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BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF SERINE PROTEASES  
IN LEISHMANIA

by

RYAN KELLS SWENERTON

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by  
Ryan Kells Swenerton

## Dedications

Over the past six and one-quarter years I have been supported, mentored, and motivated by numerous people both in and out of my scientific life. My dissertation project (and various side projects) could not have been accomplished without the direct help of Ben Kelly, my mentor for all things *Leishmania*; and Mohammed Sajid, who has continually advised me from near and far over the years. I would also like to thank Jim McKerrow, my principal investigator, for his encouragement and his leadership. Jim fosters an environment of collaboration and camaraderie in his laboratory that made working there both productive and enjoyable. In addition, I would like to thank Jim for supporting my non-research related pursuits, such as my forays into teaching and student government.

I would like to specifically dedicate this work to my family and to my wife Debbie. My family (from great-grandparents on down) has encouraged me to ask questions and to follow my interests, even the unusual ones. Through both my upbringing and genetics, my family has provided me with the curiosity and skepticism that enabled me to complete this dissertation. While in graduate school, I was fortunate to meet my partner in life, Debbie. In addition to being my constant champion both at work and at home, Debbie's work ethic, competitiveness, and love of learning have inspired me to be a better scientist and person.

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The text of this dissertation is in part (Chapter One) based on material from a review article published in *Current Opinion in Infectious Diseases* under the title “Development of protease inhibitors for protozoan infections.”

The text of this dissertation is in part (Chapter Two) based on material from a manuscript submitted to *The Journal of Biological Chemistry* under the title “The Oligopeptidase B of *Leishmania* Regulates Parasite Enolase for Immune Evasion and Virulence.”

The following individuals contributed to the data and research presented in this dissertation:

Charles Craik: contributed experimental ideas throughout this dissertation.

Richard Fetter: contributed data for Figure 3.6.

Christopher Franklin: contributed data for Figure 2.3.

Elizabeth Hansell: contributed data for Tables 2.2 & 2.3.

Benjamin Kelly: contributed experimental ideas throughout this dissertation.

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James McKerrow: contributed experimental ideas throughout this dissertation.

Katalin Medzihradsky: contributed data for Figure 2.7.

Sajid Mohammed: contributed experimental ideas throughout this dissertation.

Philip Rosenthal: contributed experimental ideas throughout this dissertation.

Judy Sakanari: contributed experimental ideas throughout this dissertation.

Shuyi Zhang: contributed to the data for Figure 2.8 & Table 2.4.

# BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF SERINE PROTEASES IN *LEISHMANIA*

Ryan Kells Swenerton

## Abstract

Proteases are a ubiquitous group of enzymes that play key roles in the life cycle of parasites and in the host-parasite relationship. They are important targets for the development of new anti-parasitic therapy. Protozoan parasites like *Leishmania* predominantly express Clan CA cysteine proteases. It was therefore unexpected to find a high level of serine protease activity in *Leishmania donovani*. Oligopeptidase B (OPB) was identified as the responsible enzyme. This was confirmed by OPB gene knockout, which resulted in the disappearance of the major serine protease activity. To delineate the role of OPB in parasite physiology, two-dimensional gel electrophoresis was carried out on OPB *-/-* versus wildtype parasites. Four protein species were significantly elevated in OPB *-/-* parasites, and all four were identified as enolase. Aside from its classic role in carbohydrate metabolism, enolase is known to bind plasminogen and function as a virulence factor for several infectious microbes. As expected, there was a striking alteration in macrophage responses to *Leishmania* when OPB was deleted. While wildtype parasites elicited little response from infected macrophages, OPB *-/-* parasites induced a massive upregulation in transcription. OPB *-/-* parasites displayed decreased virulence in the murine infection model. Additionally we have investigated the subtilisin (SUB) of *Leishmania* because it is a single-copy gene and subtilisins play important roles

in virulence of other protozoa. *Leishmania* subtilisin was deleted by gene knockout, which resulted in reduced ability to undergo promastigote-amastigote differentiation *in vitro*. EM of SUB knockout amastigotes revealed abnormal membrane structures, retained flagella, and binucleation. SUB deficient *Leishmania* displayed reduced virulence in hamster and murine infection models. Histology of spleens from SUB knockout-infected hamsters revealed the absence of psammoma body calcifications. To delineate the role of SUB in parasite physiology, two-dimensional gel electrophoresis was carried out on SUB  $-/-$  versus wildtype parasites. SUB knockout parasites showed altered regulation of the trypanothione reductase system. Trypanosomatids lack glutathione reductase, and rely on the novel trypanothione reductase system to detoxify ROSs and maintain redox homeostasis. The predominant tryparedoxin peroxidase was decreased in SUB  $-/-$  parasites, and higher molecular weight isoforms of the enzyme were present, suggesting altered processing.

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# **Chapter One:**

## **Introduction**

### **1.1. *Leishmania*: a Kinetoplastid Parasite**

The leishmaniasis are an ancient family of visceral and cutaneous parasitic diseases that have been recognized in both the Old and New Worlds for well over a thousand years. Despite this fact, the study of protozoan parasites, like *Leishmania*, was not possible until after the invention of the microscope by Van Leeuwenhoek at the end of the 17<sup>th</sup> century and the promotion of the germ theory by the works of Pasteur and Koch in the 19<sup>th</sup> century (Cox 2002). It was not until the late 19<sup>th</sup> century that the causative parasite *Leishmania* was identified. The *Leishmania* parasites responsible for Old World cutaneous leishmaniasis were first observed from a “Delhi boil” biopsy in 1885 by Sergeant Major David Cunningham (Cunningham 1885), a Scot in the British Army’s Indian Medical Service; however he did not realize what they were at the time. In 1898, while working in Turkestan (now part of Uzbekistan), Russian military surgeon Peter Borovsky was the first to correctly identify and describe *Leishmania* as the causative parasite of cutaneous leishmaniasis, known locally as the “Sart sore.” Borovsky’s work was written in Russian and therefore was not broadly distributed outside of the Russian Empire. This research did not receive widespread acknowledgement for several decades (Hoare 1938).

Credit for the discovery of *Leishmania* causing visceral disease has historically gone to Sir William Leishman, a Scottish pathologist in the British Army (Leishman 1903), and Colonel Charles Donovan, an Irish physician with the Indian Medical Service (Donovan 1903), both of whom published in 1903. The causative agent of Old World visceral leishmaniasis, *Leishmania donovani*, is named for these two scientists. The

*Leishmania* parasites responsible for New World leishmaniasis were first identified by Adolfo Lindenberg in São Paulo, Brazil in 1909 (Lindenberg 1909).

*Leishmania spp.* parasites are now known to be kinetoplastid parasites in the trypanosomatid family. Kinetoplastids are flagellated protozoa that are distinguished from other Euglenozoa by the presence of a single large DNA-containing mitochondrion termed the kinetoplast (Honigberg 1963). This mass of extranuclear DNA is often located near the base of the flagellum and was therefore initially thought to be involved in locomotion, thus the organelle's name. The trypanosomatid family also includes *Trypanosoma cruzi* (the cause of Chagas' Disease in humans), *Trypanosoma brucei ssp.* (the cause of African Sleeping Sickness in humans and Nagana in animals), and *Crithidia fasciculata* (a parasite of arthropods) (Figure 1.1). This family of parasites represents a major global disease burden and has historically been an obstacle for economic development in endemic regions.

The genus *Leishmania* is comprised of vector-borne parasites of vertebrates including over 30 species that infect mammals. The insect vector of *Leishmania* is the female phlebotomine sandfly. In the Old World, sandflies of the genus *Phlebotomus* are the vector of *Leishmania* (Sergent, Sergent et al. 1921); whereas in the New World, sandflies of the genus *Lutzomyia* are the vector (Deane and Deane 1954). *Leishmania* is a dimorphic parasite with a relatively simple life cycle (Figure 1.2). Within the sandfly midgut, *Leishmania* multiply in the promastigote stage. This stage of the parasite is spindle-shaped with an anterior flagellum, and measures 10-20  $\mu\text{M}$  in length with a diameter of 2.5-5  $\mu\text{M}$  (Figure 1.3). These parasites migrate to the proboscis of the sandfly where they differentiate into the infective metacyclic promastigote form. This



form has an elongated body and flagellum and no longer divides (Peters and Killick-Kendrick 1987).

When an infected sandfly bites a potential host, the parasites are passed into the host's tissue as the sandfly takes its blood meal. These parasites disseminate in the host's tissue and are taken up by phagocytic cells of the immune system. Within these cells *Leishmania* differentiate into the amastigote stage. This stage of the parasite is oval and aflagellated with a diameter of 2.5-5  $\mu\text{M}$ . Amastigotes reside and multiply within parasitophorous vacuoles in the infected host cells and affect different host organs depending on the parasite species. When a naïve sandfly takes a blood meal from an infected host, the parasites are passed to the sandfly where they differentiate into the promastigote stage, thus completing the parasite's life cycle (Peters and Killick-Kendrick 1987).

## **1.2. The Leishmaniases**

The diseases caused by the protozoan parasite *Leishmania* were recognized thousands of years prior to the discovery of the parasite itself. In the Old World descriptions of a disease, now believed to be leishmaniasis, are found on tablets from the library of King Ashurbanipal of Assyria dating back to 650 BCE. The Arab physician Avicenna described in detail the Balkh sore (leishmaniasis from northern Afghanistan) in the 10<sup>th</sup> century CE. The ancient disease kala azar (Sanskrit for black fever) in India is now considered to be the visceral form of leishmaniasis. In the New World, sculptures from the 5<sup>th</sup> century CE. depict disfiguring skin lesions likely caused by leishmaniasis. In the 16<sup>th</sup> century CE, Spanish missionaries described several diseases from seasonal

agricultural workers in the Andes that are now believed to be cases of leishmaniasis (Cox 2002). Many of the current local names for leishmaniasis in endemic countries have been used to describe the disease for hundreds of years. It was not until the early 20<sup>th</sup> century CE that the parasite *Leishmania* was discovered to be the etiological agent of these diseases.

Today it is known that *Leishmania spp.* cause multiple human diseases around the world including cutaneous, mucocutaneous and visceral leishmaniases. *Leishmania* infects approximately 12 million people in 88 countries. There are an estimated 1.5-2 million new cases annually and 350 million people at risk of infection (Figure 1.4). The form of the disease depends on the species of *Leishmania* as well as the host's immune status, and is characterized by whether the parasites establish a cutaneous infection or spread to the mucosa or viscera. Cutaneous infections in humans are caused by *Leishmania* species including *L. mexicana* and *L. major* and are characterized by self-healing skin lesions. This form of the leishmaniasis accounts for about 75% of infections. Mucocutaneous leishmaniasis is a rare disease that is caused by *L. braziliensis* and *L. guyanensis*. Visceral infections in humans are caused by parasites in the *L. donovani* complex, namely *L. donovani* and the related *L. infantum*. In these infections the hepatic and splenic macrophages are infected by the parasite. These visceral infections, if untreated, result in hepatosplenomegaly and death. Due to the lack of safe and effective treatments for this disease, leishmaniasis is classified by the World Health Organization as Tropical Disease Research Category I, an emerging or uncontrolled disease (Scientific Working Group on Leishmaniasis. Meeting (2004: Geneva Switzerland) and

UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. 2004).

### **1.3. Chemotherapy**

The lack of an effective vaccine for leishmaniasis has made chemotherapy the primary method for controlling the disease. While there are several drugs currently used to treat leishmaniasis, the use of these drugs is limited significantly by cost, toxicity, and growing drug resistance. For decades pentavalent antimonials (SbV) have been the mainstay of leishmaniasis chemotherapy. Despite their longtime use, the mechanism of action of SbV is not well understood. Current research suggests that antimony compromises the thiol redox potential of *Leishmania* (Wyllie, Cunningham et al. 2004) and that it may mediate DNA fragmentation, thereby causing an apoptosis-like response in the parasites (Lee, Bertholet et al. 2002). Resistance to SbV drugs has been on the rise over the past two decades, and these drugs have been rendered obsolete in some endemic regions such as the Indian state of Bihar (Sundar 2001).

Other important drugs for treatment of leishmaniasis include amphotericin B, miltefosine, paromomycin, and pentamidine. Amphotericin B (AmB) is a systemic anti-fungal drug that is now in use to effectively treat leishmaniasis. AmB interact with ergosterols in the membranes of fungi and *Leishmania*, compromising membrane integrity (Goad, Holz et al. 1984). Resistance to this drug has not yet become a problem; however, AmB is expensive and is quite toxic with severe side effects (Ouellette, Drummelsmith et al. 2004). Miltefosine is an anti-cancer drug that has recently become a potent treatment of leishmaniasis. Miltefosine is an alkyl phospholipid that is believed to

induce an apoptosis-like cell death in the parasite by interfering with alkyl-lipid metabolism and signal transduction (Lux, Heise et al. 2000). This drug was first shown to be effective against *Leishmania* in the 1980s (Croft, Neal et al. 1987); however, it has just recently come into use as a treatment for leishmaniasis and resistance has not yet become an issue. Paromomycin is an aminoglycoside antibiotic that was first seen to be antileishmanial in the 1960s. In bacteria, paromomycin binds to the ribosome and inhibits protein synthesis (Croft and Coombs 2003). This drug may also target the ribosomes of *Leishmania* (Maarouf, Lawrence et al. 1995), however the mechanism of action is not currently understood. Paromomycin is relatively inexpensive and is currently being used to successfully treat visceral leishmaniasis in India (Sundar, Jha et al. 2007). Pentamidine has been used a treatment for leishmaniasis for over 40 years. Widespread use of this drug has increased in India where SbV is no longer effective. In these regions, the cure rate of pentamidine has declined considerably, indicating that resistance may be occurring (Jha, Singh et al. 1991). Pentamidine is believed to inhibit arginine and polyamine biosynthesis and to accumulate in the mitochondrion (Basselin, Denise et al. 2002). Interestingly, pentamidine has also been shown to inhibit a putative virulence factor in trypanosomes—the cytosolic serine protease, oligopeptidase B (Morty, Troeberg et al. 1998). Because proteases represent an intriguing set of chemotherapeutic targets in *Leishmania*, research is currently underway to elucidated which of these proteases are virulence factors and how best to rationally design or identify their inhibitors.

#### **1.4. Proteases in *Leishmania***

Proteases are a ubiquitous group of enzymes functioning in nearly all biological phenomena. In protozoan parasites, proteases play key roles in life cycle transition, host invasion, parasite immune evasion, and the pathogenesis of parasitic diseases (Sajid and McKerrow 2002; McKerrow, Caffrey et al. 2006). For these reasons, proteases have been identified as druggable targets for the development of new anti-parasitic therapy. Protease inhibitors are currently used to treat HIV infection and hypertension (Vacca, Dorsey et al. 1994; Smith, Allen et al. 2006), and proteases have been targeted for the treatment of other diseases including Hepatitis C, cancer, diabetes, and osteoporosis (Jedinak and Maliar 2005; Reesink, Zeuzem et al. 2006; Choy and Lam 2007; Stoch and Wagner 2008). Proteolytic enzymes are divided into four major groups based on their catalytic mechanism: aspartic, cysteine, serine, and metalloproteases. These groupings are further subdivided into clans and families based on structural similarities and the order of the catalytic residues. The molecular evolution of proteases shows a striking dichotomy between the predominant Clan CA cysteine proteases of protozoa and primitive metazoa, and the dominant trypsin family (S1A) serine proteases of vertebrates (Rawlings, Morton et al. 2008).

Cysteine proteases are abundant in *Leishmania* and have become potential chemotherapeutic targets as well as candidates for vaccine development. The Clan CD metacaspase (a distant relative of caspase not found in mammals) of *L. major* has been reported to be essential for nucleus and kinetoplast segregation in replicating pro- and amastigotes. Metacaspase null parasites are not viable making this enzyme a potential drug target (Ambit, Fasel et al. 2008). Multiple cathepsin B- and cathepsin L-like cysteine proteases (Clan CA) are also present in *Leishmania*. Gene disruption of the

cathepsin L-like proteases of *L. mexicana* (*lmcpb* genes) has shown that while these enzymes are not required for *Leishmania* replication or differentiation *in vitro*, they are virulence factors. Macrophage infectivity of *lmcpb* null parasites is reduced by 80% (Mottram, Souza et al. 1996). The amastigote-specific cathepsin L-like protease of *L. chagasi* (*Ldccys2*) has also been found to play a role in macrophage infection and intra-macrophage survival. *Ldccys2* null parasites are inviable and antisense RNA inhibition of *Ldccys2* reduces parasite virulence *in vitro* (Mundodi, Kucknoor et al. 2005). Recent evidence has indicated that *L. mexicana* cysteine proteases modulate the Th1 response in mice during infection. Therefore, inhibiting *Leishmania* cysteine proteases may prevent the parasite from suppressing the host's protective immune response (Buxbaum, Denise et al. 2003). Molecular models of *L. major* cathepsin B- and L-like proteases have been generated and used for *in silico* screening. Potential protease inhibitors were identified, including six with  $IC_{50}$ s below 50 $\mu$ M against *L. major* cpB. Three of these inhibitors, hydrazide compounds, prevented parasite proliferation at 5-50 $\mu$ M *in vitro* (Selzer, Chen et al. 1997).

Possibly the most studied *Leishmania* protein is the major surface glycoprotein (GP63), a metalloprotease (Clan MA). This enzyme is the most abundant surface protein on *Leishmania* and is expressed on promastigotes and amastigotes of all species studied. The crystal structure for this enzyme was solved over a decade ago (Schlagenhauf, Etges et al. 1998). GP63 has several known functions including the binding and breakdown of complement component C3, resulting in parasite resistance to complement-mediated lysis (Brittingham, Morrison et al. 1995). GP63 deficient mutants of *L. major* exhibit sensitivity to complement-mediated lysis and a delay in lesion formation *in vivo* (Joshi,

Kelly et al. 2002). Additionally, GP63 has been shown to protect *Leishmania* from antimicrobial peptides such as mammalian  $\alpha$ - and  $\theta$ -defensins, magainins and cathelicidins (Kulkarni, McMaster et al. 2006). These peptides represent an important arm of the innate immune system. Effective non-toxic inhibitors of GP63 are not readily available; however, novel peptide inhibitors of GP63 based on myristoylated alanine-rich C kinase substrate (MARCKS) have been developed with an  $IC_{50}$  of approximately  $1\mu M$  (Corradin, Ransijn et al. 2002). GP63 is clearly a major factor in *Leishmania*'s virulence; thus, identification and design of GP63 inhibitors are a high priority for the control and treatment of leishmaniasis.

While the vast majority of peptidase research in *Leishmania* has focused on the metallo- and cysteine proteases, recent studies have illustrated the importance of serine proteases and identified aspartic protease activity in this parasite. A *Leishmania* cathepsin D-like aspartic protease (Clan AA) has just been identified and is postulated to be important in promastigote proliferation. The aspartic protease inhibitor, diazo-acetyl-norleucinemethylester was found to inhibit *L. mexicana* proliferation with an  $LD_{50}$  of  $22\mu M$  at 72 hours (Valdivieso, Dagger et al. 2007). Several serine proteases have been identified in *Leishmania* including homologues to those that are putative virulence factors in the trypanosomes. Serine protease inhibitors benzamidine, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and a sea anemone-derived Kunitz-type inhibitor were all found to be leishmanicidal and induced changes in the ultrastructure of the parasite's flagellar pocket (Silva-Lopez, Morgado-Diaz et al. 2007). *L. amazomensis* oligopeptidase B (Clan SC) has recently been homology modeled. The three dimensional model that was generated will serve as a basis for evaluating this enzyme as a target for structure based

drug design (de Matos Guedes, Carneiro et al. 2007). Ongoing research is continually expanding our knowledge of *Leishmania*'s serine proteases and demonstrating them to be potential drug targets.

Recently, sequencing of the *L. major* and *L. infantum* genomes were completed by The Wellcome Trust's Sanger Institute Pathogen Sequencing Unit (Ivens, Peacock et al. 2005). In addition, the genomes of *L. braziliensis* and several *Trypanosoma* species are in various stages of completion and annotation. Informatics analysis of the *Leishmania* genomes has revealed approximately 8,300 protein-coding genes and dozens of pseudogenes. Among these predicted genes are at least 21 genes for serine proteases and several serine protease pseudogenes (Figure 1.5), representing 0.25% of the genome. The serine protease repertoire of *Leishmania* contains proteases from at least 8 families representing 7 different clans. Almost half of these proteases are from Clan SC, including 3 from the S9A prolyl oligopeptidase family. *Leishmania* also has a single member of the Clan SB subtilisin S8 family. Members of these serine protease families are known to be virulence factors in other protozoan parasites (McKerrow, Rosenthal et al. 2008).

