JASON MARC GOLDSTEIN

The Isolation, Characterization and Cloning of Three Novel Peptidases From *Streptoccocus gordonii*: Their Potential Roles in Subacute Bacterial Endocarditis (Under the Direction of JAMES TRAVIS)

Streptococcus gordonii is generally considered a benign inhabitant of the oral microflora yet is a primary etiological agent in the development of subacute bacterial endocarditis (SBE), an inflammatory state that propagates thrombus formation and tissue damage on the surface of heart valves. Colonization and adherence mechanisms have been identified, yet factors necessary to sustain growth remain unidentified. Strain FSS2 produced three extracellular aminopeptidase activities during growth in neutral pHcontrolled batch cultures. The first included a serine-class dipeptidyl-aminopeptidase, an x-Pro DPP (Sg-xPDPP) found as an 85 kDa monomer by SDS-PAGE while appearing as a homodimer under native conditions. Kinetic studies indicated a unique and stringent x-Pro specificity comparable to the DPPIV/CD26 and lactococcal x-Pro DPP families. Isolation of the full-length gene uncovered a 759-amino acid polypeptide with a mass of 87,115 Da and theoretical pI of 5.6. Significant homology was found with PepX gene family members from *Lactobacillus* ssp. and *Lactococcus* ssp., and putative streptococcal x-Pro DPPs. The second activity was a putative serine-class arginine aminopeptidase (Sg-RAP) with some cysteine-class characteristics. It was found as a protein monomer of 70 kDa under denaturing conditions. Nested PCR cloning enabled the isolation of a 324 bplong DNA fragment encoding the protein's 108 amino acid N-terminus. Culture activity profiles and N-terminal sequence analysis indicated the release of this protein from the cell surface. Homology was found with a putative dipeptidase from Streptococcus progenes and non-specific dipeptidases from Lactobacillus heleveticus and Lactococcus

lactis. The third peptidase belonged to an extracellular, metallo-class dipeptidase found as a 55 kDa monomer as determined by SDS-PAGE and gel filtration analysis. Kinetic studies indicated degradation of various hydrophobic dipeptides except for an x-Pro sequence. Lesser activity was detected against the N-terminus of hydrophobic tripeptides. Isolation and sequence analysis of the full-length gene indicated a 467-amino acid polypeptide with a mass of 51,114 Da and theoretical pI of 4.8. Homology was found with the PepV gene family coding for cytoplasmic, non-specific dipeptidases from *Lactobacillus* and *Lactococcus* ssp. Collectively, these aminopeptidases may serve as critical factors during arginine acquisition, degradation of proline peptides, hydrolysis of di-, tri- and oligopeptides and maintenance of amino acid pools. During SBE pathology this peptidase system would serve to sustain growth *in vivo*, functioning in proteolysis of host protein and potentially modulating biologically active peptides.

INDEX WORDS: *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus sanguis*, *Streptococcus viridans*, infective endocarditis, subacute bacterial endocarditis, native valve endocarditis, bacterial inflammation, viridans streptococci, protease, proteinase, aminopeptidase, dipeptidase, dipeptidyl-peptidase, PepX, PepV, ADI, RAP

THE ISOLATION, CHARACTERIZATION AND CLONING OF THREE NOVEL PEPTIDASES FROM STREPTOCOCCUS GORDONII: THEIR POTENTIAL ROLES IN SUBACUTE BACTERIAL ENDOCARDITIS

by

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DEDICATION

This Dissertation is dedicated to my family:

My parents Nathan Goldstein and Sybil Goldstein. For their love and compassion all my life. Their unconditional emotional, intellectual and financial support forms the basis for all my accomplishments.

Karen. For her love, encouragement, honesty and promise of a full life.

To the memory of my grandparents Herman and Hilda Goldstein, and Jack and Frieda Solin.

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PREFACE

The experimental work described in this dissertation was performed in the laboratory of James Travis at the Department of Biochemistry and Molecular Biology, The University of Georgia, Athens, Georgia, USA from January 1997 to June 2001.

These studies represent original work by the author and have not been submitted in any other form to another university. This dissertation discusses the data of three manuscripts, Chapter 2 published by Infection and Immunity in September 2001, Chapter 3 was submitted to Infection and Immunity during September 2001 and was under review, and Chapter 4 was in manuscript form at the time this dissertation was submitted to Graduate Faculty of the University of Georgia. Where use was made of the work of others it has been duly noted in the text.

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ABBREVIATIONS

- α_1 -PI-human α_1 -proteinase inhibitor (also known as α_1 -antitrypsin)
- ABE-acute bacterial endocarditis
- ADI arginine deiminase
- AT III human antithrombin III
- ATCC American Tissue Culture Collection
- ATR –acid tolerance response
- CK -carbamate kinase
- CP -carbamoyl phosphate
- DFP -di-isopropyl fluorophosphate
- DPP –dipeptidyl peptidase
- DTT dithiothreitol
- E-64 L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
- EDTA -ethylenediaminetetraacetic acid
- FPLC fast protein liquid chromatography
- GAPDH-glyceraldehyde-3- phosphate dehydrogenase
- GSR- growth stress response
- HNE -human neutrophil elastase
- HPLC high pressure liquid chromatography
- IE -- infective endocarditis

IL -interleukin

- IVET *in vivo* expression technology
- LTA lipoteichoic acid
- MALDI-TOF matrix assisted laser desorption ionization-time of flight
- MW -molecular weight
- NTBE nonbacterial thrombotic endocarditis
- NVE -native valve endocarditis
- ORF open reading frame
- OCT ornithine carbamoyltransferase
- PAAP reticuloendothelial system
- PAGE polyacrylamide gel electrophoresis
- PCR -polymerase chain reaction
- PEPV -- di/tri-peptidase
- pI –isoelectric point
- PMSF -phenylmethylsulfonyl fluoride
- pNA -para-nitroanilide
- PVDF -polyvinylidene difluoride
- PVE –prosthetic valve endocarditis
- RAP arginine aminopeptidase
- RES reticuloendothelial system
- Sar –N-methyl glycine
- SBE –subacute bacterial endocarditis
- SDS -sodium dodecyl sulfate

Suc -succinyl

TFA - trifluoroacetic acid

TLCK -N- α -*p*-tosyl-L-lysine-chloromethyl ketone

TPCK -N-α-p-tosyl-L-phenylalanine-chloromethyl ketone

TPP -tripeptidyl-peptidase

XP-DPP – x-prolyl dipeptidyl peptidase

Z -benzyloxycarbonyl

CHAPTER 1

INTRODUCTION

Infective Endocarditis

The disease state referred to as infective endocarditis (IE) has many faces and encompasses a plethora of symptoms and manifestations in humans. The resulting infection can originate from a variety of microbial species and fluctuate between several degrees of intensity. Jean-Baptiste Bouillaud introduced the term *endocarditis* between 1824 and 1835 (Major, 1945). Yet it was William Osler who studied the disease extensively, and in his hallmark Goulstonian lectures of 1885 provided major contributions to the knowledge of the natural history, pathogenesis and pathology of the disease (Osler, 1909). Additionally, the efforts of other contemporary scientists: Lehnarz (Major, 1945), Blumer (Blumer, 1923) and Beeson *et.al.* (Blumer, 1923) shaped the medical perception of IE into the 20th Century. The post-antibiotic knowledge of the disease was first summarized in 1955, upon Kerr's comprehensive publication on subacute bacterial endocarditis (SBE) (Kerr, 1955).

Before the advent of antibiotics, IE was invariably fatal. With the discovery of anti-microbial agents, patients could make a full recovery before extensive tissue destruction and systemic infection occurred. Additionally, the improvements in surgical techniques and valve replacement have warded off the disease's death sentence. Yet the emergence of antibiotic-resistant microorganisms and shifting epidemiological groups (increased median age, increased proportion of acute cases resulting from nosocomial

1

infections, lowered mortality of children with congenital heart disease, etc.) make this life-threatening entity a continuing reality (Mounzer et al., 2000). Currently the overall mortality rate remains just over 10% (Watanakunakorn and Burkert, 1993). Various studies in developed countries have estimated the incidence of IE to be 0.7 to 6.8 cases per 100,000 person years. This would result in the range of 4000 to 15,000 new cases per year in the United States (Hogevik et al., 1995) (Drangsholt, 1998).

IE is caused by microbial infection of the endothelial lining of the heart and is characterized by a lesion, known as the vegetation, which usually develops on the heart valve but can appear elsewhere on the endocardium. The disease is classified upon two sets of criteria that are defined by: 1) the condition and type of infected valve and 2) the relative virulence of the infecting organism. Infection of a heart valve that was either previously normal or damaged by congenital or acquired disease is termed native valve endocarditis (NVE). Infection of an artificial heart valve is termed prosthetic valve endocarditis (PVE) (Sande et al., 2001). Figure 1.1 diagrams the microbial etiologies upon both types of valves. The second classification encompasses subacute bacterial endocarditis (SBE) and acute bacterial endocarditis (ABE). SBE progresses over a duration of more than six weeks and is usually caused by organisms of low virulence possessing limited ability to infect other types of tissue. The disease begins insidiously with the presentation of fever, malaise and spleen enlargement, followed by more complicated symptoms (Hermans, 1982). On the other hand, ABE is most often caused by more pathogenic organisms (i.e. Staphylococcus aureus) capable of producing infection at several other body sites. This form develops in less than six weeks and follows a rapidly changing clinical course that presents an uncomplicated diagnosis

(Korzenioswki and Kaye, 1992). NVE can be characterized as acute or subacute infections while PVE appears to result from organisms associated with ABE in the early stage of infection followed by those from SBE in latter stages (Sande et al., 2001). Those organisms that are associated with ABE and early PVE derive mostly from the skin flora (coagulase-negative and coagulase-positive *Staphylococci*, *Pseudomonas* ssp. and fungi). Intravenous drug users pose a high risk for ABE and those organisms that cause drug-related endocarditis most frequently originate from the addict's skin (Tuazon and Sheagren, 1974).



Figure 1.1 Categorization of Selected Microbial Etiologies of Native and Prosthetic Valve Endocarditis (Mounzer et al., 2000).

NVE, native valve endocarditis; PVE, prosthetic valve endocarditis

IDU, intravenous drug use; GNR, gram-negative rods

Subacute Bacterial Endocarditis

Unlike ABE, which can occur spontaneously on normal endothelium, those organisms that promote SBE are dependent upon native valves with previous damage. Therefore most patients who develop IE have some type of pre-existing heart condition. Those cardiac abnormalities that compromise valve integrity and that pose a relatively high risk for SBE include: cyanotic congenital heart disease, aortic valve disease, mitral regurgitation and stenosis, patent ductus arteriosus, ventricular septal defect, coarctation of the aorta, prosthetic heart valves and previous damage from IE (Dajani et al., 1991). An increase in the elderly population has put this group at higher risk for infection and increased mortality based upon underlying degenerative defects and calcified heart tissue (Werner et al., 1996). These phenomena ultimately result in the factors that define the epidemiology and pathology of this disease. This can be defined by 1) sites and nature of endocardium that promotes pathogenesis, 2) type and source of infecting microorganism found during bacteremia, 3) insults or procedures which initiate the infection and 4) extra-cardiac complications associated with SBE.

The development of SBE requires a complex interaction between the vascular endothelium of the host, its hemodynamic response and the presence of circulating bacteria. Normal endothelium is non-thrombogenic and poorly receptive to attachment by most bacterial species (Rodgers et al., 1983). The sterile vegetation termed nonbacterial thrombotic endocarditis (NTBE) must develop prior to infection. The force of a circulatory jet stream presumably denudes the endothelial surface exposing the basement membrane, namely a collagen matrix. This is an environment that stimulates hemostasis, leading to the deposition of a fibrin/ platelet clot (Lopez et al., 1987). These microscopic

thrombi may embolize away harmlessly or may be stabilized and grow by deposition of fibrin and more platelets to form macroscopic vegetations (Sande et al., 2001). The genesis of NTBE and the potential of bacteria to circulate upon those sites are dependent on physical properties of the heart. The frequency of involvement of each valve is directly proportional to the mean blood pressure upon it, thus the left side of the heart is more often involved than the right (Lepeschkin, 1952). Vegetations are usually located on the downstream side of anatomic abnormalities in the heart or great vessels. Vegetations usually arise at a site where blood flows from a high-pressure source (e.g. the left ventricle) through a narrow orifice (e.g. stenotic aortic valve) into a low-pressure sink (e.g. aorta) (Sande et al., 2001). Experiments revealed that bacteria carried in an aerosol flowing through a constricted tube into an area of low pressure were deposited into the walls of a tube immediately beyond the constriction due to Venturi pressure effects and turbulence (Rodbard, 1963). Figure 1.2 presents the most common locations and incidences of vegetations that predispose SBE. A high velocity regurgitation jet stream (depicted by the arrow) is shown to pass through an incompetent aortic valve into a lowpressure sink (A). Similar situation occurs at the mitral valve (B), while jet flow lesions occur at the chordae tendineae (C) and left atrium (D). 85% of SBE cases involve the left-sided valves, with the aortic (15-26% frequency), mitral (38-45%) and aortic/ mitral (23-30%) most often affected (Buchbinder and Roberts, 1972).

The bacterial species that have best evolved to colonize and infect sterile vegetations are inextricably linked to areas in the body that appear healthy and have no apparent pathologies. The mandatory presence of NTBE and benign nature of etiological bacteria categorize SBE as an opportunistic disease. The disease process requires

microbial colonization to the endocardial surface followed by multiplication and persistence at that site. Those species can efficiently adhere to the sterile vegetation, resist host defense mechanisms and retrieve materials for growth, can potentially



Figure 1.2 The Anatomic Location of Vegetations in SBE (Rodbard, 1963)

A, aortic valveB, mitral valveC, chordae tendineaeD, atrial endocardiumhigh-velocity regurgitant stream

establish a niche on the traumatized endocardium. The range of microbial species that can cause IE is quite large, yet only a few microorganisms account for infections of native valves and even fewer are responsible for SBE. On native valves Streptococci ssp. and coagulase-positive Staphylococci cause more than 80% of cases. Table 1.1 displays the frequency of various organisms to cause NVE. Streptococci produce far more cases than other organisms, with the α hemolytic, viridans streptococci group playing the biggest role. These bacteria are ubiquitous, normal inhabitants of the oropharynx and considered low-grade pathogens, rarely causing disease outside of the bloodstream. Phylogenetically-related members within the mitis group of the viridans (S. mitis, S. sanguinis, S. gordonii and S. oralis) represent 45% of all NVE cases and 70% of SBE (Roberts et al., 1979). Their ability to cause disease rests in a tendency to enter the bloodstream and adhere to the endocardium rather than a reliance on virulence factors. Bacteremia from the oral cavity often follows dental procedures associated with bleeding that includes: tooth extraction (60% of procedures resulting in bacteremia), periodontal surgery (88%), brushing teeth or irrigation (40%) and tonsillectomy (35%) (Durack, 1995). Bacteria that inhabit the gastrointestinal and genitourinary tracts, S. bovis (Group D streptococci) and Enterococcus faecalis, account for 20% of NVE cases. Their isolation in culture is often associated with diagnostic and surgical procedures of those systems (Durack, 1995). The bulk of remaining NVE cases result from the invasive Staphylococcus aureus that originate from the skin and enter the bloodstream via intravenous drug usage or IV catheters in a nosocomial environment. As a professional pathogen expressing numerous virulence factors, S. aureus infects undamaged tissue and generally leads to ABE on the right side of the heart (Watanakunakorn et al., 1973).

Organism	NVE%
Streptococci	60
Viridans, α-hemolytic	35
Streptococcus bovis	10
Enterococcus faecalis	10
Other streptococci	<5
Staphylococci	25
Coagulase-positive	23
Coagulase-negative	<5
Gram-negative aerobic bacilli	<5
Fungi	<5
Miscellaneous bacteria	<5
Diphtheroids	>1
Other anaerobes	<1
Rickettsiae	<1
Chlamydiae	<1
Polymicrobial infection	<1
Culture-negative endocarditis	5-10

Table 1.1 Frequency of Various Organisms Causing Native Valve Endocarditis(Sande et al., 2001)

A feature of viridans streptococci is their ability to play a significant role on the pathogenesis of SBE, yet display a low invasive capacity. Minimal pathogenicity, coupled with a small inoculum, is probably responsible for the long delay between the initiation of the infectious process and the clinical manifestations of the disease. Agglutinating, complement-fixing, bactericidal antibodies and cryoglobulins have been identified in patients with SBE (Cordeiro et al., 1965). Their presence suggests that the immune system plays an important role in the pathogenesis and clinical course of endocarditis. The immune system in these patients is altered in three major areas: (1) hypergammaglobinemia caused by specific polyclonal B-cell hyperactivity, (2) the presence of circulating antigen-antibody complexes and (3) the immune stimulation of an inflammatory response directed against the patient's tissues (Phair and Clarke, 1979). Injury of tissue caused by circulating immune complexes has been responsible for many of the clinical symptoms. The presence of aggregates and end organ damage lead to the development of glomerulonephritis in infected patients (Keslin et al., 1973). Peripheral manifestations of SBE include: purpuric lesions, Osler nodes, Roth spots and subungal hemorrhages. These may be due, in part, to a vasculitis caused by circulating immune complexes (Alpert et al., 1976). Cerebral emboli, kidney infarction and mycotic aneurysms develop in more than a third of patients with SBE (Morgan and Bland, 1959).

The Vegetation and Biofilm Growth

The threat posed by viridans streptococci is a consequence of their physical exodus from the oropharynx and skill in adapting to a new microenvironment. This transition is contingent upon the presence of a biofilm and the ability to maintain its integrity during infection. Within dental plaque, bacterial species specifically attach to different surfaces and co-aggregate with specific partners (Whittaker et al., 1996). Regardless of its location, the bacterial biofilm exists as a structured community of cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton et al., 1999). In the case of SBE, sessile (community bound) bacteria of the plaque become planktonic (free living individual) during transit through circulation. After encountering the vegetation, they colonize the surface and start to re-exist as sessile in the presence of numerous individuals within protected surroundings. On one hand, the lifestyle change from plaque resident to heart dweller is a journey of great consequence and peril. On the other hand and in a more simplistic view, the planktonic phase is merely a means to an end from one surface to another.

It has become increasingly evident that the oral cavity can act as the origin for the dissemination of bacteria to the heart, lungs and peripheral blood capillary system, and is common less than 1 min after an oral procedure (Kilian, 1982). The oral cavity has several barriers to bacterial penetration from dental plaque into the tissue, including the surface epithelium, defensins, electrical barrier, antibody-forming cells and the reticuloendothelial system (RES) (Loesche, 1994). Those organisms involved in transient bacteremia are usually eliminated by the RES within minutes and are generally asymptomatic to the host (Kilian, 1982). Nevertheless, multiple animal models (rats, rabbits, and pigs) subjected to trauma have histological evidence of endocarditis under experimental conditions (Durack et al., 1973). A contentious debate has surfaced pertaining to the correlation between dental procedures and susceptibility to SBE. Over 50% of all endocarditis cases are not associated with either a procedural or infectious event 3 months prior to developing symptoms (Li et al., 2000). A population-base study

found no increased prevalence for IE among dental patients with or without valvular abnormalities, concluding that bacteremia is part of daily low-grade bacteremia, coincidental with brushing and chewing, and therefore few cases would be preventable with antibiotic prophylaxis (Strom et al., 1998). A newly proposed causal model predicts that an early bacteremia may prime the endothelial surface of the heart over many years and promote valve thickening. This would render the valve susceptible to adherence and colonization by a later bacteremia that would culminate over a few weeks into fulminant infection (Drangsholt, 1998).

There are three steps critical for infection of the sterile vegetation leading up to SBE pathology. Bacterial adherence, platelet activation and fibrin overlaying are generally described in the schematic of Figure 1.3. The end result is friable white or tan masses of variable sizes that are situated along the lines of valve closures. Their composition includes clumps of microorganisms, a meshwork of fibrin-platelet bridges, occasional erythrocytes and a few leukocytes (Ferguson et al., 1986). Unimpaired bacterial growth can result in extremely high colony counts of 10^9 to 10^{10} bacteria per gram of tissue (Durack, 1975). Microbial adhesion that can be mediated by a variety of surface components and receptors, acts as a virulence factor for the colonization of the endothelium. Adherence rates upon damaged aortic valve leaflets were measured for several species and after *Enterococci* ssp., viridans streptococci showed the highest rates with 400x greater adherence than E. coli (Gould et al., 1975). Dextran (hydrated extracellular polysaccharide) has been implicated in attachment to dental enamel but more importantly has been shown to correlate directly with the ability to produce IE in the rabbit model (Scheld et al., 1978). The ubiquitous presence of fibronectin on damaged

tissue enables a surface binding protein, Fim A, to aid in colonization. 80% of IE streptococci express this binding protein on the cell surface to reduce, ostensibly to coat the surface with host protein and minimize a host immune response (Kuusela et al., 1985). Additionally, the streptococcal cell wall component, lipoteichoic acid (LTA), has been implicated as a fibronectin receptor (Nealon et al., 1986). Endothelial damage exposes the primary components of valve connective tissue, type I and IV collagens, and the Yad A adhesin is able to exploit such conditions (Herzberg, 1996). Another mechanism was elucidated in *S. gordonii* in which two cell-wall associated proteins were found to bind immobilized laminin, another target present during cell damage (Sommer et al., 1992).

Those bacteria that successfully bind the endocardium trigger a localized thrombosis that is the result of both host response and bacterial clotting factors. Preexisting fibrin from the sterile vegetation and an inflammatory cytokine response to foreign bacteria, initiate thromboplastin formation by leukocytes that further propagates clotting (van Ginkel et al., 1979). Figure 1.4 shows the active role that viridans play in the activation of platelets that results in fibrin layering constituting macroscopic vegetations. Because greater than 60% of *S. sanguinis* strains activate human platelets *in vitro*, the described mechanism was elucidated by Herzberg et.al. for this particular viridans streptococci member (Herzberg, 1996). The cell is thought to come into close proximity of the platelet via interaction between a cell surface adhesin (SsaB) and an unidentified ligand of the platelet. Platelet aggregation is stimulated by the action of a platelet aggregation-associated protein (PAAP) binding to the platelet $\alpha_2\beta_1$ integrin. An epitope on PAAP mimics the integrin's physiological ligands, collagen and von Willenbrand factor, thus activating the platelet. The effect is morphological changes, surface expression of receptors for stimulators, followed by degranulation whereupon clotting factors and fibrinogen are released, as well as ATP that is potentially hydrolyzed by a cell surface ATPase on S. *sanguinis*. The pro-coagulant effect is potentiated by platelet display of the fibrinogen receptor, GPIIb-IIIa integrin. The resulting fibrin-platelet network increases in mass as cells colonize and expand layer upon layer of vegetation.



Figure 1.3 Pathogenesis of Infected Vegetation (Mandell and Korzeniowski, 1998)



Figure 1.4 Platelet Activation by Viridans Streptococci (Herzberg, 1996)

The newly colonized vegetation represents a unique biofilm environment that is surrounded by antimicrobial dangers. Planktonic cells, resulting from transient bacteremia or released from friable pieces of vegetation, will generally succumb to immune surveillance. The situation inside the fibrin barrier is quite different. Figure 1.5 illustrates colonies of unspecified viridans streptococci emeshed in the fibrin-platelet meshwork. Those organisms that can resist antimicrobial defense mechanisms multiply rapidly in the vegetation, soon reaching high numbers and then entering a stationary growth phase. As the vegetation enlarges, the colonies are gradually buried below the accumulating layers, and large numbers of cells $(10^9 \text{ to } 10^{15} \text{ per gram of tissue})$ are the consequence of the unimpeded thrombus growth (Durack and Beeson, 1972). A phenomenon of sessile biofilm communities is their ability to withstand host immune responses in regard to leukocyte access and impeded diffusion of materials, compounded with slowed growth of the cells. The vegetation provides the bacteria with a "protected or privileged site" in which polymorphonuclear leukocytes penetrate poorly and are unable to check colony growth. Polymeric substances are known to retard the diffusion of antibiotics (Ishida et al., 1998) and solutes generally diffuse at a slower rate within biofilms than in water (Stewart, 1998). Antimicrobial oxidants produced from the oxidative bursts of phagocytes may poorly penetrate inside the vegetation, thwarting further attempts to destroy biofilm organisms (Costerton et al., 1999). Bacteria are thought to metabolize slowly in the biofim due to their extremely high densities. It is probable that certain populations experience varied metabolic states with survival in slow growing or starved states (Brown et al., 1988; Korzenioswki and Kaye, 1992).

Variable biofilm growth aids in the antibiotic resistance of bacteria, particularly on the efficacy of cell-wall active drugs, i.e. penicillins, cephalosporins and vancomycin. The vast majority of viridans streptococci strains are sensitive to penicillin and derivatives when treated in the planktonic state (Wilson et al., 1995). These phenomena are the logic behind long courses regiments of antibiotics. Nevertheless, recurrent infection (defined as the return of the symptoms of the disease and positive cultures of the blood after 6 months of treatment with an effective agent) occurred in 9.5% of SBE patients (Pankey, 1959). Most relapses occur within a few weeks of the end of treatment, but viable bacteria can persist in apparently healed vegetations for several months before a late relapse. SBE patients remain at permanent risk for relapse after the infection is curred due to compromised integrity of the endocardium associated with vegetation burden on the valve surface as well as the tissue damage from chronic inflammation at the endothelium (Sande et al., 2001).



