo, J.A., and Travis, J. (2005). Characterization of an extracellular dipeptidase from *Streptococcus gordonii* FSS2. Infect. Immun. *73*, 1256-1259.

Kawalec, M., Potempa, J., Moon, J.L., Travis, J., and Murray, B.E. (2005). Molecular diversity of a putative virulence factor: purification and characterization of isoforms of an extracellular serine glutamyl endopeptidase of *Enterococcus faecalis* with different enzymatic activities. J. Bacteriol. *187*, 266-275.

Oleksy, A., Golonka, E., Banbula, A., Szmyd, G., Moon, J., Kubica, M., Greenbaum, D., Bogyo, M., Foster, T.J., Travis, J., and Potempa, J. (2004). Growth phase-dependent production of a cell wall-associated elastinolytic cysteine proteinase by *Staphylococcus epidermidis*. Biol. Chem. *385*, 525-535.

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Characterization of an Extracellular Dipeptidase from *Streptococcus gordonii* FSS2

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PepV, a dipeptidase found in culture fluids of *Streptococcus gordonii* FSS2, was purified and characterized, and its gene was cloned. PepV is a monomeric metalloenzyme of approximately 55 kDa that preferentially degrades hydrophobic dipeptides. The gene encodes a polypeptide of 467 amino acids, with a theoretical molecular mass of 51,114 Da and a calculated pI of 4.8. The *S. gordonii* PepV gene is homologous to the PepV gene family from *Lactobacillus* and *Lactococcus* spp.

Streptococcus gordonii is an oral streptococcus that is important in both the formation of dental plaque and the production of infective endocarditis. Previous work with *S. gordonii* FSS2, a strain isolated from the blood of an endocarditis patient (10), has revealed a variety of cell-associated and extracellular glycosidolytic and proteolytic activities, thought to be important in the growth of this organism in the dental plaque and endocarditis vegetation environments (4, 5, 6, 11). In recent studies of extracellular proteinases made by this strain, an x-prolyl dipeptidyl peptidase (xPDPP) and an arginine aminopeptidase have been purified and characterized and the genes for both enzymes have been cloned and examined (4, 5). Here we report similar studies on a third such enzyme, *S. gordonii* PepV. *S. gordonii* PepV is a member of the PepV dipeptidase family, previously found in *Lactobacillus* and *Lactococcus* spp. (3, 7, 14, 15).

Materials and methods. *S. gordonii* FSS2 (previously *Streptococcus sanguinis* FSS2 [6]) was described earlier (6, 10, 11). Other oral (viridans group) streptococci (see Fig. 3) were provided by Vincent Fischetti, Rockefeller University. *S. gordonii* FSS2 was grown with pH control as previously reported (4, 5).

Enzyme assays measured both tripeptidase and dipeptidase activities of *S. gordonii* PepV (hereafter, PepV refers to *S. gordonii* PepV unless indicated otherwise; "*S. gordonii*" is added only when necessary to avoid ambiguity as to which version of PepV is meant). During purification (see Fig. 1) and most inhibitor studies (see Table 2), the tripeptidase activities of crude samples and purified PepV were measured in a two-step enzymatic reaction, with H-Ala-Phe-Pro-pNA (Sigma) as the substrate. PepV and the substrate (1 mM), both in assay buffer A (50 mM Tris, 1 mM $CaCl_2$ [pH 7.8]), were incubated; products of this reaction were NH_3^+-Ala-COO- and NH_3^+-Phe-Pro-pNA. Excess *S. gordonii* xPDPP (100 ng) then was added to release the pNA reporter group, and A_{405} was measured in a plate reader. Since *S. gordonii* xPDPP is a serine proteinase (4), potential inhibition of PepV by serine class inhibitors (diisopropylfluorophosphate, phenymethylsulfonyl fluoride, 3,4-dichloroisocoumarin, and TLCK [*Nα-p*-tosyl-L-lysine chloromethyl ketone]; see Table 2) was examined in an independent dipeptidase assay that measured PepV-dependent release of ninhydrin-positive material using Leu-Gly as the substrate (2). Inhibitors were obtained from Sigma, Roche Molecular Biochemicals, or Calbiochem, except for Anstatin, which was a gift from Mirjana Grujic, Jozef Stefan Institute, Lubljana, Slovenia. For studies of cleavage specificity (see Table 1), hydrolysis of dipeptides, tripeptides, and higher peptides was measured in a high-performance liquid chromatography (HPLC) assay. The enzyme was incubated with the experimental peptide (enzyme-to-substrate ratio, 1:1,000) in 100 μl of 100 mM Tris, pH 7.8, for 2 h at 37°C, and the products were analyzed by HPLC.