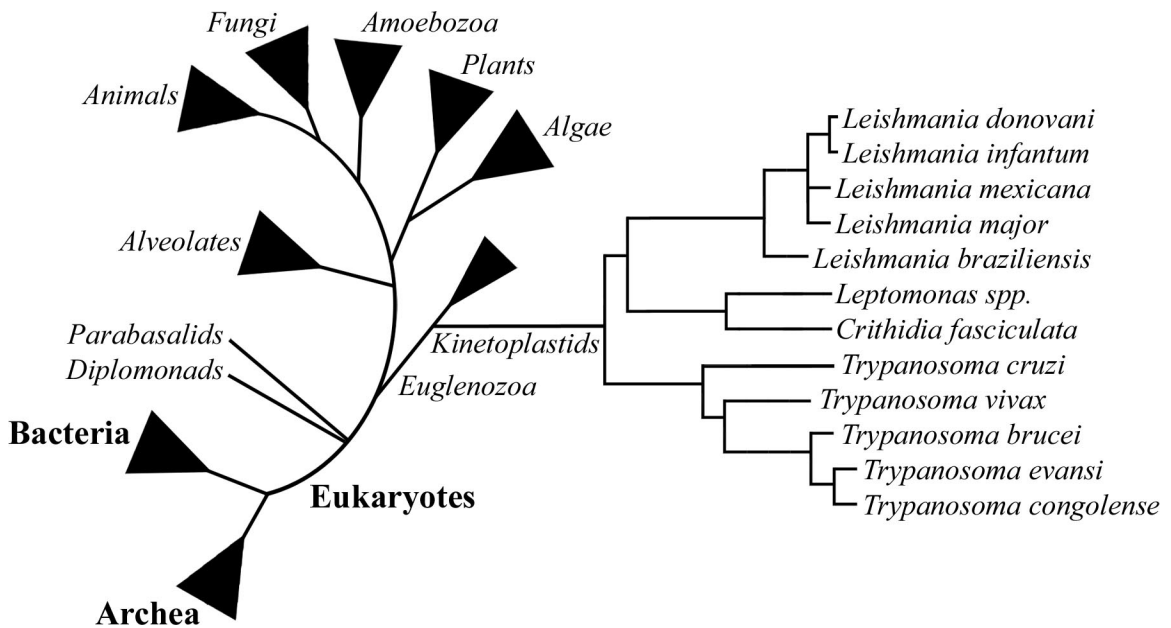


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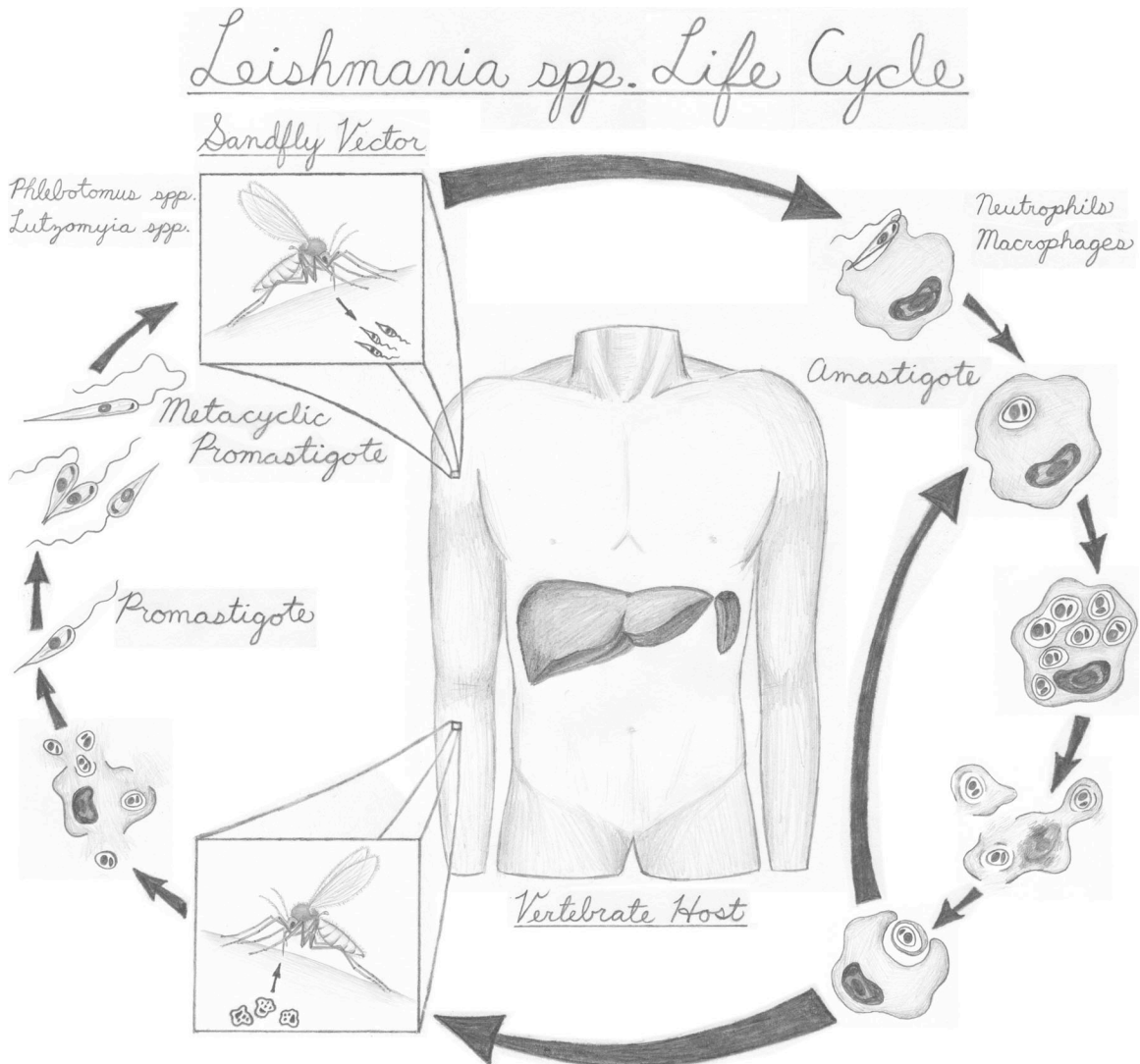
**Figure 1.1: The Phylogenetic Tree of Life**

*Leishmania* is a genus of protozoan parasites that infect vertebrates. These parasitic eukaryotes belong to the Kinetoplastida order of the Euglenozoa. Kinetoplastids are flagellated parasites characterized by the presence of a single large DNA-containing mitochondrion termed the kinetoplast. The Trypanosomatidae family of the Kinetoplastida contains parasite genera including *Trypanosoma* (species of which cause African Sleeping Sickness and Chagas' Disease), *Crithidia* (an arthropod parasite), and *Leishmania*. The genus *Leishmania* contains over 30 different species, many of which infect mammals. Of particular significance are the *L. donovani* complex (which cause visceral disease), the *L. mexicana* and *L. major* complexes (which cause cutaneous disease), and the *L. braziliensis* complex (which is capable of causing mucocutaneous disease).



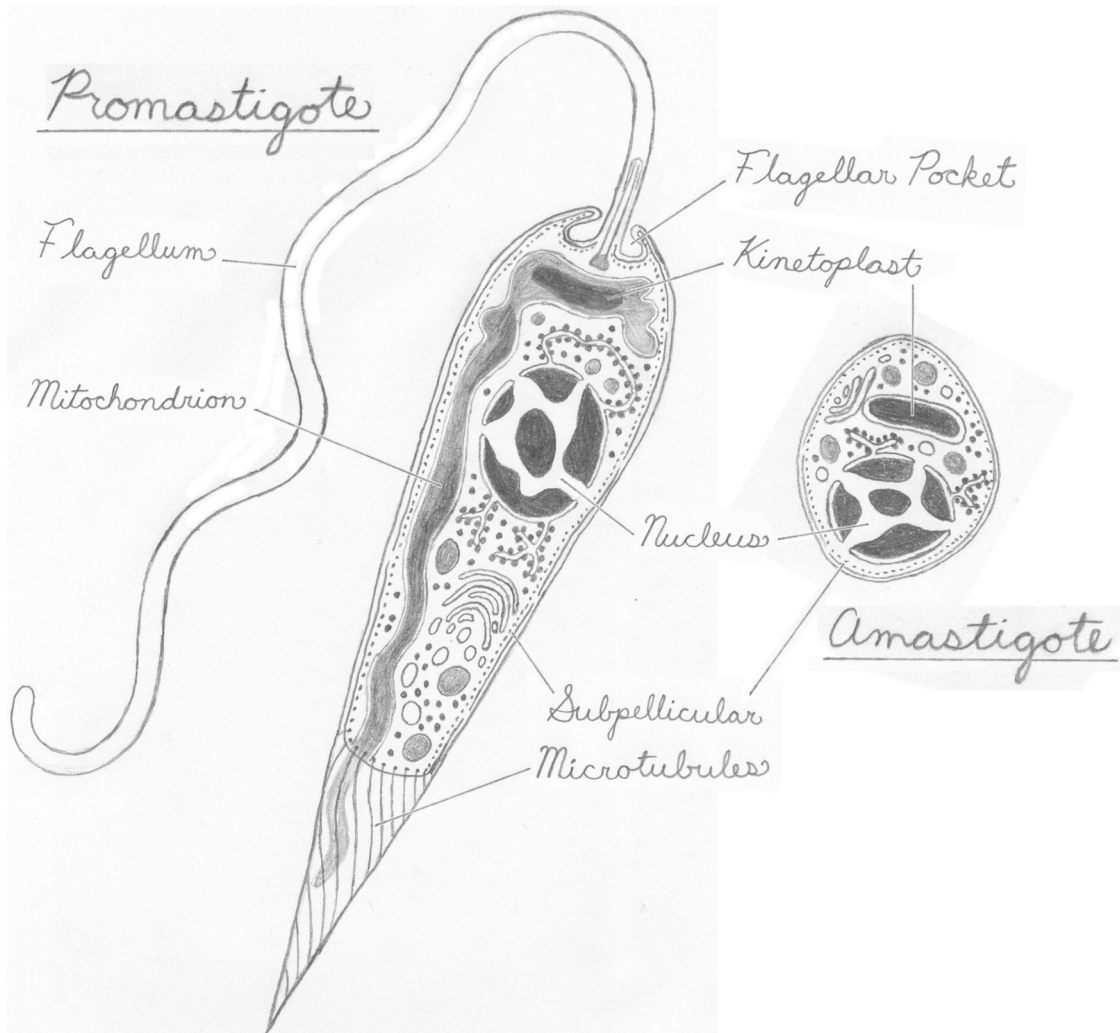
## Figure 1.2: The Life Cycle of *Leishmania*

The *Leishmania* parasite is spread between vertebrate hosts by insect vectors, sandflies of the genera *Phlebotomus* and *Lutzomyia*. When an infected sandfly bites a vertebrate host to take a blood meal, the sandfly transmits metacyclic promastigotes into the bite site. These parasites are phagocytosed by the host's neutrophils and macrophages. Within the host cell, *Leishmania* differentiates into the amastigote form. Amastigotes are round aflagellates. These parasites multiply within a parasitophorous vacuole, eventually causing the host cell to undergo apoptosis. The resultant apoptotic bodies (containing amastigotes) are then taken up by new phagocytic cells. The parasites establish a cutaneous infectious or can spread to the liver and spleen. When a naïve sandfly bites the infected vertebrate, amastigotes will be taken up with the blood meal. In the hindgut of the sandfly, *Leishmania* differentiates into the promastigote form. Promastigotes are elongated, spindle-shaped flagellates. These parasites multiply in the hindgut and eventually migrate to the foregut and proboscis of the sandfly where they stop dividing and become infectious metacyclic promastigotes.



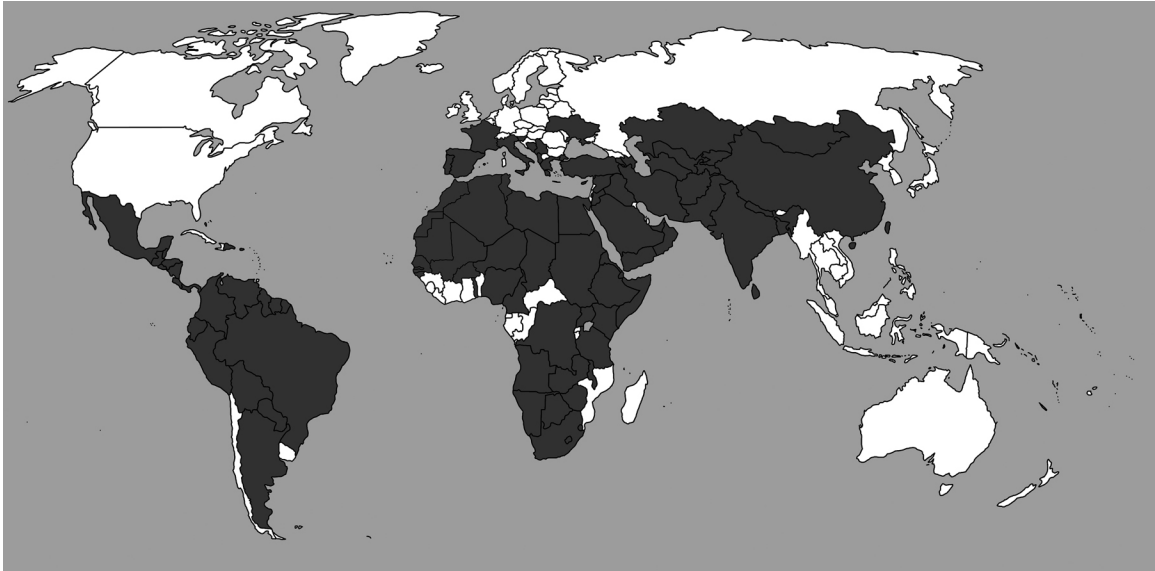
**Figure 1.3: Morphology of the *Leishmania* Parasite**

*Leishmania* is a dimorphic parasite. The promastigote form (which includes metacyclic promastigotes) is an elongated spindle with a single flagellum. This form of the parasite measures 10-20 $\mu$ M in length with a diameter of 2.5-5 $\mu$ M. The flagellum is between 1-2x the length of the cell body. The flagellum extends out of the flagellar pocket (the main site of endo- and exocytosis) at the anterior end of the parasite. This structure is present in a reduced state in the amastigote form. The amastigote stage of *Leishmania* has no exterior flagellum and is oval with a diameter of 2.5-5 $\mu$ M. Both forms of the parasite contain a nucleus and a kinetoplast, a single large DNA-containing mitochondrion. This extranuclear DNA forms mini- and maxi-circles within the kinetoplast. In the amastigote form, the kinetoplast is highly condensed. Beneath the plasma membrane of the parasite is a layer of subpellicular microtubules.



**Figure 1.4: Global Map of Leishmaniasis**

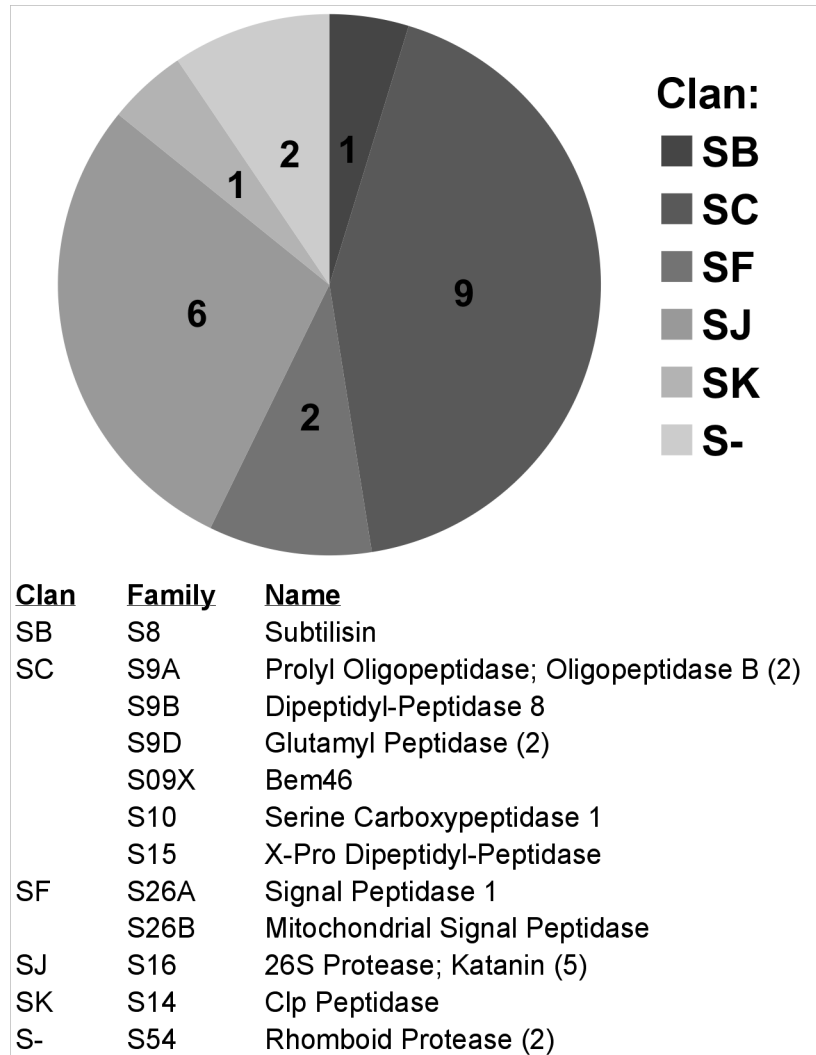
*Leishmania* is found in parts of at least 88 countries (dark gray) around the world, particularly in tropical and subtropical regions. Cutaneous leishmaniasis is endemic in areas ranging from the rain forests of Central and South America to the deserts of Africa, Asia and the Middle East. Visceral leishmaniasis is also present in most of these areas, with more than 90% of cases occurring in India, Bangladesh, Sudan, and Brazil. Leishmaniasis is also present in southern and Mediterranean Europe, and has been reported in south Texas in the U.S.A. (Maloney, Maloney et al. 2002).



Adapted from the public domain CIA Political Map of the World (Robinson projection).

**Figure 1.5: The Serine Protease Repertoire of *Leishmania***

The completed genomes of *L. major* and *L. infantum* (GeneDB) reveal that *Leishmania* has approximately twenty-one distinct serine protease genes representing six different clans. The genomes also include several protease pseudogenes (not shown). Clan SC is the dominant clan in terms of overall number. Interestingly, *Leishmania* has a single Clan SB protease and two Clan S-rhomboid proteases.



## **Chapter Two:**

### **The Oligopeptidase B of *Leishmania* Regulates Parasite Enolase for Immune Evasion and Virulence**

## **2.1. Introduction**

### **2.1.1. The Clan SC oligopeptidase B, a parasite virulence factor.**

Oligopeptidase B (OPB) is a member of the S9A prolyl oligopeptidase family of Clan SC serine proteases. This enzyme was first isolated from *E. coli* in 1975 by Pacaud and Richaud (Pacaud and Richaud 1975) and has since been described in the prokaryotes *Salmonella enterica*, *Mycobacterium tuberculosis*, and *Treponema dentocula* (Heiman and Miller 1978; Fenno, Lee et al. 2001; Camus, Pryor et al. 2002). In the eukaryotes, OPB was first isolated from *Trypanosoma cruzi* in 1978 by Bongertz and Hungerer (Bongertz and Hungerer 1978). It was initially termed alkaline peptidase because it was maximally active at a basic pH (Ashall 1990). This is in stark contrast to the lysosomal Clan CA cathepsins, common in the trypanosomes, which are active in acidic environments. Sequencing of alkaline peptidase revealed that this enzyme belongs to the prolyl oligopeptidase family (Burleigh, Caler et al. 1997). Prolyl oligopeptidases are enzymes present in organisms ranging from bacteria to mammals (Rennex, Hemmings et al. 1991; Yoshimoto, Kanatani et al. 1991). Alkaline peptidase was subsequently renamed oligopeptidase B to reflect that it is a family S9A member, with the “B” referring to the enzyme’s preference for cleaving substrates after basic amino acid residues instead of after proline like prolyl oligopeptidase (Yoshimoto, Tabira et al. 1995). OPB has been described in several of the *Trypanosoma* species and is present in *Leishmania* (Kornblatt, Mpimbaza et al. 1992; de Andrade, Santoro et al. 1998; Morty, Lonsdale-Eccles et al. 1999; Ivens, Peacock et al. 2005).

OPB, like all members of Clan SC, has a catalytic triad in the order Ser-Asp-His. In this triad the serine acts as the nucleophile during catalysis, histidine functions as a



proton donor, and aspartic acid orients the imidazole ring of histidine (Rawlings, Morton et al. 2008). While the crystal structure of OPB has not yet been solved, the structure of prolyl oligopeptidase has been known for over a decade. Family S9A proteases are cylindrical proteins with two large domains: a C-terminal catalytic domain with an  $\alpha/\beta$  hydrolase fold and an N-terminal seven-bladed  $\beta$ -propeller domain (Fulop, Bocskei et al. 1998). The  $\beta$ -propeller sits on top of the catalytic domain and partially blocks access to the active site of the enzyme. This conformation sterically prevents large substrates from entering the active site, thereby restricting the enzyme to the cleavage of small and unstructured oligopeptides. In addition to being generally inactive against globular substrates, these enzymes are insensitive to high molecular weight protease inhibitors such as aprotinin, serpins, and  $\alpha$ 2-macroglobulin (Guo, Lamb et al. 1998).

OPB is a known virulence factor for several pathogenic protozoa. It has been identified in multiple trypanosomatid species including *T. cruzi*, *T. brucei*, and *T. evansi* (Morty, Lonsdale-Eccles et al. 1999). These enzymes represent a subfamily of potential chemotherapeutic targets, as inhibitors have been shown to be trypanocidal (Morty, Troeberg et al. 1998; Bal, Van der Veken et al. 2003; Silva-Lopez, Morgado-Diaz et al. 2007). In *T. cruzi* OPB plays an important role in the invasion of non-phagocytic host cells. OPB transiently increases  $Ca^{+2}$  levels in host cells through an unknown signaling ligand and recruits lysosomes to the host cell surface to facilitate parasite entry (Burleigh, Caler et al. 1997). This process is blocked by OPB inhibitors and neutralizing antibodies (Burleigh, Caler et al. 1997). Targeted deletion of *T. cruzi* OPB resulted in reduced virulence in the murine infection model (Caler, Vaena de Avalos et al. 1998). The related *T. cruzi* prolyl oligopeptidase has also been implicated in tissue invasion by degrading

components of the extracellular matrix (Yoshida 2006). In addition, the OPBs of both *T. brucei brucei* and *T. evansi* were shown to be released into the host bloodstream by dying parasites during an infection (Morty, Lonsdale-Eccles et al. 2001). Since these enzymes are insensitive to inhibition by plasma serpins and  $\alpha$ 2-macroglobulin, they remain catalytically active in the host. During *T. evansi* infection OPB has been shown to degrade host peptide hormones, such as atrial natriuretic factor, and is therefore believed to play a role in disease pathogenesis (Morty, Pelle et al. 2005). Despite this research, a natural OPB substrate has yet to be identified in the trypanosomatid parasites and thus the physiological function of the enzyme remains unknown.