Figure 1.5 Colonies Emeshed in Fibrin-Platelet Vegetation (Durack, 1975)

The Mitis Group, *Streptococcus gordonii* and Isolate FSS2

The classification, serological typing and nomenclature associated with S. gordonii and related species has been a matter of ambiguity for microbiologists and epidemiologists alike. The term "viridans" has been synonymous with either oral streptococci or the ability of bacteria to execute the partial clearing (α -hemolysis) of erythrocytes on agar plates. The adaptation of the second definition has helped to distinguish them from pathogenic streptococci that perform complete hemolysis (βhemolysis). The definition of oral streptococci has refered to those species comprising the normal human flora of the oropharynx and occasionally the gastrointestinal and genitourinary tracts (Whiley and Beighton, 1998). Sherman provided the first accepted classification of the primary divisions of the genus Streptococcus, namely pyogenic, viridans, lactic and enterococcus. This was based upon biochemical characteristics, surface antigens and hemolysis. It was also reported that the Lancefield grouping system, used for the majority of streptococci, was not useful for classifying the viridans streptococci (Sherman, 1943). The Lancefield markers A (S. pvogenes), B (S. agalactiae), C (animal pyogenes), etc., were developed as convenient cell-surface antigenic markers for serotyping the genus. Despite antigenic variation among the viridans streptococci, a common group antigen was discovered as group H strains that fell between pyogenic and viridans physiological criteria (Lancefield, 1933). The identity of this antigen was later found as glycerol teichoic acid (Hamada et al., 1979). Further classifications have been numerous and contradictory, mainly due to ambiguity and poor reproduction of biochemical tests, followed by differences in international nomenclature. Recently, the techniques of genotypic data, mostly in DNA base pairing and 16S rRNA
sequence analyses, have enabled the construction of a definitive classification of the *Streptococcus* genus. Figure 1.6 outlines the phylogenetic relationships among 34 streptococcus species by 16S rRNA gene sequence analysis. The four groups that encompass oral streptococci, "anginosus", "mitis", "mutans" and "salivarius" are differentiated from pyogenic and other pathogenic groups, as well as previously know streptococci that are currently classified as *Enterococcus* and *Lactococcus* (Whiley and Beighton, 1998). The four oral groups now include 18 species that can be separated further into strain-specific biovars (Kawamura et al., 1995).

Based on 16S rRNA homology, *S. gordonii* is a member of the mitis group. These 6 species that comprise this group are found within two phylogenetic branches, 1) *sanguinis* and *parasanguinis* and 2) *mitis, pneumoniae, oralis* and *gordonii*. Yet based on biochemical criteria (cell wall polysaccharide constituents, murein linkage, carbohydrate fermentation) *S. gordonii* behaves more like *S. sanguinis* than more closely related members. A recent study of 151 viridans strains has identified *S. gordonii* as a new species from previously identified subspecies and biotypes. *S. sanguinis* is currently divided into *S. sanguinis sensu stricto* and *S. gordonii* (Kilian et al., 1989).

Statistical analysis for IE etiologies generally fails to provide a detailed description of viridans streptococci. This has been the result of taxonomic confusion and convoluted biochemical differentiation. A recent study has provided insight into the association between individual species and their relative contributions to disease (Douglas et al., 1993). The identities of 47 strains of oral streptococci, collected from 42 confirmed cases of IE, were revealed. The most common species identified were *S. sanguinis sensu stricto* (32%), *S. oralis* (30%) and *S. gordonii* (13%). Other related



Figure 1.6 Phylogenetic Relationships among 34 Streptococcus Species by 16S rRNA

Gene Sequence Analysis (Whiley and Beighton, 1998)

species, including *S. mitis* and *S. parasanguinis*, were less common. The authors speculate that the frequency of the IE producing species in examined cases is the result of certain shared pathogenic traits, namely dextran-mediated adhesion, platelet aggregation and adherence to connective tissue. Extracellular dextran is produced from sucrose by all three species and evidence suggests it promotes experimental endocarditis in the rabbit model (Baddour et al., 1989). Aforementioned PAAP of *S. sanguinis* is another pathogenic feature although its presence within other species and their potential to activate platelets has not been discussed.

Two strains from the *S. sanguinis* group (SSG), FSS2 and L50, were compared based upon their ability to aggregate both human and rat platelets. *S. sanguinis* FSS2, reclassified as *S. gordonii* FSS2 upon reevaluation by new biochemical testing (Harty et al., 2000), was isolated from the blood culture of a patient with endocarditis (Manning et al., 1994). Strain L50, provided by Dr. M. Herzberg was isolated from the dental plaque of a healthy volunteer. Both strains were shown to bind both insoluble human fibrinogen and fibronectin. But FSS2 was more active than L50 in all assays, especially a 200-fold greater adherence rate to human platelets, and a threefold difference with fibrinogen and platelet-fibrin clots. FSS2 aggregated both human and rat platelets to a final value of 85-90%, measured in lag times of 8 min. and 0.5 min, respectively. L50 was unable to induce any measurable platelet activation. Although L50 was capable of producing endocarditis in the rat model, infection with strain FSS2 produced significantly larger vegetations and embolic spread that manifested principally as multifocal renal abscess.

for initial colonization but contributes to the pathologies associated with thrombosis (Manning et al., 1994).

Metabolism and the Arginine Deiminase Pathway

S. gordonii, in addition to other Gram-positive streptococci, produce lactic acid as anaerobic fermentation of glucose-6-phosphate. sole end product in the the Homofermentation also occurs on N-acetyl-glucosamine, esculin, amygdalin, arbutin, fructose, galactose, lactose, maltose, sialicin and trehalose. Like most other oral streptococci, S. gordonii can convert sucrose into dextran polymers that constitutes a sticky, glycocalyx coat and structural element within dental plaque (Whiley and Beighton, 1998). Although thriving amidst the plaque environment characterized by low oxygen tension, viridans streptococci are facultative (aerotolerant) anaerobes that are relatively insensitive to the lethal effects of oxygen. Cells absorb the element via the flavoprotein oxidase system to produce H₂O₂, and dispose of this toxic metabolite using peroxidase as opposed to the catalase system. The inability of S. gordonii to utilize oxygen as a final electron acceptor, the absence of porphyrin and cytochrome-containing proteins and the failure to express TCA specific enzymes, commit ATP production by substrate-level phosphorylation. Therefore, 85-90% of glucose is metabolized by homofermentation as the result of limited biosynthetic capability. Glycolysis of one glucose results in the net gain of 2 ATPs and 2 pyruvates, followed by homolactic fermentation that produces 2 lactate molecules and the oxidation of NADH for recycling. In contrast, heterofermentation yields only 1 ATP per glucose along with ethanol, CO_2 and minor acid products. Homofermenters produce twice the cell biomass as heterofermenters using the same quantity of glucose. The consequence of this more

efficient fermentation is the commitment release of protons and a rapid fall in intracellular and biofilm pH as lactic acid concentration increases (Brock et al., 1994).

The catabolism of arginine provides *S. gordonii* with an alternative energy source to carbohydrates. Among the Gram-positives and members of the Bacillus group, the arginine deiminase (ADI) pathway accounts for the majority of arginine degradation (Baumberg, 1993). The pathway has been identified in members of the genera: Streptococcus, Bacillus, Clostridium and Lactobacillus, and among viridans members: *S. sanguinis*, *S. mitis* and *S. gordonii* (Floderus et al., 1990).

The ADI Pathway (Figure 1.7) consists of three enzymatic steps that produce intermediates and end products that serve critical aspects of growth physiology, metabolism and survival in the plaque environment. Analysis of the catabolic pathway can illustrate four advantages that are conferred to Gram-positive bacteria. Firstly, one molecule of ATP is generated from a single arginine via substrate-level phosphorylation in the final reaction involving carbamate kinase. This underscores the fact that lactic acid bacteria are unable to carry out electron-transport phosphorylation (Fisher, 1993). This is perhaps the most important and fundamental role of the pathway when accessibility to carbohydrates is low and energy demands remain high.



17	Arginine deiminase	Arginine + $H_2O \rightarrow citrulline + NH_4'$
18	Ornithine carbamoyltrans- ferase (catabolic)	Citrulline + $P_i \rightarrow$ ornithine + carbamoyl phosphate
19	Carbamate kinase	Carbamoyl phosphate + ADP \rightarrow ATP + NH ₄ ⁺ + HCO ₃ ⁻

Figure 1.7 The Arginine Deiminase (ADI) Pathway (Cunin et al., 1986)

Secondly, the production of ornithine from citrulline, via the ornithine carbamoytransferase (OCT) reaction, is an essential step in the catabolism of arginine, proline and glutamate biosynthesis, and arginine intracellular transport. OCT can proceed in both directions with a steady-state equilibrium that lies in conversion towards citrulline Ornithine is therefore either a short-lived intermediate or exported from the cell. In resting *S. sanguinis* 903 cells, ornithine was released from cells upon incubation with free arginine, arg-containing peptides or saliva. When extracellular citrulline is supplied to cultures, the compound is neither transformed into ornithine nor involved in cellular uptake (Abdelal, 1979).

The ADI pathway confers a third advantage to cells when pools of carbamoyl phosphate (CP) appear as a result of citrulline lysis. The acceptance of free phosphate during OCT catalysis provides the carbamoyl group with a high-energy phosphate bond. This has the potential to drive several metabolic events for the cell during substrate-level phosphorylation reactions. Phosphoryl donation enables the production of glucose-1phosphate, facilitating intracellular glycogen stores that maintain viability during carbohydrate starvation and contributing to glycan formation that forges cellular adhesion and biofilm formation. The enhanced production of glucose-6-P, as a result of ATP and CP, permits additional substrate to enter the glycolytic pathway. Additionally, CP contributes to glucose uptake via the cellular phosphotransferase system. Further substrate-level phosphorylation drives UMP/pyrimidine synthesis as well as other phoshorylation events for cell growth. Perhaps the most notable event occurs during the recycling of ADP to ATP. The action of carbamate kinase (CK) on CP produces ATP, ammonia and carbon dioxide upon hydrolysis. Studies with S. faecalus reveal that for accelerated exponential growth, the simultaneous presence of glucose and arginine is necessary (Simon et al., 1982). Under glucose-limiting conditions in continuous culture, the fermentation of glucose in the presence of arginine enables S. sanguinis to maintain efficient growth. The ability of cells to convert arginine into biomass and/or intracellular glycogen explains how this organism can survive and dominate mixed cultures grown in glucose-limited chemostats (Floderus et al., 1990).

Non-mutans streptococci (predominantly viridans streptococci members) are pioneer bacteria for dental plaque formation and predominate the microbial flora of plaque. S. gordonii, S. oralis, S. mitis and S. sanguinis have been studied based upon their adaptation and tolerance in acidic microenvironments established by more acidogenic such as lactobacilli and mutans streptococci. Upon acidification at pH 4.0, S. bacteria gordonii increases (H+)-ATPase-driven pumps and ADI activities, as well as upregulation of stress proteins (Takahashi and Yamada, 1999). Ammonia production at the termination point of the ADI pathway serves to neutralize protons generated by lactate, protect acid sensitive cells and contribute to a temporary pH rise in the saliva. It is believed that enamel demineralization (an etiological factor for dental caries) results from the inability of bacteria to generate sufficient base from salivary substrates rather than excessive glycolysis on the tooth surface (Abelson and Mandel, 1981). Arginine, in comparison to the other amino acids, is the most effective at counteracting periodic acid shifts. Ammonia production can occur at low pH values and arginine catabolism occurs at a pH well below the minimum for viridans growth (pH 4.7) and glycolysis (pH 3.7) in complex media.

Amino Acid and Peptide Transport

The cellular benefits of the ADI Pathway are ultimately limited to the availability of free arginine to the growing cell. The acquisition of additional amino acids for biosynthetic and regulatory systems follows a closely related scenario. This is a function of 1) mechanisms and efficiency of amino acid and peptide transport, 2) the presence of free arginine and peptides/ proteins in the surrounding environment (plaque, saliva, thrombic vegetation or bloodstream) and 3) the success of converting arginine-based substrates into a suitable form for transport (i.e. proteolytic activity).

Arginine transport is regulated by low and high affinity transport systems (Cunin et al., 1986). Viridans streptococci have two known systems for arginine import: 1) a high affinity uniporter and 2) the lower affinity arginine/ornithine antiporter. When cells are grown in the presence of high glucose they require small quantities or arginine for biosynthetic purposes rather than energy production. An Arg uniporter is driven by pmfcoupled facilitated transport. It represents a constitutively active, scavenger system for anabolic (peptide or pyrimidine synthesis) rather than catabolic utilization of the limited arginine (Konings et al., 1989). An Arg/Orn antiporter has been characterized from lactic acid bacteria supplemented with galactose and arginine (Konings et al., 1989). This low affinity transporter accounts for the majority of extracellular arginine import at concentrations $>10^{-6}$ M. It is composed of an Arg/Orn binding protein and a putative membrane-carrier protein. This system requires neither exogenous metabolic energy nor a readily available proton motive force. The driving force is supplied by a concentration gradient formed by intracellular ornithine and extracellular arginine (Konings et al., 1989). The antiporter system has been described in S. sanguinis and has been implicated in metabolite exchange (Poolman et al., 1987). This antiporter follows a ping-pong mechanism for residue exchange and is dependent on high intracellular pools of ornithine (42 i M) and dissociation values which favor binding of arginine at common binding sites at the cell surface (K_d Arg= 6.6 i M vs orn=62 i M). Although affinity constants for the antiporter are significantly lower than for the pmf-driven uniporter, the lower affinity

system provides the cell with a high turnover of arginine that is critical during carbohydrate depletion or environments deficient in suitable fermentable substrates.

Utilization of other types of amino acids is more reliant upon pmf-driven transport and ATP/phosphate-donor linked transport. The transport of branched chain, bulky and neutral amino acids are tightly coupled to the pmf and therefore highly dependent on the regulation of intracellular pH (Konings et al., 1989). The driving force behind the import of charged residues lies in the metabolic energy from glycolysis or the ADI pathway. In comparison to antiporter exchange, rates of transport are two orders of magnitude greater in the presence of an energy source (Poolman et al., 1987).

Many distinct systems of peptide transport exist between bacterial species and can be defined by the kinetics and character of the translocated substrate. The import mechanisms elucidated in Gram-positive lactate bacteria have been almost exclusively from lactococci and lactobacilli. Research has therefore been focused on the amino acid/ peptide content of dairy starter cultures and their applications in food processing. Peptide uptake by lactococci is an energy-requiring process and is abolished by inhibitors of a membrane-bound ATPase (Boven and Konings, 1987). By 2000, 15% of the *S. gordonii* DL1 genome was sequenced by the Australian Institute of Dental Research (AIDR) and the University of New South Wales. 15 of the 171 ORFs coding putative proteins of known function represent ATP-binding cassette (ABC)-transporters as well as oligopeptide-binding lipoproteins (permeases) that are thought to contribute to this type of active transport. Lactococci have been shown to possess separate transport systems for di-, tri-, and oligopeptides that are necessary for transport of peptides 4 to 8 residues (Kunji et al., 1993). But growth studies on peptide-containing media and uptake of ¹⁴[C-] labeled peptides have indicated the size exclusion limit for substrates across the membrane is four to five residues in length (Law, 1978). In *S. gordonii*, a solute-binding-protein complex consisting of membrane-bound lipoproteins was shown to be necessary for the binding and subsequent uptake of hexa- and heptapeptides (Jenkinson et al., 1996).

Distinct transport systems for di-, oligo- and anionic peptides have been identified in *E. faecalis*. The dipeptide system displays greater affinity and specificity than either the oligopeptide or amino acid transporters (Nisbet and Payne, 1982). *S. sanguinis* ATCC 10556 maintains a pathway for the degradation and uptake of XPro-Y tripeptides which permits the import of X-Pro upon proteolysis (Cowman and Baron, 1997). Free L-Pro cannot be translocated into *L. lactis* cells (Smid et al., 1989). Although this phenomenon has not been published for viridans streptococci, generation of this dipeptide form could be a means to circumvent such a constraint. In general, oral streptococci show extreme selectivity towards those peptides that are degraded and thus imported. Salivary peptides containing high concentrations of Arg and Pro are externally cleaved by *S. mitis* and *S. sanguinis*, thereby reducing the need for specific transport systems that cater to longer forms of these peptides (Rogers et al., 1991).

Hypothesis for Proteolytic Activity in the Vegetation

S. gordonii exists in one temporary environment (bloodstream) and two permanent ones (oral and vegetation environments) as a circumstance of SBE etiology. The consequence of this phenomenon is both the intermittent and variable access to resources. Growth in culture is fastidious due to excess supplies of glucose, peptides, amino acids, cofactors and salts. A standard, pH-controlled culture environment (complex, undefined media with 50 mM glucose and 3.5 mM arginine) provides accelerated growth, reaching late-logarithmic phase within 7 hours. This is a stark contrast to the oral flora where doubling times are approximately 3-7 hours (Sissons et al., 1995). This type of quantification in tissue is technically difficult because of the lack of reproducable animal models and the presence of varying biofilm microenvironments. Stationary growth in the vegetation is characterized by varying degrees of metabolic activity in which younger colonies are oriented toward the surface and continue to proliferate, while older, embedded colonies are largely quiescent or dead [Durack, 1972] #66]. Nevertheless, any degree of in vivo survival is highly dependent on the success of the individual and whole colony. This can be measured in the extraction of sufficient quantities of nutrients from the local environment. In the oropharynx these can derive from the carbohydrate-rich meals of the host and to a lesser extent saliva, gingival crevicular fluid and contents of the damaged epithelium. The oral cavity is subject to periodic high loadings of dietary carbohydrate with a subsequent low pH as the carbohydrate is metabolized. During periods of host fast the access to dietary carbohydrates is limited, and a more neutral pH occurs through buffering of saliva. Under these conditions the bacterium must scavenge nutrients from salivary and plaque components, and it has been shown that peptidase and glycosidase enzymes become upregulated and increasingly important (Harty et al., 2000). These starvation conditions can be parallelled to life inside the vegetation whereupon fibrinogen and penetrable constituents of plasma become significant energy sources. Table 1.2 compares the known concentrations of key elements for the growth of S. gordonii FSS2 within such relevant physiological environments. This table provides only a rough estimation of the

changing conditions facing bacteria and hint at their potential for the impediment of growth in the vegetation.

Table 1.2. Potential Resources and Growth Factors Pertinent to S. gordonii FSS2

Growth in Physiological Environments. (Hunter, 1958; Miller, 1936; Oster, 1953; Stein and Moore, 1954; Sunderman and Boerner, 1950; Wolding, 1955).

	saliva/	plasma	fibrinogen-vegetation		
	plaque		matrix		
glucose	200-400ì M/	5.3 mM	diffusion dependent		
	100-1000 mM range (meal				
	dependent)				
arginine	arginine trace/		8% by mass		
	trace (fasting)-meal				
	dependent				
proline	trace/	243 ì M	3.7% by mass		
	2nmol/mg (fasting)-meal				
	dependent				
Ca ²⁺	1-2.5mM (fasting)-meal	2.4 mM	diffusion dependent		
	dependent				
pН	5.17(meal)	7.1	7.1+buffering capacity of		
	6.77 (fasting)		charged residues		

Periods of starvation under adverse conditions become a new reality for *S. gordonii* in the vegetation. Proteolytic enzymes are postulated to be an intricate part in the new paradigm for survival. Their indispensable role in other disease states has been well documented. As the primary agent in development of the chronic inflammatory disease, periodontitis, *P. gingivalis* produces a large array of proteolytic enzymes that have subsequently been the most investigated aspect of the Gram-negative species, both as a means of nutrient acquisition and as virulence factors in modulating the host immune response (Travis et al., 2000). The severity of infections by *S. pyogenes*, an epidemiological factor in streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) cases, has been linked to proteolytic factors. These findings implicate a cysteine protease (SpeB) in host-pathogen interactions via regulation of the expression of GAS virulence genes (Kansal et al., 2000).

Studies of proteolytic activities from viridans streptococci (with the exception of *S. pneumoniae*) are scarce and their implications in the progression of SBE are nonexistent. Among members of the mitis group, an adhesin-degrading proteinase (Lamont and Rosan, 1989) and IgA1-specific proteinase (Labib et al., 1978), both from *S. sanguinis*, have been identified. Additionally, a trypsin-like endopeptidase from *S. oralis* (Lo and Hughes, 1996) and a neutral, metallopeptidase from *S. parasanguinis* (Froeliger et al., 1999) were uncovered. *S. gordonii* has representatives, with a 98 kDa serine proteinase capable of hydrolyzing collagen and fibrinogen (Juarez and Stinson, 1999) followed by a putative zinc metalloproteinase (EMBL T11548) and intracellular sortase involved in protein maturation (EMBL Q9F0P0). *S. gordonii* FSS2 has exhibited both cell-surface and secreted activities against chromogenic and fluorogenic substrates for thrombin, collagenase, Xa, chymotrypsin, Hageman, plasmin, kallikrein and Ca²⁺dependent protease (Harty et al., 2000). Cell-free culture supernatant was also tested for proteolytic activity against whole proteins. Figure 1.8a shows time-based degradation of A α and B β chains of human fibrinogen while the zymogram in Fig.1.8b reveals several discrete activities capable of the broad hydrolysis of denatured collagen (gelatin). Both these and several of the synthetic substrate extracellular activities are significantly increased upon rise in the terminal pH of batch culture.

The hypothetical model for bacterial growth in the SBE vegetation supports a state of nutrient stress and a general stress response (GSR) in which environmental cues have dramatic effects on cellular activity. One such likely response is an exaggerated drive for the acquisition of peptides and amino acids. Those endoproteinase activities that have been partially characterized could potentially aid the remodeling and fragmentation of the surrounding protein matrix. However, enzymes responsible for the turnover of smaller peptides and amino acids which could benefit cellular transport systems and the ADI pathway have not yet been identified. This dissertation describes the isolation, characterization, cloning and sequence analysis of three peptidases secreted by *S. gordonii* FSS2 that can potentially meet these challenges.

A.







A, Fibronogen degradation assay, 1ì g crude supernatant protein: 20 ì g human fibrinogen, 37C; markers, crude supernatant, t(hrs)=0, 1, 2, 7 and 24. B, Gelatin zymograph, 10% substrate in SDS-PAGE, 100 ì g crude supernatant protein, 37C 15hrs.

CHAPTER 2

NOVEL EXTRACELLULAR X-PROLYL DIPEPTIDYL-PEPTIDASE (xPDPP) FROM *STREPTOCOCCUS GORDONII FSS2*: AN EMERGING SUBFAMILY OF VIRIDANS STREPTOCOCCAL X-PROLYL DPPs¹

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Abstract

Streptococcus gordonii is generally considered a benign inhabitant of the oral microflora yet is a primary etiological agent in the development of subacute bacterial endocarditis (SBE), an inflammatory state that propagates thrombus formation and tissue damage on the surface of heart valves. Strain FSS2 produced several extracellular aminopeptidase and fibrinogen-degrading activites during growth in culture. In this report we describe the purification, characterization and cloning of a serine-class dipeptidylaminopeptidase, an x-Pro DPP (Sg-xPDPP), produced in a pH-controlled batch culture. Purification of this enzyme by anion-exchange, gel filtration and hydrophobic interaction chromatography yielded a protein monomer of approximately 85 kDa, as shown by SDS-PAGE under denaturing conditions. However under native conditions, the protein appeared to be a homo-dimer based upon gel filtration and PAGE. Kinetic studies indicated that purified enzyme had a unique and stringent x-Pro specificity that is comparable to both the DPPIV/CD26 and lactococcal x-Pro DPP families. Nested PCR cloning from an S. gordonii library enabled the isolation and sequence analysis of the full-length gene. A 759-amino acid polypeptide with a theoretical molecular mass of 87,115 Da and calculated pI of 5.6 was encoded by this gene. Significant homology was found with the PepX gene family from *Lactobacillus* and *Lactococcus* ssp. and putative x-Pro DPPs from other streptococcal ssp. Sg-xPDPP may serve as a critical factor for the sustaining bacterial growth in vivo and furthermore, aid in the proteolysis of host tissue that is commonly observed during SBE pathology.

Introduction

Streptococcus gordonii, classified in the *S. mitis* group of oral streptococci, is a well studied member of the viridans family of streptococci (53). These primary colonizers serve a necessary role in the establishment of microbial communities that are characteristic of healthy dental plaque. Although considered benign inhabitants of the oral microflora, viridans members have been implicated in the systemic disease, infective endocarditis (IE) (9, 26). The progression of this disease state requires: 1) trauma (congenital or disease-related) to the endothelial valve surface such that it is predisposed to colonization, 2) adhesion of organisms to the modified valve surface after their entry into the bloodstream via the oral cavity and 3) the propagation of infected vegetations consisting of a fibrin-platelet meshwork (50). Despite the uniform susceptibility of these streptococci to β -lactam antibiotics and their lack of classical streptococcal virulence factors, they can cause life-threatening disease and/or chronic inflammation with periods of latency and several defined stages (10, 14)

The ability of these organisms to colonize biofilm surfaces at two distinct microenvironments has prompted studies of their dynamic metabolism and patterns of gene expression. *Streptococcus sanguinis*, studied as model for viridans pathogenesis, expresses cell surface adhesins and PAAP (platelet aggregation-associated proteins) that facilitate both colonization and thrombosis at the infected lesion (12, 15). In addition, metabolic activities have been shown to dictate the availability of cell-surface receptors manifested during the recruitment of planktonic bacteria to plaque or the attachment to a new biofilm surface (4, 12). Upon entry into the bloodstream, bacteria undergo a shift in

pH from mildly acidic plaque (6.0-6.5) to the neutral pH (7.3) of the blood (41). In vivo

expression technology (IVET) was employed on the *S. gordonii* rabbit model of IE to detect genes activated in the new environment with the alkaline shift in pH correlating with enhanced bacterial growth, upregulation of the *msrA* oxidative stress gene (52) and the induction of genes encoding carbohydrate metabolism enzymes, protein transporters and cell surface proteins (22). The expression and secretion of glycosidase and peptidase activities, as examined in pH-controlled batch cultures, was found to be down-regulated by acid growth conditions and up-regulated by growth in a neutral pH environment supplemented with serum (13) Chemostat growth also uncovered a pH-dependent thrombin-like activity which was considered more important in the tissue model than on tooth surfaces (32). This could reflect selective pressure for the organism to adapt novel enzymatic mechanisms for a changing environment.

It is presumed that *S. gordonii* obtains necessary protein nutrients from salivary glycoproteins in the oral cavity, while utilizing plasma proteins when growing on heart surfaces. This use of plasma proteins by oral streptococci as carbon/nitrogen sources would ostensibly benefit growth in the vegetation. Certainly, proteolytic and peptide transport systems for viridans members have been described (1,7,17, 18, 25, 45, 54), and it has been shown that small peptides can be imported into *S. sanguinis* while those exceeding size limitation would require further hydrolysis by endo- and exopeptidases present on the surface or secreted by these cells (8). One such activity that facilitates these metabolic requirements, a dipeptidyl peptidase with Gly-Pro-pNa hydrolyzing activity, has been detected in culture supernatant of *S. gordonii* (8, 19) yet has presently

remained undefined. In this report we describe the purification, characterization and cloning of a novel extracellular x-Pro dipeptidyl-peptidase (*Sg*-xPDPP) that derives from *S. gordonii* FSS2, a strain previously isolated from the bloodstream of an SBE patient.