PepV was purified from the culture fluid of a 15-liter stirred culture (pH was maintained at 7.5) in early stationary phase, at which time all glucose had been metabolized and approximately 25% of the total PepV activity was extracellular (data not shown). Cells were removed, and 80% ammonium sulfate was added to the culture fluid. The precipitate was collected, dialyzed, and chromatographed on DE-52. Active fractions were successively chromatographed on Superdex-75 HR 10/30, phenyl-Sepharose HP, Mono-Q HR 10/10, Cibacron Blue Sepharose CL-B6, Gly-Pro Sepharose, and Mono-P 5/5. Details of the purification scheme are available from the authors. Enzyme purity and molecular weight were assessed by Tris-HCl–Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12), silver staining (1), and gel filtration.

To clone the PepV gene, DNA from *S. gordonii* FSS2 was purified (Purgene; Gentra, Minneapolis, Minn.). The N-terminal peptide sequence of PepV (TIDFKAEVEKRREAL), obtained by Edman degradation, was used to search an *S. gordonii* database (ftp://ftp.tigr.org/pub/data/s_gordonii), resulting in identification of a 1,401-bp open reading frame encoding PepV. Subsequently, two PCR primers encoding the N and C termini were synthesized (5′-AGTGGATCCATGACAATTG ATTCTAAAGC-3′ and 5′-TTTGGATCCTTATTTGATTAG

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Molecular Diversity of a Putative Virulence Factor: Purification and Characterization of Isoforms of an Extracellular Serine Glutamyl Endopeptidase of *Enterococcus faecalis* with Different Enzymatic Activities

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A previously identified gene *sprE* of *Enterococcus faecalis* strain OG1 was shown to encode an extracellular serine protease that appears to belong to the glutamyl endopeptidase I staphylococcal group. A single form of SprE with a molecular mass of 25 kDa and a pH optimum between 7.0 and 7.5 was isolated from culture supernatant of wild-type *E. faecalis* strain OG1RF (TX4002); this form was apparently generated by cleavage of the Ser^{-1}-Leu^{1} and Arg^{230}-Leu^{231} peptide bonds of the secreted zymogen. In contrast, the culture supernatant of the gelatinase-null mutant, TX5264, with a nonpolar deletion of *gelE* which encodes the *E. faecalis* gelatinase, was found to contain several forms of SprE proteolytically processed on both the N and C termini; in addition to a full-length zymogen and a truncated zymogen, three mature forms of the SprE proteinase, Leu^{1}-Ala^{237}, Ser^{-1}-Glu^{227}, and Leu^{1}-Glu^{227}, were identified. As with the V8 proteinase of *Staphylococcus aureus*, the closest homologue of SprE, all of the active forms cleaved specifically Glu-Xaa peptide bonds but with substantially different efficiencies, while none was able to hydrolyze peptide bonds with Asp in the P1 position. The most active of all these enzyme forms against several substrates, including human fibrinogen and β-chain insulin, was the Ser^{-1}-Glu^{227} ($^{-1}$S-SprE) isolated from TX5264; $^{-1}$S-SprE, in contrast to other forms of SprE, was unstable at 37°C, apparently due to autodegradation. In conclusion, our results demonstrate that *sprE* encodes a highly specific serine-type glutamyl endopeptidase, the maturation of which is dependent on the presence of gelatinase. In the absence of gelatinase activity, the aberrant processing of pro-SprE results in the appearance of a "superactive" form of the enzyme, $^{-1}$S-SprE.

Enterococci, often viewed primarily as human commensals and even used as probiotics, have become problematic nosocomial pathogens, at least in part because of their increasing resistance to many antibiotics and their ability to infect the growing pool of severely debilitated and/or immunocompromised patients who undergo prolonged antibiotic therapy (27, 37–39). Several groups have recently undertaken a search for enterococcal virulence factors in an effort to devise new solutions to the problems caused by these bacteria (20, 25). Included among these may be enterococcal proteinases, as enzymes of this class have been previously suggested to be important virulence factors for other bacterial pathogens. Examples include the V8 proteinase of *Staphylococcus aureus* involved in septicemia (2, 14, 44) and its homologue GluSE from *S. epidermidis*, found to be important for slime production and, consequently, biofilm formation by this bacterium in vitro (36, 43). Furthermore, the cysteine endopeptidase SpeB of *Streptococcus pyogenes* (8, 9, 16, 29–31) and proteases of *Porphyromonas gingivalis* (3, 4, 24, 42, 45), *Yersinia* spp. (22, 28, 54–56), and *Pseudomonas aeruginosa* (10, 17, 23) have all been implicated as virulence factors.