### **2.1.2. Enolase has moonlighting functions on the cell surface.**

Enolase is a well-known metalloenzyme that functions in carbohydrate metabolism in a number of organisms. It was described by Lohmann and Meyerhof in 1934 and has since been found to reversibly catalyze 2-phosphoglycerate into phosphoenolpyruvate, the ninth step of the glycolysis (Lohman and Meyerhof 1934). As a glycolytic enzyme, enolase is localized to the cytosol; however, recent studies have uncovered that in a variety of eukaryotic organisms, including yeast and mammals, enolase can localize to different parts of a cell such as the cell surface and the nucleus (Pancholi 2001; Ferguson, Parmley et al. 2002). It is now known that enolase has multiple moonlighting functions within the cell that do not require its glycolytic enzymatic function. In both bacterial and mammalian cells, enolase has been found on the outer surface of the plasma membrane where it is able to bind and activate plasminogen (Nakajima, Hamanoue et al. 1994; Pancholi and Fischetti 1998). Enolase-

bound plasminogen is converted to plasmin and is able to degrade fibrin. This process allows cells to concentrate proteolytic activity on their surface and can assist in their penetration of tissues and the extracellular matrix. Cell surface enolase and plasminogen have been implicated in several autoimmune disorders (Moodie, Leaker et al. 1993; Pratesi, Moscato et al. 2000), and are believed to play a role in tumor progression and metastasis (Chang, Liu et al. 2006).

In addition to autoimmune disorders and cancer, cell surface enolase is known to be a virulence factor for multiple infectious organisms including pathogenic bacteria and protozoa. Plasminogen activation is also known to aid in pathogen invasion of tissues. This process plays a role during infections by *Bacillus anthracis* (Agarwal, Kulshreshtha et al. 2008), *Streptococcus pneumoniae* (Attali, Durmort et al. 2008), *Toxoplasma gondii* (Ferguson, Parmley et al. 2002), and *Plasmodium* (Pal-Bhowmick, Vora et al. 2007). Recent studies have indicated that the plasminogen-plasmin system is also important for the virulence of *Leishmania* (Avilan, Calcagno et al. 2000). *L. mexicana* enolase has been cloned and characterized (Quinones, Pena et al. 2007), and it has been shown to localize to the parasite cell surface where it can bind to and activate host plasminogen (Vanegas, Quinones et al. 2007). Furthermore, *L. mexicana* infection of plasminogen knockout mice resulted in smaller lesion size with isolated foci of parasites, compared to scattered and diffuse infections in the lesions of wildtype mice (Maldonado, Marina et al. 2006). This research indicates that *Leishmania* uses cell surface enolase to interact with the host's plasminogen-plasmin system and that this process contributes to parasite virulence. Previous research has not addressed how *Leishmania* controls cell surface enolase levels or how this process is regulated.

### **2.1.3. *Leishmania* expresses a high level of serine protease activity.**

Clan CA cysteine proteases represent the dominant group of proteolytic enzymes for most protozoa and primitive metazoa, while trypsin family (S1A) serine proteases are dominant in vertebrates (Rawlings, Morton et al. 2008). It was therefore surprising to find that *Leishmania donovani*, *L. major*, and *L. mexicana* (but not the related kinetoplastid parasite, *Trypanosoma cruzi*) express high levels of serine protease activity in parasite extracts. The broad-spectrum serine protease inhibitor PEFABLOC was found to inhibit replication of *L. donovani* promastigotes in culture (Figure 2.1). In addition, studies performed on *L. amazonensis* have shown that the serine protease inhibitors benzamidine, TPCK, and ShPI-I (a Kunitz-type inhibitor from the sea anemone *Stichodactyla helianthus*) reduce parasite viability *in vitro* (Silva-Lopez, Morgado-Diaz et al. 2007).

**In this study we describe the identification and characterization of *Leishmania*'s dominant serine protease, oligopeptidase B. Native and recombinant enzyme was purified and biochemically characterized. The single-copy oligopeptidase B gene was deleted by targeted gene replacement. The role of oligopeptidase B was then analyzed both in *in vitro* culture, as well as in the murine model of infection. To delineate a specific role of OPB in the *Leishmania* infectious life cycle, two-dimensional gel electrophoresis was compared between wildtype and OPB *-/-* parasites. We found that OPB regulates levels of parasite enolase and is required for proper immune evasion.**

## **2.2. Results**

### **2.2.1. *Leishmania donovani* expresses high levels of serine protease activity.**

In a search for cysteine protease activity similar to that reported in *L. major* and *L. mexicana* (Mottram, Coombs et al. 2004), the fluorescent peptide substrate, Z-Pro-Arg-AMC, detected high levels of protease activity in extracts of *L. donovani*. However, this activity was inhibited by class-specific serine protease inhibitors such as PEFABLOC and chloromethyl ketone peptides, and was resistant to the cysteine protease inhibitor E-64. Additionally, this activity was observed at pH 8.0 and was absent at pH 5.5, a profile consistent with serine protease activity. Because Clan CA cysteine proteases are the major proteolytic activity in other protozoan parasites (Que and Reed 2000; Sajid and McKerrow 2002; Rosenthal 2004), a comparison was made between the activity found in *L. donovani* and protease activity against the same substrate in another intracellular kinetoplastid parasite, *Trypanosoma cruzi*. In contrast to *L. donovani*, *Trypanosoma cruzi* parasites did not express significant levels of serine protease activity (Figure 2.2A). Oligopeptidase B activity was detected in both the promastigote and axenic amastigote stages of *Leishmania*, however this activity was significantly elevated in the amastigote stage (Figure 2.2B).

### **2.2.2. Purification of the serine protease activity of *L. donovani* identifies oligopeptidase B by mass spectrometry.**

In order to identify the serine protease responsible for the observed proteolytic activity, the enzyme was purified from *L. donovani* utilizing Z-Pro-Arg-AMC as a substrate. The soluble fraction of *L. donovani* lysate was fractionated by Q-sepharose ion-exchange chromatography in a pH 8.0 Tris-HCl buffer. Activity assays were performed

on eluted fractions and one peak of activity was observed. SDS-PAGE of the fraction #60, the maximal active fraction, was performed (Figure 2.3). Tandem mass spectrometry was performed on bands excised from this gel. The trypsin-generated peptides from the 75-90 kDa excised band were identified by informatics analysis as consistent with *Leishmania* oligopeptidase B (predicted molecular weight: 83.1 kDa). This analysis yielded 23 peptides mapping to OPB with amino acid coverage of 36% and a probability based MOWSE score of 1053.

### **2.2.3. *L. donovani* OPB was cloned, sequenced, and recombinantly expressed in *P. pastoris*.**

The gene encoding *Leishmania donovani* OPB was cloned. Sequencing yielded a 2,196 bp (731 amino acid) gene. This sequence was submitted to GenBank with accession number GQ491028. Comparisons of *L. donovani* OPB to other trypanosomatid OPBs are summarized in Table 2.1. This gene was cloned into pPICZ- $\alpha$  A and recombinantly expressed in *Pichia pastoris*. Recombinant protein was purified using a hydrophobic interaction column followed by ion-exchange chromatography. Purified OPB protein was maximally active at pH 9.5 (Figure 2.4). The hydrolysis of Z-Pro-Arg-AMC by purified OPB was tested in the presence of a panel of protease inhibitors and divalent cations (Table 2.2). This enzymatic activity was completely abolished by antipain, PEFABLOC (AEBSF, a broad based serine protease inhibitor), PPACK (biotin-X-D-Phe-Pro-Arg-CMK), leupeptin (reversible inhibitor of trypsin-like proteases), and TLCK (irreversible trypsin inhibitor). Benzamidine (inhibitor of trypsin-like proteases) was found to partially inhibit OPB. The catalytic domain of family S9A proteases is

known to be covered by a seven-bladed  $\beta$ -propeller that prevents the entrance and hydrolysis of larger substrates (Rawlings, Morton et al. 2008). It therefore followed that the large molecular weight serine protease inhibitors  $\alpha$ 1-Antitrypsin, aprotinin, and soybean trypsin inhibitor (SBTI) did not inhibit OPB. Additionally, TPCK (irreversible chymotrypsin inhibitor) and PMSF (similar to PEFABLOC) had no effect on OPB activity. As expected, inhibitors of cysteine, aspartic and metallo-proteases did not appreciably inhibit OPB activity. Divalent cations  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$ , and  $\text{Mn}^{+2}$  partially reduced OPB activity while  $\text{Zn}^{+2}$  completely blocked activity.

#### **2.2.4. Substrate specificity profiling of recombinant OPB reveals substrate preference.**

Positional scanning synthetic combinatorial libraries (PS-SCLs) have been successfully employed to determine P1-P4 subsite preferences for several proteases (Choe, Leonetti et al. 2006; O'Brien, Mackey et al. 2008). Complete libraries for each of the P1-P4 positions were used, containing 160,000 tetrapeptide substrates (Figure 2.5). The PS-SCL showed that OPB has a strong preference for lysine or arginine residues at P1. OPB can accommodate most amino acids in the P2-P4 positions; however, there is a preference for glycine in P2 and proline in P4. Bulky hydrophobic groups such as tyrosine, phenylalanine, and tryptophan are least preferred. The optimal substrate motif for OPB, as predicted by PS-SCL is—P4: P/X, P3: X, P2: G/X, P1: K/R.

This substrate profile was verified by testing a panel of fluorogenic peptide substrates including both good and poor predicted substrates (Table 2.3). OPB cleavage of each substrate is normalized to the  $V_{\text{max}}$  of the Z-PR-AMC substrate. Only substrates

with either a lysine or an arginine in the P1 position were cleaved. As predicted by PS-SCL analysis, the substrate Z-GGR-AMC was preferred by OPB.  $K_{cat}$  and  $K_m$  were determined for both Z-GGR-AMC and Z-GPR-AMC. This data demonstrates that Gly-Gly-Arg is a better substrate and validates the results of the PS-SCL. Compared to Z-GGR-AMC, Glut-GR- and Z-R-AMC were moderate and poor substrates, respectively. Thus, fluorogenic peptide hydrolysis was more efficient when greater than one substrate-binding site was occupied

### **2.2.5. Oligopeptidase B *-/-* parasites lose the major serine protease activity.**

The published *L. major* and *L. infantum* genomes (GeneDB) as well as a study performed on *L. amazonensis* (Guedes, Rezende et al. 2007) indicate that *Leishmania spp.* contain a single copy of OPB per haploid genome. This was confirmed to be true in *L. donovani* and *L. major* by Southern blot analysis of genomic DNA (data not shown). Deletion of both alleles of the single-copy gene was carried out by two rounds of targeted gene replacement and confirmed by southern blot (Figure 2.6A). To confirm the identity of the major serine protease activity of *L. donovani* as due to oligopeptidase B, OPB +/- and OPB *-/-* parasites were compared to wildtype (Figure 2.6B). Serine protease activity measured by Z-PR-AMC was completely lost in the OPB *-/-* parasites. Furthermore, episomal replacement of the OPB gene on a pXG plasmid resulted in recovery of this serine protease activity.

OPB +/- and *-/-* parasites successfully differentiated in culture back and forth between promastigote and axenic amastigote forms. OPB *-/-* promastigotes (but not amastigotes) had a statistically significant growth delay in culture, however at stationary



phase (day 5 post-split) these parasites reached the same density as their wildtype counterparts. These knockout parasites also efficiently infected J774 murine macrophages in culture (data not shown).

#### **2.2.6. OPB $-/-$ parasites accumulate inactive enolase isoforms.**

During infection by *T. cruzi* OPB is used to indirectly stimulate transient  $\text{Ca}^{+2}$  levels in host cells. This results in the recruitment of lysosomes to the plasma membrane and allows the parasite to penetrate non-phagocytic cells (Burleigh, Caler et al. 1997). *Leishmania* does not employ this strategy and is restricted to phagocytic cells of the immune system. We next sought to uncover the function of OPB in this genus of parasite. Two-dimensional gel electrophoresis was used to study the main differences in protein expression between wildtype and OPB  $-/-$  parasites (Figure 2.7A). Four major protein spots were significantly more intense in OPB  $-/-$ . Mass spectrometry analysis indicated that all four were consistent with *Leishmania* enolase. These spots differ by their isoelectric points in a step-wise manner suggesting that they represent different phosphorylation states of enolase. In the malaria parasite *Plasmodium yoelii* different phosphorylated isoforms of enolase indicated localization of enolase to the parasite membrane (Pal-Bhowmick, Vora et al. 2007). It was recently found that inactive forms of enolase are found on the plasma membrane of *L. mexicana* (Quinones, Pena et al. 2007). We therefore tested OPB  $-/-$  lysates for enolase enzymatic activity by detecting the conversion of 2-phosphoglycerate to phosphoenolpyruvate spectrophotometrically (Figure 2.7B). As predicted, there was no increase in active enolase; thus the increase in enolase isoforms detected by 2-D gel analysis did not represent active enzyme.

### **2.2.7. OPB is necessary for *Leishmania* to silently infect macrophages.**

It is known that *Leishmania* parasites are able to evade the host immune response, at least initially, when establishing an infection (Bogdan and Rollinghoff 1998). Macrophages infected with several species of *Leishmania* do not significantly alter their gene expression profiles over the first 24 hours post-infection (Zhang and McKerrow 2009). To test if the accumulation of inactive enolase by OPB *-/-* parasites interferes with detection by macrophages we performed microarray analysis of gene expression by bone marrow-derived macrophages infected with *Leishmania*. Macrophages were infected with wildtype or OPB *-/-* parasites, or were uninfected. RNA was collected at 0, 2, 6, 12, and 24 hours post-infection. These RNAs were dye-coupled and hybridized onto Mouse Exonic Evidence-Based Oligonucleotide (MEEBO) Arrays. Array data were analyzed for statistically significant genes using the Statistical Analysis of Microarray (SAM) software. Fold-change was calculated compared to uninfected cells (Figure 2.8A & B). Using a false discovery rate threshold of <1.0% and a minimum fold-change of 2 over uninfected, only 23 genes were significantly up- or down-regulated in wildtype *Leishmania*-infected macrophages; however, a striking 495 genes were significantly up-regulated in OPB *-/-* *Leishmania*-infected macrophages. Heat maps of clustered arrays illustrate the significant up-regulation of macrophage genes following infection by OPB *-/-* parasites compared to wildtype-infected and uninfected controls (Figure 2.8C). Families of proteins that were significantly upregulated in the OPB *-/-* infections include proteins involved in cytokine secretion, signal transduction, and the inflammatory response (Table 2.4).

### **2.2.8. Loss of OPB results in delayed lesion formation in mice.**

BALB/c mice were infected subcutaneously into their left hind footpads with either wildtype or OPB *-/-* parasites. Footpad swelling was measured weekly (Figure 2.9). Swelling was evident in the wildtype-infected mice after 7 weeks; however, significant swelling was not observed in mice infected with OPB *-/-* parasites until after 14 weeks. The OPB *-/-* infections were not self-limiting and continued to increase footpad swelling, but the lesion size was consistently 7 weeks delayed compared to the wildtype infections.

## **2.3. Discussion**

*Leishmania* parasites are responsible for a major global health problem, leishmaniasis, which affects tens of millions of people worldwide. While different *Leishmania* species produce a spectrum of clinical disease, all are characterized by parasite invasion and survival within host macrophages. The initial entry into the macrophage likely occurs when parasites are introduced into the skin through the bite of a sandfly vector. The resulting inflammatory response to the sandfly saliva brings macrophages and parasites into contact. In the case of the most serious *Leishmania* infection, visceral leishmaniasis caused by *Leishmania donovani* and related species, parasite-infected macrophages are found in numerous viscera, including the liver, spleen, and bone marrow.

Proteases are key virulence factors in the pathogenesis of diseases caused by *Leishmania* and related kinetoplastid parasites (Mottram, Coombs et al. 2004). A noteworthy biochemical difference between *Leishmania* parasites and the closely related

*Trypanosoma cruzi* is the discovery, reported here, of unexpectedly high levels of serine protease activity. While proteases represent a significant genomic complement of protozoa and metazoa, the Clan CA cysteine proteases predominate in invertebrates (Sajid and McKerrow 2002).

In this study, we identified oligopeptidase B (OPB) as the predominant serine protease of the *Leishmania* parasite. Initially, proteolytic activity was identified in *L. donovani* lysate using a Z-Pro-Arg-AMC substrate, and was believed to be due to a Clan CA cysteine protease. This activity was determined to be a serine protease by its vulnerability to PEFABLOC and its preference for an alkaline pH (Figure 2.2A). To identify the serine protease responsible for this proteolytic activity, the protein of interest was purified from *L. donovani* lysate by ion-exchange chromatography followed by SDS-PAGE and mass spectrometry (Figure 2.3). Two Clan SC family S9A proteases were found in the maximally active fraction, oligopeptidase B and prolyl oligopeptidase (POP). It was hypothesized that OPB was the enzyme of interest due to the fact that OPBs typically cleave after basic residues and POPs are post-proline-cutting enzymes.

To confirm that OPB was the main serine protease of *Leishmania*, the *OPB* gene was deleted from the *L. donovani* and *L. major* genomes by homologous recombination (Figure 2.6). As expected the proteolytic activity was abolished in the *OPB* *-/-* parasites. *OPB* was then cloned onto the *Leishmania* expression vector pXG and episomally added back to knockout parasites. The expected proteolytic activity returned to these parasites, giving hard proof that *OPB* was responsible for *Leishmania*'s high serine protease activity. Previous studies (Silva-Lopez, Morgado-Diaz et al. 2007) and our work (Figure 2.1) have shown that serine protease inhibitors such as TPCK, ShPI-I, benzamidine, and

PEFABLOC reduce parasite viability *in vitro*. Our research has shown that OPB -/- parasites are viable as both promastigotes and amastigotes, thus these inhibitors are likely acting through an additional serine protease.

OPB from *L. donovani* was cloned and recombinantly expressed in *Pichia pastoris*. This enzyme was found to have a high level of sequence identity to other *Leishmania* and *Trypanosoma* OPBs (Table 2.1). Like these other OPBs, *L. donovani* OPB has a C-terminal catalytic domain and a predicted N-terminal  $\beta$ -propeller domain, which was predicted to restrict large substrates from the proteolytic active site. As expected, *L. donovani* OPB was unable to digest large substrates such as casein, hemoglobin, and bovine serum albumin. PS-SCL profiling revealed that this enzyme requires a basic amino acid in the P1 position, which is characteristic for this type of enzyme (Kanatani, Masuda et al. 1991). The P2 position was found to have a strong preference for Gly (Figure 2.5). Previous studies have found that Gly is acceptable for other OPBs; however unlike *L. donovani* OPB, a basic residue is typically preferred (Morty, Lonsdale-Eccles et al. 1999). Mapping of the trypanosomatid OPB S1 and S2 subsite pockets by de Matos Guedes *et al.* revealed that S2 in the trypanosomes has two acidic residues, whereas S2 in the *Leishmanias* has only one acidic residue together with a neutral or basic residue (de Matos Guedes, Carneiro et al. 2007). This difference in S2 charge would explain the *L. donovani* OPB preference for Gly over Arg and Lys. OPB preference for P3 and P4 residues was found to be more flexible than for P1 and P2. In the P3 position Ser, Gly, Ala, Asn, Pro and Thr are each acceptable. In the P4 position Pro is preferred. In all four positions tested, Tyr and Trp were the least tolerated residues.

The results of the PS-SCL were confirmed by evaluating the cleavage of a panel

of synthetic substrates by OPB (Table 2.3). As predicted, Z-Gly-Gly-Arg-AMC was found to be the ideal substrate. In addition, OPB exhibited a preference for substrates at least two amino acids in length, a preference documented in other OPBs (Nishikata 1984). *L. donovani* OPB is optimally active at an alkaline pH and is resistant to large molecular weight inhibitors (Table 2.2) as has been previously reported (Barrett, Rawlings et al. 2004). Interestingly, OPB is resistant to PMSF but is sensitive to PEFABLOC, a PMSF analog. This characteristic could be used in differentiating OPB activity from that of other serine proteases. Unlike trypanosome OPBs, the *Leishmania* OPB is not highly sensitive to DCI and shows reduced activity in the presence of divalent cations (Morty, Authie et al. 1999).

Previous research has not been able to identify the natural substrate for OPB in trypanosomatid parasites. We sought to biologically characterize the function of OPB in *Leishmania*. We found that three species (*L. donovani*, *L. major*, and *L. mexicana*), representing three of the main *Leishmania* complexes, exhibited high levels of OPB when compared to *T. cruzi* (Figure 2.2B). While biochemical studies have been performed on OPB from several trypanosome species, the majority of the biological characterization of OPB from a trypanosomatid has been performed on *T. cruzi* (Burleigh, Caler et al. 1997). *T. cruzi*, like *Leishmania*, is an intracellular parasite within the host; however, unlike *Leishmania*, *T. cruzi* infects non-phagocytic cells. OPB from *T. cruzi* has been found to play an important role in the invasion of these non-phagocytic cells. It is known that OPB transiently increases  $\text{Ca}^{+2}$  levels in host cells, and that this results in the recruitment of lysosomes to the host cell surface for parasite entry; however, the direct action of OPB in this process has not yet been found. It is believed that OPB acts through an unknown

signaling ligand or parasite cell surface receptor (Caler, Vaena de Avalos et al. 1998).