Experimental Procedures: Materials

H-Gly-Pro-*p*Na, H-Arg-Pro-*p*Na, H-Ala-Ala-*p*Na, N-Suc-Gly-Pro-*p*Na, L-Pro-*p*Na, H-Gly-Arg-pNa, H-Gly-pNa, Sar-Pro-Arg-pNa, Di-isopropyl fluorophosphate (DFP), N α *p*-tosyl-L-lysine-chloromethyl ketone (TLCK), $N\alpha$ -*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), iodoacetamide, O-phenanthroline, aprotinin, bestatin, apstatin, β mercaptoethanol, Gly-Pro-Arg-Pro-amide, sexual agglutination peptide, sleep inducing peptide, substance P, des-Arg¹ bradykinin, bradykinin, kallidin, lymphocyte activating pentapeptide fragment and fibrin polymerization inhibitor were obtained from Sigma. Pefabloc SC, phenylmethylsulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin and E-64 from Boehringer Mannheim. Z-Ala-Pro-pNa, H-Ala-Ala-pNa, H-Ala-Phe-pNa, H-LyspNa, H-Arg-pNa, protein kinase C fragment, Arg-Pro and fibrin inhibitory peptide from Bachem. Protein kinase c peptide from American Peptide Company. Human RANTES, MIP-1 β and GM-CSF from PeproTech. H-Glu-(NHO-Bz) pyrollidide and diprotin A from Calbiochem. α_1 proteinase inhibitor (α_1 -PI) and α -2 macroglobulin were gifts from Athens Research and Technology, Athens, Ga. Viridans streptococci strains were a gift from Dr. Vincent Fischetti at The Rockefeller Institute.

Experimental Procedures: Methods

Bacterial Growth. *S. gordonii* FSS2 (previously *S. sanguinis* FSS2 (13,29) was stored and maintained (at -80°C) as previously described (32). Frozen cells were

inoculated into autoclaved medium containing 20 g/L trypticase peptone (BBL), 5 g/L yeast extract (Difco), 2 g/L NaCl, 0.1 g/L CaCb, 4 g/L K₂HPO₄ and 1 g/L KH₂PO₄. 10 g/L glucose and 05 g /L L-arginine were sterile filtered and subsequently added. A static culture (200 ml) was grown overnight at 37°C and used to inoculate a 4 L starter culture, in turn used to inoculate a 15 L stirred batch culture which was grown in an atmosphere of 5% CO₂ and 95% N₂ at 37°C with the pH held constant at 7.5 by addition of 5 M KOH using a pH controller (Cole Parmer). Cultures were harvested in early stationary phase at a point when the bacteria had metabolized all available glucose and the addition of base had ceased.

Enzymatic Assay. Amidolytic activities of crude samples and purified protease were measured using the substrate H-Gly-Pro-pNa (1 mM final concentration) in assay buffer A (50 mM Tris, 1 mM CaCb, pH 7.8) at 37°C. Assays were performed in 0.1 ml on 96 well plates using a thermostated microplate reader, and the release of p-nitroaniline was measured at 405 nm. (Spectramax, Molecular Devices). Inhibition assays involved pre-incubation of pure enzyme with inhibitor for 10 min at 37°C followed by measurement of residual activity. Additional pNa substrates were treated identically.

Enzyme Purification 14.5 L of cell-free culture filtrate was obtained after centrifugation (20 min, 4 $^{\circ}$ C at 6000 x g) of the batch culture. Proteins in the filtrate were precipitated over several hours at 4 $^{\circ}$ C with ammonium sulfate to a final concentration of 80% and the precipitate pelleted by centrifugation at 8000 x g for 40 min. Protein pellets were obtained upon centrifugation as previously described. Pellets were resuspended in 200 ml of buffer A and dialyzed over 2 days (4 $^{\circ}$ C), with changes, against 40 volumes of the same buffer. All column chromatography steps were performed at 4 $^{\circ}$ C except FPLC

separations that were done at room temperature. The dialyzed fractions was applied to a DE52 (Whatman) column (2.5 x 30 cm, 150 ml) equilibrated with buffer A. The column was washed with 3 column volumes of buffer A at 1ml/min. A gradient from 0 to 1M NaCl in buffer A was applied over a total volume of 700 ml. Peak activity that eluted was pooled and concentrated by ultrafiltration to 32ml, using a 10K membrane (Filtron). The concentrated sample was divided into three equal fractions that were separately loaded onto a SuperdexTM-75 (Amersham Pharmacia Biotech) HR 10/30 equilibrated with gel filtration buffer (50 mM Tris, 200 mM NaCl, 1 mM CaCb, 0.02% sodium azide, pH 7.8) at 1 ml/min. Peak activities of all runs were combined and concentrated to 22 ml. Ammonium sulfate was added to the fraction in order to give a final salt concentration of 1 M. This sample was then applied to a phenyl-Sepharose HP column (1.5 x 9 cm, 15 ml) equilibrated with 200 mM potassium phosphate-1M ammonium sulfate (pH 7.5) at 0.5 ml/min. The column was washed with high salt buffer (120 ml) until reaching an A_{280} baseline and then subjected to a 250 ml gradient of 1.0 M to 0.0 M ammonium sulfate utilizing 50 mM potassium phosphate (pH 7.5). Active fractions were pooled and dialyzed against 20 volumes of buffer A over a 24 hr period. The dialyzed sample was concentrated to 37 ml and then applied to a Mono Q HR 10/10 FPLC column (Amersham Pharmacia Biotech) that was equilibrated with buffer A. The column was washed with equilibration buffer until the base line stabilized. A linear gradient (0-500 mM NaCl in buffer A) over 100 ml, was applied, and peak enzyme activity eluted between 200-250 mM NaCl. This activity was pooled, dialyzed against 10 volumes of buffer B (25 mM Tris, 5 mM CaCb, pH 7.4) and applied to a Cibacron blue Sepharose CL-6B column (1 ml) equilibrated with buffer B. Activity flowed through the matrix and was collected,

while bound, non-specific protein was eluted in a high salt buffer B. Final purification to remove additional contaminants involved the use of a Gly-Pro Sepharose affinity column (1 ml) Enzyme obtained from the previous step was loaded onto the column, previously equilibrated with buffer B. It was then washed with 10 column volumes of this buffer and a linear gradient (0-1 M NaCl in buffer B) over 30 ml was applied. Peak activity eluted between 150-200 mM NaCl and was concentrated to 2.5 ml using a 10K MicrosepTM.

Protein Determination. Protein concentration was determined using a bicinchoninic acid BCA reagent kit (Sigma) according to the manufacturer's protocol.

Electrophoresis and Autoradiography. Enzyme purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel and the Tris-HCl/Tricine buffer system, according to Schägger and von Jagow (49). Nondenaturing gel electrophoresis (48) in a 4-20% gradient gel and gel filtration was used to obtain the MW of the native protein. To confirm the identity of *Sg*-xPDPP as a serine protease, five micrograms of pure enzyme was incubated with 500nCi of [1,3-³H] DFP for 30 min at 25 °C in 20 mM HEPES, pH 7.5. Cold DFP (10 mM final concentration) was used to quench the reaction and labeled protein was run on SDS-PAGE, followed by fixing in 2,5-diphenyloxazole, drying of the gel and exposure to x-ray film (XAR, Eastman Kodak) over a 96 hr period. For amino-terminal sequence analysis, *Sg*-xPDPP was resolved by SDS-PAGE, followed by electroblotting to a polyvinylidene difluoride membrane using 10 mM 3-(cyclohexylamino) propanesulfonic acid, 10% methanol, pH 11(30). The blot was air dried and the band was submitted for sequencing.

Enzyme Kinetics and Specificity. Kinetic values were measured using H-Gly-Pro-pNa at varying concentrations ranging from 20 μ M to 10mM, with a fixed enzyme concentration at 2 nM, in 100 mM Tris, pH 7.8, at 37 °C. V_{max} and K_m values were obtained through Hyperbolic Regression Analysis (shareware from J.S. Easterby, University of Liverpool, UK). Specificity studies utilized *Sg*-xPDPP incubated with 5 μ g of peptide in a 1:1000 molar ratio. Reactions occurred in 100 μ L volumes with 100 mM Tris, pH 7.8, at 37 °C for 2 hours. Digestions were terminated by acidification with 10 μ L of 10 M HCl, followed by centrifugation (10,000 x g, 5 min). The entire supernatant was applied to a reverse phase high pressure liquid chromatography using a LC-18 column (25 x 4.6 mm, 5 μ m)(Supelco) equilibrated with 0.1% TFA in HPLC grade water and developed with an acetonitrile gradient (0-80% in 0.08% TFA over 50 min). Peaks were manually collected and analyzed by mass spectrometry.

Mass Spectrometry. Peptides and native *Sg*-xPDPP were analyzed by MALDI-TOF on a Hewlett-Packard G2030A mass spectrometer. The instrument was operated at an accelerating voltage of 28 kV, an extractor voltage of 7 kV, and a pressure of 7 x 10^{-7} Torr. Samples were dissolved in sinapanic acid and ionized from the probe tip using a nitrogen laser source. Calibration was performed using mixtures of peptides/proteins of known molecular masses.

N-terminal and Internal Sequencing. Proteins and peptides were sequenced by Edman degradation in a model Procise-cLC sequencer (PE Biosytems, Foster City, CA) operated using the manufacturer's protocol. For internal sequence, proteins were in-gel digested with trypsin (Promega, sequence grade) and the peptides extracted (46), with their masses being determined by reflectron MALDI-TOF mass spectrometry using a Bruker Daltonics ProFlex instrument (42). Selected peptides were sequenced by the Edman Degradation method.

Cloning of S. gordonii xPDPP Gene. DNA from *S. gordonii* was purified using the Purgene DNA Isolation Kit (Gentra, Minneapolis, MN) according to manufacturer's instructions. The N-terminal fragment of *xPDPP* gene was obtained using a PCR approach and degenerate primers designed to the N-terminal (MRYNQY) and the internal (NVIDWL) peptides. Two putative N-terminal primers and one internal primer (all containing BamHI sites) were synthesized (nt-1-dpp; 5'-CGC<u>GGATCC</u>ATGCGNTAYAAYCARTA-3', nt-2-dpp;

5'-CGCGGATCCATGAGRTAYA AYCARTA-3' and int-1-dpp; 5'-CGCGGATTCARCCARTCDATNACRTT-3', respectively). PCR was performed using the Pwo DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN), 1 µg of S. gordonii DNA and 500 ng of the primers (94°C for 1 min, 65°C for 1 min, 72°C for 1 min, 32 cycles). A single 965 bp-long PCR product was obtained, gel-purified, digested with Bam HI, subcloned into the Bam HI site of pUC19 and sequenced. The obtained sequence was used to search the unfinished S. gordonii database available at The Institute Genomic Research (ftp://ftp.tigr.org/pub/data/s_gordonii/). of (TIGR) Several overlapping contigs were found and this approach resulted in the identification of a 2280 bp-long open reading frame encoding S. gordonii xPDPP. Subsequently, two PCR primers encoding the N-and C-terminus of DPPIV synthesized were (5'-AGTGGATCCATGCGTTACAACCAGTATAG-3' 5'-TTTGGA and TCCTCAAGATTTCTTGTGAGGC-3') and used in the PCR to obtained full-length DNA fragment encoding DPPIV. The 2298 bp-long PCR product was gel-purified, digested with Bam HI, subcloned into the Bam HI site of pUC19 and sequenced on both strands. The obtained sequence was deposited in GeneBank (accession number AY032733).

Southern Blot Analysis. DNA from S. gordonii PK48, DL1, PK2585, S. parasanguinis PK2584, S.mitis J27, S. oralis J21, PK34 and S. salivarius ATCC 27945 and was purified using the Purgene DNA Isolation Kit (Gentra, Minneapolis, MN) according to manufacturer's instructions. Four µg of bacterial DNA was digested with each of the restriction enzymes BamHI, EcoRI, HindIII or PstI overnight at 37° C. Digestion products were separated in 0.7% agarose gels using Standard procedure (Sambrook). Gels were soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min followed by neutralization solution (1.5 M Tris pH 7.5, 1.5 M NaCl) for 1 hour. Separated DNA was transferred onto a nylon membrane (Amersham-Pharmacia Biotech) by capillary transfer and subsequently fixed by cross-linking (Stratalinker, Stratagene Inc.). Membranes were prehybridized in 0.5 M phosphate buffer pH 7.2, 7% SDS and 1 mM EDTA at 50C for 1 hour, ³²P-labelled DPP cDNA probe was added and incubated overnight. After hybridization, non-specifically bound radioactivity was removed by a single wash in 2xSSC and 0.1% SDS at room temperature for 20 min followed by two subsequent washes in 0.5xSSC and 0.1%SDS at 50C for 20 min. Membranes were exposed to X-ray films using enhancing screens at -80C for 1-4 hours.

Results

Growth and Culture Activity. Preliminary experiments indicated the presence of an extracellular Gly-Pro-pNa activity in culture media (Fig. 2.1). Detection of this amidolytic activity in cell-free filtrate was observed during growth in culture supplemented with 50 mM glucose and 3.5 mM arginine, and was dependent on a pH maintained in the range of 6.0-8.0. Growth and activity from a 1 L pH-controlled (pH 7.5) batch culture were monitored at hourly intervals. Detection of cell-associated Gly-Pro-pNa activity occured early in logarithmic phase, as it paralleled the growth curve, and continued to increase until early stationary phase. Cell-free activity appeared later in log phase and sharply increased during early stationary phase. The profiles of both activity forms began to diverge during later stationary phase, between the 8th and 10th hours of growth. As cell-associated activity decreased by approximately 25%, extracellular activity steadily increased by roughly 15%. The trend of enzyme shedding continued beyond the shown timepoints (not shown) at a cost to activity found on the cell. These observations occurred at a time when bacteria had metabolized all available glucose and the addition of base had ceased. This phenomenon may be the result of the permeability in the cell envelope, cell wall turnover and/ or peptidoglycan rearrangement due to autolysin activities.

Further growth experiments were conducted under similar conditions but varied in culture environments with respect to pH control, carbohydrates, arginine, Ca²⁺, strain type and addition of serum (Fig. 2.2). Measurements were made at the 10th hour of growth when stationary phase was evident by lack of sugar consumption. The activity profiles during varied pH-controlled conditions provided evidence for a pH-dependent

secretion mechanism. Cell-associated Gly-Pro-pNa activity was detected under all conditions, with the least activity measured in culture devoid of pH control (final pH of 4.4) and greatest activity found in pH 7.0-controlled culture. Cultures at pH 6.3, 7.5 and 8.0 were similar. Appreciable extracellular activity was evident at pH 7.0 and 7.5, and at roughly equal values. Although growth at pH 7.0 accounted for the greatest total Gly-Pro-pNa activity, cells at pH 7.5 contributed their highest proportion of total activity (51%) to the secreted form. The remaining cultures were controlled at pH 7.0 for standardization and optimum enzyme expression. Excess supplemented arginine (50 mM) provided the greatest level of cell-associated activity while it appeared to inhibit the secretion of protease. Under this condition, less than 2% of the extracellular form was found versus the 3.5 mM arginine culture. The 25 mM glucose culture was unable to reach the appropriate stationary phase in the 10 hr. timeframe and thus cannot be accurately compared. The secretion of enzyme seemed dependent on the addition of Ca²⁺ (mM) since culture without any exogenously supplied to the media could only produce cell-surface enzyme. The addition of 25% fetal bovine serum to the starting media provided a decrease in total activity with no significant difference in the proportion of secreted activity. Mice-passaged S. gordonii FSS2 assays showed that these cells produced slightly less total Gly-Pro-pNa activity while a substantial decrease in extracellular activity (10% of total) occurred. Although, an overall decrease in total activity was seen in galactose-supplemented cultures, this type of sugar neither repressed nor activated the release of activity from cells, indicating the absence of a glucose repressor.



Fig. 2.1. *S. gordonii* **FSS2** growth and activity curve from a pH 7.5-controlled culture supplemented with 50 mM glucose and 3.5 mM arginine. Samples (1 ml) were removed from culture at fixed timepoints and cells were removed from media by centrifugation (5 min, 4 °C, 13,000 x g) followed by 2x wash and resuspension into initial volume with non-supplemented media. Symbols: , whole culture; •, cell-free culture fluid; , whole cells. Assays were performed as previously described using 30ì L of sample.



Fig. 2.2. Extracellular and cell-surface *Sg*-xPDPP activities under variable growth conditions. Samples (1 ml) were removed from culture at early stationary phase and cells were removed from media by centrifugation (5 min, 4 °C, 13,000 x g) followed by 2x wash and resuspension into initial volume with non-supplemented media. Symbols: \blacksquare , cell-free culture fluid; \square , whole cells. Assays were performed as previously described using 30ìL of sample. Total cell and cell-free activities for 1L cultures were measured as OD/min and adjusted for cell density.

Enzyme Purification. Earlier growth experiments indicated the presence of an extracellular Gly-Pro-pNa activity in culture media. Detection of this amidolytic activity in cell free filtrate was dependent upon growth maintained in a pH range of 6-7.5 (unpublished results). In this report, maximum activity was achieved using a 14.5 L batch culture held at pH 7.5 by addition of base and harvested during early stationary growth. An 80% ammonium sulfate precipitation concentrated extracellular proteins to a workable volume despite the large decrease in enzymatic yield. The following chromatographic steps (DE52 anion-exchange, S-75 gel filtration and Phenyl Sepahrose hydrophobic chromatography) successfully removed high and low molecular weight contaminants, media pigments and peptides/amino acid away from the Gly-Pro-pNa activity, with a minimal drop in yield. The Mono-Q step increased specific activity by 16 fold, although the yield was reduced fifty percent. CB Sepharose chromatography served as a negative step, enabling activity to flow through the matrix while higher affinity proteins and GAPDH(40) remained bound. The final step, utilizing Gly-Pro Sepharose, resulted in a single, sharp peak of purified protein. Concentrated fractions corresponded to high specific activity, a greater than 6000-fold purification and 1% yield from the starting batch culture (Table 2.1).

Step	Volume	Total Activity a	Total Protein	Specific Activity	Purific ation	Yield
	ml	Units ^a	mg	units/mg	fold	%
Culture filtrate	14,500	134,117	58,000	2.3	1	100
Ammonium sulfate	320	47,663	9504	5.0	2	36
DE52 anion exchange	32	41,717	3290	12.7	6	31
Superdex 75	22	38,063	2168	17.6	8	28
Phenyl Sepharose	37	35,708	1125	31.7	14	27
Mono-Q, FPLC ^b	10	17,063	35	487.5	211	13
CB Sepharose,	15	4180	7	593.0	258	4
Gly-Pro Sepharose, FPLC	3	1370	0.97	14,153	6153	1

Table 2.1 Purification of S. gordonii x-Pro DPP

^aBased on enzymatic activity using H-Gly-Pro-pNa, where 1 unit= 1 μ mol of pNa released per sec. ^bFPLC, fast protein liquid chromatography

Physical Properties. SDS-PAGE analysis of the purified enzyme (Figure 2.2) showed a single protein band, as judged by both Coomassie and silver staining (latter not shown) with an approximate mass of of 85 kDa. Analysis of the protein by MALDI revealed a laser intensity peak that corresponded to a mass of 86,977.6 Da. Protein analyzed on a 4-20% native gradient gel and Superdex 200 gel filtration indicated a molecular weight between 150-200 kDa, providing evidence for homodimer formation in the native state. This was ablated by heat treatment, SDS denaturation, and MALDI analysis. This suggested the dimer was stabilized by weak ionic/ electrostatic conditions. Isoelectric focusing (data not shown) produced a pI of 4.9 for the native protein. The Gly-Pro-*p*Na activity was optimum at pH 8.0 with activity detected over a broad phosphate buffer range of 5.0 to 10.0. The optimum temperature for Gly-Pro-*p*Na hydrolysis was determined to be between 45-50 °C. The enzyme in its pure form was unstable over a 15h period at 37 °C and 25 °C (complete loss of activity,) and 4 °C (75% loss of activity) while storage at -20 °C over several months exhibited minimal activity loss.

Figure 2.3 SDS-PAGE of fractions from the purification of *S. gordonii* x-Pro DPP and the autoradiography of the purified peptidase. *Lane 1*, 15 ig of molecular mass markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa). The following lanes contained boiled, reduced samples: *lane 2*, 140 ig of 80% ammonium sulfate precipitate; *lane 3*, 102 ig of eluted peak from DE52 anion exchange; *lane 4*, 98 ig of peak from Superdex 75 gel filtration wash; *lane 5*, 55 ig of eluted peak from Phenyl Sepharose; *lane 6*, 39 ig of eluted peak from Mono-Q anion exchange; *lane 7*, 15 ig of CB Sepharose flow through; *lane 8*, 6 ig of purified *Sg*-xPDPP from Gly-Pro Sepharose; *lane 9*, autoradiograph of $[^{2}$ H]-DFP-labeled enzyme exposed for 96 h to x-ray film.


Enzyme Specificity. Of the fourteen chromogenic endo- and aminopeptidase substrates tested on purified enzyme (Table 2.2), only three (H-Gly-Pro-pNa, H-Ala-Pro-pNa and H-Arg-Pro-pNa) were rapidly hydrolyzed. Weaker activity (< 10%) was detected against H-Ala-Ala-pNa. A maximum rate of hydrolysis occurred against H-Gly-Pro-pNa and Michaelis-Menten kinetics were measured using this substrate. A Vm of 15.2 $\mu mol~min^{-1}$ and K_m of 378 $\,\mu M$ was observed with 2 nM of enzyme. To obtain further information on cleavage specificity, various peptides were tested as substrates for Sg-xPDPP (Table 2.3). Proteolysis was confirmed on peptides between 4 to 17 residues with a Proline residue in the P₁ position. A major impediment for cleavage was the presence of Pro in the P_1 '. There were no additional restrictions for amino acids present in the P₂ and P₁'sites. The general formula for proteolysis was deduced as NH₂-Xaa-Pro(Ala)- \downarrow -Yaa-(Xaa)_n, where Yaa could represent any residue except Pro. The combined data for synthetic and peptide substrates fit a profile that was consistent with previous data for Gram-positive bacterial x-Pro-dipeptidyl aminopeptidases (11, 20, 21, 28, 33, 34, 36) and Gram-negative DPPIV families (2, 56). Extended time incubation with peptides failed to yield additional peptide fragments, indicating the absence of contaminating aminopeptidases. endopeptidase activity or Proteolysis of CC chemokines (RANTES and MIP-1 β), which presented an unblocked x-Pro N-terminus, was not observed under identical conditions as peptide substrates. The inability of SgxPDPP to catalyze endo-specific proteolysis was tested on whole protein substrates (azocasein,

Table 2.2 Relative amidolytic activity of S. gordonii x-1	Pro DPP	against
various substrates		

Substrate (pNa)	Relative
	activity
	(%)
H-Gly-Pro	100
H-Ala-Pro	81
H-Arg-Pro	63
H-Ala-Ala	8
H-Gly-Arg	0
H-Ala-Phe	0
H-Arg	0
H-Lys	0
H-Ala	0
H-Gly	0
L-Pro	0
Suc-Gly-Pro	0
Z-Ala-Pro	0
Sar-Pro-Arg	0

¹Activity against H-Gly-Pro-*p*Na hydolysis taken as 100

Substrate	Sequence	
Sexual Agglutination Peptide	Arg-Gly-Pro-Phe-Pro-Ile	
Protein Kinase C Fragment	Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-	
Substance P	Asn-Val Arg-Pro-↓-Lys-Pro-↓-Gln-Gly-Leu-Met	
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	
des Arg ¹ Bradykinin	Pro-Pro-↓-Gly-Phe-Ser-Pro-Phe-Arg	
Dipeptide A	Arg-Pro	
Sleep Inducing Peptide	Trp-Ala-↓-Gly-Gly-Asp-Ala-Ser-Gly-Glu	
Fibrin Polymerization Inhibitor	Gly-Pro-↓-Arg-Pro	
Fibrin Inhibitory Peptide	Gly-Pro-↓-Arg-Pro-Pro-Glu-Arg-His-Gln-Ser	
Protein Kinase C Peptide	Gly-Pro-↓-Lys-Thr-Pro-Gln-Lys-Thr-Ala-Asn- Thr-Ile-Ser-Lys-Phe-Asp-Cys	
Kallidin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	
Lymphocyte Activating Pentapeptide	Leu-Pro-Pro-Ser-Arg	

Table 2.3. Cleavage specificity of S. gordonii x-Pro DPP on peptide substrates

 \downarrow -cleavage site

gelatin, collagen type IV, fibrinogen). Internal digestion was not observed after standard incubations and SDS-PAGE analysis (data not shown).

Inhibition and Activation Studies. Autoradiography of the purified Sg-xPDPP radiolabeled with $[1,3-^{3}H]$ DFP (Figure 1, lane 9) positively identified the protease as a member of the serine class. Further studies with class-specific inhibitors (Table 2.4) supported this assignment. Sensitivity was observed to serine protease inhibitors (Pefabloc, PMSF, and 3,4 Dichloroisocoumarin), while cysteine-class (iodoacetamide and E64) and metallo-class (EDTA and 1,10-orthophenanthroline) had little or no effect on activity. Compounds specific for Aminopeptidase B and Leucine Aminopeptidase (Bestatin) and Aminopeptidase P (Apstatin) were not inhibitory. However, Sg-xPDPP was highly sensitive to the DPPIV/CD26 specific inhibitors, H-Glu-pyrollidide and Diprotin A(Ile-Pro-Ile), as evidenced by IC₅₀ values that approximated 100 µM. Human plasma inhibitors, α -1-proteinase inhibitor and α -2-macroglobulin, had no effect on enzymatic activity. Sg-xPDPP activity was also inhibited by the heavy metal ions Zn^{2+} and Co^{2+} , at 0.5 and 5 mM, respectively. Complete inactivation occurred in the presence of 1% SDS detergent. Activity was unaffected by reducing agents and urea. Gly-Pro-Arg-Pro-amide served as a competitive inhibitor while the dipeptide, Gly-Pro, provided no inhibition. In contrast, Gly-Gly moderately stimulated pNa hydrolysis.