Enterococcus faecalis has long been known to produce gelatinase (coccolinase; EC 3.4.24.30) (GelE) (1, 21, 32, 38, 51, 58), a 30-kDa extracellular metalloendopeptidase encoded by the *gelE* gene (58). Downstream from *gelE*, an open reading frame called *sprE*, coding for a putative serine protease (GenBank accession No Z12296), was identified (57). While gelatinase activity and the *gelE* gene have been utilized in a number of studies, including epidemiological ones (11, 18, 26, 35, 61–63), and in animal models of infection (15, 53), suggesting a possible role in microbial virulence and host response (33), until recently, little has been done to investigate *sprE* and the possible role of the predicted SprE protein or the presence of any other proteolytic activities in *E. faecalis*. Qin et al. described the *fsr* locus, a regulatory system of *E. faecalis* (41, 47, 48) that is homologous to the *S. aureus agr* locus (49) that encodes a quorum sensing system regulating cotranscription of *gelE* and *sprE* from the common promoter (47, 48). The deduced amino acid sequence of SprE shows a high degree of similarity to those of staphylococcal glutamyl endopeptidases, including V8 (49% similarity, 27% identity) (66) and GluSE (49% similarity, 26% identity) (43), but this predicted enzyme has not been purified or characterized.

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Growth phase-dependent production of a cell wall-associated elastinolytic cysteine proteinase by *Staphylococcus epidermidis*

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Abstract

Staphylococcus epidermidis, a Gram-positive, coagulase-negative bacterium is a predominant inhabitant of human skin and mucous membranes. Recently, however, it has become one of the most important agents of hospital-acquired bacteriemia, as it has been found to be responsible for surgical wound infections developed in individuals with indwelling catheters or prosthetic devices, as well as in immunosupressed or neutropenic patients. Despite their medical significance, little is known about proteolytic enzymes of *S. epidermidis* and their possible contribution to the bacterium's pathogenicity; however, it is likely that they function as virulence factors in a manner similar to that proposed for the proteases of *Staphylococcus aureus*. Here we describe the purification of a cell wall-associated cysteine protease from *S. epidermidis,* its biochemical properties and specificity. A homology search using N-terminal sequence data revealed similarity to staphopain A (ScpA) and staphopain B (SspB), cysteine proteases from *S. aureus.* Moreover, the gene encoding *S. epidermidis* cysteine protease (Ecp) and a downstream gene coding for a putative inhibitor of the protease form an operon structure which resembles that of staphopain A in *S. aureus*. The active cysteine protease was detected on the bacterial cell surface as well as in the culture media and is apparently produced in a growth phase-dependent manner, with initial expression occurring in the mid-logarithmic phase. This enzyme, with elastinolytic properties, as well as the ability to cleave α,PI, fibrinogen and fibronectin, may possibly contribute to the invasiveness and pathogenic potential of *S. epidermidis.*

Keywords: cysteine protease; elastase; staphopain; *Staphylococcus epidermidis.*

Introduction

Among members of the normal bacterial flora of human skin, skin glands and mucous membranes, *Staphylococcus epidermidis* is a predominant species, widely spread throughout the cutaneous ecosystems. This coagulase-negative bacterium, previously regarded as a commensal organism of low virulence, has recently been recognized as an emerging etiological agent of numerous clinical conditions. *S. epidermidis* is an opportunistic pathogen that requires a predisposed/susceptible host in order to change from a normal inhabitant of human skin to an infectious agent. Most of the infections are hospital-acquired and inflict those individuals with either implanted medical devices or undergoing surgical intervention, as well as immunosupressed patients (Blum and Rodvold, 1987; Kloos and Bannerman, 1994). The pathologies caused by *S. epidermidis* range from bacterial keratitis, postoperative wound infections, rejection of indwelling foreign devices (such as prosthetic joints, cardiac valves, intravascular and peritoneal dialysis catheters or intraocular lenses) to a bacteremia often developed in immunocompromised patients (Baddour et al., 1987; Blum and Rodvold, 1987; Mack, 1999; Tabbara et al., 2000). As many *S. epidermidis* isolates are multi-antibiotic resistant, such infections are very serious and can even be fatal.

The pathogenicity of *S. epidermidis* is mainly due to its ability to form biofilms on the surfaces of indwelling synthetic devices as well as damaged heart valves (Bayston and Rodgers, 1990). The hydrophobic nature of the cell surface is crucial for bacterial adherence during initial colonization of plastic or metal foreign bodies, while in the later stages a copious amount of extracellular polysaccharide material is synthesized, forming a protective multilayered biofilm preventing the clearance of bacteria by host defense mechanisms and making infection difficult to eradicate (Blum and Rodvold, 1987).

In comparison to the closely related *S. aureus*, *S. epidermidis* produces a very limited number of tissue-damaging secretory factors such as exoenzymes and toxins. The analysis of *S. epidermidis* strain ATCC 12228 genome revealed that with exception of haemolysins beta and delta, genes encoding enterotoxins, leukotoxins and hemolytic alpha and gamma toxins are absent (Zhang et al., 2003). The lack of numerous extracellular virulence factors, which are typical hallmarks of *S. aureus* infections, may explain why the infections caused by *S. epidermidis* are of subacute or chronic type (Vuong and Otto, 2002). Degradation of host connective tissues components and other proteins as well as modifications of bacterial cell surface proteins by secretory proteases is known to contribute to invasiveness of *S. aureus*. It is