As *Leishmania* is only able to enter host cells via phagocytosis, OPB does not function in host cell entry as it does in *T. cruzi*. To determine the pathway by which OPB acts in *Leishmania* we employed 2-dimensional gel analysis. Four enolase isoforms were identified as the major protein targets of OPB in *Leishmania*. These isoforms accumulated in the OPB *-/-* parasites, as compared to wildtype (Figure 2.7). While enolase functions in carbohydrate metabolism in a number of organisms, it has also recently been shown to function as a virulence factor in bacterial pathogens such as *Bacillus anthracis* and *Streptococcus pneumoniae* (Agarwal, Kulshreshtha et al. 2008; Attali, Durmort et al. 2008) as well as in protozoan parasites like *Toxoplasma gondii* and *Plasmodium* (Ferguson, Parmley et al. 2002; Pal-Bhowmick, Vora et al. 2007). In fact, in *Leishmania* parasites, surface associated enolase was previously documented and shown to bind host plasminogen (Vanegas, Quinones et al. 2007). In addition, *Leishmania* was found to be less infection in plasminogen-knockout mice, supporting the role of enolase-plasminogen interactions in *Leishmania*'s virulence (Maldonado, Marina et al. 2006).

Plasminogen activated to the protease plasmin or binding to plasminogen receptors may facilitate parasite entry into macrophages or prevent fibrin deposition on extracellular parasites. However, once in the macrophage, retention of enolase might negatively impact parasite survival by activating macrophages. This hypothesis was supported by showing that OPB *-/-* *Leishmania* caused activation of infected macrophages with expression of TLR-2, TGF- $\beta$  receptor, TNF receptors, and interferon-activated proteins (Table 2.4 and Figure 2.8). This is in stark contrast to wildtype *Leishmania* that elicits little, if any, macrophage reaction. The proposed role of OPB

during host infection is illustrated in Figure 2.10. A defect in the ability of *Leishmania* to enter or reside within host macrophages without macrophage activation would explain the significant delay in footpad swelling seen in the murine experimental model of OPB -/- *Leishmania* infection when compared to wildtype parasites. While not absolutely necessary for disease pathogenesis, OPB is required for full virulence.



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**Table 2.1: Comparison of predicted OPB proteins from trypanosomatids.**

The homologous OPB proteins predicted in five *Leishmania* and three *Trypanosoma* species are compared. Amino acid identities were compared to the sequence of *L. donovani* OPB. All of the proteins were found to be between 714 and 731 amino acids in length, between 80.7 and 83.5 kDa in mass, and to contain the conserved canonical clan SC catalytic triad.

**Comparison of Predicted OPB Proteins from Trypanosomatids**

Species:	AA Identity:	Length (AA):	kDa:	Catalytic Triad:
<i>L. donovani</i>	100.0%	731	83.1	S577, D662, H697
<i>L. infantum</i>	99.7%	731	83.1	S577, D662, H697
<i>L. major</i>	95.5%	731	83.0	S577, D662, H697
<i>L. amazonensis</i>	90.3%	731	83.5	S577, D662, H697
<i>L. braziliensis</i>	86.5%	731	83.1	S577, D662, H697
<i>T. cruzi</i>	63.2%	714	81.0	S562, D647, H682
<i>T. brucei brucei</i>	62.1%	715	80.7	S563, D648, H683
<i>T. evansi</i>	61.8%	715	80.7	S563, D648, H683

**Table 2.2: Inhibition of OPB enzymatic activity by protease inhibitors and divalent cations.**

OPB was tested for sensitivity to class-specific protease inhibitors and common divalent cations. OPB activity was measured by an activity assay at pH 8.0 using the Z-PR-AMC substrate. The enzyme was pre-incubated with an inhibitor or cation for 10 minutes at the concentrations indicated. Substrate was then added and hydrolysis was measured by AMC hydrolysis. Residual activity is the percent of OPB activity remaining as compared to pre-incubation with solvent-only controls.

**Inhibition of OPB Enzymatic Activity by Protease Inhibitors and Divalent Cations**

Target Protease	Inhibitor	Concentration	Residual Activity
Serine	$\alpha$ 1-Antitrypsin	1 $\mu$ g/mL	100%
	Antipain	100 $\mu$ M	0%
	Aprotinin	1 $\mu$ g/mL	100%
	Benzamidine	1mM	27%
	3,4-DCI	50 $\mu$ M	88%
	PEFABLOC	2mM	0%
	PMSF	1mM	100%
	PPACK	10 $\mu$ M	0%
	Leupeptin	100 $\mu$ M	0%
	SBTI	10 $\mu$ g/mL	100%
	TLCK	100 $\mu$ M	0%
TPCK	100 $\mu$ M	100%	
Threonine	Bortezomib	10 $\mu$ M	100%
Cysteine	CA-074	1 $\mu$ M	96%
	Cystatin	1 $\mu$ g/mL	100%
	E-64	10 $\mu$ M	85%
	K11777	100 $\mu$ M	100%
	DTT	100 $\mu$ M	100%
Aspartic	Pepstatin A	1 $\mu$ M	100%
Metallo	EDTA	5mM	100%
	<b>Cations</b>	<b>Concentration</b>	<b>Residual Activity</b>
	CaCl <sub>2</sub>	10mM	69%
	MgCl <sub>2</sub>	10mM	73%
	MnCl <sub>2</sub>	10mM	73%
	ZnCl <sub>2</sub>	10mM	0%

**Table 2.3: Substrate specificity of OPB.**

To test the predictive power of the PS-SCL, fluorogenic substrates were obtained that were predicted to be either good or poor matches by the PS-SCL results. Substrates were grouped and listed by decreasing initial cleavage velocities. Percent activity is normalized to the initial velocity of observed using the Z-PR-AMC substrate. All assays were performed at pH 8.0 using 20  $\mu\text{M}$  substrate (except for those used to calculate  $K_{\text{cat}}$  and  $K_{\text{m}}$  values). The PS-SCL predicted that the ideal P2 amino acid would be Gly. Therefore Z-GGR-AMC and Z-GPR-AMC, that differ only in the P2 position, were compared as substrates for OPB and the kinetic parameters were calculated. These assays were performed at substrate concentrations ranging from 80 to 0.2  $\mu\text{M}$ .

Substrate Specificity of OPB							Substrate Specificity of OPB						
Blocking Group	P5	P4	P3	P2	P1	Activity	Blocking Group	P5	P4	P3	P2	P1	Activity
	Z		G	G	R	197%		H				A	0%
NtBoc			L	G	R	108%	Ac	D	E	V	D		0%
	Z		G	P	R	104%	Z		V	A	D		0%
	Z			P	R	100%	Ac	W	E	H	D		0%
	Z		V	V	R	92%	Z		L	L	E		0%
Glut				G	R	91%	Suc		A	A	F		0%
	Z			R	R	89%	Suc	P	S	P	F		0%
	Z			L	R	78%	H			G	F		0%
Boc			L	R	R	69%	H				L		0%
Ac				F	R	62%	Z		G	G	L		0%
Ac	K	Q	K	L	R	51%	Z		A	E	N		0%
	Z			F	R	50%	Ac		A	S	N		0%
	Z	A	A	R	R	40%	Ac		A	V	N		0%
Boc		R	V	R	R	31%	Suc	G	P	L	G	P	0%
Ac	F	R	S	L	K	23%	Suc			L	Y		0%
	Z				R	1%	Suc	L	L	V	Y		0%
							TFA				Y		0%

Kinetic Parameter	Z-GPR-AMC	Z-GGR-AMC
$K_{\text{m}}$ ( $\mu\text{M}$ )	7.76	3.08
$K_{\text{cat}}$ ( $\text{S}^{-1}$ )	4.25e4	6.83e4
$K_{\text{cat}}/K_{\text{m}}$ ( $\text{S}^{-1}\mu\text{M}^{-1}$ )	0.55e4	2.22e4

**Table 2.4: Gene families upregulated following OPB -/- infection.**

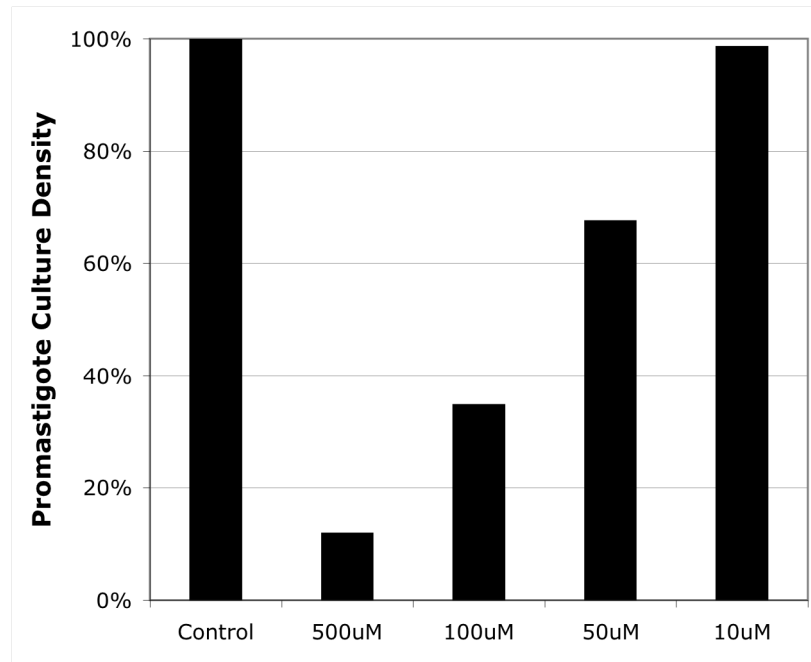
Gene families upregulated following OPB -/- infection. DAVID analysis was performed to identify families and pathways of proteins that were significantly upregulated in macrophages infected by OPB knockout *Leishmania*. Protein families that were over-represented in the hit set with a fold change greater than 2.5 and a p-value less than 0.05 are listed.

**Gene Families Upregulated Following OPB -/- Infection**

Protein Family	Count	Fold Change	P-Value
Cytokine secretion	3	15.36	0.014
Positive regulation of Ras protein signal transduction	4	14.62	0.002
Positive regulation of cell adhesion	4	11.37	0.004
Positive regulation of small GTPase mediated signal transduction	4	10.24	0.006
Regulation of protein secretion	4	8.53	0.010
Cytokines and Inflammatory Response	3	8.09	0.047
Positive regulation of phosphorylation	4	7.87	0.012
Positive regulation of phosphate metabolic process	4	7.31	0.015
Protein secretion	6	6.40	0.002
Inositol or phosphatidylinositol phosphatase activity	5	4.46	0.024
Ubiquitin thiolesterase activity	6	3.48	0.027
Ubiquitin-specific protease activity	6	3.33	0.033
Small conjugating protein-specific protease activity	6	3.19	0.038
Protein ubiquitination	6	3.13	0.041
Thiolester hydrolase activity	7	3.01	0.027
Vasculature development	13	2.77	0.002
Growth factor activity	7	2.73	0.042
Blood vessel development	12	2.58	0.006
Blood vessel morphogenesis	10	2.56	0.016

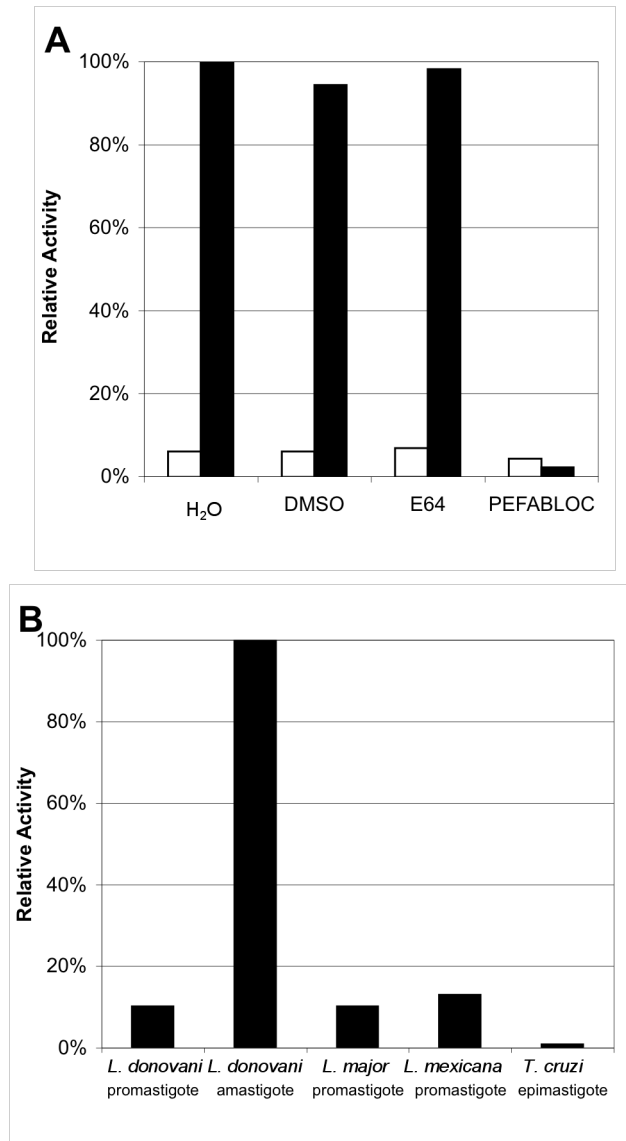
**Figure 2.1: PEFABLOC reduces the replication rate of *L. donovani* in culture.**

*L. donovani* promastigotes were split 1:20 into M199 containing different concentrations of the broad-spectrum serine protease inhibitor PEFABLOC. At 48 hours post-split, the cell density was measured and compared to an untreated control. PEFABLOC reduced the promastigote culture density in a dose-dependant manner.



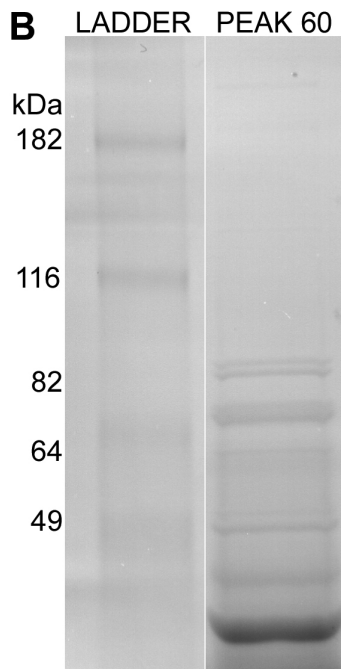
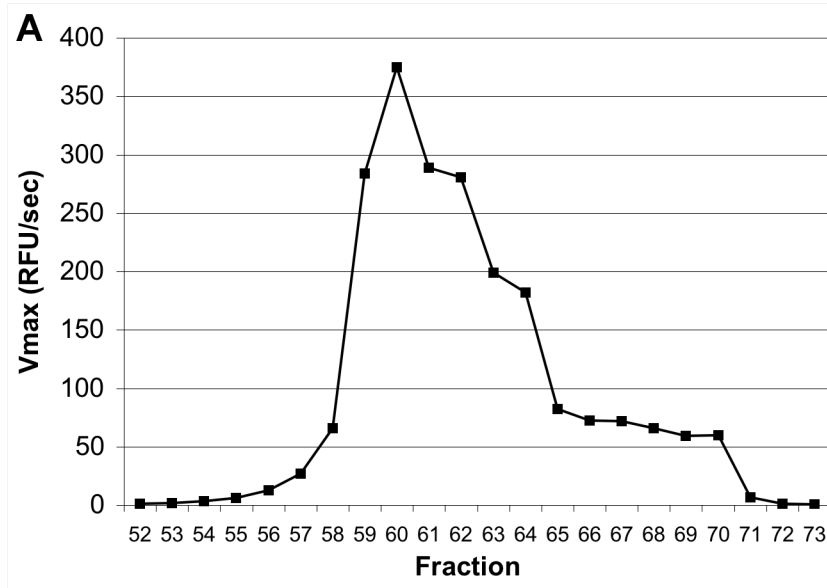
**Figure 2.2: Identification of high serine protease activity in *Leishmania* lysate.**

- A. The soluble fraction of *L. donovani* lysate was tested for protease activity using a Z-PR-AMC substrate. Activity assays were performed at pH 5.5 (empty bar) and at 8.0 (solid black bar), pH levels that are generally preferred by cysteine and serine proteases, respectively. The activity observed was tested for sensitivity to the cysteine protease inhibitor E-64 and the serine protease inhibitor PEFABLOC. Activities were normalized to the protease activity observed at the indicated pH without inhibitor.
- B. OPB activity was compared between three species of *Leishmania* promastigotes, *L. donovani* axenic amastigotes, and *T. cruzi* epimastigotes. Equivalent amounts of protein from lysed parasites were measured for OPB activity at pH 8.0 using the Z-PR-AMC substrate. All three *Leishmania* promastigotes had activity levels greater than 10-fold higher than the level observed from *T. cruzi*. *L. donovani* amastigotes contained 10-fold higher OPB activity than of that found in the promastigote form.



**Figure 2.3: Purification scheme and identification of OPB from *L. donovani* lysate.**

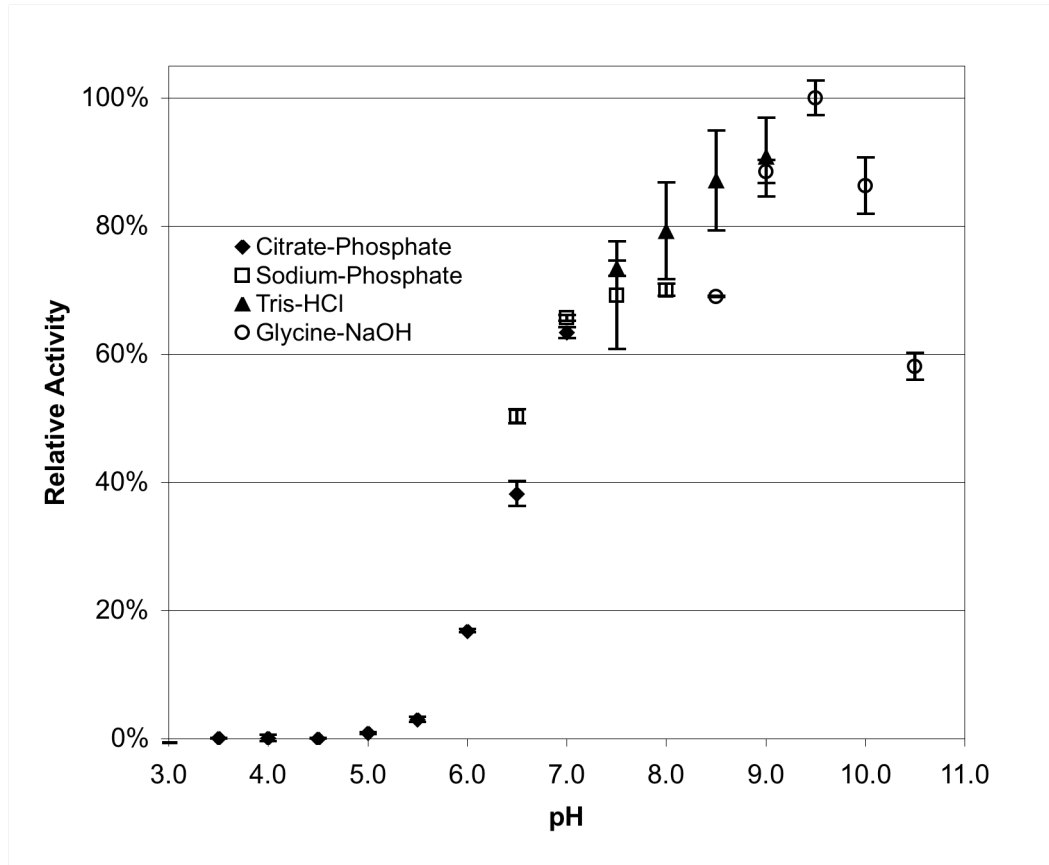
- A. The soluble fraction of *L. donovani* promastigote lysate was fractionated by Q-sepharose ion-exchange chromatography. OPB-containing fractions were identified by testing for cleavage of Z-PR-AMC at pH 8.0. Activity was plotted against fraction number and a single peak of activity was observed.
- B. The maximally active fraction, #60, was resolved on an SDS-PAGE gel. Major bands were excised from the gel and the proteins were identified by tandem mass spectrometry. The protein contained in the excised gel from 75-90 kDa was identified as OPB. OPB has a predicted molecular weight of 83.1 kDa.