Inhibitor	Concentration	% Residual Activity
DFP	10 mM	0
Petabloc	I mM	55
PMSF	5 mM	51
3,4-	5 mM	0
Dichloroisocoumarin		
TLCK	10 mM	96
ТРСК	10 mM	100
Aprotinin	600 µM	97
Bestatin	250 μM	99
Apstatin	1 mM	100
H-Glu-pyrollidide	100µM	56
	1 mM	11
Diprotin	100 µM	47
	1 mM	10
E-64	1 mM	85
Iodoacetamide	1 mM	100
EDTA	1 mM	106
1,10-	10 mM	99
Orthophenanthroline		
L-Cysteine	5 mM	100
β-mercaptoethanol	5 mM	87
Gly-Gly	5 mM	117
Gly-Pro	10 mM	105
Gly-Pro-Arg-Pro-	4 mM	62
amide		
Co2+	500 μM	57
Zn2+	5 mM	42
Ca2+	5 mM	103
SDS	1%	7
Urea	2 mM	100

Table 2.4. Inhibition profile of S. gordonii x-Pro DPP

Sg-xProDPP Sequence Analysis and Gene Structure. N-terminal and internal sequences facilitated the construction of degenerate primers. PCR against an FSS2 library resulted in the isolation of a 960bp fragment that represented the N-terminus of the protein. This product was used to search the genomic clone of a relatively uncharacterized S. gordonii strain in the Unfinished Microbial Genomes database, TIGR. An ORF of 2280bp was identified and was found to correspond to the Sg-xPDPP based upon the conservation of protein-derived peptides. Primers were designed from the electronic sequence and a PCR approach was used to obtain a full-length DNA fragment encoding DPPIV. A 759-amino acid polypeptide with a theoretical molecular mass of 87,115 Da and calculated pI of 5.6 was encoded by this ORF. This is in agreement with the observed experimental mass while the native state dimer is produced by association of two identical monomers. There is no evidence for post-translational modifications of the gene product. The homology search (Figure 2.3) performed using NCBI TBLASTN tool against EMBL, DDBJ, GenBank and PDB databases indicated that Sg-xProDPP is a new member of the X-Pro dipeptidyl-peptidase S15 family grouped in the SC clan of serine peptidases (5). This family has previously consisted of peptidases that derive from lactic acid bacteria (Lactobacillus ssp. and Lactococcus ssp.), known as PepX (51). SgxProDPP shares the closest homology with putative genes uncovered in *Streptococcus* pneumoniae (72% identity) and Streptococcus mutans (57% identity) while maintaining only 48% and 34% identity with Lactococcus and Lactobacillus, respectively. SgxProDPP displays evolutionary divergence from bacterial members of the DPPIV (S9) family, Flavobacterium meningosepticum (10% identity) and Porphyromonas gingivalis (11% identity), although sharing functional similarity. The active site serine has been identified in the PepX gene of *L. lactis* as Ser 348 inside the motif GKSYLG (6). Thus, by analogy Ser 347 of Sg-xProDPP is likely to be the active site residue. Streptococcal xProDPPs and PepX have nearly identical residues flanking the catalytic motif, except for a hydrophobic amino acid replacing a charged Lys in the second position. Based upon homology to SC peptidases with Ser-Asp-His active site nomenclature, Asp 574/576 and His 733 of Sg-xProDPP are likely candidates for the catalytic triad (44). A computer assisted search for protein localization predicted neither signal sequence sites nor transmembrane regions in the protein (37). Although lacking an export signal and predicted to be cytoplasmic, activity detected on the cell surface and within supernatant revealed over 50% of activity to be extracellular at pH 7.5.

Southern Blot analysis revealed Sg-xPDPP to be a single copy gene (Fig. 2.5). A 2.3 kb ORF was examined in the electronic database for the presence of promoter elements and additional genes. Three open reading frames were revealed and designated ORF 1 (760 codons), ORF 2 (277 codons) and ORF 3 (236 codons). Sg-xPDPP is coded by ORF 1, while ORFs 2 and 3 are oriented upstream on complementary strands in reverse directions. A ribosome-binding site was identified 5 bp upstream of the start codon. The putative transcription start site was found 33 bp from the start Met as well as the TATA (-10 box) and -35 region promoters. Previous growth experiments with glucose and galactose revealed that the metabolism of glucose did not alter the total activity profile. The absence of sequence indicating a glucose repression operon corresponds with this observation. ORF 2 is 118 bp upstream from the start codon of ORF 1. The protein specified by ORF 2 has close to 70% identity with a hypothetical 30.9 kDa protein found in the PepX 5' region of L. lactis (31, 38) and shares significant

homology to proteins in the aforementioned databases.

Fig. 2.4. Multiple sequence alignment of *S. gordonii* x-Pro DPP (*Sg* xPDPP), putative streptococcal Pep X genes and other bacterial homologues. Sequences of *Sg*-xPDPP (Sgord) deduced from FSS2 genome, putative x-Pro DPPs from gene products of *Streptococcus pneumoniae* (Spneu) and *Streptococcus mutans* (Smut), cloned x-Pro DPP from *Lactococcus lactis lactis* 763 (Llac763), *Lactococcus lactis cremoris* (Lcrem), *Lactobacillus delbrueckii lactis* (Ldel17290), and cloned DPPIV from *Flavobacterium meningosepticum* (Fmenin) and *Porphyromonas gingivalis* (Pging) were aligned using the ClustalW multiple sequence alignment tool according to homology modeling (grey boxes indicate similarity and black boxes indicate identity). The putative catalytic residues (serine, aspartic acid and histidine) are denoted (*). The arrow marks a consensus motif flanking the active site serine in members of the x-Pro DPP family (*Pep X*).

Sgord Spneu Smut Llac763 Lcrem Lde17290 Fmenin Pging	1 1 1 1 1 1	MRYNQYSYTKASEDVMIDELARLGFTIQTTNSPKENLHHIQKILFRYQDVN MRFNQYSYTNFPKENVISELKKCGFDLQNTANHKDSLETFLRRFFTYQDTN MKYNQYSYIGTSVSQAEKELKELGFQISSQKTNKANLAFVSQVYFHNPDKD MRFNHFSIVDKNFDEQTAELDQLGFRWSVFWDEKKILKDFLIQSPSD MRFNHFSIVDKNFDEQTAELDQLGFRWSVFWDEKKILKDFLIQSPSD MKFNHFSIVDKNFDEQTAELDQLGFRWSVFWDEKKILKDFLIQSPSD MKYNQYAYVETSPEKATEELLAINELPENYSSLSFSELLAVITGNVLAEATTRQAKD MKKKIFSLSIAVVAFHGLSAQEITLDKIYSGQYRAKGIS.GTASLNDGENVATIP. MKRPVIILLLGIVTMCAMAQTGDKPVDLKEITSGMFYARSAGRGIRSMPDGEHVTEMNRE
Sgord Spneu Smut Llac763 Lcrem Lde17290 Fmenin Pging	53 53 48 48 58 57 61	.YVLSSWVADQKTDLLTFFQSDKQLTEEVFYTVALQVLGFAPFVDFDDVTAFCKEI .YPLSILAADKKTDLLTFFQSEDELTADIFYTVAFQLLGFSYLVDFEDSDVFRKET .DVFKSIIADSQTDLATFLHSDRELTEEIFYTIALQLLEFTPYIDFDE.AKTFIKHS MTALQATAELDVIEFLKSS.IELDWEIFWNIALQLLDFVPNFDFFIGKAFEYAKNS MTDLQATAELDVIEFLKSS.IELDWEIFWNIALQLLDFVPNFDFFIGKAFEYAKNS .AKLAEFAVDQTDLAAFLLDTP.TATTASQFANVALQLLGYHPNYDYSLTDPLTCGKKH .TGIAKYSYKTSQKEKNIVDGSFQGYTFSNDESKILLQKSS.Q.SIYRHS RTAIVRYNYASGKAVDTLFSVERARECPFKQIQNYEVSSTGHHILLFTDME.SIYRHS
Sgord Spneu Smut Llac763 Lcrem Lde17290 Fmenin Pging	108 108 103 103 116 104 118	HFPITYGNILENLYQLLNTRTKLGNTLIDQLVSEGFIPESNDYHFFNGKSLA GFPIIYGDLIENLYQLLNTRTKKGNTLIDQLVSDGLIPEDNDYHYFNGKSLA HFPIIFQPKHFLLNFYQLLGTRTKNGMTLIDKLVSQGFLPADNHYRYFNGKSLA NLPQIEAEMTTENIISAFYYLLCTRKKTGMILVEHWVSEGLLPLDNHYHFFNDKSLA NLPQIEAEMTTENIISAFYYLLCTRKKNGMILVEHWVSEGLLPLDNHYHFFNDKSLA ALPAFKDLTSKEELIFTFYRLLNTRSKNGQILLDVMAGKGYFTQFWGEGKFMFFNGKSLP FLGKFEVKDLKSRTVVSLNNANWIQEPKFSPDGSKVAFIADNNLF YRAAVYDYDVRRNLVKPLSEHVGKVMIPTFSPDGRMVAFVRDNNIF
Sgord Spneu Smut Llac763 Lcrem Ldel7290 Fmenin Pging	160 162 160 160 176 149 164	TFSSHEAIREVVYVESRVDTDGDGKPDLVKVSIIRPSYEGQVPAVMTASPYHQGTND TFSNQDVIREVVYVESRVDTDQKGLSDLVKVSIIRPRFDGKIPAIMTASPYHQGTND TFDTNALIREIVYVEAPLDTDKDGQLDLIKVNIIRPQTKAKLPVVMTASPYHQGTND TFDSSLLEREVLWVESPVDSEQRGENDLIKIQIIRPKSTEKLPVVMTASPYHLGIND TFDSSLLEREVLWVESPVDSEQRGENDLIKIQIIRPKSTEKLPVVMTASPYHLGIND VFDTSQVIREVVYVQSDLDTDGDGKGDLLPVTVFRPVESQDQLKVPALYTASPYFGGIID YQDLNTGKITQITTDGKKNEIINGLGDWVYEEEFGHADYYQWNKAGDALVFVRF IKKFDFDTEVQVTTDGQINSILNGATDWVYEEFFGVTNLMSWSADNAFLAFVRS
Sgord Spneu Smut Llac763 Lcrem Ldel7290 Fmenin Pging	217 219 217 217 236 203 218	KASDKALHNMNVDLSCKNPRTITVQESSIQTIEPQGQASLVEKAEEKLGHIG.SYTLNDY KASDKALYKMEGELEVKLPHKIELEKPQLNLVQPQGKAELIAEAEEKLTHINSSYTLNDY KGADKKLHQMEGELTVKKAATITVTDSHFQALQVPSSNLPISPAQESFSYID.SYTLNDY KANDLALHDMNVELEEKTSHEIHVEQKLPQKLSAKAKELPIVDKAPYRFTHGWTYSLNDY KANDLALHDMNVELEEKTSHEIHVEQKLPQKLSAKAKELPIVDKAPYRFTHGWTYSLNDY NVKTNHNVDENLTDATTWTNPKYVAKPLVKSPAPSDQDVPATELATGQSSYGLNEY DERKVPEINIPIYYQNLYPKLMTYKYPKAGEENSAVTAYLYQLSSGKSAQLNFG.SSEKY DESAVPEYRMPMYEDKIYPEDYTYKYPKAGEKNSTVSLHLYNVADRNTKSVSLP.IDADG
Sgord Spneu Smut Llac763 Lcremx4 Ldel7290 Fmenin	276 277 278 277 277 292 262	LLPRGFANLYVSGVGTKDSEGMMTSGDYQQIEAYKNVIDWLNGRCRAFTDHTRQREIKAT FLPRGFANLYVSGVGTKDSTGFMTNGDYQQIEAYKNVIDWLNGRCRAFTDHTRQRQVKAD FLARGFANIYVSGVGTKDSDGFMTSGDYAQIESFKAVIDWLNGRTTAYTSHKRDKRVMAD FLTRGFASIYVAGVGTRSSDGFQTSGDYQQIYSMTAVIDWLNGRARAYTSRKKTHEIKAS FLTRGFASIYVAGVGTRSSDGFQTSGDYQQIYSMTAVIDWLNGRARAYTSRKKTHEIKAS LLARGFASVFSGAIGNRHGDGIRITGSPEETISQKEVIEWLTGDRVAYTDRTRRFETKAS

Fmenin262YIPQLFQTNANDEIVVATANRHQNKVDLLKVNTKTAAVSKLFTETDNAWIETDNLT.MEFPging277YIPRIAFTDNADELAVMTLNRLQNDFKMYYVHPKSLVAKLILQDMNKRYVDSDWIQALKF

Sgord	336	WSNGKVATTGISYLGTMSNGLATTGVDGLEVIIAEAGISSWYNYYRENGLVTSPGGYPGE
Spneu	337	WSNGKVATTGLSYLGTMSNGLATTGVDGLEVIIAEAGISSWYNYYRENGLVTSPGGYPGE
Smut	338	WTNGLVATTGKSYLGTMSTGLATTGIKELKVIIAESAISSWYDYYRENGLVCSPGGYPGE
Llac763	337	WANGKVAMTGKSYLGTMAYGAATTGVEGLELILAEAGISSWYNYYRENGLVRSPGGFPGE
Lcrem	337	WANGKVAMTGKSYLGTMAYGAATTGVEGLEVILAEAGISSWYNYYRENGLVRSPGGFPGE
Ldel7290	352	WCSGNVCMTGRSYLGTLQIAIATTGVKGLKTVVSEAAISSWYDYYRENGLVRSPGGFPGE
Fmenin	321	LDDNSFLWASERDGHRHLYWYDAAGKLKKOVSKGDWEIINYYGYNPKTKEVYIQTTEKGS
Pging	337	TAGGGFAYVSEKDGFAHIYLYDNKGVMHRRITSCNWDVTKLYGVD.ASGTVFYQSAEESP
Sgord	396	DFESLTELTYSRNLLAGEYLRHNQAYQAYLDQQRKDLERETGDYNQFWHDRNYLIHADKV
Spneu	397	DFDSLAELTYSRNLLAGDYIRGNEAHQADLEKVKAQLDRKTGDYNQFWHDRNYLINAHKV
Smut	398	DTDVLTELTYSRNLAAGDYLRNNAQYQKMLAEQVKQIDRTSGDYNQFWQDRNYLPHAHKI
Llac763	397	DLDVLALTYSRNLDGADFLKGNAEYEKRLAEMTAALDRKSGDYNQFWHDRNYLINTDKV
Lcremx4	397	DLDVLALTYSRNLDGADFLKGNAEYEKRLAEMTAALDRKSGDYNQFWHDRNYLINTDKV
Lde17290	412	DLDVLALTYSRNLDGADFLKGNAEYEKRLAEMTAALDRKSGDYNQFWHDRNYLINTDKV
Fmenin	381	DMDKLAEVCQS.NLWDGGNFTAKKAYEAEQAELLAAQDRATGQYSDFWESRNYRHHTDGI
Pging	396	INKVSKLNIN
Sgord	456	KAEVVFTHGSQDWNVKPLHVYNMFHALPAH.IKKHLFFHNGAHVYINNWQSIDFRESMNA
Spneu	457	KAEVVFTHGSQDWNVKPLHVYOMFHALPTH.IHKHLFFHNGAHVYMNNWQSIDFRESINA
Smut	458	KAHVVYTHGLQDWNVKPNQVYYIFNALPEE.IOKHIFLHOGOHVYMHNWQSIDFRESMNA
Llac763	457	KADVLIVHGLQDWNVTPEQAYNFWKALPEG.HAKHAFLHRGAHIYMNSWQSIDFSETINA
Lcrem	457	KADVLIVHGLQDWNVTPEQAYNFWKALPEG.HAKHAFLHRGAHIYMNSWQSIDFSETINA
Ldel7290	471	KCSWISVHGLNDWNVKPKNVYKIWQKVKOLPVKSHIFLHOGPHYMMNNLVSIDFTDLMNL
Fmenin	423	AKVPTKY.ILKDANGKDVKELONNDDLLNK.LKSDNFIAK.EFITIPN.AAG.DOMNA
Pging	437	AMTPTVVSVFRSKDAKELRTLEDNVALRER.LKAYRYNPK.EFTIKT.QSG.LELNA
Sgord	515	L SKKLIGHSSDFDLPPVIWQDNSQAQNWMSLDDFGNQEDYSHFHLGKGSQE RN
Spneu	516	L TKKLLGQFTDFOLPTVIWQDNTAPOTWLSLDNFGGQENCETFSLGQEEQAIQN
Smut	517	L SEELLGLQNHFOLPTIIWQDNSQVOTWKKLNNFG.SHQTRQFSLGQEKKIDN
Llac763	516	YFVAKLLDRDLNLNLPPVILQENSKDQVWTMMNDFG.ANTQIKLPLGKTAVSFAQFDN
Lcrem	516	YFVAKLLDRDLNLNLPPVILQENSKDQVWTMMNDFG.ANTQIKLPLGKTAVSFAQFDN
Lde17290	531	WFVHELLEVSNGAYEQWPKVMIQDNLEADEWHAESDWASDLGQASLYLPTADGDLSTVEN
Fmenin	476	WMIKPKNFDPAKXYPVFMFQYSGPGSQQVANSWDGGNGIWFDMLAQKCYLVVCVDG
Pging	491	WIVKPIDFDPSRHYPVLMVQYSGPNSQQVLDRYSFDWEHYLASKCYVVACVDG
Sgord Spneu Smut Llac763 Lcrem Lde17290 Fmenin Pging	570 571 573 573 591 532 544	* * * RYSDEDYNRFAKSYOVFKNELFEGKTOQITLDWTLEQDLFINGPAKL QYPDKDFERYGKTYOTENTELYQGKANQITINLPVTKDLHLNGRAQL HYPSSDFKLYSDUHAFKHDLFLKKANEIAIDDFIQEDWLVNQQIKL NYDDETFKKYSKDFNVFKKDLFENKANEAVIDLEPSMLTINGPVEL HYDDETFKKYSKDFNVFKKDLFENKANEAVIDLEPSMLTINGPVEL GTGQLTFTDLGGT DFK KQGISETDWEYQFISGEEKWAKASLFESEFILPTTLVGRPKV RGTGFRGTKYKKVTYKNLGKYEIEDQTAAKWUGNQSYVDKSRI RGTGARGEEWRKCTYMQLGVFESDDQIAAATAIGQLPYVDAARI
Sgord Spneu Smut Llac763 Lcrem Lde17290 Fmenin Pging	617 618 620 620 651 576 588	KLRLKSSTNKGLISAQILDYGPAKRLTPIPS LEPRVMDNGRYMLDNLMELP.FADIPH NLRIKSSTNKGLLSAQILEFGQKKYLQPYPAI NLTLKSSSNKGLLSAQVLDYGQKKRFSDLPTILEHNSIDNGQNESREALRELP.FKKAPY ELRLKLNDTKGFLSAQILDFGQKKRLEDKVRVKDFKVLDRGRNFMLDDLVELP.LVESPY ELRLKLNDTKGFLSAQILDFGQKKRLEDKARVKDFKVLDRGRNFMLDDLVELP.LVESPY RVRVAANKTVGQLSVALVDLGTRQRLTATEK FARGNQPFGYRFGADSLQEFVPDKA KA GIFGWSYGGYTTLMSLCRG.NGTFKAGIAVAPVTNWRFYDSIYTERFLGTPQENKDGY GIWGWSYGGYTTLMSLCRG.NGTFKAGIAVAPVADWRFYDSVYTERFMRTPKENASGY
Sgord Spneu Smut Llac763 Lcrem Lde17290 Fmenin Pging	676 677 679 679 711 633 645	* RVITKGFINLQNRTDLLTVEEVVPNQM ELSFELQPTIKKKKDQLRJVLYTDFEHTV RIITKGVINLQNRTDLLTIEDIFPNKMM TITFNLQASIYOLOKGDSLRIVLYTDFEHTV QLVTKGFTNLQN.QSLLTVSDLKADBWF TIKFELQPTIYHLEKADKLRVILYSTDFEHTV QLITKGFTNLQN.QNLLTVSDLKADBWF TIKFELQPTIYHLEKADKLRVILYSTDFEHTV KLITKAHMNLQNYQDMKQPSKLEAGQFV DLEFELQPTYYTLPAGAKLGLIIYSTDQGMTK DLNSPTTYAKLLKGKFTLIHG.TADDNV HLQNNMEFSEALTQNKKQFDFMAYPDKNHSII KMSSALDVASQLQGNLIIVSG.SADDNV HLQNTMLFTEALVQANIPFDMATYMDKNHSIY

Sgord	736	RDKTDYHLSVDMEHSSLSLPHKKS
Spneu		<u> </u>
Smut	737	RDNSNYILVVDLAKSTIEIPIA
Llac763	738	RDNRKVTYEIDLSQSKLIIPIESVKN
Lcrem	738	RDNRKVTYEIDLSQSKLIIPIESVKN
Ldel7290	771	RPLETEDYTVDLAGTALLLYRK
Fmenin	692	GGNTRPQLYEKMTNYILENL
Pging	704	GGNTRYHLYIRKAKFLFDNL

Genetic Analysis of Sg-xPDPP in S. gordonii FSS2 and Viridans Streptococci. A Southern Blot of the S. gordonii FSS2 genome (Fig. 2.5) reveals a single hybridizing band for four of the restriction enzyme digests under high stringency conditions. All digests show single bands as would be expected for a single copy gene that does not harbor any digest sites within its sequence. Strains representing the major serovars of S. gordonii, as well as mitis group members, S. parasanguinis, S. mitis and S. oralis, and related viridans member, S. salivarius, were screened for xPDPP homologues (Fig 2.6). The protein was conserved in all S. gordonii strains tested and in S. mitis, but was not detected in S. parasanguinis, S. oralis and S. salivarius. In the strains in which xPDPP was detected, the probe hybridized with a 3.0-kb HindIII fragment, except that in S. gordonii PK48 the band was 7.0-9.0-kb. There is little evidence here for variability in the *xPDPP* gene and it appears highly conserved in representative strains of *S. gordonii*. The difference with PK48 band size may be due to inaccessability of genomic DNA to the restriction endonuclease, while the decrease in hybridization intensity is probably the result of lower DNA sample quantity. We believe the fainter S. mitis intensity is due to less specific hybridization, hence sequence divergence from S. gordonii. The inability for other strains to maintain homologues is consistent with phylogenetic relationships discussed in Figure 1.6. Although, other mitis group members and S. salivarius lack a close genetic homologue to xPDPP, protein sequence analyses with S. pneumoniae and S. *mutans* make it highly probable for protein homologues among these members.

M 1 2 3 4



Fig. 2.5. Copy Number of *S. gordonii* x-Pro DPP (*Sg- xPDPP*) gene in strain
FSS2 as revealed by restriction endonuclease analysis. Lanes: M, markers (kb) 5.0,
3.0, 2.0, 1.5 and 1.0; 1. BamHI, 2. EcoRI, 3. HindIII, 4. Pst 1.

M 1 2 3 4 5 6 7 8 9



Fig. 2.6. Conservation of *Sg-xPDPP* in viridans streptococcus (mitis group).
Southern blot of HindIII-digested chromosomal DNA probed with 2.3-kb clone of *Sg-xPDPP*. Lanes: M, markers (kb) 5.0, 3.0, 2.0, 1.5 and 1.0; *S. gordonii* 1. FSS2, 2. PK48,
3. DL1, 4. PK2585, 5. *S. parasanguinis* PK2584, 6. *S.mitis* J27, 7. *S. oralis* J21,8.
PK34, and 9. *S. salivarius* ATCC 27945.

Discussion

This report describes the purification, characterization, cloning and sequence analysis of an extracellular x-Pro dipeptidyl peptidase from *S. gordonii* FSS2. This activity has been described in studies concerning tripeptide degradation systems of *S. sanguinis* and *S. mutans* (8) and collagen degradation by *S. gordonii* Challis in the IE model (19). Our work presents the first biochemical and genetic studies on purified enzyme. Filtrate obtained from a pH-controlled culture of *S. gordonii* FSS2 enabled the purification of *Sg*-xPDPP by over 6000 fold. The preparation was determined to be homogenous and consisted of two identical, non-covalently attached monomers in the native state. Inactivation studies revealed a serine-class catalytic mechanism in which DPPIV-family inhibitors appear to be the most effective. Specificity studies were conducted using para-nitroanalides and peptides smaller than 20 residues. Limited proteolysis was observed upon incubation with either substrate. The requirement of Pro or Ala in the penultimate position and prohibition of Pro in the P1' site define the specific nature of this peptidase.

Collectively, the biochemical data are consistent with DPPIV-like peptidases studies from *Streptococcus mitis* (11), *Streptococcus thermophillus* (33) and *Streptococcus salivarius* (35). Comparison of the *Sg*-xPDPP protein sequence with cloned x-Pro dipeptidyl peptidases reveal significant homology (41% average identity) with *PepX* gene products of lactic acid bacteria (31, 34). Putative genes for x-Pro dipeptidyl peptidase were identified in unfinished microbial genomes for *S. mutans* and *S. pneumoniae* and maintained 57% and 72% identity to *Sg*-xPDPP, respectively. The consensus sequence, GXSYLG, is present at the active site of PepX and streptococcal

members. Phylogenetically-related viridans members, *S. pneumoniae* and *S. gordonii*, maintain hydrophobic residues at the second position which are a contrast to basic residues conserved in other species. The PepX gene and streptococcal analogue appear to have diverged from a common ancestor that bears little sequence homology to the CD26/DPPIV family of eukaryotic and gram negative peptidases. *Sg*-xPDPP closely resembles members of the S15 family (EC 3.4.14.11) yet our discovery of streptococcal members with remarkable evolutionary conservation may warrant a change in nomenclature.

The localization of peptidases outside of the cell is considered to be a necessary mechanism for the degradation and intracellular transport of endopeptidase-liberted peptides. Currently, all reported x-Pro DPP members from Gram-positive bacteria have been either associated with cytoplasmic, membrane or whole cell extracts (24). SgxPDPP represents the first peptidase from this group isolated from an extracellular source. Culture conditions revealed the presence of 1 mM Ca²⁺ rather than trace levels in complex media to be necessary for export from the cell. The presence of this cation may alter membrane permeability, non-specifically, or enhance an unknown Ca²⁺-dependent mechanism for secretion. Additionally, excess arginine (50 mM) greatly reduced levels of the secreted protein. The presence of the free amino acid and/or the utilization of the ADI pathway as a continued source of energy could inactivate an export mechanism while still promoting the cell-surface localization of the protease. Sequence analysis did not reveal a N-terminal signal sequence, LPXTG motif (39) or post-translational modifications that could account for translocation from the cytoplasm. We found the gene for Sg-xPDPP to be single copy, ruling out the possibility of an altered form of the

peptidase. Previous reports have suggested that such enigmatic transport could be the result of "specific leakage" of cytoplasmic aminopeptidase from cells (51) or the shedding of protease/peptidoglycan complexes as a consequence of cell wall turnover (19). In S. gordonii G9B, extracellular protein profiles were altered by changes in pH, growth medium composition and rate of growth (23). Additionally, the secretion of two cytoplasmic proteins from S. gordonii, a GAPDH (40) and collagenase (19) were observed upon growth at a constant, neutral pH. In studies conducted by Harty et. al., specific activities of extracellular proteases (thrombin-like, Hageman-factor and collagenase) from FSS2 increased several fold upon a shift in pH from 6.5 to 7.5 (13). This data is consistent with maximum detection of our Sg-xPDPP activity at a controlled pH of 7.5. A similar level activity was observed at pH 7.0 but accounted for lower proportion of the secreted form. Furthermore, the peak of Gly-Pro-pNa activity occurred during early stationary phase when excess glucose had been exhausted. The alkaline environment of the buffered vegetation contacting the bloodstream may be more suitable for enzyme release from the cell. An extracellular peptidase would therefore better serve the nutritional requirements of the bacteria in a fibrin/ECM surrounding versus the carbohydrate-rich oral environment.