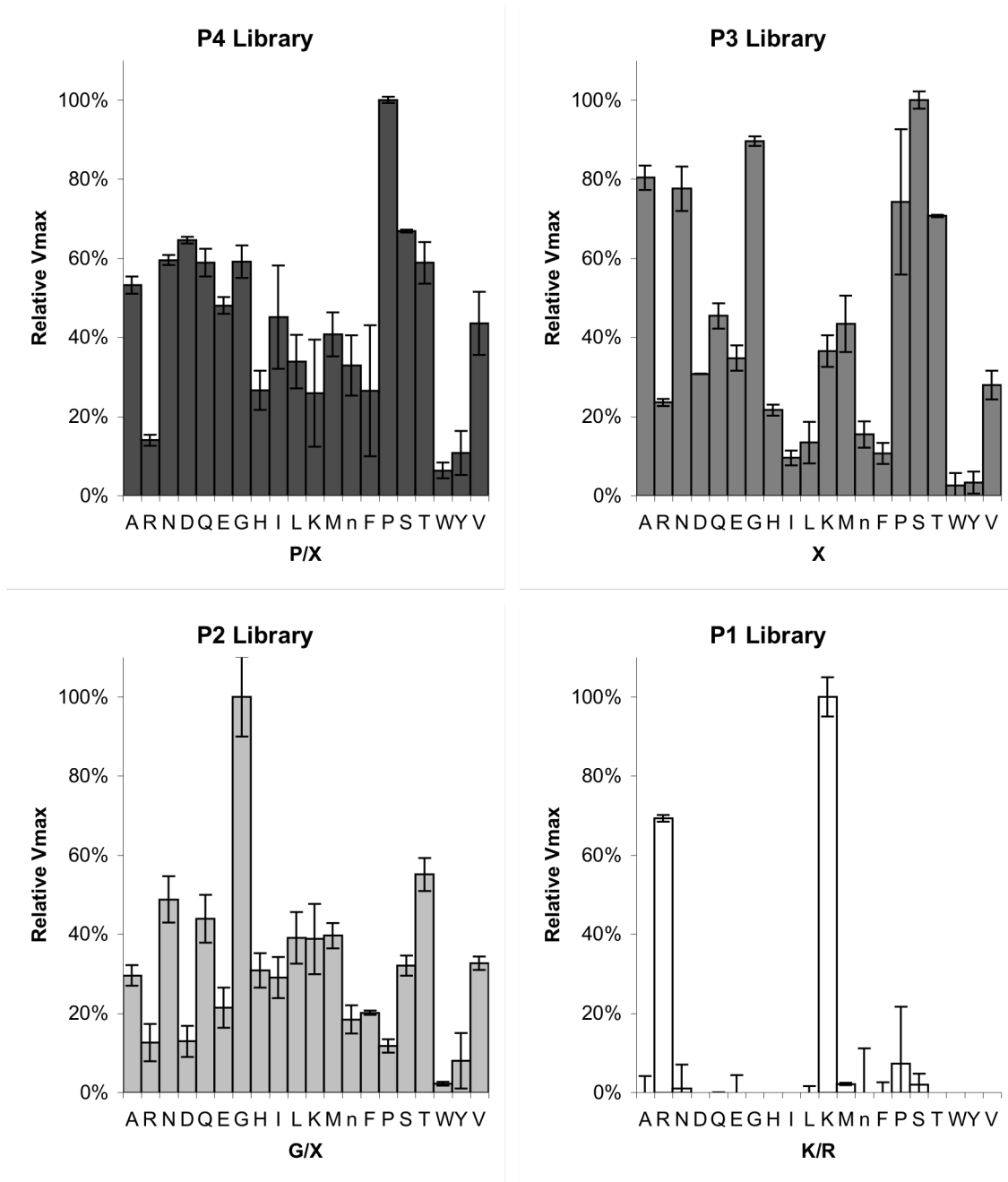
**Figure 2.4: Determination of the pH preference of OPB.**

Purified OPB was tested for pH preference by measuring the hydrolysis of Z-PR-AMC in different pH buffers. Activity was compared at pH values 3.0-10.5 with a 0.5 pH unit step size in 100 mM Citrate-Phosphate buffer (pH 3.0-7.0), Sodium-Phosphate buffer (pH 6.5-8.0), Tris-HCl buffer (pH 7.5-9.0), and Glycine-NaOH buffer (pH 8.5-10.5).



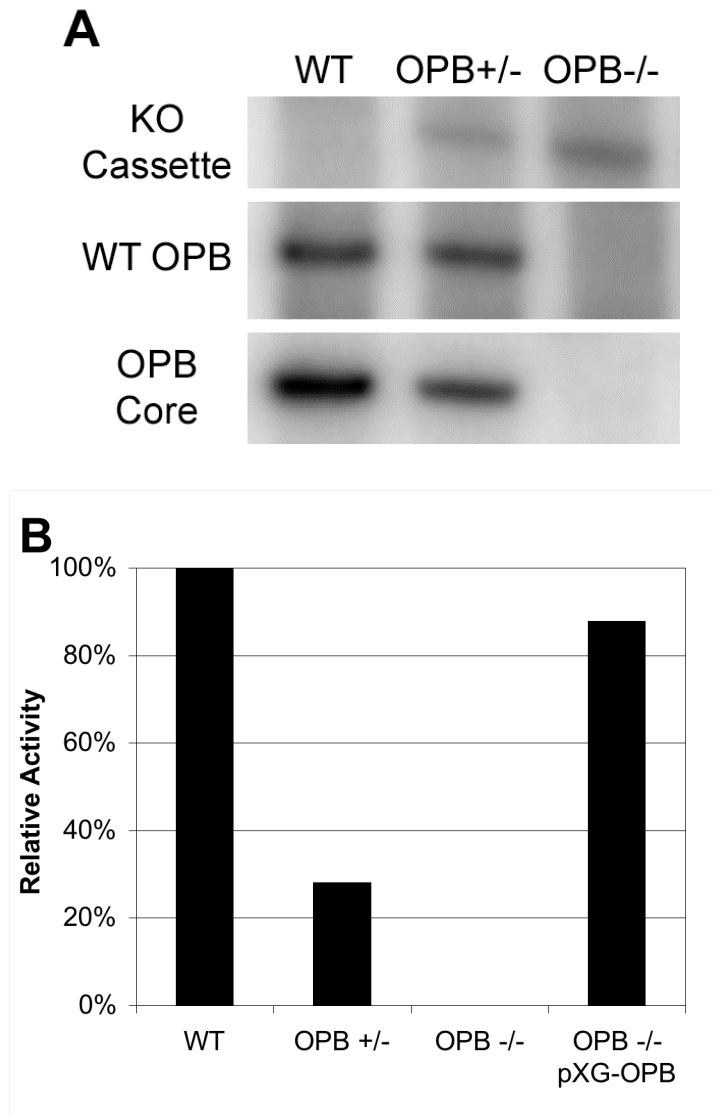
**Figure 2.5: Tetrapeptide substrate specificity profiling of OPB using a positional scanning synthetic combinatorial library.**

Subsite preference was determined using P1-P4 combinatorial libraries. All of the substrates contained an ACC fluorogenic leaving group. Assays were performed in triplicate and error bars denote the mean  $\pm$ S.D. Activity levels were normalized to the maximum mean value observed for each library. The X-axis indicates the amino acid held constant at each position for a given library, designated by the one-letter code (with “n” representing norleucine). The determined preference for each subsite is in bold below the X-axis (“x” indicates that multiple amino acids are permissible in the given subsite).



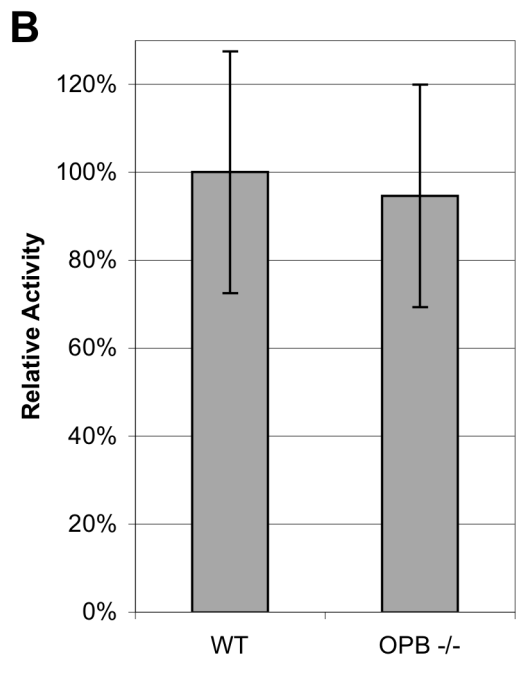
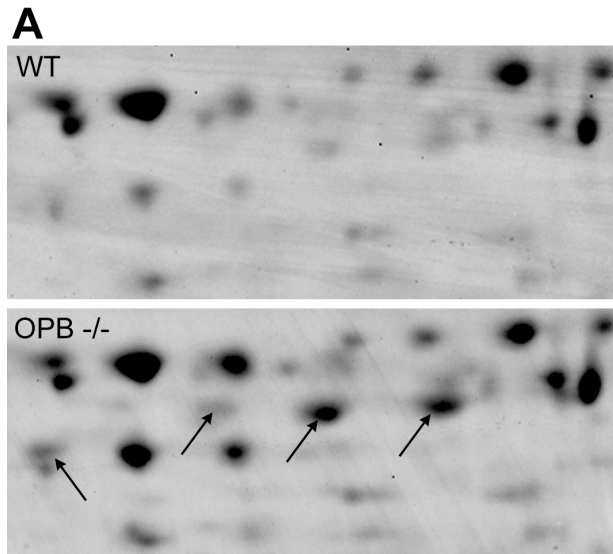
**Figure 2.6: Genetic and enzymatic analysis of OPB knockouts.**

- A. Successful deletion of both genomic copies of the *OPB* gene was determined by Southern blot analysis. Digested genomic DNA from wildtype, single-copy, and double-copy knockouts were analyzed at the *OPB* locus for the presence of either the wildtype locus or the knockout cassette. The blot was stripped and re-probed for the *OPB* gene itself to ensure that the gene was not relocated to another site in the genome.
- B. Lysates from wildtype, single-copy, and double-copy knockouts were analyzed for OPB enzymatic activity using the Z-PR-AMC substrate at pH 8.0. Additionally, OPB was episomally added back to the OPB double knockout and measured for enzymatic activity.



**Figure 2.7: Proteomic analysis of OPB knockout parasites.**

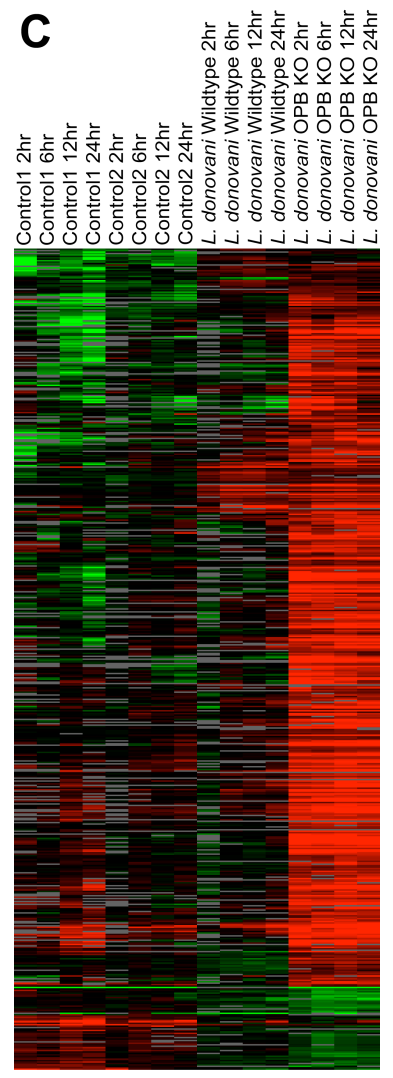
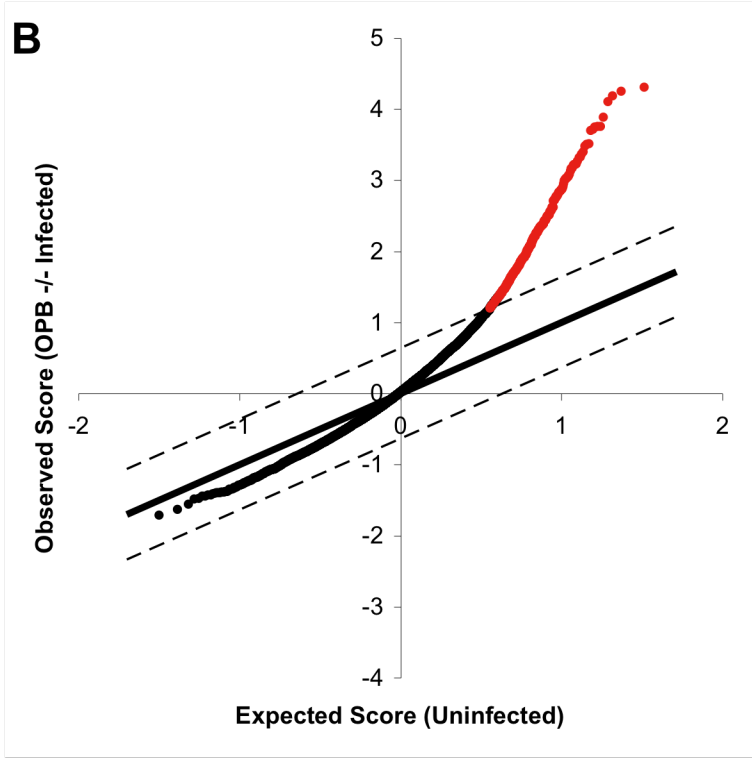
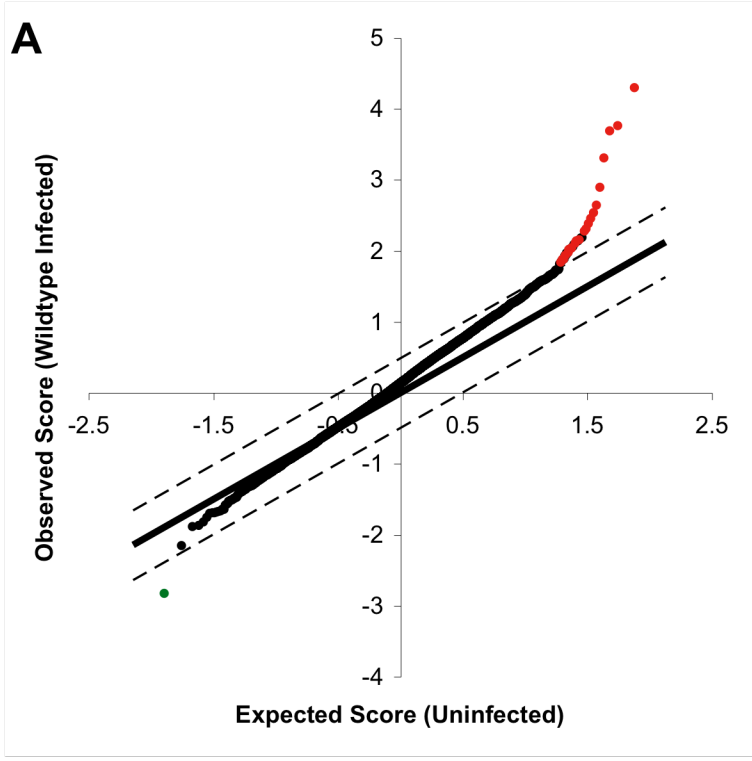
- A.** Two-dimensional gel electrophoresis was used to compare lysates from wildtype and OPB double knockout parasites. Arrows indicate four spots that were significantly more intense in lysate from the knockout parasites. The intensity increase was observed on replicate gels. The protein in these spots were identified by mass spectrometry and were all found to be enolase.
- B.** To confirm that the accumulated isoforms of enolase found in the OPB knockout parasites did not represent increased glycolytic enolase, the glycolytic pathway function of enolase was analyzed by measuring the conversion of 2-phosphoglycerate to phosphoenolpyruvate spectrophotometrically. Error bars indicate the S.D. of three separate experiments.



**Figure 2.8: Gene expression of macrophages infected by OPB knockout parasites.**

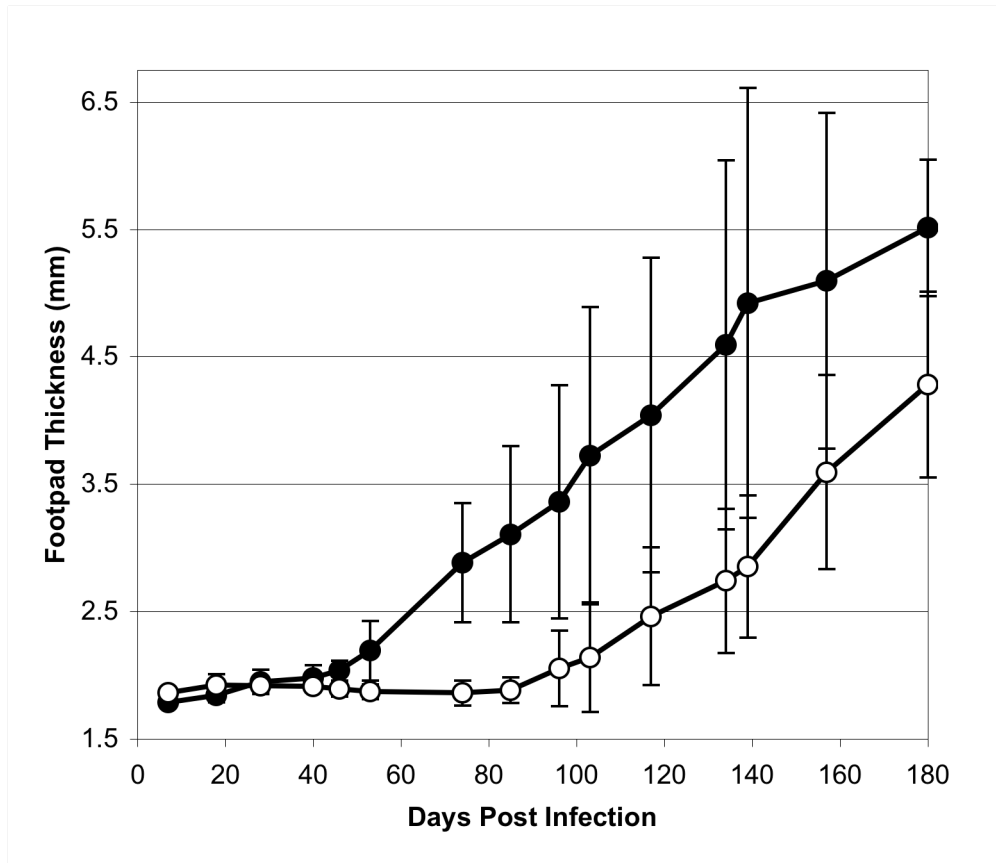
Murine bone marrow-derived macrophages were infected with either wildtype or OPB *-/-* parasites for 0, 2, 6, 12, and 24 hours. Gene expression from these macrophages was analyzed by microarray.

- A. Array data for wildtype infections were analyzed to find statistically significant changes in gene expression compared to uninfected macrophages using a false discovery rate threshold of <math><1.0\%</math> and a minimum fold-change of 2. The X-axis indicates the expression level of a given gene in the uninfected control macrophages. The Y-axis indicates the expression level observed in the infected macrophages. Genes with a significant change in expression after *Leishmania* infection are colored red for upregulation and green for down-regulation. In the wildtype infections only 23 genes were differentially expressed (false discovery rate = 0.00%).
- B. Array data for OPB *-/-* infections were also analyzed. In contrast to the few genes differentially expressed in the wildtype infections, in the OPB *-/-* infections there were 459 significantly upregulated genes (false discovery rate = 0.67%).
- C. Heat maps of the array data were clustered to illustrate the differences between the different time courses. The OPB knockout-infected macrophage arrays show a significant increase in gene expression (red) compared to the uninfected and wildtype *Leishmania*-infected macrophage arrays.



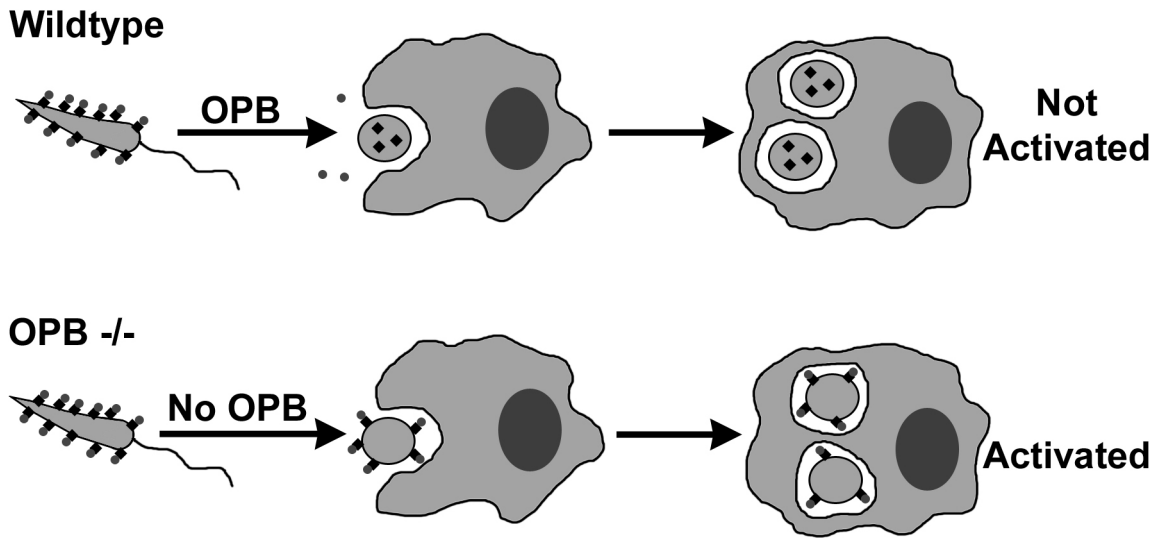
**Figure 2.9: Mouse infections by wildtype and OPB  $-/-$  *Leishmania*.**

BALB/c mice (n=5) were infected subcutaneously in the left hind footpads with wildtype (solid circle) or knockout (empty circle) parasites. Footpad swelling was measured weekly and the footpad thickness was plotted over time. Error bars indicate the S.D. between the 5 mice in each group. The mice were sacrificed at 180 days post infection. No swelling was observed in the right hind (uninfected) footpads.



**Figure 2.10: OPB regulates surface enolase and macrophage activation.**

This schematic details the proposed function of *Leishmania* OPB during infection of a vertebrate host. In a wildtype infection, surface enolase (black diamonds) binds host plasminogen (dark gray circles) on the parasite cell surface. As the parasite differentiates into an amastigote, OPB is upregulated thereby clearing surface enolase and plasminogen. The amastigotes replicate undetected within the macrophage. In an OPB  $-/-$  infection, when the parasite differentiates into the amastigote stage, enolase and plasminogen are retained on the cell surface. This infection is detected by the macrophage resulting in increased macrophage gene transcription and reduced parasite virulence.