The biological significance of Sg-xPDPP outside of the cell would serve a threefold purpose, 1) the production of proline-containing dipeptides that could subsequently be transported into the cell, 2) a synergy with other secreted proteases that would permit the degradation of host proteins and 3) the modification of bioactive peptides at the endothelium. *L. lactis* and other lactic acid bacteria are unable to import free Pro which must be transported in a peptide-bound configuration (24). A tripeptide

transport system was identified in *S. sanguinis* that permitted the assimilation of free amino acids by membrane-bound and intracellular proteolysis. It was further concluded that x-Pro dipeptides were insufficient for transport (8). This is counterintuitive to the action of Sg-xPDPP that results in high concentrations of extracellular dipeptides. In *S. gordonii*, uptake could be driven by an inwardly directed chemical gradient maintained by fluctuating levels of extracellular and cell-associated products of peptide hydrolysis. The limited cleavage specificity of Sg-xPDPP prohibits the independent proteolysis of constituents within and surrounding the thrombotic vegetation.

Neither type IV collagen nor fibrinogen were suitable substrates for the enzyme. This contrasts the findings by Juarez and Stinson that reports a Gly-Pro-pNa activity capable of endopeptidase attack on native collagen (19). Culture supernatant of FSS2 yielded several endopeptidase activities capable of degrading denatured collagen, fibrinogen and azocasein. Two additional extracellular aminopeptidases, an Arg-specific aminopeptidase (RAP) and PepV di/tri-peptidase (manuscripts in preparation), would participate in the acquisition of small peptides from the protein meshwork surrounding the bacteria.

Although Sg-xPDPP achieved proteolysis of all suitable peptides tested, there is an apparent restriction of substrate size. Three cytokines tested (RANTES, MIP-1 β and GM-CSF), presenting unblocked x-Pro motifs, failed to undergo cleavage upon extended incubations and increased E:S molar ratios. This is in contrast to the activities of DPPIV/CD26 members which facilitate the N-terminal proteolysis of these larger substrates despite their high catalytic similarity to the Pep X members (16, 43). Sequence divergence in specificity pockets and the presence of adhesion domains, absent in Gram-

positive peptidases, may contribute to this phenomenon. The removal of two dipeptides from substance P was observed and this heptapeptide product has been found to display biological activity more potent that the intact peptide (55). Additionally, the concerted action of an extracellular Arg aminopeptidase and Sg-xPDPP produce a truncated form of bradykinin that has lost essential residues for receptor activation (3). The combined effect of these modifications may result in local changes in vascular permeability and smooth muscle contraction at the infected endothelium. The truncation of fibrin inhibitory peptides by Sg-xPDPP could have consequences on thrombus formation and the overall growth of the vegetation. The soluble tetrapeptide (GPRP), representing the N-terminus of the fibrin α -chain, blocks the polymerization of fibrin monomers leading to blocked polymerization, decreased clotting and increased clotting times (27). The minimum structural unit (GPR) is required for inhibitory action. Sg-xPDPP may inactivate circulating inhibitor in the growing thrombus and alter the balance between polymerization/fibrinolysis in favor of the growing vegetation. Studies with the micepassaged FSS2 strain indicated levels of cell-surface activity close similar to the basic laboratory strain. Levels of extracellular Sg-xPDPP were roughly 4x lower in the micepassaged strain. This may indicate an alteration in secretion mechanism or mutation upon entry into an animal model. Although this data was counterintuitive to the secretion hypothesis, regrowth in media could induce new stress responses or alter gene expression in a manner inconsistent with in vivo human colonization. Future studies will focus on the expression and knockout of Sg-xPDPP in order to evaluate its relative contributions to streptococcal virulence and survival at the site of infection.

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CHAPTER 3

AN EXTRACELLULAR ARGININE AMINOPEPTIDASE (RAP) FROM

STREPTOCOCCUS GORDONII FSS2¹

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Abstract

Streptococcus gordonii is generally considered to be a benign inhabitant of the oral microflora. However, it is also a primary etiological agent in the development of subacute bacterial endocarditis (SBE) producing thrombus formation and tissue damage on the surface of heart valves. Strain FSS2 of S. gordonii has been found to produce several extracellular aminopeptidase and fibrinogen-degrading activites during growth in a pHcontrolled batch culture. In this report we describe the purification, characterization and partial cloning of a predicted serine-class arginine aminopeptidase (RAP) with some cysteine-class characteristics. Isolation of this enzyme by anion-exchange, gel filtration and IEF chromatography yielded a protein monomer of approximately 70 kDa, as shown by MALDI-TOF mass spectrometry, gel filtration and SDS-PAGE under denaturing conditions. Nested PCR cloning enabled the isolation of a 324 bp-long DNA fragment encoding the108 amino acid N-terminus of RAP. Culture activity profiles and N-terminal sequence analysis indicated the release of this protein from the cell surface. Homology was found with a putative dipeptidase from Streptococcus pyogenes and non-specific dipeptidases from Lactobacillus heleveticus and Lactococcus lactis. We believe that RAP may serve as a critical factor for arginine acquisition during nutrient stress in vivo and also in the proteolysis of host proteins and peptides during SBE pathology.

Introduction

Streptococcus gordonii is a frequently researched member of the viridans family of oral streptococci (55). Viridans streptococci are primary colonizers of human dental plaque where they serve a critical role in the establishment of microbial communities associated with good oral health. Although considered benign inhabitants of the oral microflora, members have been implicated in the systemic disease, subacute bacterial endocarditis (SBE) (8, 33). The progression of this disease state requires: 1) trauma (congenital or inflammatory) to the endothelial valve surface such that it is predisposed to colonization, 2) adhesion of organisms to the modified valve surface after their entry into the bloodstream via the oral cavity and 3) the propagation of infected vegetations consisting of bacteria in a fibrin-platelet meshwork (52). Despite the uniform susceptibility of these organisms to β -lactam antibiotics and their lack of classical streptococcal virulence factors, they can cause a chronic inflammation and/or lifethreatening disease with periods of latency and recalcitrant infection (9, 18).

The ability of these organisms to colonize biofilm surfaces within two distinct microenvironments evoked studies into their patterns of gene expression and dynamic metabolism. *Streptococcus sanguinis*, studied as a model for viridans pathogenesis, is known to express cell surface adhesins and a platelet aggregation-associated protein (PAAP) that facilitate both colonization and thrombosis (16, 19). Upon their entry into the bloodstream, bacteria undergo a shift in pH from mildly acidic in dental plaque (6.0-6.5) to neutral pH (7.3) of the blood (44). This is significant since proteins extracted from *Streptococcus oralis* grown in batch culture and analyed by 2D electrophoresis indicates 39 proteins with altered expression at pH 5.2 vs. 7.0 (56). Indeed, *in vivo* Expression

Technology (IVET) conducted on the *S. gordonii* rabbit model for IE to detect genes activated in the new environment indicated an upregulation of the *msrA* oxidative stress gene (54) and the induction of genes encoding carbohydrate metabolism enzymes, protein transporters and cell surface proteins (26). The expression and secretion of glycosidase and peptidase activities, as examined in pH-controlled batch cultures, was found to be down-regulated by acid growth conditions and up-regulated by growth in a neutral pH environment supplemented with serum (17).

Survival in vivo is dependent on the ability of the bacterium to remove sufficient quantities of nutrients from its environment. It is presumed that S. gordonii meets these needs by degrading salivary carbohydrates and glycoproteins in the oral cavity, while utilizing plasma proteins when growing on heart valve surfaces. The vegetation biofilm is known to retard the diffusion of antibiotics (24) and solutes generally diffuse into the interior at slower rates than in water (51). Thus, access to carbohydrates and plasma proteins within the vegetation remains problematic. During such conditions where carbohydrates are scarce, oral streptococci can catabolize arginine via the arginine deiminase (ADI) pathway, resulting in the synthesis of both ATP and other essential metabolic precursors (7, 14). The utilization of this amino acid is then prioritized so that the transport and metabolism of arginine-containing peptides may become particularly important. Amino acid and peptide transport systems for viridans streptococci members have been described (2, 5, 10, 25, 31), and it has been shown that amino acids and small peptides are readily imported while those exceeding size limitations require hydrolysis by endo- and exopeptidases either present on the surface or secreted by these cells (6). One such activity that could meet the transport and metabolic challenges in vivo is an arginine

aminopeptidase. Such an enzyme has previously been described from cell wall and cytoplasmic preparations of *S. mitis* and *S. sanguinis* (1, 12, 21) but has eluded detailed investigations. Here, we describe the purification, characterization and partial cloning of the first reported extracellular arginine aminopeptidase (RAP) derived from *S. gordonii* FSS2, a strain previously isolated from the bloodstream of an SBE patient (38).

Experimental Procedures: Materials

H-Gly-Pro-pNa, H-Gly-Arg-pNa, H-Gly-pNa, Ile-Pro-Arg-pNa, L-Leu-pNa, Lys-ArgpNa, L-Pro-pNa, Sar-Pro-Arg-pNa, Di-isopropyl fluorophosphate (DFP), N α -p-tosyl-Llysine-chloromethyl ketone (TLCK), iodoacetamide, O-phenanthroline, amastatin. apstatin, bestatin, β -mercaptoethanol, L-Cysteine, L-Arginine, L-Lysine, Gly-Pro, Gly-Gly, angiotensin III, fibronectin binding inhibitor, platelet aggregation inhibitor, sexual agglutination peptide, substance P, thymopentin fragment, bradykinin, lymphocyte activating pentapeptide fragment and fibrin polymerization inhibitor were obtained from Sigma. Pefabloc SC, 3,4-dichloroisocoumarin and E-64 from Boehringer Mannheim. Anistatin-related peptide, Bz-Arg-pNa, H-Arg-pNa, H-Ala-pNa, H-Ile-pNa, H-Lys-pNa, H-Val-pNa and protein kinase C substrate were from Bachem. Protein kinase c peptide was purchased from American Peptide Company and H-Glu-(NHO-Bz) pyrollidide from Calbiochem. α_1 Proteinase inhibitor (α_1 PI) and α -2 macroglobulin were purchased from Athens Research and Technology, Athens, Ga.

Experimental Procedures: Methods

Bacterial Growth. *S. gordonii* FSS2 (previously *S. sanguinis* FSS2 (17, 38)) was stored and maintained (at -80°C) as previously described (40). Frozen cells were inoculated into autoclaved medium containing 20 g/L trypticase peptone (BBL), 5 g/L

yeast extract (Difco), 2 g/L NaCl, 0.1 g/L CaCb, 4 g/L K₂HPO₄ and 1 g/L KH₂PO₄. Ten g/L glucose and 0.5 g /L Larginine were filter sterilized and subsequently added. A static culture (200 ml) was grown overnight at 37°C, and a 15L batch culture further inoculated and grown in an atmosphere of 5% CO₂ and 95% N₂ at 37°C with the pH held constant at 7.0 by addition of 5M KOH. Cultures were harvested in early stationary phase at a point when the bacteria had metabolized all available glucose and the addition of base was no longer required.

Enzymatic Assay. Amidolytic activities of crude samples and purified protease were measured using the substrate H-Arg-pNa (1 mM final concentration) in Buffer A (50 mM Tris, 1 mM CaCb, pH 7.8) at 37°C. Assays were performed on 96 well plates (0.1 ml) using a thermostated microplate reader, and the release of p-nitroaniline was measured at 405 nm. (Spectramax, Molecular Devices). Inhibition assays involved pre-incubation of pure enzyme with inhibitor for 10 min. at 37°C, followed by measurement of residual activity.

Enzyme Purification 4 L of cell-free culture filtrate was obtained after centrifugation (20 min., 4 °C at 6000 x g) of the batch culture. Proteins in the filtrate were precipitated with $(NH_4)_2SO_4$ over several hours at 4 °C to a final concentration of 80% and the precipitate pelleted by centrifugation at 8000 x g for 40 min. Pellets were resuspended in 30 ml of buffer A and dialyzed over 2 days (4 °C), with changes, against 40 volumes of the same buffer. The dialyzed material was applied to a DE52 (Whatman) column (2.5 x 30 cm, 150 ml) equilibrated with buffer A, which was then washed with 3 column volumes of buffer A at 1ml/min. All protein that eluted in the flow-through was pooled and concentrated by ultrafiltration. The concentrated sample (10 ml) was loaded

onto and eluted from a SuperdexTM-75 (Amersham Pharmacia Biotech) HR 10/30 equilibrated with gel filtration buffer (50 mM Tris, 200 mM NaCl, 1 mM CaCb, 0.02% sodium azide, pH 7.8) and eluted with the same buffer at 1 ml/min. Peak activities were combined and concentrated. The sample (10 ml) was then applied to a Mono Q HR 10/10 FPLC column (Amersham Pharmacia Biotech) equilibrated with buffer A. The column was washed with equilibration buffer until the base line stabilized, a linear gradient (0-500 mM NaCl in buffer A) applied, and peak enzyme activity eluted between 200-250 mM NaCl. After pooling and concentration of active fractions, protein was dialyzed against 25 mM N-methyl piperazine, pH 5.3 and loaded onto a Mono-P 10/5 FPLC chromatofocusing column equilibrated the same buffer. The column was then washed with four column volumes of equilibration buffer and a pH gradient developed with 40 ml of 10 x diluted Polybuffer 74 (Amersham Pharmacia Biotech) adjusted to a pH of 3.8. Peak activity was pooled and concentrated to a volume of 2.0 ml.

Protein Determination Protein concentration was determined using a bicinchoninic acid BCA reagent kit (Sigma) according to the manufacturer's protocol.

Electrophoresis. Enzyme purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel and the Tris-HCl/Tricine buffer system, according to Shagger and von Jagow (48). For aminoterminal sequence analysis, RAP was resolved by SDS-PAGE, followed by a polyvinylidene difluoride electroblotting to membrane using 10 mΜ 3-(cyclohexylamino) propanesulfonic acid, 10% methanol, pH 11 (39). The blot was airdried and subjected to sequencing.

Enzyme Kinetics and Specificity. Kinetic values were measured using H-ArgpNa and other substrates at varying concentrations [25 μ M to 2 mM], with a fixed enzyme concentration at 34 nM, in 100 mM Tris, pH 7.8, at 37 °C. V_{max} and K_m values were obtained through Hyperbolic Regression Analysis (shareware from J.S. Easterby, University of Liverpool, UK).

Specificity studies utilized RAP incubated with 5 μ g of peptide in a 1:1000 [E:S] molar ratio. Reactions were done in 100 μ l volumes with 100 mM Tris, pH 7.8, at 37 °C for 2 hours. Digestions were terminated by acidification with 10 μ l of 10 M HCl, followed by centrifugation (10,000xg, 5 min.). The entire supernatant was applied to a reverse phase high pressure liquid chromatography using an LC-18 column (25 x 4.6 mm, 5 μ m)(Supelco) equilibrated with 0.1% TFA in HPLC grade water and developed with an acetonitrile gradient (0-80% in 0.08% TFA over 50 min.). Peaks were manually collected and analyzed by mass spectrometry.

Mass Spectrometry. Peptides and native RAP were analyzed by MALDI-TOF on a Hewlett-Packard G2030A mass spectrometer. The instrument was operated at an accelerating voltage of 28 kV, an extractor voltage of 7 kV, and a pressure of 7 x 10^{-7} Torr. Samples were dissolved in sinapanic acid and ionized from the probe tip using a nitrogen laser source. Calibration was performed using mixtures of peptides/proteins of known molecular masses.

Internal Sequencing. Proteins and peptides were sequenced by Edman degradation in a model Procise-cLC sequencer (PE Biosytems, Foster City, CA) operated using the manufacturer's protocol. To obtain internal sequences, proteins were in-gel digested with trypsin (Promega, sequence grade) and the peptides extracted (47), with

their masses being determined by reflectron MALDI-TOF mass spectrometry using a Bruker Daltonics ProFlex instrument (45). The selected peptides were sequenced by Edman Degradation.

Cloning of DNA fragment encoding N-terminal region of RAP. DNA from S. gordonii was purified using the Purgene DNA Isolation Kit (Gentra, Minneapolis, MN) according manufacturer's instructions. The to internal peptide sequence (DLTADGSTLFGR) was used to search the unfinished S. gordonii database available at Genomic Research (TIGR) (ftp://ftp.tigr.org/pub/data/s gordonii/). The Institute of Briefly, DNA sequences were imported into Microsoft Word and searched for a matching DNA sequence encoding the peptide using wild cards at degenerate code positions. One matching contig was found that contained a DNA sequence encoding the N-terminus and internal fragment of RAP. Subsequently, two PCR primers were synthesized (5'-AGAGGATCCATGAAGAAAATACTGCTC-3' and

5'-GAA<u>GGATCC</u>GTTACTTCCG TTGTCAC-3') and used in the PCR to obtain a DNA fragment encoding the N-terminal fragment of RAP. PCR was performed using the Pwo DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN), 1 μ g of *S. gordonii* DNA and 500 ng of the primers (94°C for 1 min, 65°C for 1 min, 72°C for 1 min, 32 cycles). A single 340 bp-long PCR product was obtained, gel-purified, digested with Bam HI, subcloned into the Bam HI site of pUC19 and sequenced. The obtained sequence was deposited in GeneBank under accession number AY052829.

Southern Blot Analysis. Four µg of bacterial DNA was digested with each of the

restriction enzymes BamHI, BgIII, SacI, SpeI, EcoRI, HindIII or PstI overnight at 37° C. Digestion products were separated in 0.7% agarose gels using Standard procedure (Sambrook). Gels were soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min followed by neutralization solution (1.5 M Tris pH 7.5, 1.5 M NaCl) for 1 hour. Separated DNA was transferred onto a nylon membrane (Amersham-Pharmacia Biotech) by capillary transfer and subsequently fixed by cross-linking (Stratalinker, Stratagene Inc.). Membranes were prehybridized in 0.5 M phosphate buffer pH 7.2, 7% SDS and 1 mM EDTA at 50C for 1 hour, ³²P-labelled DPP cDNA probe was added and incubated overnight. After hybridization, non-specifically bound radioactivity was removed by a single wash in 2xSSC and 0.1% SDS at room temperature for 20 min followed by two subsequent washes in 0.5xSSC and 0.1%SDS at 50C for 1-4 hours.

<u>Results</u>

Growth and Culture Activity. Preliminary experiments indicated the presence of an extracellular H-Arg-*p*Na activity in culture media. Detection of this amidolytic activity in cell free filtrate was observed during growth in culture supplemented with 50 mM glucose and 3.5 mM arginine and was dependent on a pH maintained in the range of 6.0-7.0. Growth and activity from a 1 L pH-controlled (pH 7.0) batch culture were monitored at hourly intervals (Figure 3.1). Detection of extracellular and cell-associated H-Arg-*p*Na activity did not occur until early stationary phase when bacteria had metabolized all available glucose and the addition of base had ceased. A substantial increase in activity occurred between hours 6 and 7 when extracellular activity increased fivefold while cellassociated activity had a slight increase of 1.5 fold. Examination of cultures in stationary phase revealed a constant level of H-Arg-*p*Na activity secreted into the medium, whereas activity on cell-surfaces peaked at 8 hours (approximately 1/3 of the activity found in
media) and fell to basal (pre-stationary) levels at the final time point. This latter phenomenon may be the result of the complete export of H-Arg-pNa activity from the cell surface.

Further growth experiments were conducted under similar conditions but varied in culture environments with respect to pH control, carbohydrates, arginine, Ca²⁺, strain type and addition of serum (Fig. 3.2). Measurements were made at the 10th hour of growth when stationary phase was evident by lack of glucose consumption. The activity profiles during varied pH-controlled conditions provided evidence for a dependence on pH for the expression of H-Arg-pNa activity. Cell-associated and extracellular activities were detected under all conditions, except those devoid of pH control, with the least activity measured in more alkaline cultures (pH 7.5 and 8.0). Appreciable extracellular activity was evident at pH 6.3 and 7.0. Growth at pH 7.0 accounted for the greatest total activity, while cells at pH 6.5 contributed 30% less. Growth under both these pH conditions maintained an equal proportion of total activity (70-75%) to the secreted form. The remaining culture conditions were controlled at pH 7.0 for standardization and optimum enzyme expression. Excess supplemented arginine (50 mM) provided the greatest level of total activity while it has little effect on the level of extracellular H-ArgpNa. The 25 mM glucose culture was unable to reach the appropriate stationary phase in the 10 hr. timeframe and thus cannot be accurately compared. The expression of enzyme seemed dependent on the addition of 1 mM Ca²⁺ since culture without exogenously supplied Ca²⁺ could only produce cell-surface enzyme. Only a slight increase in the secreted form was detected under this condition. The addition of 25% fetal bovine serum to the starting media provided a drastic decrease (2.5x) in total activity with a significant drop $(^{1}/_{3})$ in the proportion of secreted activity. Mouse *S. gordonii* FSS2 assays showed no significant difference in activity profile. Although, an overall decrease in total activity was seen in galactose-supplemented cultures, this sugar neither repressed nor activated the release of activity from cells. This indicates a potential absence of a glucose repressor.



Fig. 3.1. *S. gordonii* FSS2 growth and activity curve from a pH 7.0-controlled culture supplemented with 50 mM glucose and 3.5 mM arginine. Samples (1 ml) were removed from culture at fixed timepoints and cells were removed from media by centrifugation (5 min, 4 °C, 13,000 x g) followed by 2x wash and resuspension into initial volume with non-supplemented media. Symbols: , whole culture OD; \blacklozenge , cell-free culture fluid; , whole cells. Assays were performed as previously described using 30iL of sample.



Fig. 3.2. Extracellular and cell-surface RAP activities under variable growth conditions. Samples (1 ml) were removed from culture at early stationary phase and cells were removed from media by centrifugation (5 min, 4 °C, 13,000 x g) followed by 2x wash and resuspension into initial volume with non-supplemented media. Symbols: , cell-free culture fluid; \Box , whole cells. Assays were performed as previously described using 30i L of sample.

Enzyme Purification. Maximum activity for the purpose of isolation was achieved using a 4 L batch culture held at pH 7.0 with added base and harvested during early stationary growth. An 80% ammonium sulfate precipitation concentrated extracellular proteins to a workable volume despite the large decrease in enzymatic yield. A negative DE52 anion-exchange step then permitted the recovery of most of the remaining RAP activity. S-75 gel filtration aided primarily in the removal of high and low molecular weight contaminants, media pigmentation and peptide/amino acid components. Mono-Q chromatography provided sufficient anionic affinity for RAP binding to allow for an increase in specific activity by 6 fold after desorption. The final step, which utilized a Mono-P chromatofocusing column, resulted in a single, defined peak of purified protein, with the concentrated enzyme corresponding to a specific activity of 1835 units/mg of protein, a greater than 3500 fold purification and roughly 3% yield from the starting batch culture (Table 3.1).

Step	Volume	Total Activity ^a	Total Protein	Specific Activity	Purifi cation	Yield
	ml	<i>Units^a</i>	mg	units/mg	fold	%
Culture filtrate	3900	8375	15,990	0.524	1	100
Ammonium sulfate	43	1707	433	3.94	7.52	20
DE52 anion exchange	10	3284	176	18.7	36	40
Superdex 75	10	1941	8	241.3	461	23
Mono-Q, FPLC ^b	1	449	0.3	1496	2855	5.5
Mono-P, FPLC	2	232	0.12	1835	3506	2.8

Table 3.1. Purification of S.gordonii RAP

^aBased on enzymatic activity using H-Arg-pNa, which 1 unit= 1 μ mol of pNa released per sec. ^bFPLC, fast protein liquid chromatography

Physical Properties. SDS-PAGE analysis of the purified enzyme showed a single protein band, as judged by both Coomassie (Figure 3.4) and silver staining (not shown) with an approximate mass of 70 kDa. Analysis of the protein by MALDI revealed a laser intensity peak that corresponded to a mass of 69,695.6 Da, while analytical Superdex 200 gel filtration indicated a protein size of 64 kDa and, therefore, a monomeric structure. Isoelectric focusing produced a pI of 5.6 for the native protein. The HArg-*p*Na activity was optimum at pH 6.5 with activity detected over a broad phosphate buffer range of 4.0 to 10.0. The enzyme in its pure form was unstable after a 24h period at either 37 °C or 25 °C while affected minimally at 4 °C. However, storage at -20 °C over several weeks resulted in a major loss in enzyme with complete inactivation after a 2 months period.



Fig. 3.3. SDS-PAGE of fractions from the purification of *S. gordonii* **arginine aminopeptidase** . *Lane 1*,7 molecular mass markers (phosphorlase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa). The following lanes contained boiled, reduced samples: *lane 2*, 80% ammonium sulfate precipitation; *lane 3*, flow through from DE52 anion exchange; *lane 4*, peak from Superdex 75 gel filtration wash; *lane 5*, eluted peak from Mono Q; *lane 6*, purified RAP (arginine aminopeptidase) from Mono-P.

Enzyme Specificity. Of the fourteen chromogenic endo- and aminopeptidase substrates tested on the purified enzyme (Table 3.2), only H-Arg-*p*Na was rapidly hydrolyzed. Weaker activity (< 10%) was detected against H-Lys-*p*Na and HLeu-*p*Na. When Michaelis-Menten kinetics were measured using H-Arg-*p*Na as substrate, a V_m of 17.44 µmol min⁻¹ and K_m of 51 µM were determined.

To obtain further information on cleavage specificity, various peptides (2-13 aa) were tested as substrates for RAP (Table 3.3). Significantly, all proteolysis was restricted to peptides with an arginine residue at the N-terminus, with preferential cleavage occuring when a hydrophobic or uncharged residue occupied the 2^{nd} site. There were no apparent additional restrictions for amino acids present downstream of the cleavage site. Extended time incubation with peptides failed to yield additional peptide fragments, indicating the absence of endopeptidase activity or contaminating aminopeptidases. The inability of RAP to catalyze endo-specific proteolysis as tested on whole protein substrates (azocasein, gelatin, collagen type IV, fibrinogen), further supported the role of the purified enzyme as an exopeptidases with the potential to aid endopeptidases.