## **Chapter Three:**

### ***Leishmania* Subtilisin is a Maturase for the Trypanothione Reductase System and Contributes to Disease Pathology**

### 3.1. Introduction

#### 3.1.1. The Clan SB subtilisin, a parasite virulence factor.

Clan SB serine proteases were among the first proteolytic enzymes to be studied in great detail. The first documented enzyme of this clan was found in 1948 by Eeg-Larsen and Linderstrom-Lang when they investigated digestion of ovalbumin by the bacterium *Bacillus licheniformis* (Eeg-Larsen, Linderstrom-Lang et al. 1948). The enzyme was later named subtilisin by Ottensen and Svensen after they isolated it from *B. subtilis* in 1970 (Perlmann and Lorand 1970). The crystal structure of the enzyme was solved in 1969 and was found to have a unique tertiary structure compared to traditional serine proteases such as pancreatic chymotrypsin (Wright, Alden et al. 1969). Subtilisin was also one of the first enzymes to undergo mutagenesis in the laboratory, when Polgár and Bender chemically converted the catalytic serine into a cysteine (Polgar and Bender 1966). In the subsequent decades subtilisin was cloned and re-engineered extensively (Wells, Ferrari et al. 1983; Wells and Estell 1988). This work has made subtilisin extremely important in our understanding of proteolytic mechanisms.

We currently place the subtilisins and related subtilases in the S8 family within Clan SB. This family is further segregated into subfamily S8A, which includes prokaryotic and eukaryotic peptidases such as the bacterial subtilisins, subtilisin-like proteases from *Plasmodium*, and the proprotein convertase site-1; and subfamily S8B, which includes eukaryotic endopeptidases such as the yeast protease kexin and the animal protease furin. The S8 proteases are secreted or membrane-bound endopeptidases with the exception of tripeptidyl-peptidase II, which is a cytosolic exopeptidase of mammals (Barrett, Rawlings et al. 2004). Subtilisins are expressed as proenzymes, with their pro

segments near their N-termini where they are required for proper protein folding (Strausberg, Alexander et al. 1993).

Subtilisin, like all Clan SB serine proteases, has a catalytic triad in the order Asp-His-Ser. The function of this triad is similar to that of the Clan SC proteases described in Chapter Two. In subfamily S8A the catalytic residues often occur in the motifs **Asp**-Thr/Ser-Gly, **His**-Gly-Thr-His, and Gly-Thr-**Ser**-Met-Ala-X-Pro. In contrast, in subfamily S8B the catalytic residues usually occur within **Asp**-Asp-Gly, **His**-Gly-Thr-Arg, and Gly-Thr-**Ser**-Ala/Val-Ala/Ser-Pro (Rawlings, Morton et al. 2008). Subtilisin is a large, single-domain protease unlike the two-domain structures of chymotrypsin and oligopeptidase B. The protease fold of subtilisin is comprised of a central seven-stranded  $\beta$ -sheet that is surrounded by nine  $\alpha$ -helices and two C-terminal  $\beta$ -sheets. The catalytic triad is positioned near the surface of the enzyme, allowing the protease to cleave globular substrates (Robertus, Alden et al. 1972).

The S8A bacterial subtilisins are relatively nonspecific proteases, although there is some evidence to suggest that there is some subsite preference in the S1 and S4 pockets (Gron, Bech et al. 1996). Analysis of the crystal structures of these enzymes indicated that the S1 and S4 pockets have the most prominent cavities, with S1 preferring large, hydrophobic side chains and S4 preferring hydrophobic side chains (Estell, Graycar et al. 1986; Gron and Breddam 1992). In contrast to the bacterial subtilisins, the S8A site-1 proteases have relatively strong substrate specificities. These enzymes, also known as subtilisin kexin-isozyme-1s, are major processing enzymes of the endoplasmic reticulum/Golgi lumen (Seidah, Mowla et al. 1999). Site-1 protease is known to autocatalytically cleave off its pro region to self-activate. This enzyme also activates

sterol regulatory element binding protein (SREBP) and processes pro-brain-derived neurotrophic factor (proBDNF) into BDNF (Seidah and Chretien 1999; Mowla, Farhadi et al. 2001). Analysis of the cleavage site patterns in several *in vitro* site-1 substrates (including site-1 autocatalysis, SREBP, proBDNF, and the envelope proteins of Lassa and Crimean Congo hemorrhagic fever viruses) revealed a strong P4 preference for Arg and a P2 preference for small hydrophobic or aliphatic residues. The P1 and P3 amino acid preferences are comparatively relaxed, but there is a slight preference against Pro, Cys, and acidic residues at P1 and for basic residues at P3 (Lenz, ter Meulen et al. 2001; Vincent, Sanchez et al. 2003). The S8B proprotein processing enzymes, kexin and furin, have strict substrate specificities. Kexin, the major processing protease of yeast, requires basic residues in both the P1 and P2 positions with a strong P1 preference for Arg (Julius, Brake et al. 1984). It is a transmembrane protein that functions as a maturase for  $\alpha$ -factor and killer toxin by cleaving their precursors at dibasic amino acid residues (Leibowitz and Wickner 1976; Mizuno, Nakamura et al. 1989). The furins, which are the homologues of kexin in animals, are a large group of prohormone or proprotein convertases. These enzymes process various proproteins in the trans-Golgi network, including growth factors and receptors (Creemers, Siezen et al. 1993). Like kexin, the furins require basic residues in both the P1 (preferentially Arg) and P2 positions, however the furins also have a strong preference for Arg at the P4 position (Hosaka, Nagahama et al. 1991).

Subtilisin-like proteases are known to be virulence factors for several pathogenic protozoa. These enzymes have been identified in multiple apicomplexa including *Plasmodium spp.*, *Toxoplasma gondii*, and *Babesia divergens*. *Plasmodium* is known to

rely on proteases for several key life cycle functions, including cysteine proteases required for hemoglobin digestion (Rosenthal, Sijwali et al. 2002). This genus of parasite encodes three distinct subtilisin-like proteases. The best characterized of these proteases is SUB1, an essential enzyme in merozoites that is released from the exoerythrocytic vacuole within erythrocytes (Blackman, Fujioka et al. 1998). SUB1 is an autocatalytic maturase (Sajid, Withers-Martinez et al. 2000) that processes serine repeat antigen (SERA) proteins and facilitates the rupture of erythrocytes and the egress of invasive merozoites (Yeoh, O'Donnell et al. 2007). A second *Plasmodium* subtilisin, SUB2, is responsible for invasion of erythrocytes and the release of antigens from the parasite cell surface (Uzureau, Barale et al. 2004). This enzyme is believed to accumulate in the parasite's dense granules and to be responsible for the shedding of merozoite surface protein-1 and apical membrane antigen-1 (Barale, Blisnick et al. 1999; Howell, Well et al. 2003). The third *Plasmodium* subtilisin, SUB3, was identified in the parasite's genome; however, very little has been revealed about this subtilase (Le Roch, Zhou et al. 2003). *Toxoplasma gondii* also has multiple subtilisin-like enzymes that are implicated in virulence. *T. gondii* SUB1 has been localized to the microneme, an apical secretory and adhesion organelle, and is hypothesized to be involved in the processing of several micronemal proteins (Miller, Binder et al. 2001). SUB2 is a putative maturase in the rhoptry organelles. This gene could not be disrupted in tachyzoites, suggesting that it is an essential protein (Miller, Thathy et al. 2003). The published trypanosomatid genomes reveal that this family of parasites also possesses a subtilisin-like serine protease (GeneDB).

### 3.1.2. The trypanothione reductase system.

Throughout its life cycle, *Leishmania* must cope with a variety of oxidative stresses. In addition to maintaining its own redox homeostasis, this parasite must withstand oxidants in the sandfly gut as well as the burst of reactive oxygen species (ROS) within host macrophages. Despite these insults, antioxidant defense in *Leishmania* and other kinetoplastids is surprisingly weak (Flohé and Harris 2007). For almost all eukaryotes the glutathione reductase and thioredoxin reductase systems serve as the main pathways by which redox is maintained. These pathways are not present in the kinetoplastids. In fact, the known trypanosomatid genomes do not contain genes for glutathione reductase, thioredoxin reductase, catalase, or selenocysteine-glutathione peroxidases (Ivens, Peacock et al. 2005; Krauth-Siegel and Comini 2008). The kinetoplastids instead rely on a novel pathway to control redox homeostasis that is based on the low molecular mass dithiol trypanothione. This pathway requires multiple essential enzymes that may be targets for antiparasitic chemotherapy (Flohé, Steinert et al. 2002).

Trypanothione was discovered in 1985 by Fairlamb and Cerami (Fairlamb and Cerami 1985). This spermidine analogue is an essential cofactor in kinetoplastids for the detoxification of hydroperoxides. In 1997 the trypanothione reductase system was discovered by Nogoceke *et al.* in *Crithidia fasciculata*. This work uncovered a unique cascade of reductases that catalyze trypanothione-dependant peroxide metabolism (Nogoceke, Gommel et al. 1997). In the kinetoplastids, peroxide reduction is linked to the oxidation of NADPH by this novel reduction system (Figure 3.1). NADPH is oxidized by the enzyme trypanothione reductase. This enzyme is able to convert oxidized

trypanothione into reduced trypanothione. Tryparedoxin uses reduced trypanothione to reduce a terminal peroxidase, either tryparedoxin peroxidase or peroxidoxin. These peroxidases function to convert hydroperoxides into water and their corresponding alcohols (Krauth-Siegel and Comini 2008).

Tryparedoxin peroxidase and peroxidoxin belong to the peroxiredoxins, a family of enzymes found in organisms from bacteria to mammals (Rhee, Kang et al. 1999). The first member of this family to be discovered was the thioredoxin peroxidase of *S. cerevisiae* by Kim *et al.* in 1988 (Kim, Kim et al. 1988). These peroxidases have since been identified and characterized in the trypanosomes (Wilkinson, Temperton et al. 2000; Tetaud, Giroud et al. 2001) and in several species of *Leishmania* (Levick, Tetaud et al. 1998; Flohe, Budde et al. 2002). Trypanosomatid peroxidoxins contain two conserved cysteine domains, which classify these proteins as 2-Cys peroxidoxins. In *Leishmania*, the tryparedoxin peroxidases are encoded by a multi-copy gene array that is of varying length in different species; however, peroxidoxin is encoded by a single-copy gene in these species (GeneDB). In *L. infantum*, peroxidoxin was found to be confined to the mitochondrion while the tryparedoxin peroxidases were found to be cytoplasmic. These enzymes are believed to have complementary roles in protecting the parasites from oxidative damage (Castro, Sousa et al. 2002). Knockdown of either tryparedoxin peroxidase or peroxidoxin in *Leishmania* was found to increase the parasite's sensitivity to oxidants. Conversely, overexpression of these enzymes resulted in enhanced resistance to hydroperoxides (Lin, Hsu et al. 2005). The lack of a redundant glutathione or thioredoxin reductase system in *Leishmania* and the absence of the trypanothione

reductase system in humans make this pathway an attractive target for antiparasitic drugs and future research.

### **3.1.3. The *Leishmania* genomes encode a unique subtilisin-like protease.**

The known *Leishmania* genomes each encode a single Clan SB subtilisin-like serine protease. This protease is unlike the subfamily S8B kexins and furins. Due to the fact that mammals lack subtilisin homologues and because subtilisins are virulence factors for apicomplexan parasites, this enzyme is a potential chemotherapeutic target. Much like the subtilisins of *Plasmodium* and *Toxoplasma*, *Leishmania* subtilisin may be a maturase for the parasite's endogenous proteins.

**In this study we describe the identification and phenotypic characterization of the *Leishmania* subtilisin protease. Subtilisin from *L. donovani* was cloned and sequenced. The single-copy subtilisin gene was deleted by targeted gene replacement. The role of subtilisin was then analyzed both in *in vitro* culture, as well as in the animal models of infection. To delineate a specific role of SUB in the *Leishmania* infectious life cycle, two-dimensional gel electrophoresis was compared between wildtype and SUB *-/-* parasites. We found that SUB is required for proper promastigote to amastigote differentiation and that it regulates the terminal peroxidases of the trypanothione reductase system.**

## **3.2. Results**

### **3.2.1. *L. donovani* SUB uses a non-canonical catalytic triad.**



The gene encoding *Leishmania donovani* SUB was cloned as described above. Sequencing yielded a 5,235 bp gene. This sequence was submitted to GenBank with accession number GU230888. The resultant 1,744 amino acid protein has an estimated molecular weight of 184.7 kDa. This protein has a predicted signal peptide (SignalP V2.0 HMM probability of 0.995) with a cleavage site between amino acids 38-39 (0.421 probability), and a probable C-terminal transmembrane helix between amino acids 1709-1731 (TMHMM v. 2.0). The published *L. major* SUB (GeneDB: LmjF13.1040) shares this general layout; however, SUB from the more closely related *L. infantum* (a subspecies from within the *L. donovani* complex) has a C-terminal truncation after amino acid 1192 (LinJ13\_V3.0940). The Pfam predicted Subtilase family core for *L. donovani* SUB is between amino acids 86-414. Comparisons of *L. donovani* SUB to other trypanosomatid SUBs are summarized in Table 3.1. Interestingly, *L. donovani* SUB has a non-canonical catalytic triad with the catalytic Glu in place of the standard Asp due to a single C to G base pair change. *L. infantum* SUB also has Glu in place of the Asp, indicating that this adaptation may be specific to parasites in the *L. donovani* complex. The SUB catalytic core amino acid sequences are relatively conserved within the *Leishmania* species; however, these sequences have diverged considerably from those of the trypanosomes, with only a 40% identity between the genera.

In order to determine the subfamily of *Leishmania* subtilisin, the catalytic core sequence was compared to the cores of other Clan SB, family S8 family members. Core sequences were aligned using ClustalW2 (EMBL-EBI) and a phylogenetic tree was generated (Figure 3.2). The *Leishmania* SUBs group with the subfamily S8A proteases, which include the eukaryotic Site-1 peptidases and the bacterial subtilisins. This

distinguishes *Leishmania* SUB from the *Toxoplasma* and *Plasmodium* SUBs and from the subfamily S8B kexins and furins. Site-1 peptidases are restricted to metazoan organisms and are known to process sterol regulatory element binding proteins (SREBPs), which are not found in trypanosomatids (Barrett, Rawlings et al. 2004).

### **3.2.2. *L. donovani* and *L. major* subtilisins were recombinantly expressed in *P. pastoris*.**

The catalytic cores of both the *L. donovani* and the *L. major* subtilisins were cloned into the pPICZ- $\alpha$  A vector and confirmed by sequencing. These constructs were then electroporated into *Pichia pastoris* strain X33 for recombinant expression. After induction, the recombinantly expressed SUBs were harvested from the supernatant and concentrated. Protease activity was evaluated in Tris-HCl pH 7.5 using three synthetic fluorogenic peptides (Figure 3.3): Z-RWPL-AMC, Z-VFRSLK-AMC, and Z-RVRR-AMC. These peptide substrates were chosen because of the likelihood that subtilisin prefers a P4 Arg. No substrate cleavage was observed with supernatant taken from X33 wildtype *P. pastoris*. For each *L. donovani* and *L. major* SUB cores, three clones were independently evaluated for protease activity. All six of these clones were found to cleave the tested substrates. Five of these six clones preferred the RVRR substrate and all six clones showed low activity for the other two substrates.

### **3.2.3. SUB $-/-$ parasites have defects in promastigote to amastigote differentiation.**

The published *L. major* and *L. infantum* genomes (GeneDB) indicate that *Leishmania* spp. contain a single copy of SUB per haploid genome. This was confirmed

to be true in *L. donovani* and *L. major* by Southern blot analysis of genomic DNA (data not shown). Deletion of both alleles of the single copy gene was carried out by two rounds of targeted gene replacement in *L. donovani* and confirmed by southern blot (Figure 3.4). Only one allele could be deleted from *L. major* despite multiple attempts at targeting the second allele.

Wildtype, SUB +/-, and -/- *L. donovani* promastigotes were cultured at 27°C in M199. These parasites replicated at a rate comparable to wildtype parasites. To test for the ability to differentiate into axenic amastigotes, stationary phase (day 5 post-split) promastigotes were split 1:10 in FBS at 37°C. Wildtype and SUB +/- parasites differentiated readily, however SUB -/- did not (Figure 3.5). The SUB -/- parasites remained as elongated, flagellated spindles. These cells did not form aggregates of cells typically seen in axenic amastigote cultures. To test for differentiation under lower dilution conditions, stationary phase SUB -/- promastigotes were diluted 1:2 in FBS at 37°C. After 4 days, some of the cells appeared to differentiate; however, cell aggregation did not occur.

#### **3.2.4. Electron microscopy of SUB -/- amastigotes revealed abnormal ultrastructures.**

Wildtype and SUB -/- axenic amastigotes from the low dilution differentiation were processed for transmission electron microscopy. Wildtype axenic amastigotes had typical amastigote morphology—a rounded cell body measuring approximately 3 µm in diameter, no external flagellum, and a condensed electron-dense kinetoplast (Figure 3.6A). SUB -/- axenic amastigotes exhibited many abnormalities (Figure 3.6B-D). While

most of these cells had rounded cell bodies, many were still elongated and spindle-shaped. Those that were rounded often had invaginations in the plasma membrane. Additionally, many of these cells were binucleated. Unlike the wildtype amastigotes, the SUB *-/-* cells also had multiple flagellar cross sections including flagella appearing in the cytoplasm, outside of their expected location within the flagellar pocket. These images indicate that the SUB *-/-* cells were not successfully differentiating into amastigotes.

### **3.2.5. SUB regulates levels of peroxidases from the trypanothione reductase system.**

Site-1 peptidases and the subtilisins from apicomplexan parasites such as *Toxoplasma* and *Plasmodium* are maturases that process proteins within vesicles (Barale, Blisnick et al. 1999; Miller, Thathy et al. 2003; Rawlings, Morton et al. 2008). The presence of a signal peptide and C-terminal transmembrane domain on *Leishmania* SUB indicates that it may perform a similar function. We employed two-dimensional gel electrophoresis to study the main differences in protein expression and processing between wildtype and SUB *-/-* parasites (Figure 3.7). Spots that differed in intensity between the wildtype and SUB *-/-* parasites were selected for peptide sequencing by mass spectrometry. The identities of these proteins are presented in Table 3.2. Interestingly, nine distinct spots were found to be tryparedoxin peroxidases, the terminal peroxidases of the trypanothione reductase system. In the *Leishmania donovani* complex, this family of peroxidases is comprised of three cytoplasmic tryparedoxin peroxidases, TXNPx1-3 (TRYP1-3), and a mitochondrial peroxidoxin, Prx (Flohé and Harris 2007). These enzymes are 2-Cys peroxiredoxins and have complementary roles in parasite protection against oxidative stress (Castro, Sousa et al. 2002). TXNPx1 and 3 share 99% amino

acid identity and have 94% identity to TXNPx2 (GeneDB). Wildtype *L. donovani* had high levels of TXNPx1 and 3 forming a single doublet of spots. The level of TXNPx2 was low and Prx was not detectable. In the SUB *-/-* parasites, the wildtype TXNPx1/3 doublet decreased and two new TXNPx1/3 doublets were present at a higher molecular weight and at a higher molecular weight with a lower pI. TXNPx2 and Prx levels were elevated in these parasites, which could compensate for the decreased level of wildtype TXNPx1/3.

### **3.2.6. SUB deficient parasites are more sensitive to oxidative damage.**

Proteomic analysis of wildtype versus SUB *-/-* parasites revealed abnormal levels of the terminal peroxidases of the trypanothione reductase system. It was then predicted that SUB deficient parasites would be more sensitive to oxidative damage. *L. donovani* SUB *-/-* parasites have an upregulation of Prx which may act compensatorily to protect the parasites from oxidants. To test if SUB deficient *Leishmania* promastigotes are more sensitive to hydroperoxides, *L. donovani* (Figure 3.8A) and *L. major* (Figure 3.8B) wildtype, SUB deficient, and SUB add-back parasites were split into different concentrations of *tert*-butyl hydroperoxide (*t*-bOOH). As expected for *L. donovani*, only a slight increase in hydroperoxide sensitivity was noted in the SUB deficient parasites, and this increased sensitivity was not exhibited by the add-back parasites. In addition, the *L. major* SUB deficient promastigotes were much more sensitive to hydroperoxides than the wildtype culture. This sensitivity was mitigated by the addition of the add-back construct.

### **3.2.7. Loss of SUB results in delayed lesion formation in mice and the absence of psammoma body lesions in hamsters.**

Within the host, *Leishmania* is exposed to a variety of oxidative stresses particularly within host macrophages. It was predicted that SUB deficient parasites would have reduced virulence in animal infection models due to the altered regulation of the trypanothione reductase system. Indeed, SUB deficient *Leishmania* were found to be less virulent in both the mouse and hamster systems.

BALB/c mice were infected subcutaneously into their left hind footpads with either wildtype or SUB deficient parasites. Footpad swelling was measured weekly (Figure 3.9). Swelling was evident in the wild-type infected mice after 7 weeks; however, significant swelling (compared to the contralateral footpad) was not observed in mice infected with SUB deficient parasites until after 14 weeks. The SUB deficient infections were not self-limiting and continued to increase footpad swelling, however the lesion size was consistently 7 to 8 weeks delayed compared to the wildtype infections.

For the visceral leishmaniasis infection model, male Golden Syrian hamsters were infected intraperitoneally with wildtype or SUB deficient parasites. At 200 days post-infection, the hamsters were sacrificed and their spleens were sectioned and histologically examined. Spleens were enlarged in both wildtype- and knockout-infected animals. All wildtype-infected hamsters' spleens contained psammoma body calcifications (Figure 3.10A & B), indicative of granulomatous lesions that occur in visceral leishmaniasis (Wilson, Innes et al. 1987; Kahl, Byram et al. 1991). Strikingly, no psammoma bodies were observed in the spleens of hamsters infected with SUB deficient *Leishmania* (Figure 3.10C & D).