Table 3.2. Relative amidolytic activity of S. gordonii RAP against various substrates

Substrate (pNa)	Relative activity
H-Arg	100
H-Lys	5
H-Ala	0
H-Gly	0
H-Ile	0
L-Leu	7
L-Pro	0
H-Val	0
H-Gly-Arg	0
H-Gly-Pro	0
Lys-Arg	0
Bz-Arg	0
Ile-Pro-Arg	0
Sar-Pro-Arg	0

¹Activity against H-Arg-pNa hydrolysis taken as 100

Table 3.3. Cleavage Specificity of S. gordonii arginine aminopeptidase on Peptide Substrates

Substrate	Sequence		
Tyrosine Kinase Substrate	Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala- Arg-Gly		
Anistatin Related Peptide	Arg-Cys-Arg-Val-His-Cys-Pro		
Fibronectin Binding Inhibitor	Arg-↓-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro		
Sexual Agglutination Peptide	Arg-↓-Gly-Pro-Phe-Pro-Ile		
Protein Kinase C Substrate	Arg-↓-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-		
Platelet Aggregation Inhibitor	Arg-↓-Gly-Glu-Ser		
Peptide 7	Arg-↓-Gly		
Thymopentin Fragment	Arg-Lys-Asp-Val-Tyr		
Peptide 9	Arg-↓-Phe		
Substance P	Arg-↓-Pro-Lys-Pro-Gln-Gly-Leu-Met		
Bradykinin	Arg-↓-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg		
Dipeptide A	Arg-↓-Pro		
Angiotensin III	Arg-↓-Val-Tyr-Ile-His-Pro-Ile		
Fibrin Polymerization Inhibitor	Gly-Pro-Arg-Pro		
Kallidin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg		
Lymphocyte Activating Pentapeptide	Leu-Pro-Pro-Ser-Arg		

 \downarrow , cleavage site

Inhibition and Activation Studies. Studies with class-specific inhibitors (Table 3.4) supported the assignment of a serine-class mechanism with some cysteine-class characteristics. Moderate inhibition was observed using serine protease inhibitors (Pefabloc and to a lesser extent, DFP and 3,4-Dichloroisocoumarin). However, cysteineclass inhibitors, iodoacetamide and E64, had little or no effect on activity while pCMB, a sulfhydryl-sensitive reagent, was a potent inhibitor of RAP, indicating the possibility of a functional cysteine residue near the vicinity of the active site. Reducing agents (cysteine and β -mercaptoethanol) stimulated activity by approximately 50% and had a more pronounced effect at lower enzyme concentrations. With metallo-class inhibitors, 1,10orthophenanthroline provided no inhibition and EDTA actually stimulated RAP activity in a similar manner as the reducing agents. Activity was inhibited by the heavy metal ions Zn^{2+} and Co^{2+} at 1mM but was increased in the presence of lower mass mono- and L-arginine served as a competitive inhibitor while L-lysine gave divalent cations. minimal inhibition, providing further evidence for the strict specificity in the P_1 position. Compounds specific for eukaryotic arginine aminopeptidase/ Aminopeptidase B (EC 3.4.11.6) and Leucine Aminopeptidase (Bestatin) as well as glutamyl aminopeptidase/ aminopeptidase A (Amastatin) were not inhibitory even at high concentrations. However, RAP was sensitive to the DPPIV-specific inhibitors, H-Glu-pyrollidide, and the Aminopeptidase P-specific inhibitor, Apstatin, as evidenced by 98% and 50% inhibition, respectively, at 100 μ M. Human plasma inhibitors, α -1-proteinase inhibitor and α -2macroglobulin, had no effect on enzymatic activity.

These results are consistent with previous studies conducted on cell-associated arginine aminopeptidases from *S. sanguinis* (12) and *S. mitis* (21).

Table 3.4. Inhibition profile of S. gordonii RAP

Inhibitor	Concentration	% of Residual Activity
DFP	5 mM	75
D (11	10 mM	56
Petabloc	5 mM	40
A (D' 11)	10 mM	31
3,4-Dichloroisocoumarin	2 mM	62
Amastatin	1 mM	89
Bestatin	l mM	68
Apstatin	100µM	50
H-Glu-pyrollidide	100µM	2
E-64	500 µM	98
Iodoacetamide	5 mM	91
EDTA	5 mM	131
1,10-Orthophenanthroline	1 mM	99
pCMB	2 mM	0
L-Cysteine	5 mM	148
L-Arginine	5 mM	30
L-Lysine	5 mM	72
β-mercaptoethanol	1 mM	160
Gly-Gly	5 mM	92
Gly-Pro	5 mM	104
Na+	200 mM	166
K+	5 mM	131
Ca2+	5 mM	150
Mg2+	1mM	93
SDS	5%	4
Urea	2 M	82

RAP Sequence Analysis. The failure to generate a protein sequence from an apparently blocked N-terminus required the sequencing of internal peptides after trypsin digestion. This partial structure resulted in the isolation of a peptide DLTADGSTLFGR which was used to design a degenerate primer. In turn, this was employed to search the genomic clone of an *S. gordonii* strain in the Unfinished Microbial Genomes Database, TIGR. A matching contig (bvs 3948) was identified which comprised an ORF of 324 bp and included the internal sequence of RAP. N and C-terminal sequences facilitated the construction of degenerative primers and PCR against a FSS2 library resulted in the isolation of the 324 bp product that constituted a partial-length DNA fragment. Southerm Blot analysis was performed with the clone and revealed RAP to be a single copy gene. A 108-amino acid polypeptide with a theoretical molecular mass of 11,934 Da and representing approximately 17% of the sequence of the intact protein was encoded by this ORF (Figure 3.4).

The sequence was analyzed for the presence of Gram-positive post-translational modifications. Predictions indicated an intracellular, positively charged N-terminus, a hydrophobic membrane insertion domain and a putative site for signal peptide cleavage (43). This translocation mechanism is well conserved among *Bacillus* spp. (50) and has been identified in *B. subtilis* alkaline protease (49) and Bacillopeptidase F (57). In RAP, an export signal sequence of 14 hydrophobic residues is located downstream from the N-terminus, followed by a polar region (denoted by QACS) that provides a cleavage site after alanine.

The finding of an export signal supports the data for extracellular localization in

culture experiments. A protein homology search was performed with the 108 amino acid fragment using CMR BLAST against EMBL, DDBJ, GenBank and PDB databases. Results indicated that RAP shares 65% identity and 82% similarity with an unpublished report of a gene encoding an amylase binding protein B (abpB) from *S. gordonii* (36). RAP and abpB have predicted transmembrane domains, share nearly identical export signals and have masses of approximately 70 kDa upon secretion from the cell. RAP maintains lesser homology with a putative dipeptidase (TIGR#NTLO1SPL1617) from *Streptococcus pyogenes* (44% identity), dipeptidase DA from *Lactococcus lactis* (34% identity) and non-specific dipeptidase A from *Lactobacillus helveticus* (31% identity).



Fig. 3.4. N-terminal sequence of *S. gordonii* arginine aminopeptidase (*RAP*) deduced from FSS2 genome. Single underlined sequence represents the predicted transmembrane domain. The bold letters mark a conserved signal peptide cleavage site for Gram-positive bacteria. Double lines represent the internal fragment used to search the electronic database and generate a 340 bp PCR product.

Genetic Analysis of Sg-RAP in S. gordonii FSS2 and Viridans Streptococci. A Southern Blot of the S. gordonii FSS2 genome (Fig. 3.6) reveals a single hybridizing band for seven of the restriction enzyme digests under high stringency conditions. All digests show single bands as would be expected for a single copy gene. Digests were subjected to electrophoresis, blotted, and probed for the RAP gene utilizing the 0.34 kb clone corresponding to the N-terminus of the protein.



1 2 3 5 6 7 4

Fig. 3.5. Copy Number of S. gordonii RAP (Sg-RAP) gene in strain FSS2 as Revealed by restriction endonuclease analysis. Lanes: 1. BamHI, 2. BgIII, 3. EcoRI, 4. HindIII, 5. PstI, 6. SacI, 7. SpeI.

Discussion

This report describes the purification, characterization, partial cloning and sequence analysis of an arginine aminopeptidase from *S. gordonii* FSS2. While this activity has been described in studies concerning arginine proteolysis by *S. sanguinis* and *S. mitis* (1, 12, 13, 20, 21, 37), the results presented here are the first biochemical evidence of an extracellular arginine aminopeptidase (RAP), as well as the only study of the molecular structure of a viridans streptococcal arginine aminopeptidase. The enzyme, which was obtained from a pH-controlled culture, was purified over 3500 fold and determined to be a homogenous monomeric protein with a molecular size of 70 kDa. Specificity studies conducted using both *p*-nitroanilides and peptides smaller than 13 residues, indicated only an exopeptidase activity upon incubation with either type of substrate. Although minor Lys and Leu cleavage occured, efficient aminopeptidase activity required an Arg in the first position with a non-polar or uncharged residue preferred in the second site. Inactivation studies propose a serine-class catalytic mechanism in which x-Pro specific inhibitors appear to be the most effective.

Collectively, the biochemical studies on RAP are consistent with those on arginine aminopeptidases purified from *S. mitis* ATCC 9811 and *S. sanguinis* ATCC 903. These enzymes represented the first Aminopeptidase B/ arginyl-exopeptidase activities isolated from a bacterial source. Previous work has identified two activities, a broad substrate aminopeptidase and an Arg-specific aminopeptidase, each capable of hydrolyzing arginine-2-naphthylamide when using cytoplasmic and cell wall/ membrane fractions in cultures undergoing late exponential-early stationary growth (12, 21). These molecular forms differ from each other with respect to their physical properties,

inhibition studies, immunochemical properties and cellular localization. RAP is most closely identified with the Arg-specific, lower molecular weight form that is associated with the cell wall and membrane, found to be sensitive to heavy metals, and classified as a cysteine protease based upon inhibition studies. The inhibition of the S. mitis arginine aminopeptidase by pCMB and metals is suggested to be indicative of a sulfhydryl group located near the active site and essential for enzymatic activity (21). Additionally, it was reported that the stimulating effect of reducing agents was evidence for cysteine residue(s) prone to oxidation (12). However, the sensitivity of RAP to serine-class inhibitors, together with general unresponsiveness to cysteine-class inhibitors, questions such a characterization. The majority of bacterial aminopeptidases, including the broad substrate Arg-aminopeptidase, are members of the metallo-class and excluding the PepC family, bacterial cysteine aminopeptidases are a rarity. The elucidation of crystal structures and site-directed mutatgenesis experiments on bacterial prolyl-iminopeptidase (PIP) family members indicated that they are serine peptidases resistant to fluoride compounds, activated by reducing agents and sensitive to mercurial and heavy metal These features have provided ambiguity during characterization and have salts. improperly classified them as members of the cysteine class without conclusive evidence (27). PIP genes from Bacillus coagulans (28) and Lactobacillus lactis (29) were modeled upon elucidation of the three dimensional structure of Xanthomonas campestris, a model for the PIP family. The data obtained indicated that the active site was comprised of the catalytic triad Asp58, Ser101, and His 267, with Cys62 located at the bottom of the active site about 5Å from the nucleophilic serine, presumably accounting for its cysteine-class

behavior (41). However, in the case of RAP, the absence of both a complete sequence and analogous reactive cysteine, provide inconclusive evidence for serine classification.

The pattern of substrate cleavage exhibited by RAP indicates an aminopeptidase with a strict specificity for arginine in the first position and a restriction against positively charged (Arg or Lys) or polar (Cys) residues in the second. However, proteolysis is favored when non-polar or hydrophobic amino acids are present in that position. There is neither constraint for residues downstream of the hydrophobic site nor for peptide length, although longer peptides and proteins were not tested.

The unique specificity of RAP for Arg and Pro-containing substrates may represent a new paradigm for aminopeptidase catalysis. The selectivity of RAP for arginine distinguishes it from eukaryotic Aminopeptidase B, which cleaves, equally well, arginine or lysine from the N-terminus (23). Proteolysis with proline in the second position is generally reserved solely for members of the Aminopeptidase P family (4). Nevertheless, RAP functioned on three peptides containing Pro in the second position. Furthermore, inhibition was observed with both Apstatin and H-Ghu-pyrollidide, two compounds that target those peptidases that can accept a Pro in their specificity pockets.

This report provides the initial sequence data for a viridans streptococcal arginine aminopeptidase. A homology search using the partial sequence of RAP revealed significant homology with a group of dipeptidases from Gram-positive sources. An unclassified, putative dipeptidase from *S. pyogenes* shares the most identity (44% identical; 60% similar) and predicted a protein of about 498 amino acids. It was also suggested to be a cell surface protein with an export signal (VSYACT) similar to that of RAP (VAQACS). Both *L. lactis* and *L. helveticus* express biochemically identical

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dipeptidases, pepDA (459 aa) and cytosol non-specific dipeptidase 3.4.13.18 (474aa), which are approximately 30% identical and 47% similar to RAP (11). These enzymes are biochemically distinct from RAP, specific for dipeptides of variable composition and isolated from the cytoplasm of prokaryotic cells (34, 35).

Currently, all reported viridans arginine aminopeptidase members have been either associated with the cytoplasm, cell wall or cell membrane (13, 20). The apparent export signal discovered in RAP represents the first extracellular protease from *S. gordonii*. Similar to *Bacillus* ssp. exported proteins, RAP maintains N-, H- and C-regions in its unprocessed form. The consensus cleavage site (QACS), between residues 24-27, most closely resembles those sites in *Bacillus* spp. proteins. Extracellular proteases from *B. subtilis* and *L. lactis* (PrsA and PrtM, respectively) maintain SACS sites that are processed via signal peptidase II (32). An oligopeptide-binding protein from *S. gordonii* also shares an analogous AACS site (25). These findings correspond to results obtained during varying culture conditions. Although total H-Arg-*p*Na activity was variable, the percentage of secreted activity was unaltered. Collectively, this data hypothesizes an export or expression mechanism that is more influenced by the phase of growth than culture composition.

Although arginine acquisition has been considered an important event inside the cell or in the space between the membrane and cell wall junction during peptide import, studies have not focused on extracellular peptidases capable of generating free arginine on the cell surface or in the surrounding environment. As opposed to broad-substrate aminopeptidases, the specific arginine aminopeptidase has been implicated in the generation of a substrate for the arginine deiminase (ADI) pathway (22). Suitable

concentrations of extracellular arginine are required for proper function of an antiporter that exchanges intracellular ornithine, a metabolite of the ADI pathway, for exogenous arginine (31, 46). The cell-surface localization of the highly specific RAP is advantageous for the preservation of an energetically favorable concentration gradient. Its close proximity to the cellular membrane systems involved in amino acid and peptide transport could help meet nutritional requirements. The secretion of RAP would then regulate free arginine concentrations proximal to the cell and serve to scavenge proteins in the local environment, while retention of the broad substrate aminopeptidase might be necessary for proteolysis of imported peptides and general protein turnover.

. In *S. gordonii* G9B, extracellular protein profiles were altered by changes in pH, media composition and rate of growth (30). The release of two cytoplasmic proteins from *S. gordonii*, a GAPDH (42) and an x-Pro DPP (15) was observed upon growth at a constant, neutral pH. This data is consistent with maximum expression of RAP activity at a controlled pH of 7.0 compared to growth at more alkaline conditions. Regardless of changes made to culture, between 70-80% of total secreted RAP activity was constantly released from the cell. Growth and activity curves revealed the peak of H-Arg-*p*Na activity to occur during early stationary phase when excess glucose had been exhausted and a switch to arginine metabolism was evident by ammonia production in culture. Furthermore, linkage between carbohydrate metabolism and peptide degradation has been established. Experiments showed the levels of Arg-specific aminopeptidase and the three enzymes of the ADI pathway were suppressed by glucose via catabolite repression in a coordinated manner (22). On the contrary, growth experiments with glucose and galactose failed to show such a carbohydrate-repression effect. A slightly alkaline

environment of the blood may be more conducive for expression and release of RAP from the cell wall compared to the more acidic surroundings within the plaque. An extracellular RAP may therefore exploit the potential of arginine as an energy source in the protein-rich vegetation versus the carbohydrate-rich oral environment.

Culture supernatant of FSS2 yielded several endopeptidase activities capable of degrading denatured collagen, fibrinogen and azocasein, and at least two additional extracellular aminopeptidases, an x-Pro DPP (15) and PepV di/tri-peptidase (manuscript in preparation). The concerted action of RAP with these activities would be beneficial for the acquisition of small peptides and exposure of new cleavage sites within the protein meshwork surrounding bacteria in the vegetation. We predict that as a consequence of intense arginine acquisition during physiological stress, RAP may catalyze proteolytic events that could exacerbate the pathology of SBE. The removal of an N-terminal arginine from bradykinin and substance P produces truncated forms that have either lost essential residues for receptor activation (3) are found to display biological activity more potent than the intact peptide (58). The combined effect of these modifications could result in local changes in vascular permeability and smooth muscle contraction at the infected endothelium. The truncation of platelet aggregation inhibitor by RAP could have consequences on thrombus formation and the overall growth of the vegetation. The soluble tetrapeptide (RGES), derived from fibrinolysis of the fibrinogen A-gamma chain, inhibits both platelet aggregation and fibrinogen binding to activated alpha IIb-beta 3 integrin (53). RAP may inactivate circulating inhibitor in the growing thrombus and alter the balance between polymerization and fibrinolysis in favor of the growing vegetation.

Future studies will focus on the expression and knockout of RAP in order to evaluate its relative contributions to streptococcal virulence and survival at the site of infection.

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CHAPTER 4

A NOVEL EXTRACELLULAR DI/TRI-PEPTIDASE (PEP V) FROM

STREPTOCOCCUS GORDONII FSS2¹

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Abstract

Generally considered a benign inhabitant of the oral microflora, Streptococcus gordonii is a primary etiological agent in the development of subacute bacterial endocarditis (SBE). Strain FSS2, isolated from an endocarditis patient, produced extracellular gelatin and fibrinogen-degrading activites, as well as an x-Pro dipeptidylpeptidase (xPDPP) and arginine aminopeptidase (RAP) during growth in culture. In this report we describe the purification, characterization and cloning of an extracellular, metallo-class dipeptidase produced in a pH 7.5 controlled batch culture. Purification of this enzyme by anion-exchange, gel filtration, hydrophobic interaction and isoelectric focusing chromatography yielded a protein monomer of approximately 55 kDa, as shown by SDS-PAGE under denaturing conditions and gel filtration analysis. Kinetic studies indicated that the purified enzyme was capable of degrading various hydrophobic dipeptides with the exception of an x-Pro sequence. Lower activity was detected against the N-terminus of hydrophobic tripeptides. Nested PCR cloning against an S. gordonii library enabled the isolation and sequence analysis of the full-length gene. A 467-amino acid polypeptide with a theoretical molecular mass of 51,114 Da and calculated pI of 4.8 was encoded by this gene. Significant homology was found with the PepV gene family that codes for cytoplasmic, non-specific dipeptidases from Lactobacillus and Lactococcus ssp. Sg-PepV may serve as a growth factor during the extracellular, cell surface or cytoplasmic hydrolysis of di- and tripeptides in vivo, and thus maintain sufficient pools of free amino acids capable of sustaining bacterial growth.

Introduction

It is widely believed that S. gordonii obtains necessary protein nutrients from salivary glycoproteins in the oral cavity, while utilizing plasma proteins when growing on heart surfaces. This use of plasma proteins by oral streptococci would presumably benefit growth in the vegetation. Proteolytic and peptide transport systems for viridans members have been described and it has been shown that amino acids and small peptides can be imported into S. sanguinis while those exceeding size limitation would require further hydrolysis by endo- and exopeptidases present on the surface or secreted by these cells (2). One such enzyme that could facilitate these metabolic requirements is a peptidase capable of non-specifically degrading dipeptides and tripeptides into free amino acids. Such an activity has been characterized from whole cell and cytoplasmic extracts of lactic acid bacteria (4,7,19,20) This activity, known as PepV, has been studied in terms of fermenting dairy bacteria concerning casein degradation and cheese ripening processes. In this report we describe the purification, characterization and cloning of a novel extracellular di/tri-peptidase (Sg-PepV) that derives from S. gordonii FSS2, a strain previously isolated from the bloodstream of an SBE patient.

Experimental Procedures: Materials

H-Ala-Phe-Pro-*p*Na, Ala-Phe, Ala-Pro, Ala-Pro-Gly, Ala-Ala, β -Ala-His, Gly-Gly, Gly-Pro, Gly-Gly-Ala, Gly-Pro-Ala, Ile-Pro, Leu-Gly, Leu-Pro, Lys-Pro, Met-Tyr, Pro-Phe, Ser-Pro, di-isopropyl fluorophosphate (DFP), N- α -*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), EDTA, EGTA, iodoacetamide, 1,10-*o*-phenanthroline, phosphoramidon, amastatin, apstatin, DTT, β -mercaptoethanol, Gly-Pro-Arg-Pro-amide, sleep inducing peptide and Cd-ninhydrin were obtained from Sigma. Phenylmethylsulfonyl fluoride (PMSF), 3,4-di-chloroisocoumarin and E-64 from Boehringer Mannheim. Z-Ala-PropNa, H-Ala-Ala-pNa, H-Ala-Phe-pNa, H-Lys-pNa, H-Arg-pNa and Arg-Pro from Bachem. Diprotin A from Calbiochem. Anstatin was a gift from Mirjana Grujic at the Jozef Stefan Institute, Ljublana, Slovenia. Viridans streptococci strains were a gift from Dr. Vincent Fischetti at The Rockefeller University.

Experimental Procedures: Methods

Bacterial Growth. *S. gordonii* FSS2 (previously *S. sanguinis* FSS2 (6,11) was stored and maintained (at -80°C) as previously described (13). Frozen cells were inoculated into autoclaved medium containing 20 g/L trypticase peptone (BBL), 5 g/L yeast extract (Difco), 2 g/L NaCl, 0.1 g/L CaCb, 4 g/L K₂HPO₄ and 1 g/L KH₂PO₄. 10 g/L glucose and 0.5 g /L L-arginine were sterile filtered and subsequently added. A static culture (200 ml) was grown overnight at 37°C and used to inoculate a 4 L starter culture, in turn used to inoculate a 15 L stirred batch culture which was grown in an atmosphere of 5% CO₂ and 95% N₂ at 37°C with the pH held constant at 7.5 by addition of 5 M KOH using a pH controller (Cole Parmer). Gultures were harvested in early stationary phase at a point when the bacteria had metabolized all available glucose and the addition of base had ceased.

Enzymatic Assay. Amidolytic activities of crude samples and purified protease were measured using the tripeptide substrate, H-Ala-Phe-Pro-pNa, at a 1 mM final concentration in assay buffer A (50 mM Tris, 1 mM CaC₂, pH 7.8) at 37°C. Samples were incubated with substrate for 10 min followed by the addition of 50 ng of Sg-xPDPP to obtain release of the Phe-Pro from the reporter group (5). Assays were performed in 0.1 ml in 96 well plates using a thermostated microplate reader, and the release of p-

nitroaniline was measured at 405 nm (Spectramax, Molecular Devices). Dipeptidase activities were determined by the Cd-ninhydrin method (3). Briefly, sample was diluted into 100 11 volume of 100 mM Tris (pH 7.8) containing 20 mM of dipeptide, followed by incubation at 37 °C for 2 hrs. The reaction was terminated by the addition of 50 11 of the Cd-ninydrin reagent (in 50% ethanol). After boiling at 100 °C for 5 min., the solution was cooled and the A_{570} was measured. Enzymatic activity was reported as the endpoint absorbance. Inhibition assays were conducted using Leu-Gly by the ninhydrin method. Pure enzyme was pre-incubated with inhibitor for 10 min at 37°C followed by measurement of residual activity.

Enzyme Purification 14.5 L of cell-free culture filtrate was obtained after centrifugation (20 min, 4 °C at 6000 x g) of the batch culture. Proteins in the filtrate were treated over several hours at 4 °C with ammonium sulfate to a final concentration of 80% and the precipitate collected by centrifugation at 8000 x g for 40 min. Pellets were resuspended in 200 ml of buffer A and dialyzed over 2 days (4 °C), with changes, against 40 volumes of the same buffer. All column chromatography steps were performed at 4 °C except FPLC separations that were done at room temperature. The dialyzed fractions were applied to a DE52 (Whatman) column (2.5 x 30 cm, 150 ml) equilibrated with buffer A. The column was washed with 3 column volumes of buffer A at 1ml/min. A gradient from 0 to 1M NaCl in buffer A was applied over a total volume of 700 ml. Peak activity that eluted was pooled and concentrated by ultrafiltration to 32ml, using a 10K membrane (Filtron). The concentrated sample was divided into three equal fractions that were separately loaded onto a SuperdexTM-75 (Amersham Pharmacia Biotech) HR 10/30 equilibrated with gel filtration buffer (50 mM Tris, 200 mM NaCl, 1 mM CaCb, 0.02%

sodium azide, pH 7.8) at 1 ml/min. Peak activities of all runs were combined and concentrated to 22 ml. Ammonium sulfate was added to the fraction in order to give a final salt concentration of 1 M. This sample was then applied to a phenyl-Sepharose HP column (1.5 x 9 cm, 15 ml) equilibrated with 200 mM potassium phosphate-1M ammonium sulfate (pH 7.5) at 0.5 ml/min. The column was washed with high salt buffer (120 ml) until reaching an A_{280} baseline and then subjected to a 250 ml gradient of 1.0 M to 0.0 M ammonium sulfate utilizing 50 mM potassium phosphate (pH 7.5). Active fractions were pooled and dialyzed against 20 volumes of buffer A over a 24 hr period. The dialyzed sample was concentrated to 37 ml and then applied to a Mono Q HR 10/10 FPLC column (Amersham Pharmacia Biotech) that was equilibrated with buffer A. The column was washed with equilibration buffer until the base line stabilized. A linear gradient (0-500 mM NaCl in buffer A) over 100 ml, was applied, and peak enzyme activity eluted between 200-250 mM NaCl. This activity was pooled, dialyzed against 10 volumes of buffer B (25 mM Tris, 5 mM CaCb, pH 7.4) and applied to a Cibacron blue Sepharose CL-6B column (1 ml) equilibrated with buffer B. Activity flowed through the matrix and was collected, while bound, non-specific protein was eluted in a high salt buffer B. Further contaminants were removed using a Gly-Pro Sepharose affinity column (1 ml) Enzyme obtained from the previous step was loaded onto the column, previously equilibrated with buffer B. It was then washed with 10 column volumes of this buffer and a linear gradient (0-1 M NaCl in buffer B) over 30 ml was applied. Peak activity eluted between 150-200 mM NaCl and was concentrated to 2.5 ml using a 10K MicrosepTM. The sample (8 ml) was then applied to a Mono Q HR 10/10 FPLC column (Amersham Pharmacia Biotech) equilibrated with buffer A. The column was washed with
equilibration buffer until the base line stabilized, a linear gradient (0-500 mM NaCl in buffer A) applied, and peak enzyme activity eluted between 200-250 mM NaCl. After pooling and concentration of active fractions, protein was dialyzed against 25 mM N-methyl piperazine, pH 5.3 and loaded onto a Mono-P 10/5 FPLC chromatofocusing column equilibrated the same buffer. The column was then washed with four column volumes of equilibration buffer and a pH gradient developed with 40 ml of 10 x diluted Polybuffer 74 (Amersham Pharmacia Biotech) adjusted to a pH of 3.8. Samples were returned to a pH of 7.8 using 2M Tris. Peak activity was pooled and concentrated to a volume of 1.0 ml.