### 3.3. Discussion

*Leishmania* parasites are dimorphic parasites that must survive and replicate in two vastly different environments: the gut of a phlebotomine sandfly and within the parasitophorous vacuole in phagocytic cells of vertebrates. Throughout this life cycle, the parasite is exposed to a variety of oxidative insults including the ROSs produced by host macrophages. Antioxidant defense is therefore extremely important for parasite survival. *Leishmania*, along with the other kinetoplastids, uses an unusual hydroperoxide metabolic pathway, the trypanothione reductase system, which employs trypanothione as the main transporter of electrons (Flohé, Steinert et al. 2002). My research showed that *Leishmania* parasites contain an unusual subtilisin-like enzyme that governs the levels of key peroxidases in the trypanothione reductase system. This serine protease represents a potential target for rational drug design, as serine proteases are known virulence factors for several parasites, including trypanosomatids (Caler, Vaena de Avalos et al. 1998; Bal, Van der Veken et al. 2003).

We have identified and cloned a novel subtilisin-like protease from the parasite *Leishmania donovani*. Sequencing of this gene revealed that it encodes a large 1,744 amino acid protein with an N-terminal catalytic domain that is approximately 400 amino acids. The *L. donovani* SUB has a high degree of identity to the other known *Leishmania* SUBs (Table 3.1), however there is very little identity between the *Leishmania* SUBs and those of the trypanosomes. A phylogenetic sorting of known subtilisin catalytic cores showed that the *Leishmania* subtilisins fall within the subfamily S8A and are most closely related to site-1 proteases (Figure 3.2).

Interestingly, *L. donovani* (and the related *L. infantum*) SUB has a non-canonical catalytic triad due to a single C to G base pair change. Clan SB serine proteases use an Asp-His-Ser triad, whereas *L. donovani* complex SUBs use a Glu in place of the Asp. Glu and Asp are identical save for one additional carbon in the side chain of Glu. Subtilisins are known to be pliable enzymes; however, a search of the MEROPS database has shown that there are currently no other known cases of Glu in the catalytic triad of a Clan SB protease (Rawlings, Morton et al. 2008). To verify that *Leishmania donovani* subtilisin is an active enzyme, the catalytic cores and predicted prepro regions of both *L. donovani* and *L. major* were recombinantly expressed in *Pichia pastoris*. Activity was recovered for both SUB cores indicating that the Glu-His-Ser catalytic triad is functional (Figure 3.3). Proteolytic cleavage only occurred when substrates were used that had Arg in the P4 position. Site-1 proteases have a strong preference for P4 Arg (Lenz, ter Meulen et al. 2001; Vincent, Sanchez et al. 2003). This strengthens the placement of *Leishmania* subtilisin in subfamily S8A.

To phenotypically characterize the function of subtilisin in *Leishmania*, the genes were disrupted in *L. donovani* and *L. major* by homologous recombination. Both alleles of the *L. donovani* gene were knocked out; however, only one allele could be deleted in *L. major* despite multiple attempts at gene targeting. This could be due to either a greater requirement for subtilisin in *L. major* or a compensatory change in *L. donovani* that allowed for the full knockout to be generated. Knockout parasites of both species grew well *in vitro* as promastigotes; however, attempts to grow the *L. donovani* SUB -/- parasites revealed a defect in their ability to differentiate (Figure 3.5). This indicates that subtilisin may be beneficial for survival in the amastigote stage or in the differentiation



process. Electron microscopy of amastigote-like cells from the SUB  $-/-$  differentiation experiments revealed that these cells were either extremely abnormal or had not fully differentiated (Figure 3.6). Commonly seen ultrastructural abnormalities included elongated cell bodies, severe membrane invaginations, binucleation, and multiple flagellar cross sections. These abnormalities are likely due to parasite distress in response to the lack of subtilisin enzyme. This research suggested that the biological role of subtilisin within *Leishmania* may be as a maturase for a protein or pathway that promotes amastigote survival.

Proteomic analysis was performed on *Leishmania donovani* wildtype and SUB  $-/-$  parasites to uncover the pathway of this enzyme. We uncovered five sets of protein spots that differed considerably between wildtype and SUB  $-/-$  parasites. All five of these sets were identified as members of the trypanothione reductase system, the terminal peroxidases of the trypanothione reductase system (Figure 3.1). At least three of the four *L. donovani* trypanothione reductases were identified (Table 3.2 and Figure 3.7) including multiple cytoplasmic trypanothione reductases (TXNPxs) and the mitochondrial peroxidase (Prx). TXNPxs from SUB  $-/-$  parasites were of higher molecular weights and one set of spots appeared at a lower pI. These results show that these peroxidases are proteolytically processed in wildtype *Leishmania*, but not in the SUB  $-/-$  cells, which would indicate that subtilisin is the maturase for these enzymes. Mass spectrometry peptide fragments for the higher molecular weight spots, but not the lower wildtype spots, included segments of the C-terminus. Subtilisin may therefore process TXNPx by removing the C-terminal end of the protein. The proposed role of subtilisin is depicted in Figure 3.11.

SUB  $-/-$  parasites also showed increased levels of Prx compared to wildtype. The function of mitochondrial Prx is believed to be complementary to that of the cytosolic TXNPx (Castro, Sousa et al. 2002), thus the increase in Prx may be a compensatory change due to the reduction of functional TXNPx. This hypothesis is supported by the fact that both alleles of the *SUB* gene could not be deleted in *L. major*. While *L. donovani* complex parasites encode three cytosolic trypanothione peroxidase genes in the TXNPx gene array, *L. major* encodes seven cytosolic trypanothione peroxidases (GeneDB). *L. major* therefore relies more heavily on these cytosolic enzymes, thereby making a sufficient compensatory increase in Prx more difficult. Reduced viability of SUB deficient *L. major* and *L. donovani* amastigotes was exhibited using the murine and hamster infection models, respectively (Figures 3.9 & 3.10). In both systems, the SUB knockout parasites had clearly reduced virulence. Our research has shown that subtilisin promotes survival of *Leishmania* amastigotes by serving as a maturase for the trypanothione reductase system, thus aiding in redox homeostasis and protecting the parasite from oxidative stresses in the host macrophage.

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**Table 3.1: Comparison of predicted SUB proteins from Trypanosomatids.**

*L. donovani* SUB was compared to the other known trypanosomatid SUB protein sequences using ClustalW2. The catalytic cores of each protein were determined by aligning them with the Pfam Subtilase family core (PF00082). Subtilisins have the catalytic triad Asp-His-Ser. *L. donovani* SUB has a non-canonical catalytic triad with a Glu in place of the standard Asp due to a single C to G base pair change. *L. infantum* SUB also has Glu in place of the Asp, indicating that this adaptation may be specific to parasites in the *L. donovani* complex. The SUB catalytic core amino acid sequences are relatively conserved within the *Leishmania* species; however, these sequences have diverged considerably from those of the trypanosomes, with only a 40% identity between the genera. *L. infantum* has a truncated C-terminus. All the trypanosomatid SUB proteins fall between 126-191 kDa.

**Comparison of Predicted SUB Proteins from Trypanosomatids**

Species:	Gene:	AA Identity:	Core Identity:	Length (AA):	kDa:	Catalytic Triad:
<i>L. donovani</i>	SUB	100.0%	100.0%	1744	184.7	E97, H130, S395
<i>L. infantum</i>	SUB	99.6%	99.7%	1192	126.2	E97, H130, S395
<i>L. major</i>	SUB	89.0%	94.5%	1722	182.7	D99, H132, S397
<i>L. braziliensis</i>	SUB	70.0%	83.0%	1785	190.7	D97, H130, S405
<i>T. cruzi</i>	SUB1	23.1%	40.3%	1430	160.5	D219, H269, S502
<i>T. cruzi</i>	SUB2	23.4%	40.1%	1408	158.0	D196, H246, S480
<i>T. brucei</i>	SUB1	24.4%	39.5%	1487	161.5	D191, H238, S476

**Table 3.2: Mass spectrometry of WT and SUB -/- *Leishmania* 2-d gels.**

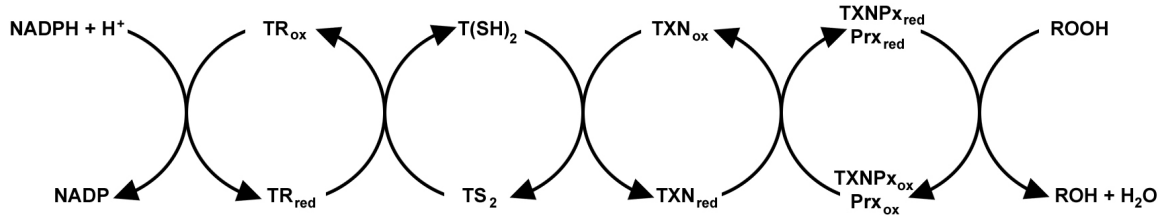
Spots that differed in intensity between the wildtype and SUB -/- parasites were selected for peptide sequencing by mass spectrometry. Nine distinct spots were found to be trypanredoxin peroxidases, the terminal peroxidases of the trypanothione reductase system. In the *Leishmania donovani* complex, this family of peroxidases is comprised of three cytoplasmic trypanredoxin peroxidases, TXNPx1-3 (TRYP1-3), and a mitochondrial peroxidoxin.

**Two-Dimensional Gel Analysis of WT and SUB -/- *Leishmania***

WT		SUB -/-		
Spot:	Protein:	Spot:	Protein:	Relative to WT:
1	no Prx detected	1	Prx	elevated
		2	Prx	elevated
3	TXNPx2	3	TXNPx2	elevated
4	TXNPx1/3	4	TXNPx1/3	decreased
5	TXNPx1/3	5	TXNPx1/3	decreased
		6	TXNPx1/3	elevated
7	no TXNPx detected	7	TXNPx3	elevated
		8	TXNPx1/2/3	elevated
		9	TXNPx1/2/3	elevated

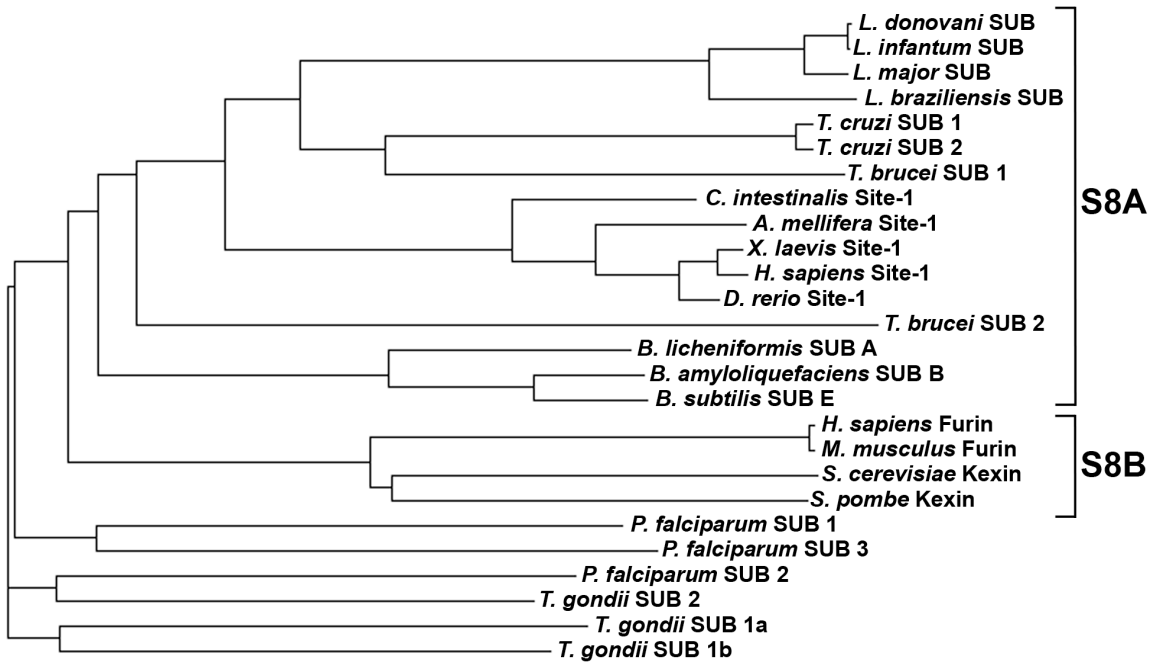
**Figure 3.1: Peroxide detoxification in the kinetoplastids.**

Reduction of hydroperoxides in the kinetoplastids is dependant on a novel detoxification system that, unlike other eukaryotes, does not use glutathione peroxidase. NADPH is oxidized by trypanothione reductase (TR). This enzyme then is able to convert oxidized trypanothione (TS<sub>2</sub>) into reduced trypanothione (T(SH)<sub>2</sub>). Tryparedoxin (TXN) uses T(SH)<sub>2</sub> as a reducing agent to reduce either tryparedoxin peroxidase (TXNPx) or peroxidoxin (Prx). These terminal peroxidases are able to convert hydroperoxides into H<sub>2</sub>O and alcohol (ROH).



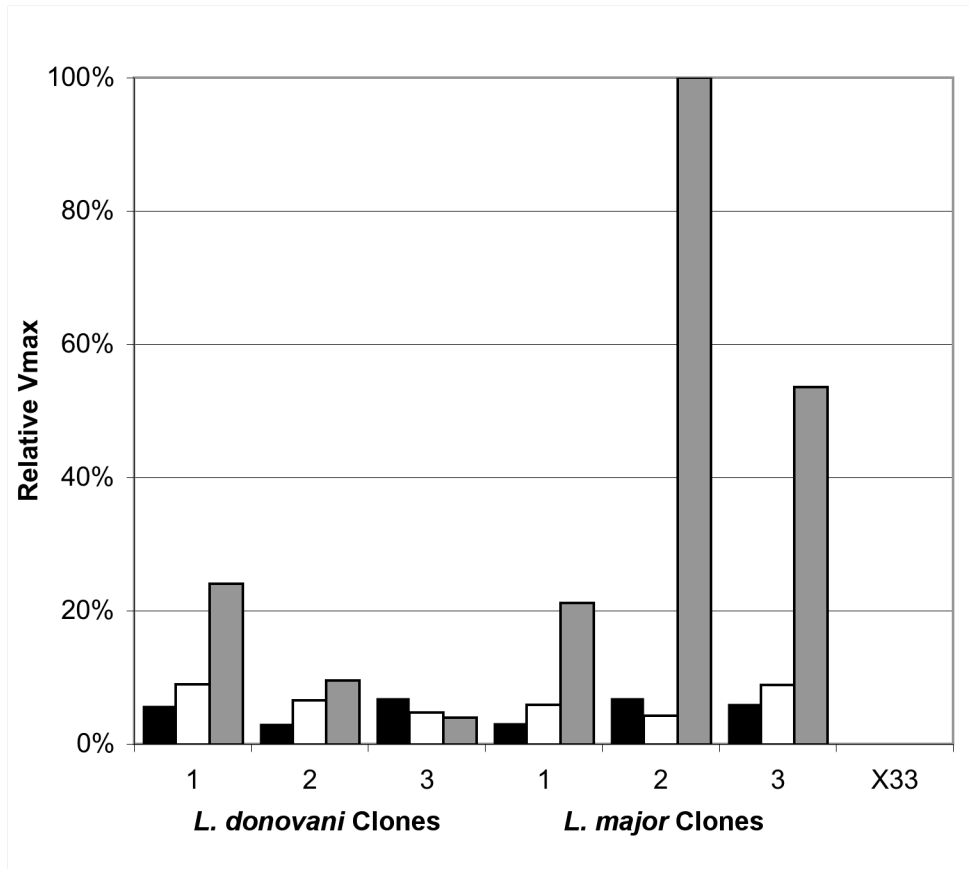
**Figure 3.2: Phylogenetic tree of family S8, Clan SB serine proteases.**

The catalytic cores of the *Leishmania* subtilisins were compared to the cores of other Clan SB, family S8 family members (Table 5.2), as determined by Pfam. Core sequences were aligned using ClustalW2 (EMBL-EBI). The *Leishmania* SUBs group with the subfamily S8A proteases, which include the eukaryotic Site-1 peptidases and the bacterial subtilisins. This distinguishes *Leishmania* SUB from the *Toxoplasma* and *Plasmodium* SUBs and from the subfamily S8B kexins and furins.



**Figure 3.3: Activity of recombinantly expressed *L. donovani* and *L. major* SUBs.**

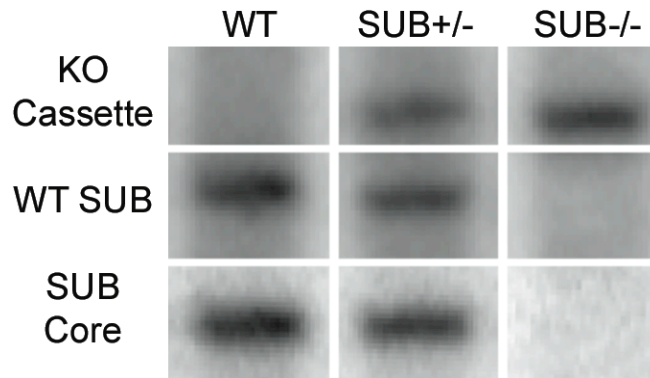
The catalytic cores of *L. donovani* and *L. major* SUBs were recombinantly expressed in *P. pastoris* X33. Purified and concentrated protease was tested for activity against three synthetic fluorogenic substrates: RWPL (black bars), VFRSLK (white bars), and RVRP (gray bars). For each SUB core, three clones were induced and tested for activity. Additionally, an X33 wildtype clone was also induced and tested. No background activity was seen in the X33 wildtype control clone.



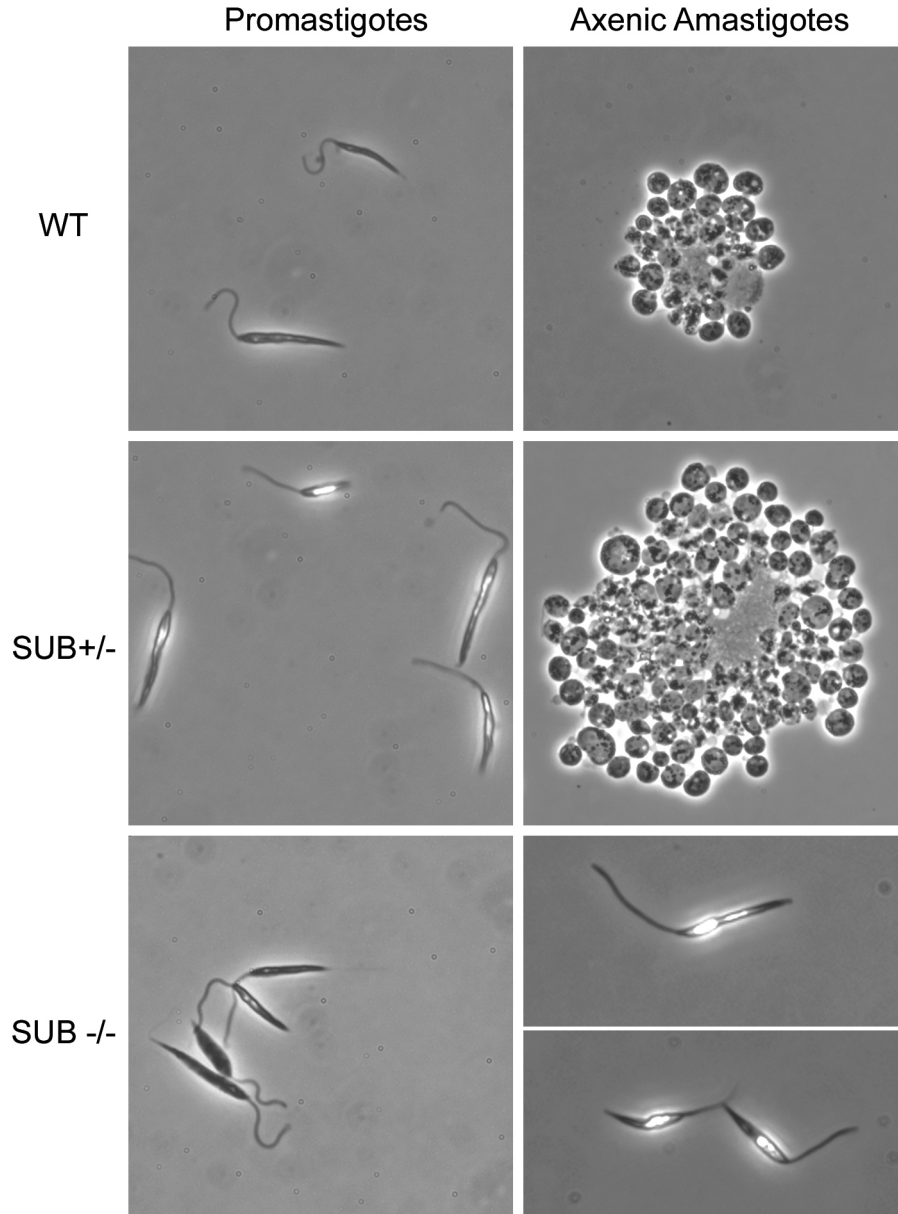


**Figure 3.4: Genetic analysis of SUB knockout *L. donovani*.**

Successful deletion of both genomic copies of the *SUB* gene was determined by Southern blot analysis. Digested genomic DNA from wildtype, single-copy, and double-copy knockouts were analyzed at the *SUB* locus for the presence of either the wildtype locus or the knockout cassette. The blot was stripped and re-probed for the core of the *SUB* gene itself to ensure that the gene was not relocated to another site in the genome.