Electrophoresis. Enzyme purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel and the Tris-HCl/Tricine buffer system, according to Schägger and von Jagow (17) followed by silver-staining analysis (1). Sepharose-200 gel filtration was used to obtain the MW of the native protein. For sequence analysis, *Sg*-PepV was resolved by SDS-PAGE, followed by electroblotting to a polyvinylidene difluoride membrane using 10 mM 3-(cyclohexylamino) propanesulfonic acid, 10% methanol, pH 11 (12). The blot was air-dried and the band was submitted for sequencing.

Enzyme Kinetics and Specificity. Specificity studies utilized Sg-PepV incubated with 5 μ g of peptide in a 1:1000 (E:S) molar ratio. Reactions occurred in 100 μ L volumes with 100 mM Tris, pH 7.8, at 37 °C for 2 hours. Digestions were terminated by acidification with 10 μ L of 10 M HCl, followed by centrifugation (10,000 x g, 5 min). The entire supernatant was applied to a reverse phase high pressure liquid chromatography using a LC-18 column (25 x 4.6 mm, 5 μ m)(Supelco) equilibrated with

0.1% TFA in HPLC grade water and developed with an acetonitrile gradient (0-80% in 0.08% TFA over 50 min). Experimental and control peaks were integrated and % cleavage was determined.

N-terminal Sequencing. Protein was sequenced by Edman degradation in a model Procise-cLC sequencer (PE Biosytems, Foster City, CA) operated using the manufacturer's protocol

Cloning of S. gordonii PepV Gene. DNA from S. gordonii was purified using the Purgene DNA Isolation Kit (Gentra, Minneapolis, MN) according to the manufacturers instructions. The N-terminal peptide sequence (TIDFKAEVEKRREAL) was used to search the unfinished S. gordonii database available at The Institute of Genomic Research (TIGR) (ftp://ftp.tigr.org/pub/data/s gordonii/). Briefly, DNA sequences were imported into Microsoft Word and searched for a matching DNA sequence encoding the peptide using wild cards at degenerate code positions. Several overlapping contigs were found and this approach resulted in the identification of a 1401 bp-long open reading frame encoding S. gordonii PepV. Subsequently, two PCR primers encoding the N-and Ctermini were synthesized (5'-AGTGGATCCATGACAATTGATTCTAAAGC-3' and 5'-TTTGGATCCTTATTTGATTAGTTCGTAG-3') and used in PCR to obtained fulllength DNA fragment encoding Sg-PepV. PCR was performed using the Pwo DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN), 1 µg of S. gordonii DNA and 500 ng of the primers (94°C for 1 min, 65°C for 1 min, 72°C for 1 min, 32 cycles). A single 1410 bp-long PCR product was obtained, gel-purified, digested with Bam HI, subcloned into the Bam HI site of pUC19 and sequenced.

Southern Blot Analysis. DNA from S. gordonii PK48, DL1, PK2585, S. parasanguinis PK2584, S.mitis J27, S. oralis J21, PK34 and S. salivarius ATCC 27945 and was purified using the Purgene DNA Isolation Kit (Gentra, Minneapolis, MN) according to manufacturer's instructions. Four µg of bacterial DNA was digested with each of the restriction enzymes BamHI, EcoRI, HindIII or PstI overnight at 37° C. Digestion products were separated in 0.7% agarose gels using Standard procedure (Sambrook). Gels were soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min followed by neutralization solution (1.5 M Tris pH 7.5, 1.5 M NaCl) for 1 hour. Separated DNA was transferred onto a nylon membrane (Amersham-Pharmacia Biotech) by capillary transfer and subsequently fixed by cross-linking (Stratalinker, Stratagene Inc.). Membranes were prehybridized in 0.5 M phosphate buffer pH 7.2, 7% SDS and 1 mM EDTA at 50C for 1 hour. ³²P-labelled DPP cDNA probe was added and incubated overnight. After hybridization, non-specifically bound radioactivity was removed by a single wash in 2xSSC and 0.1% SDS at room temperature for 20 min followed by two subsequent washes in 0.5xSSC and 0.1%SDS at 50C for 20 min. Membranes were exposed to X-ray films using enhancing screens at -80C for 1-4 hours.

<u>Results</u>

Growth and Culture Activity. Preliminary experiments indicated the presence of an extracellular peptidase activity in culture media (Fig. 4.1). Detection of this activity in cell- free filtrate was observed during growth in media supplemented with 50 mM glucose and 3.5 mM arginine and was dependent on a pH maintained in the range of 6.0-8.0. Growth and activity from a 1 L pH-controlled (pH 7.5) batch culture were monitored at hourly intervals. Detection of *Sg*-PepV occurred using Ala-Phe-Pro-*p*Na as a tripeptide

substrate. Activity was detected through the use of culture sample followed by the addition of 100 ng of pure Sg-xPDPP to hydrolyze Phe-Pro from the chromogenic group. Cell-associated activity appeared early in logarithmic phase and increased until early stationary phase. Cell-free activity appeared early in stationary phase and steadily increased over the next four hours as bacteria metabolized all available glucose and the addition of base had ceased. An increase in extracellular Sg-PepV occurred between hours 6 and 10 when activity increased two-fold. Cell-associated activity sharply increased by 17-fold between hours 3 to 7, paralleling the period of logarithmic growth. Final measurements at stationary phase revealed a constant level of cell-associated activity while the secreted form continued to increase. Extracellular activity accounted for approximately $\frac{1}{4}$ of the cell-associated activity. This phenomenon may be the result of end-stage growth characterized by peptidoglycan turnover and shedding of wall-associated proteins into the media.



Fig. 4.1. *S. gordonii* FSS2 growth and *Sg*-PepV activity curve from a pH 7.5controlled culture supplemented with 50 mM glucose and 3.5 mM arginine. Samples (1 ml) were removed from culture at fixed timepoints and cells were removed from media by centrifugation (5 min, 4 °C, 13,000 x g) followed by 2x wash and resuspension into initial volume with non-supplemented media. Symbols: , whole culture OD; \blacklozenge , cell-free culture fluid; , whole cells. Assays were performed as previously described using 30iL of sample.

PepV Purification and Physical Properties. Purification of *Sg*-PepV was the result of co-purification with *Sg*-xPDPP. The utilization of an additional, final step, isoelectric focusing chromatography (Mono-P), enabled the homogenous preparation of the enzyme. SDS-PAGE analysis of purified protein (Figure 4.2) shows a single protein band, as judged by silver staining, with an approximate mass of of 50 kDa. Protein analyzed on a Superdex 200 gel filtration (not shown) indicated a molecular weight between 50-60 kDa, providing evidence for a monomer in the native state. IEF-PAGE and Mono-P standardization indicated an approximate pI of 4.3 for the enzyme.



Figure 4.2 SDS-PAGE (silver stained) of purified *Sg*-**x**-**Pro DPP and** *Sg*-**PepV.** *Lane 1*, 5 ig of molecular mass markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; cytochrome c, 14 kDa). The following lanes contained boiled, reduced samples of pure protein from their respective final chromatography steps: *lane 2*, 500 ng of purified *Sg*-**x**PDPP from Gly-Pro Sepharose; *lane 3* 100 ng of partially purified *Sg*-PepV from Mono-P; *lane 4*, 200 ng of purified *Sg*-PepV from Mono-P.

Enzyme Specificity. Of the thirteen dipeptides tested on purified enzyme, using Cd-ninhydrin detection (Figure 4.3) and HPLC analysis (Table 4.1), five were completely hydrolyzed. They included Ala-Ala, Ala-Phe, Leu-Gly, Met-Tyr and Pro-Phe. 100% hydrolysis occurred against Leu-Gly as observed by endpoint analyses. The inability to charged and/or polar dipeptides prohibited a comprehensive obtain and test characterization of enzymatic specificity. To obtain further information on substrate cleavage, tripeptides were tested as against Sg-PepV (Fig. 4.4). Partial proteolysis was confirmed on three peptides, Gly-Gly-Ala (75% cleavage), Gly-Pro-Ala (20%) and Ala-Phe-Pro (20%). The presence of residues in the P_{2} , enabled cleavage between Gly-Gly and Gly-Pro, which were otherwise prohibited in their dipeptide forms. A major impediment for the cleavage of all dipeptides was the presence of Pro in the P₁' position. Such activity is generally reserved for the Prolidase family (21). The collective data for the proteolysis of dipeptide substrates fits a profile that is consistent with peptidases from Lactococcus lactis (7) and Lactobacillus delbrueckii (20) that belong to the PepV family. These dipeptidases hydrolyze both charged/polar and hydrophobic substrates. PepV members have been termed carnosinases based on their ability to degrade the physiological dipeptide, Ala-His. Sg-PepV was unable to utilize carnosine as a substrate. In addition, the ability of Sg-PepV to hydrolyze the N-terminus of tripeptides is comparable to a non-specific dipeptidase (pepDA) from Lactobacillus helveticus (4). Extended time incubations with longer peptides (fibrin inhibitory peptide and sleep inducing peptide) failed to yield additional fragments, indicating size limitation for activity aminopeptidases. and potential absence of contaminating



Figure 4.3. Activity of S. gordonii PepV against dipeptide substrates

Peptide	Sequence	% Cleavage
1	Ala-↓-Ala	100
carnosine	Ala-His	0
3	Ala-↓-Phe	100
4	Leu-↓-Gly	100
5	Gly-Pro	0
6	Gly-Gly	0
7	Met-↓-Tyr	100
8	Pro-↓-Phe	100
9	Ala-↓-Phe-Pro	20
10	Ala-↓-Pro-Gly	6
11	Gly-↓-Gly-↓-Ala	75
12	Gly-↓-Pro-Ala	20
Fibrin inhibitory	Gly-Pro-Arg-Pro	0
peptide Sleep inducing peptide	Trp-Ala-Gly-Gly- Asp-Ala-Ser-Gly-(0 Glu

Table 4.1. Cleavage specificity of S. gordonii PepV on peptide substrates

↓, cleavage site; % cleavage (area experimental peak/ area control peak)

Inhibition and Activation Studies. Sensitivity was observed to broad, metalloclass inhibitors (EDTA, EGTA and 1,10-orthophenanthroline), while serine protease inhibitors (DFP, PMSF and 3,4 Dichloroisocoumarin) and cysteine-class inhibitors (iodoacetamide and E64) had little or no effect on activity. Phosphoramidon, an inhibitor of metallo-class endopeptidases, was not effective against PepV. This is to be expected for metallo-exopeptidases which are unable to accommadate the inhibitor's L-rhamnose and phosphate groups in the active site. Compounds specific for Aminopeptidase N (Anstatin) and DPPIV/CD26 (Diprotin A) were not inhibitory. However, Sg-PepV was sensitive to inhibitors of Aminopeptidase P (Apstatin) and glutamyl aminopeptidase/ aminopeptidase A (Amastatin) as evidenced by sensitivity in the µM range. Sg-PepV activity was significantly inhibited by the heavy metal ion Zn^{2+} (91%) and to a lesser extent, Ca²⁺ (65%), although other cations, Mg²⁺ and Mn²⁺, stimulated activity. Reducing agents at 5 mM (DTT and β -mercaptoethanol) activated the peptidase. Urea destabilized the enzyme and complete inactivation occurred in the presence of 1% SDS detergent and 10 min. boiling.

Inhibitor Concentration		%
		Activity
DFP	5 mM	90
PMSF	2 mM	100
3,4-Dichloroisocoumarin	2 mM	97
TLCK	10 mM	100
Amastatin	350ì M	3
Anstatin	50ì M	100
Apstatin	500 µM	1
Diprotin A	500 µM	98
E-64	500 µM	157
Iodoacetamide	5 mM	100
EDTA	5 mM	2
EGTA	5 mM	6
1,10-o-phenanthroline	5 mM	0
Phosphoramidon	1mM	110
DTT	5 mM	215
β -mercaptoethanol	5 mM	130
K+	5mM	76
Ca2+	5 mM	35
Mg2+	5 mM	136
Mn2+	5mM	143
Zn2+	5 mM	9
Urea	1 mM	16
SDS	1%	1
100°C	10min.	0

Table 4.2. Inhibition profile of S. gordonii PepV

Sg-PepV Sequence Analysis. The deduced N-terminal sequence was used to search the genomic clone of a relatively uncharacterized S. gordonii strain in the Unfinished Microbial Genomes database, TIGR. An ORF of 1401bp was identified and was found to correspond to the Sg-PepV based upon the conservation of the N-terminal sequence of the protein. Primers were designed from the electronic sequence and a PCR approach was used to obtain a full-length DNA fragment encoding Sg-PepV. A 467amino acid polypeptide with a theoretical molecular mass of 51,114 Da and calculated pI of 4.8 was encoded by this gene. There is no evidence for post-translational modifications of the gene product. The homology search (Figure 4.4) performed using NCBI TBLASTN tool against EMBL, DDBJ, GenBank and PDB databases indicated that Sg-PepV is highly conserved with the non-specific dipeptidase M20 family grouped in the MH clan of metallo-peptidases. This family has previously consisted of carnosinases/ditripeptidases that derive from lactic acid bacteria (Lactobacillus ssp. and Lactococcus ssp.), known as the PepV family (7,19,20) Sg-PepV shares the closest homology with genes cloned from Lactococcus lactis (60% identity), Lactobacillus helveticus (49% identity) and Lactobacillus delbrueckii (45%) as well as a hypothetical dipeptidase from Bacillus subtilis (49%). A putative active site histidine (His 88) has been identified in the box 1 catalytic site of the PepV gene of L. helveticus. It is found inside the motif LGIIGHMDVVP (7). By analogy, His 87 of Sg-PepV is likely to be one of the active site residues. Sg-PepV and known PepV members have nearly identical residues flanking the catalytic motif, except for FA in place of the consensus IG that are two residues upstream of the catalytic histidine. Three additional regions of the Sg-PepV sequence conform to the M20 family of metallopeptidases. These regions, known as box 2, box 3 and box 4,

may represent other catalytically or structurally important domains in the protein based upon a series of conserved charges (7). Sg-PepV maintains high identity with these signature sequences, namely 69% (box 2), 57% (box 3) and 93% (box 4), and conservation of 9/9 invariable, charged residues. A recent crystal structure of PepV from Lactobacillus delbrueckii was solved to 1.8 Å by MAD (Jozic D., Bode W., Huber R., and Maskos K., unpublished). The structure consists of two domains, a large catalytic domain containing two zinc atoms at the active site and a separate small domain that forms the lid. The catalytic domain was found to have structural similarity with other zinc dependent exopeptidases. The mechanism of peptide cleavage is believed to be quite similar in these enzymes and involve a bridging hydroxyl-ion ligand acting as a nucleophile. Apart from the metal coordinating His, identified as His 87, five residues (Asp 120, Glu 151, Asp 177, Arg 351 and His 439) have been implicated in critical metal coordination and nucleophile stabilization within the catalytic pocket. Sg-PepV maintains all of these essential residues. A computer-assisted search for protein localization predicted neither signal sequence sites nor transmembrane regions in the protein (14). Although lacking an export signal and predicted to be cytoplasmic, detection during stationary phase revealed approximately 25% of Ala-Phe-Pro-pNa activity to be extracellular.

Fig. 4.4. Multiple sequence alignment of *S. gordonii* PepV (*Sg PepV*) and bacterial homologues. Sequences of *Sg*-PepV deduced from FSS2 genome and cloned PepV members from *Lactococcus lactis* MG1363 (Llactdipep), *Lactobacillus helveticus* SBT2171 (Lbhelvcarn), *Bacillus subtilus* (Bsubputpep), *Lactobacillus delbrueckii lactis* DSM7290 (Lbdelcarn) and were aligned using the ClustalW multiple sequence alignment tool according to homology modeling (grey boxes indicate similarity and black boxes indicate identity). Conserved box regions [box 1, box 2, box 3 and box 4] are in sequential order and denoted with single lines. The putative catalytic His residue within box 1 is denoted (\blacksquare). Conserved charged residues are denoted (\bigstar). Residues implicated in the catalytic pocket from the crystal structure of *L. delbrueckii* PepV are denoted (\bigstar).

SgPepV Llactdipep Lbhelvcarn	1 1 1	-MTIDSKAEVEKRREALLADLFSLLEINSERDDSKADKEHPFGPGPVQALHKFLEIAERD MTTIDFKAEVEKRKDALMEDLFSLLRIDSAMDMEHADAENPFGPGPBRKALDAFLKIAERD -MNLDYKKLAAAKKDDILRDLDELISIDSSEDLDNTSAEYPVGPGPVKAMKKFLSFAKRD
Bsubputpep Lbdelcarn	1 1	MNWEVEVIRKKEDLIRDTQEFLRINSVMDETTAGPGKPFGEGVNASLTSLLELGEKE -MDLNFKELAEAKKDAILKDLEELIAIDSSEDLENATEEYPVGKGPVDAMTKFLSFAKRD
SapenV	60	
Llactdipep	61	GYTTKNYDNYVGHFEYENGANADAEVLGIIGHLDVVPAGSGWDSNPFEPEIRN-GNLYAR
Lbhelvcarn	60	GFHIKNVDNYAGRVDYGDGEKRLGIIGHMDVVPAGDGWNTDPFKMLIKD-GKTIGR
Bsubputpep	58	GFTTKNLD <mark>C</mark> FAGHIEWGEGDDIIGVLCHVDVVP <mark>P</mark> GDGWTSDPFSAEIRN-GRIYAR
Lbdelcarn	60	GFDTENFANYAGRVNFGAGDKRLGIIGHMDVVPAGEGWTRDPFKMEIDEEGRTYGR
SgPepV	115	GS <mark>SDDKGPTMAC</mark> YYGLKIIKDLGLPVSKRVRFVVGTDEESGWQDMDYYFKHVGLPEPDFG
Llactdipep	120	GASDDKGPTVACYYALKILKELNLPLSKKIRFIVGTNEETGWADMDYYFEHCELPLPDFG
Lbhelvcarn	115	GSADDKGPA AAYYG LIIKEAGFTPKKKIDFIYGTNEETNWVG DYYLKHEPTPDQV
Bsubputpep	113	GALDDKGPTMAAFYALKI VKDMNLPLSKRVRMI I GTDEESDWRCVEHYFKHEEMPTMG
LDGelCarn	110	GSADDRGPSLIAYYGVLIDREAGF KPKKKIDF VIGINEETINWVGIDYYDKHEPIEDIV
SaPenV	175	ESDDAEEDIINGEKENITEYTHEGNENTENAKTHSETEETRENMVPESATAVVSGOI.
Llactdipep	180	FSPDAEFPIINGEKGNITEYIHESGKNAGOVUHSFKAGLAENMVPESATAVIS-GA
Lbhelvcarn	173	FSPDAEYPIINGEQCIYTLIINFKDDAQTGSVKLEKFTAGIAENVTPQKAYATISG
Bsubputpep	171	FAPDADFPIINAEKGII <mark>DASL</mark> LIPHRPNQAEPKAVLV <mark>SF</mark> QSGLRLNMVPDAAEAVIEG
Lbdelcarn	174	FSPDAEYPIINGEQGIFTLEFSFKNDDTKGDYVLDKFKAGIATNVTPQVTRATISG
SgPepv	232	PDLAGLLDAFAKEHQLQYEISTVDEET YTITTIGKSAHGSTPEDGINGGTYLALLLNQ
Liactuipep	230 229	ADLANIKEOFTKETTDINKEGNEETNCDEAKTELTCOCAHASADOWCDNAATELCKELNO
Bsubputpep	229	REET SEKOMURTTOOKGEAATENGOLII RYYCLSCHAMEPINGINAGILLCEFLOO
Lbdelcarn	230	PDLEAVKLAYESFLADKELDGSFEINDESADIVLIGQGAHASAPQVGKNSATFLALFLDQ
SgPepV	290	FDFGGDAKAYLQVAARVLHEDFTGEKLGIAYTDAKMGALSMNAGVFHFDSSKADNTIALN
Llactdipep	296	FDFADGAAAFIKVGAEKLLEDHEGEKLGTAFVDELMENTSMNAGVWSFDEN-GEGKLALN
Bsubnutnen	289 289	TELDDAGKREVOVUTDKESGDTRGKKLDIDCEDETSGELTINVGTLRVKEG-OGGELGIN
Lbdelcarn	290	YAFAGRDKNFLHFLAEVEHEDFYGKKLGIFHHDDLMGDLASSPSMFDYEHA-GKASLLNN
		▲
SgPepV	350	IRYPQGTDPKT QAGLEKVTGVVSVSLSEHGHTPHYVPTDDELVATLLSVYEKQTGLKGH
Llactdipep	355	FREPQGNSPERMQEILAKLDGVWEVELSKHLHTPHYVPMSDPLVSTLLDVYEKHTGLKGY
Lbhelvcarn	348	
Lbdelcarn	348 349	VRYPQGTDPDTMIKQVLDKFSGILDVTYNGFEEPHYVPGSDPMVQTLLKVYEKQTGKPGH
		★ ▲
SgPepV	410	E <mark>Q</mark> VIGGGTFGRLLKRGVAFGAMFPDYVNTMHQANEFTDVEDLFRAAAIYAEAIYELIK
Llactdipep	415	ETIIGGGTFGRLLERGVAYGAMFEGEPDSMHQANEMKPVENIYKAAVIYAEAIYELAK
Lbhelvcarn	408	EVVIGGGTYGRUFKIGVAFGAQPEDAPMVMHQANEYMKVDDLIDSIATYAEATYELTKEA
bsubputpep Lbdelcarn	406 409	EISIGGGTTAKSLAGVAFGPTFPGRPDSAHQKDEYTETDDLLRSTALYAQALYEDAK EVVIGGGTYGRLFERGVAFGAQPENGPMVMHAANEFMMLDDLIISIAIYAFAIYETTKDE

Genetic Analysis of Sg-PepV in S. gordonii FSS2 and Viridans Streptococci. Strains representing the major servors of S. gordonii, as well as mitis group members, S. parasanguinis, S. mitis and S. oralis, and a related viridans streptococcal member, S. salivarius, were screened for PepV homologues (Fig 4.5). The protein was conserved in all S. gordonii strains tested as well as mitis group members: S. mitis, S. parasanguinis and S. oralis. There was no apparent homologous gene in S. salivarius. The probe hybridized with a 3.0-kb HindIII fragment in S. gordonii FSS2 and DL1, while other strains exhibited positives in digests of different lengths. There is strong evidence here for the conservation of the PepV gene among S. gordonii strains and other closely related viridans streptococcci. The broad substrate specificity of PepV and its role in generating free amino acids are probably indispensable functions for these bacteria. Therefore, there is a greater likelihood for the conservation of this activity at the genetic level. We believe the fainter S. mitis and S. oralis intensity is due to less specific hybridization, hence minor sequence divergence from S. gordonii. Although, S. salivarius lacks a genetic homologue to PepV, protein sequence analyses with Lactococcus ssp. and Lactobacillus ssp. make it highly probable for protein homologues among other streptococcal members.



Fig. 4.5. Conservation of Sg-PepV in viridans streptococcus strains. Southern
blot of HindIII-digested chromosomal DNA probed with 2.3-kb clone of Sg-PepV. Lanes:
M, markers (kb) 10.0, 5.0, 3.0, 2.0, 1.5 and 1.0; S. gordonii 1. FSS2, 2. PK48, 3. DL1,
4. PK2585, 5. S. parasanguinis PK2584, 6. S.mitis J27, 7. S. oralis J21, 8. PK34, and 9.
S. salivarius ATCC 27945.

Discussion

This report describes the purification, characterization, cloning and sequence analysis of an extracellular dipeptidase, with some tripeptidase specificity, from S. gordonii FSS2. This work presents the first biochemical and genetic studies on a purified dipeptidase from this species. Filtrate obtained from a pH-controlled culture of S. gordonii FSS2 enabled the homogenous purification of Sg-PepV. An assay that employed purified Sg-xPDPP and partially purified Sg-PepV was developed with the chromogenic substrate, Ala-Phe-Pro-pNa. This unconventional technique required the tripeptidase activity of crude PepV sample to hydrolyze the alanine group, followed by the dpeptidylpeptidase activity of pure xPDPP to release the chromogenic group. The final preparation was determined to be homogenous and consisted of a single monomer in the native state. Inactivation studies revealed a metallo-class catalytic mechanism in which metal chelators and aminopeptidase inhibitors appeared to be the most effective. Specificity studies were conducted using di- and tripeptides. Non-specific proteolysis of hydrophobic substrates was observed. The impediment of cleavage with Pro in the P1' site, certain dipeptide motifs and extended peptides, define the specific nature of this peptidase.

Collectively, the biochemical data are consistent with dipeptidases studied from *Lactococcus lactis* MG1363 (7), *Lactobacillus helveticus* SBT2171 (19), *Lactobacillus delbrueckii lactis* DSM7290 (20)] and *Lactobacillus helveticus* CNRZ32 (4). Comparison of the *Sg*-PepV protein sequence with cloned peptidases reveals a significant homology (51% average identity) with PepV gene products of lactic acid bacteria. The consensus sequence, LGIIGHxDVVPAG, is present at the proposed active site of PepV. Within this motif, *S. gordonii* maintains a Phe at the fourth position and an Ala at the fifth in contrast

to the IG sequence conserved in other species. Despite this minor divergence, *Sg*-PepV maintains highly conserved charges within all 4 box motifs and preserves biochemical similarity to members of the M20 family. The elucidation of the PepV crystal structure reveals that *S. gordonii* PepV maintains those catalytic residues thought to stabilize zinc and the water nucleophile. Our discovery represents the first streptococcal member of the PepV subfamily with sequence conservation that may provide evidence for evolutionary divergence from a common Gram-positive ancestor.

Sg-PepV has a few unique characteristics that are separable from PepV members.

The localization of peptidases outside of the cell is considered to be a necessary mechanism for the degradation and intracellular transport of endopeptidase-liberted peptides. Currently, all reported PepV members and di/tripeptidases from Gram-positive bacteria have been either associated with cytoplasmic, membrane or whole cell extracts. Sg-PepV represents the first peptidase from this group isolated in an extracellular form. Sequence analysis did not reveal an N-terminal signal sequence, LPXTG motif (15) or post-translational modifications that could account for translocation from the cytoplasm. We found the gene for Sg-PepV to be single copy, ruling out the possibility of an altered form of the peptidase. Previous reports have suggested that such enigmatic transport could be the result of "specific leakage" of cytoplasmic aminopeptidase from cells (18) or the shedding of protease/peptidoglycan complexes as a consequence of cell wall turnover (8). In S. gordonii G9B, extracellular protein profiles were altered by changes in pH, growth medium composition and rate of growth (9). The secretion of two cytoplasmic proteins from S. gordonii, a GAPDH (16) and collagenase (8) were observed upon growth at a constant, neutral pH. Additionally, this type of secretion is comparable to

that observed for Sg-xPDPP during stationary growth. Altogether, these data are consistent with maximum detection of Sg-PepV activity at a controlled pH of 7.5. The appearance of cell-surface Ala-Phe-Pro-pNa activity occurred throughout logarithmic growth while the peak occured during early stationary phase when excess glucose had been exhausted. At this point, extracellular activity steadily increased and was trending toward greater levels at the final time point. Much like pH-controlled culture conditions, the alkaline environment of the buffered vegetation contacting the bloodstream may be more suitable for enzyme release from the cell compared to activities within the acidic plaque. An extracellular peptidase would, therefore, better serve the nutritional requirements of the bacteria in a fibrin/ECM surrounding versus the carbohydrate-rich oral environment.

The biological significance of the PepV subfamily is presently unknown. The study of this peptidase has been in terms of casein degradation by lactic acid bacteria. A PepV knockout strain was constructed and growth in milk was significantly slower than that of the wild type, but the strains ultimately reached the same final cell densities (7). The ability to cleave strictly dipeptides by most PepV members could make this activity non-essential for cellular activity. Complex proteolytic systems of lactococci and lactobacilli are characterized by redundant components with several activities capable of compensating for a single deficiency (10). The cleavage of β -Ala-His (carnosine) and other unusual dipeptides is crucial for strains that are auxotrophic for His, and suggests the participation of PepV in certain metabolic pathways that have nothing in common with casein degradation. Because *S. gordonii* FSS2 has no known metabolic auxotrophies nor grows in a casein environment, new roles for PepV should be explored. Analogous to

the casein model, bacteria within the biofilm could utilize PepV in conjunction with dipeptidyl-peptidases, tripeptidyl-peptidases endopeptidases, and oligopeptidases. Whether during a period of starvation in dental plaque or within the vegetation, the actions of the protease on peptide substrates may potentiate its physiological role. Biochemical and kinetic studies show a more likely role for Sg-PepV in dipeptide degradation, hence a more direct, synergistic relationship with dipeptidyl-peptidases. PepV may contribute to overall protein turnover and cellular remodeling in the cytoplasm, with the potential for processing events during protein maturation. The generation and elevated concentrations of free amino acids, either cytoplasmically or extracellularly, would benefit antiporter function for the respective residue to be translocated. Future studies will focus on the expression and knockout of Sg-PepV in order to evaluate its relative contributions to streptococcal virulence and survival at the site of infection.

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CHAPTER 5

DISCUSSION

Subacute bacterial endocarditis (SBE) is characterized by the microbial colonization of pre-existing fibrin-platelet vegetations and the subsequent onset of chronic inflammation. Bacteria are normally destroyed by phagocytes in the blood yet in those individuals with heart valve abnormalities, immune responses are generally ineffective (Korzenioswki and Kaye, 1992). This is a direct effect of a physical barrier and protective environment for oral Gram-positive anaerobes. Histological examinations of rabbits inflicted with experimental endocarditis were conducted using pathogenic strains. Lesions were found as a surface to which circulating bacteria adhere and multiply unimpeded by the host immune response. They are painted as microenvironments that ostensibly interfere with diapedesis and phagocytosis by infiltrating leukocytes. Micrographs of vegetations indicate the absence of leukocytes around those colonies embedded in a fibrin matrix, often personified as a "privileged sanctuary" [Durack, 1972 #64].

Viridans streptococci have developed defined mechanisms for colonization and thrombus formation, namely cell surface adhesins and activators of platelets (Herzberg, 1996). As an opportunistic pathogen, classical virulence factors have yet to be identified in *S. gordonii*. Determinants of virulence (i.e. pneumolysin and a complement/Ab-resistant capsule) found in the mitis-group member *S. pneumoniae*, are not in the molecular repertoire of viridans streptococci (Salyers and Whitt, 1994). As a

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consequence of its relatively benign nature and ubiquitous habitation in the oropharynx, research into *S. gordonii* has focused on exploitation of its innate pathogenesis through transgenic expression. Recombinant *S. gordonii* strains are employed as vaccine and/or protein expression vectors for insertion of foreign genes. Most notably, portions of the emm6 gene (M protein) of *S. pyogenes*, *P. gingivalis* FimA, and tetanus toxin fragment C have been expressed in dental strains and introduced as an oral vector to illicit protective immune responses (Franke et al., 2001; Medaglini et al., 2001; Sharma et al., 2001).

Yet studies into other factors that are innate to the bacterium and confer advantages during vegetation colonization and growth have been absent in the literature. Viridans members are nutritionally variant streptococci maintaining complex glycosidase and metabolic systems with no reported auxotrophies (Beighton et al., 1991). These features enable growth in complex media and assist the pioneer colonization of the tooth plaque. Proteolytic capability is a vital aspect of nutrient acquisition and comprises those mechanisms that support hardy growth (e.g. specific needs for transport and the ADI pathway). Previous reports have found *S. gordonii* FSS2 to express activities of greater significance in the vegetation vs. the plaque (Harty et al., 2000; Juarez and Stinson, 1999; Mayo et al., 1995). Significantly, none of the previously characterized proteases possess a broad substrate profile or the ability to meet the aforementioned nutritional demands of cells.

As shown in this dissertation, three amidolytic assays were employed to specifically detect proteases as potential growth factors. The *in vivo* importance of the elucidated peptidases is currently speculative and will be difficult to assess until the creation of knockout strains and study within experimental animal growth models. Furthermore, *in vitro* experiments presented here suggest that these enzymes could play a role in processing and assimilation of the protein milieu and therefore benefit *S. gordonii* during *in vivo* infection. I believe the properties of these peptidases, working in concert with several undefined endoproteinase activities, play significant roles during SBE.

Characteristics of xPDPP, RAP and PepV

Initial experiments identified several endoproteinase activities capable of acting on fibrinogen and gelatin. Attempts to purify a putative "fibrinogenase" using the chromogenic substrate N-methyl-Gly-Pro-Arg-pNa, previously identified as a thrombinlike activity, were unsuccessful. Additionally, the tripeptidyl-peptidase (TPP) substrate Ala-Phe-Pro-pNa failed to uncover its respective activity. Both synthetic substrates yielded activities in culture filtrate, whole cells and after preliminary chromatographic steps, albeit at low kinetic velocities. Recovery and pooling assays uncovered three distinct peptidase activities that acted in concert to hydrolyze the substrates (Table 5.1).

The first enzyme, in chapter 2, was characterized and termed as an xPDPP (xprolyl-dipeptidylpeptidase) based on the initial cleavage of N-methyl-Gly-Pro, secondary cleavage of Phe-Pro-pNa and primary cleavage of H-Gly-Pro-pNa. It was classified as an 87 kDa serine peptidase with a unique proline specificity that generated an x-Pro dipeptide. The activity was detected on cells in early log phase and was found in culture in late log and early stationary phases.

The second enzyme, in chapter 3, was studied by similar methodology and termed RAP (arginine aminopeptidase) due to secondary cleavage of Arg from N-methyl-Gly-Pro-Arg-*p*Na and primary hydrolysis of H-Arg-*p*Na. It was classified as a 70 kDa peptidase with cysteine-class characteristics but with partial evidence for serine classification. It was shown to have a strict preference for Arg at the N-terminus regardless of peptide length. Its detection on cells and in an extracellular form coincided during early stationary growth.

The third and final enzyme, described in chapter 4, was found to be primarily a dipeptidase with tripeptidase activity, and termed PepV. It was identified through its latter activity, the N-terminal cleavage of Ala from Ala-Phe-Pro-pNa, followed by the action of xPDPP to release the reporter group. Classified as a 51 kDa metallo-class peptidase, this enzyme showed a liberal specificity for substrates. The detection of this activity on whole cells and in media was nearly identical to xPDPP.

Based on biochemical data, all three peptidases were similar to proteolytic activities found in Gram-positive lactic acid bacteria, predominately *Lactococcus* ssp. and *Lactobacillus* ssp. Among all three enzymes, homologues were found based on sequence analysis that included *L. lactis*, *S. pyogenes* (GAS) and *S. pneumoniae*. At the genetic level, homologues were identified among other *S. gordonii* strains and evolutionary-related viridans streptococci.

	xPDPP	RAP	PepV
Molecular Weight	87 kDa	70 kDa	51 kDa
Mechanistic Class	serine	putative serine cysteine-like	metallo
Specificity	x-Pro- \downarrow -y-z _n (x-Ala; minor) (y Pro)	Argy-z _n (x=non-polar/ hydrophobic)	xy-(z) (x,y=broad) (y Pro)
Phase Detected on Cell	early log	early stationary	early log
Secretion Phase	late log/ early stationary	early stationary	early stationary
Regulation	Arg; pH; Ca_2^+ ; carbohy.	Arg; pH; Ca_2^+ ; carbohy.	?
Putative Physiological Function	Pro assimilation peptide- digestion/ modulation	ADI pathway peptide- digestion/ modulation	Free amino acids
Homologues (sequence)	<i>S. pneumoniae</i> (putative xPDPP)	<i>S. pyogenes</i> (putative dipep.)	L. lactis PepV
(Southern)	S. gordonii S. mitis	?	all tested mitis group

Table 5.1. Comparison of Sg Peptidases: xPDPP, RAP and PepV

Role of S. gordonii Peptidases in Vegetation Environment

The conditions within plaques provide variable concentrations of resources that hinge on dietary activities of the host. Despite these fluctuations, ample supplies of carbohydrates (i.e. sucrose) can be expected at mealtimes while periods of fasting predicate alternative energy sources (i.e. arginine). Table 1.2 contrasts levels of energy sources between the oral environment, plasma and vegetation. Although levels of glucose in plasma can reach 5 mM, concentrations experienced in the oral environment during meals are always greater. Furthermore, and with reasonable certainty, actual levels reaching cells in the vegetation matrix are less than 5 mM based on diffusion phenomenon inside the biofilm. Under states of nutrient stress and a general stress response (GSR), instead of growing slowly, cells may continue to proliferate, adapt to resistant forms or enter quiescent states. To grow continuously, bacteria need a constant supply of all the resources that are convertible into all the constituents necessary for cell propagation. The dogma associated with this rule provides for extracellular processing and uptake, which includes: 1) the degradation of a substance in the medium by enzymes secreted by the cell to convert certain macromolecules to utilizable forms, 2) diffusion of the resources into the cell from the environment and 3) movement of the cell to the resources (Koch, 1997). Contrary to the third point, physical confinement of deeply embedded cells (and to a lesser extent partially mobile cells closer to the surface) forces the movement of resources to the cell if growth is to continue. This, in turn, prioritizes the role of extracellular proteases if sufficient resource diffusion is to be maintained. The model for streptococcal proteases in the SBE vegetation (Figure 5.1) is believed to meet these demands. The degradation of fibrin(ogen) by secreted endopeptidases would

loosen up the matrix and generate peptide fragments. *S. gordonii* would encounter substrate at its surface or continue to employ extracellular endopeptidases for continued fragmentation. Exopeptidases, such as xPDPP, RAP and PepV, are now in a position to attack smaller peptides creating greater concentrations of substrate. Their combined effort, with one another and endopeptidases, provides one mechanism in the exaggerated drive for alternative energy sources (Arg) and biosynthetic precursors (x-Pro dipeptides and free amino acids) during periods of nutrient stress.



Figure 5.1. Model of streptococcal proteases in SBE vegetation.

Environmental Effects on S. gordonii Peptidases

Evidence to support this model has manifested in the forms of *in vivo* protein expression and the effects of pH on protein regulation and export. An environmental change encountered upon entering the bloodstream from the oral cavity is an increase in pH, 7.3 vs. 6.2, respectively. An S. gordonii CH1 genomic library was used to select for promoters upregulated by this shift (Vriesema et al., 2000; Vriesema et al., 2000). A plasmid-based selection system for the isolation of inducible promoters was designed partly in analogy to the *in vivo* expression technology (IVET) system. This chromosomal integration system had previously enabled the selected of 13 inducible promoters from S. gordonii V288 in a rabbit model of IE. The genes controlled by the isolated promoters encode proteins involved in different cellular functions, including rapid bacterial growth and resistance to host defense (Herzberg et al., 1997). Work with the pH-regulated promoter showed that S. gordonii could sense an increase in environmental pH as a signal for bacterial gene activation. Five clones with inducible promoters showed homology to promoters from the hydA gene of C. acetobutylicum, cysteine synthase gene from B. subtilis and msrA gene (methionine sulfide reductase) found in many prokaryotic and eukaryotic species (Vriesema et al., 2000; Vriesema et al., 2000).

The phenomenon of pH regulation among oral streptococci has been explored at the protein level. Initial studies focused on pH variation in the context of acid tolerance response (ATR) during growth in the oral cavity. This concept allows for the adaptation of weakly cariogenic strains (viridans streptococci) in acidic environments (< pH 5.0) facilitated during periods of excess carbohydrate fermentation by acidogenic strains (*S*. *mutans* and *Lactobacillus* spp.) (Burne, 1998). Two-dimensional gel electrophoresis showed that ATR affected the *de novo* synthesis or upregulation of 39 proteins from *S. oralis*. Soluble, cellular proteins were extracted from growth in batch culture at pH 5.2 and 7.0, and the resulting peptide mass fingerprints from the 2D-gel were compared to a genomic database from *S. pneumoniae*. The expression of proteins from several functional categories, including stress proteins and components of intermediary metabolism, were modulated as a result of growth at low pH (Wilkins et al., 2001).

The synthesis of cell-associated and secreted proteins from S. gordonii FSS2 is also influenced by pH and the availability of energy sources. Many of the major glycosidases synthesized by the bacteria were down-regulated by acid growth conditions. Studies in chemostatic, glucose limited cultures at a dilution rate of 0.10 h⁻¹ showed a pattern for specific activities hydrolyzing glycosidase and proteinase substrates. Activities were generally highest in cells grown at pH 6.5 and equal or lower in cells grown at pH 5.5 or 7.5. One exception was the Sar-Pro-Arg-pNa activity that was 5-fold higher in cells grown at pH 7.5 than in cells grown at pH 5.5 (Mayo et al., 1995). Controlling the pH at 7.5 in stirred batch cultures led to an increase in cell-associated and secreted protein concentrations, during both late exponential and stationary phases, by 68% and 125%, respectively, compared with similar cultures without pH control. Extracellular proteolytic activities were significantly increased when the pH was maintained at 6.0 or 7.5, indicating modulation of enzyme activity by pH (Harty et al., 2000). Growth at defined pH values showed the secretion of S. gordonii FSS2 GAPDH was regulated by environmental pH. The protein was primarily surface-associated at pH 6.5 but shifted to a >90% secreted form during growth at pH 7.5 (Nelson et al., 2001).

The expression and secretion data for xPDPP was consistent with previous data for an alkaline pH response. Total pNa activity (cell surface + extracellular) reached a maximum at pH 7.0 and the greatest amount of secreted activity occurred at pH 7.5. Shifts in pH to 6.3 or 8.0 permitted the release of less than 10% activity found at 7.5. Total activity with no pH control (final pH 4.5) was 5x less than control at pH 7.0. Additionally, both extracellular xPDPP and PepV activities were increased during late exponential and stationary phase, identical to the properties of Sar-Pro-Arg-*p*Na observed in batch culture. The maximum expression of RAP occurred at a lower pH range (pH 6.3 and 7.0) while activity was dwarfed at pH 7.5 and 8.0, and non-existent during the absence of pH control. The secretion mechanism for RAP was independent of a direct pH effect but more likely a proteolytic event on the signal peptide. A comparable signal sequence was absent in xPDPP or PepV but additional target sequences that could account for export have been identified. They include lipoprotein attachment motifs, serine-rich repeats, LPxTG anchor motifs, choline-binding sites and transmembrane pentapeptide sequences (Bateman et al., 2000; Hayashi and Wu, 1990; Pallen et al., 2001; Tettelin et al., 2001). Such mechanisms for protein secretion in Gram-positive bacteria have been intensively studied among B. subtilis, S. pyogenes and S. pneumoniae upon elucidation of complete genome sequences Their results have provided new insights into the complexity and function of extracellular enzyme systems to provide essential nutrients while facilitating the colonization of host tissues. Unfortunately, and as previously observed with S. gordonii GAPDH, all aforementioned targeting sequences do not appear in xPDPP and PepV. The potential exists for autolysin activity in the cell wall that appears during early stationary growth. This would lead to accelerated

peptidtoglycan turnover and non-specific secretion of proteins as a consequence of their cell surface localization. Nevertheless, this theory fails to explain reasons for association of xPDPP and PepV (both predicted to be cytoplasmic) with the cell wall, as well as the effects of pH and Ca protein export. A last attempt to understand this phenomenon is based on the influence of protein charge on the late stages of secretion. The high density of negative charges on the cell surface, due to teichoic acids and LTA, endow the cell wall with an ion-exchange properties. The physico-chemical properties of the wall would then determine the nature of interactions between secreted proteins and the wall. Experiments using engineered α -amylase variants in *B. subtilis* led the development of a hypothesis in which secretory proteins with an overall positive charge would interact strongly with the cell wall while those of an overall neutral or negative charge would be repelled, thus escaping considerable wall-associated degradation and being readily secreted from the cell during peptidoglycan turnover (Stephenson et al., 2000). Anionexchange properties were observed for xPDPP, PepV and GAPDH during chromatography procedures. The pI values for xPDPP, 5.6 (theoretical) and 4.9 (experimental), PepV, 4.8 (theoretical) and 4.32 (experimental), and GAPDH 5.14 (theoretical), provide for negatively charged proteins at a neutral pH and candidates for repulsion. Additionally, the ability of the cell wall to bind and concentrate protons and cations was found to be necessary for membrane function and efficient secretion and folding of Bacillus ssp. secretory proteins. The dependence on culture supplemented Ca²⁺ for the detection of extracellular xPDPP may be indicative of these findings. The concentration of free protons and their potential to modify charges at the cell wall could
explain the pH-dependent effect on protein secretion. Definitive proof of Sg peptidases in this model will await mutation experiments on their surface charges.

Synergy Between S. gordonii Peptidases and Their Potential Physiological Effects

The first indication of cooperation between the peptidases occurred during their purification procedures. It became evident during kinetic measurements with Sar-Pro-Arg-pNa (xPDPP and RAP) and Ala-Phe-Pro-pNa (PepV and xPDPP), that multiple activities provided a bi-phase response. Perhaps the best example of this synergy was the inability of xPDPP to process bradykinin unless the N-terminal arginine was first removed by RAP. xPDPP provides RAP, exopeptidases and endopeptidases with the ability to process or completely degrade peptides with proline residues. Because Pro serves as an important regulatory signal in determining the lifetime of biologically active peptides and acts as critical structural elements in a protein's structure, its presence in a polypeptide chain restricts its susceptibility to most peptidases (Yaron and Naider, 1993). The ability of xPDPP to facilitate N-terminal x-Pro cleavage, RAP to accommodate Pro in the P1' and PepV to hydrolyze dipeptides with Pro at their N-terminus, are significant events for the assimilation of Pro during proteolysis.

A proline residue occurs at a specific invariant position in several cytokines, growth factors and tachykinins, including IL-1 (Ala-Pro), IL-2 (Ala-Pro), bradykinin (Arg-Pro), substance P (Arg-Pro), growth hormone (Phe-Pro), granulocyte colony stimulating factor (Ala-Pro) and insulin growth factor (Gly-Pro). The concerted actions of aminopeptidase P and DPPIV from human platelets have been implicated in the local control of these hormones and a general blood homeostasis (Scharpe et al., 1991). RAP and xPDPP can work in a similar fashion upon N-Arg-Pro peptides, thus subverting

physiological responses. This is most probable on small peptides with high Arg or Pro content at their N-terminus. Substrates meeting these criteria should have an effect on normal circulatory or immune function to be physiologically relevant during SBE. Three potential targets have been identified as tachykinins, namely bradykinin, substance P and neuropeptide K (Hafstrom et al., 1993). Tachykinins are neuropeptides that act as putative mediators of inflammation, potent vasodilators and secretagogues, excite neurons and promote the contraction of smooth muscles (Hafstrom et al., 1993). At physiological concentrations substance P (RPKPQQFFGLM) has been shown to prime PMN chemiluminescence and increase the migration of polymorphonuclear cells. Neuropeptide K (RDADSSIEKQ..) also effects the in vitro migration of leukocytes, further indicating the involvement of neuropeptides in the genesis of inflammation (Partsch and Matucci-Cerinic, 1992). Bradykinin (RPPGFSPFR) is responsible for many effects in leukocytes including the release of other inflammatory mediators, such as cytokines, prostaglandins, leukotrienes, and reactive oxygen species. In the blood vessel, the local release of kinins by this processing system may induce the diapedesis of neutrophils by opening endothelial cell junctions (Bockmann and Paegelow, 2000). Nterminal cleavage of any single peptide or combination could potentially limit or abolish biological activity. This would affect soluble mediators and limit their interaction with cooresponding receptors, impairing cellular functions thus normal during an inflammatory response. The net result could strengthen the "privileged sanctuary" of the vegetation by impairing the migration of leukocytes to the nidus of infection.

Summary and Conclusions

In this dissertation, three proteolytic activities novel to S. gordonii were identified and intensively researched in the context of survival during subacute bacterial endocarditis infection. Microbiological, biochemical and molecular biological techniques were used to identify, purify, characterize, clone and analyze three proteins which account for these activities: an x-Pro Dipeptidyl-Peptidase (Chapter 2), an Arginine Aminopeptidase (Chapter 3) and a Di/Tri-Peptidase V (Chapter 4). We propose that these enzymes support the colonization and survival of this organism within the vegetation, and subsequently, help sustain a chronic inflammatory state at the endocardium. First, the expression and cellular export (specific and non-specific) of the peptidases follow behavior patterns during nutrient stress and alteration in pH that have been observed during in vivo studies and in vitro culture growth. Second, due to the specific and cooperative nature of these peptidases, degraded proteins and peptides could be processed to a suitable size (< 4 residues) for uptake by this organism. This is reflected in their abilities to degrade proline-containing substrates, release free arginine and generate amino acids non-specifically. Finally, their synergistic action upon the processing bioactive peptides could lead to cardiovascular and immune-deficient effects observed in experimental endocarditis animal models and pathology studies.

In conclusion, it has been determined that:

A.) Sg-xPDPP is an 87 kDa homodimer belonging to the serine protease class. It is a dipeptidyl-peptidase which has strict specificity for x-Pro (to a lesser degree x-Ala) at the N-terminus of peptides. It is biochemically related to the DPPIV/CD26

family and genetically similar to the PepX family from lactic acid Gram-positive bacteria.

- B.) Sg-RAP is an approximately 70 kDa monomer with mixed characteristics of serine and cysteine protease classes. It is an aminopeptidase with strict specificity for arginine and a moderate specificity for residue types in the P1'. Partial cloning permitted the loose comparison by sequence to Gram-positive dipeptidases.
- C.) Sg-PepV is a 51 kDa monomer belonging to the metallo protease class. It is primarily a dipeptidase (with some tripeptidase activity) with broad specificity for amino acid types. It is biochemically and genetically related to the PepV family of dipeptidases from lactic acid Gram-positive bacteria.
- D.) All three peptidases were isolated from the culture media under pH-controlled conditions. RAP appears to be secreted to the cell surface and released from the cell by a signal peptidase mechanism during early stationary phase, whereas xPDPP and PepV are secreted and released from the cell in late exponential through mid stationary phase by an alkaline-pH response of unknown mechanism.
- E.) The concerted proteolysis by the three peptidases on synthetic and natural substrates indicate their potential roles in the sustenance of viable cells during SBE infection. These include: generation of new sites for endoproteinases enabling the maximum degradation of host protein, scavenging of dipeptides and amino acids for metabolic processes (i.e. ADI pathway), reorganization of fibrin matrix and modulation of bioactive peptides that have physiological significance at the infected site.

F.) In vitro conditions used to simulate certain characteristics of the *in vivo* environment (alkaline pH and Ca²⁺ concentrations) suggest greater potential for expression and cellular export within the vegetation environment of SBE.

Future Research

- A.) Expression of xPDPP for structural studies. There is much interest in solving the crystal structures of PepX enzymes for elucidation of the specificity pocket and inhibitor design. To this end, I have begun a collaboration with Prof. Wolfram Bode of the Max Planck Institute for Biochemistry in Martinsried, Germany, to crystallize this peptidase.
- B.) Obtaining the full-length sequence of RAP upon completion of the *S. gordonii* electronic database.
- C.) Submission of purified peptidases to future analysis by the Australian Proteome Analysis Facility (<u>http://expassy.proteome.org.au</u>). Expression of enzymes under various growth conditions and strains could be determined by this method.
- D.) Generation of antibody to peptidases would enable localization studies on the cell *in vitro* and in the vegetation *in vivo*.
- E.) Knockout strains for single mutants or multiple mutants of the peptidases would contribute to an understanding of the physiological importance of the peptidases to growth and colonization upon experiments in an IE animal (rabbit) model.
- F.) Elucidation of putative endopeptidases responsible for fibrin(ogen) degradation.

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