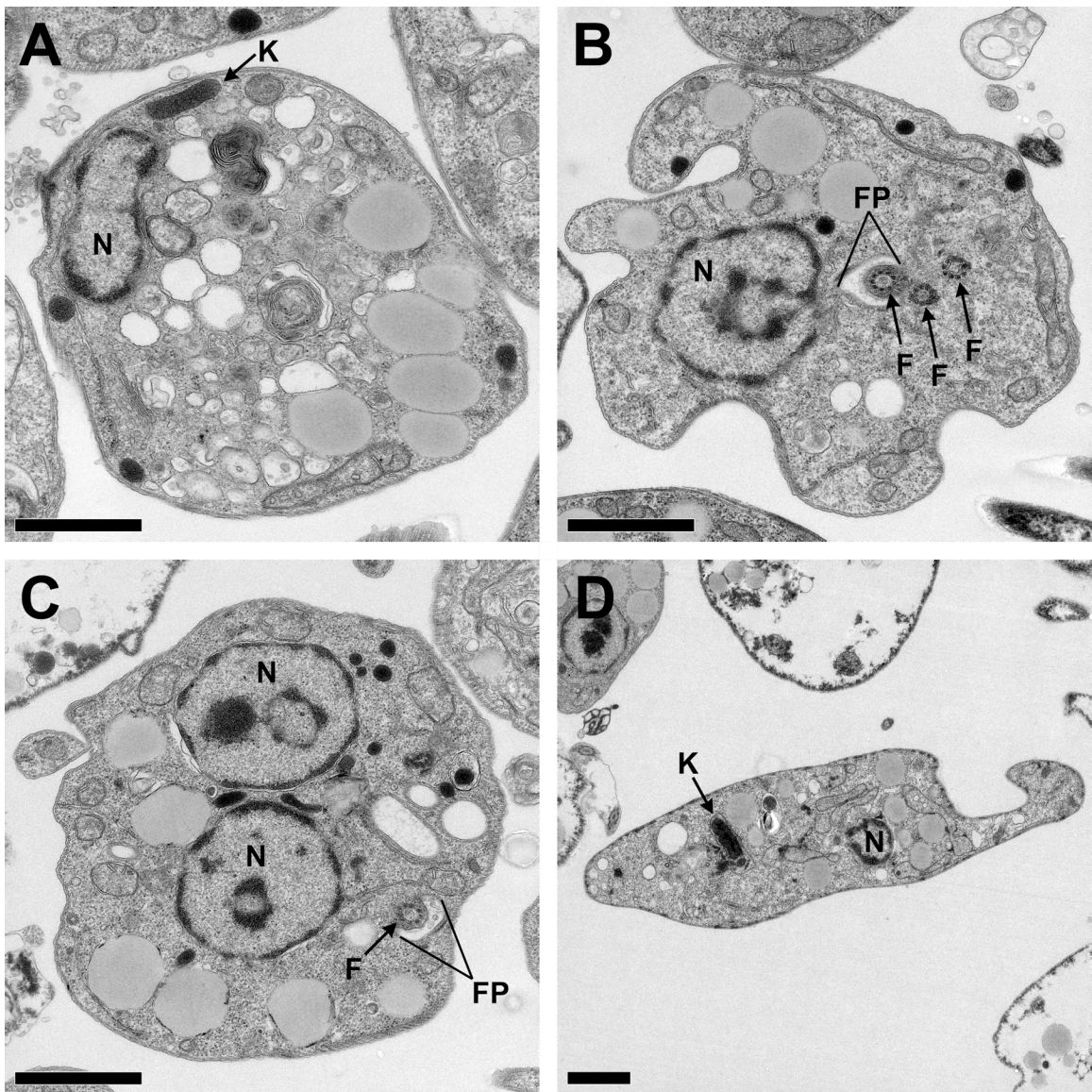


**Figure 3.5: SUB  $-/-$  *L. donovani* is defective in pro- to amastigote differentiation.** Wildtype, SUB  $+/-$ , and  $-/-$  *L. donovani* promastigotes were cultured successfully at 27°C in M199. To test for the ability to differentiate into axenic amastigotes, stationary phase (day 5 post-split) promastigotes of each culture were split 1:10 in FBS at 37°C. Wildtype and SUB  $+/-$  parasites differentiated readily, however SUB  $-/-$  did not. The SUB  $-/-$  parasites remained as elongated, flagellated spindles. These cells did not form aggregates of cells typically seen in axenic amastigote cultures.



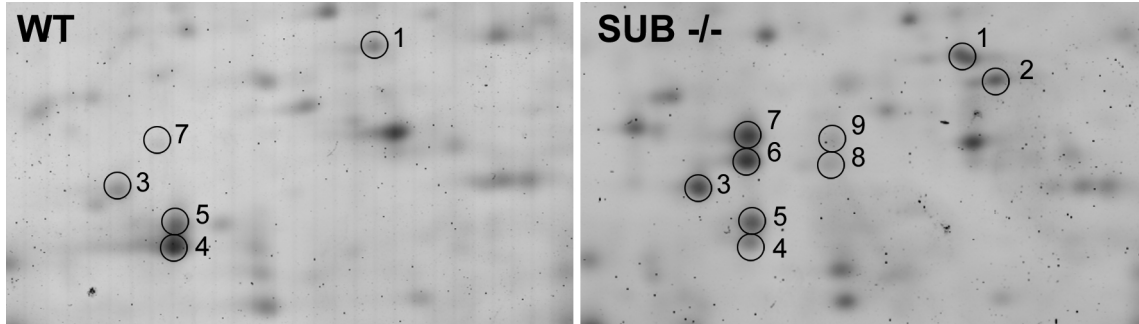
**Figure 3.6: SUB  $-/-$  *L. donovani* amastigotes are ultrastructurally abnormal.**

Wildtype axenic amastigotes (A) had normal rounded cell bodies measuring approximately 3  $\mu\text{m}$  in diameter (scale bar = 1  $\mu\text{m}$ ) with no external flagellum and a condensed electron-dense kinetoplast. SUB  $-/-$  axenic amastigotes (B-D) exhibited many abnormalities. While most of these cells had rounded cell bodies, many were still elongated and spindle-shaped (D). Those that were rounded often had invaginations in the plasma membrane (B). Additionally, many of these cells were binucleated (C). Unlike the wildtype amastigotes, the SUB  $-/-$  cells also had multiple flagellar cross sections including flagella appearing in the cytoplasm (B), outside of their expected location within the flagellar pocket. (N = nucleus, K = kinetoplast, FP = flagellar pocket, F = flagellum)



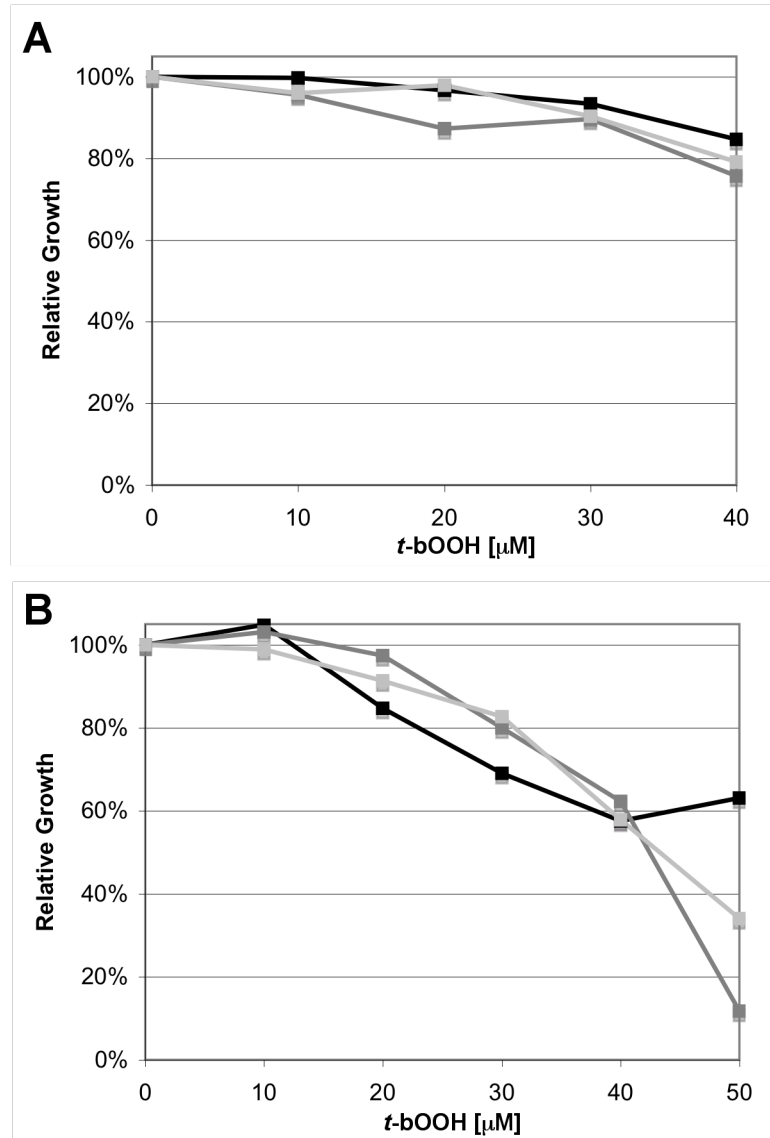
**Figure 3.7: Two-dimensional gel analysis of WT and SUB -/- *Leishmania*.**

Wildtype *L. donovani* had high levels of TXNPx1 and 3 forming a single doublet of spots (WT spots 4 & 5). The level of TXNPx2 was low (spot 3) and Prx was not detectable (spot 1). In the SUB -/- parasites, the wildtype TXNPx1/3 doublet decreased (SUB -/- spots 4 & 5) and two new TXNPx1/3 doublets were present at a higher molecular weight (spots 6 & 7) and at a higher molecular weight with a lower pI (spots 8 & 9). TXNPx2 (spot 3) and Prx levels (spots 1 & 2) were elevated in these parasites.



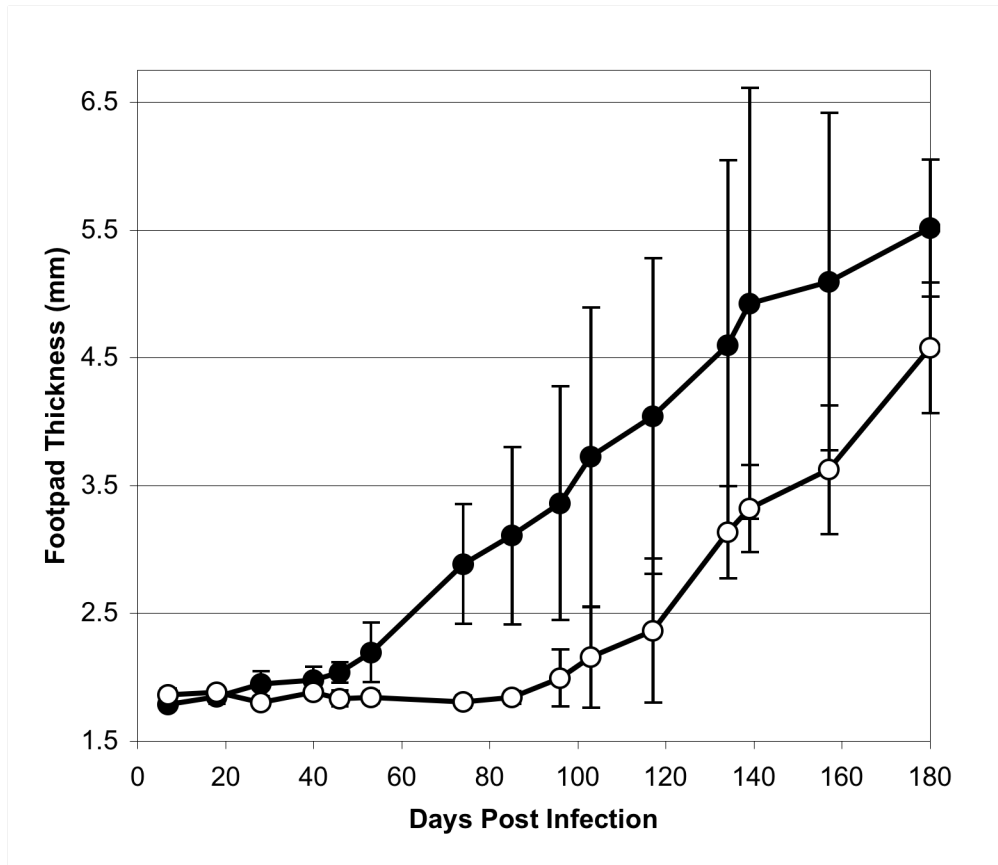
**Figure 3.8: Effect of hydroperoxide on *Leishmania* replication.**

*Leishmania donovani* (A) and *Leishmania major* (B) promastigote replication was measured in the presence of varying concentrations of *t*-bOOH. Wildtype (black squares), SUB deficient (dark gray squares), and SUB add-back (light gray squares) were grown for five days and counted on a Multisizer 3 COULTER COUNTER. Values are expressed at the percent culture density relative to the untreated controls.



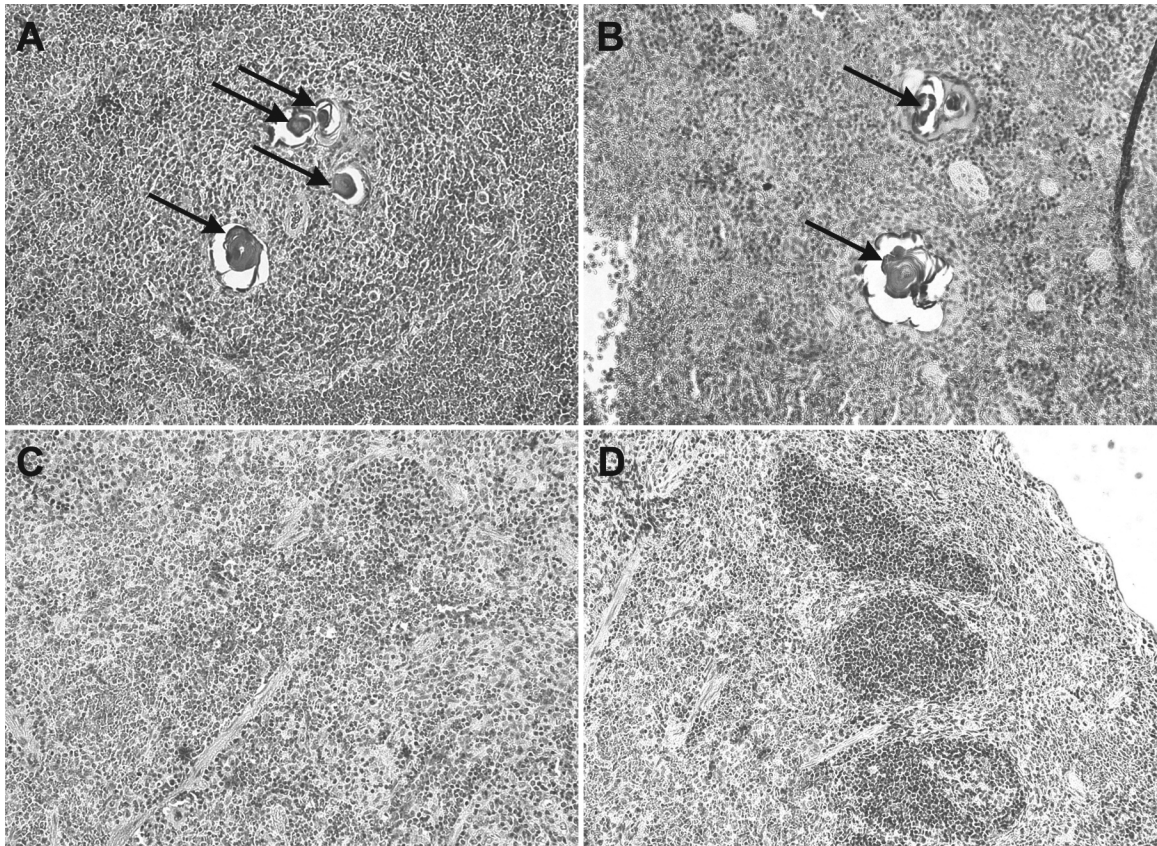
**Figure 3.9: Mouse infections by wildtype and SUB-deficient *Leishmania major*.**

BALB/c mice (n=5) were infected subcutaneously in the left hind footpads with wildtype (solid circle) or SUB +/- (empty circle) parasites. Footpad swelling was measured weekly and the footpad thickness was plotted over time. Error bars indicate the S.D. between the 5 mice in each group. The mice were sacrificed at 180 days post infection. No swelling was observed in the right hind (uninfected) footpads.



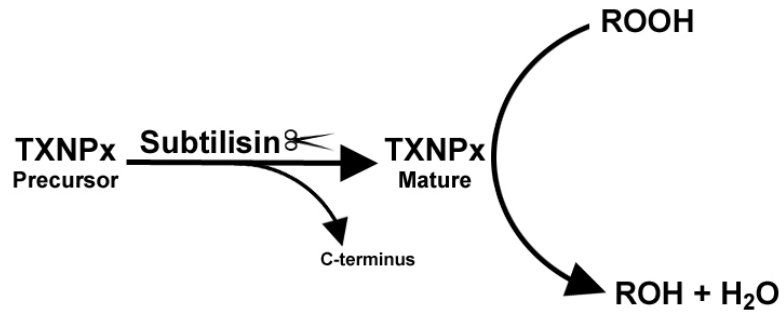
**Figure 3.10: Hamster infections by wildtype and SUB  $-/-$  *Leishmania donovani*.**

Male Golden Syrian hamsters (n=3) were infected intraperitoneally with wildtype or SUB  $-/-$  *L. donovani*. At 200 days post-infection, the hamsters were sacrificed and their spleens were sectioned, H & E stained, and histologically examined. All wildtype-infected hamsters' spleens (A & B) contained psammoma body calcifications (arrows). No psammoma bodies were observed in the spleens of hamsters infected with SUB deficient *Leishmania* (C & D).



**Figure 3.11: Subtilisin is the maturase for the trypanedoxin peroxidases.**

This schematic details the proposed function of *Leishmania* SUB. When promastigotes differentiate into amastigotes within the vertebrate host, they are phagocytosed by host macrophages. Within the parasitophorous vacuoles of these macrophages, the amastigotes must survive a burst of reactive oxygen species. *Leishmania* uses the trypanothione reductase system for antioxidant defense. The terminal peroxidases of this system, TXNPxs, are processed by subtilisin into their mature forms via the removal of their C-termini. The mature TXNPxs are then able to convert hydroperoxides into alcohol and water.





**Chapter Four:**  
**General Discussion and Future Directions**

#### **4.1. Implications of this research and potential future directions.**

Recent advances in parasite molecular biology and bioinformatics have enabled us to strategically identify and study *Leishmania* proteins as therapeutic targets. Parasite proteases are viable drug targets, as many of them are required for the pathogenic life cycle of the parasite. We have identified two serine proteases that are virulence factors for *Leishmania*: a Clan SC subfamily S9A oligopeptidase B and a Clan SB subfamily S8A subtilisin. These enzymes have very different roles in the parasite's infectious life cycle, yet both are required for full virulence. Our research provides new insights into the pathways and functions of these two enzymes. Additionally, we have paired both classical biochemical isolation and identification of these enzymes with targeted gene deletion followed by phenotypic analysis. We also have characterized the functions of these enzymes using proteomics and microarray analyses, and we have determined subsite specificities using synthetic substrates and a positional scanning synthetic combinatorial library.

Oligopeptidase B (OPB) has been identified as the main serine protease activity from *Leishmania* lysates. This enzyme has been characterized biochemically and its role in *Leishmania* biology has been uncovered. OPB regulates levels of parasite enolase, a protein that moonlights as a virulence factor on the parasite cell surface by binding host proteins such as plasminogen. We propose that tight control of cell surface enolase/plasminogen levels by OPB during infection of the host enables *Leishmania* to disseminate from the sandfly bite site and to infect macrophages undetected. Future research into this pathway would include determining if OPB acts directly on enolase, or if OPB acts upstream of enolase through an intermediate. Enolase isoforms build up in

OPB  $-/-$  parasites. These isoforms potentially represent different phosphorylation states of the protein. The phosphorylation states of enolase have been shown to determine the protein's subcellular localization in *Plasmodium* (Pal-Bhowmick, Vora et al. 2007). Additional research is needed to determine how the accumulated enolase isoforms differ in OPB knockouts compared to wildtype *Leishmania*. Subcellular localization of enolase isoforms should also be tracked during different stages of the parasite life cycle and infection.

*Leishmania mexicana* has been shown to have reduced virulence in plasminogen deficient mice (Maldonado, Marina et al. 2006). This reduction in virulence is likely due to the inability of the parasite to bind host plasminogen to its cell surface using enolase. We have shown that OPB modulates parasite enolase and that OPB  $-/-$  parasites have reduced virulence in the mouse model. It would therefore be interesting to examine the virulence of OPB  $-/-$  parasites in plasminogen  $-/-$  mice. We would predict that the virulence of OPB  $-/-$  *Leishmania* would not be additionally reduced (as compared to wildtype *Leishmania*) in the plasminogen  $-/-$  mice, because plasminogen is acting downstream of OPB in the OPB/enolase/plasminogen system.

OPB from *T. cruzi* has been heavily studied and is known to play a role in host cell entry by modulating recruitment of lysosomes to the host cell surface through  $\text{Ca}^{+2}$  signaling. This process is induced by *T. cruzi* OPB through an as yet unknown cell surface ligand or receptor (Burleigh, Caler et al. 1997). The interaction of *T. cruzi* OPB with this parasite's enolase should be investigated. Cell surface enolase may be the receptor involved in the upregulation of  $\text{Ca}^{+2}$  levels in host cells. Enolase may also be binding to host plasminogen in this process and signaling the host cells via a plasminogen

receptor. *T. cruzi* virulence should therefore be tested in plasminogen  $-/-$  mice. It is predicted that *T. cruzi*, like *Leishmania*, will have reduced virulence in mice deficient in plasminogen.

*Leishmania* subtilisin (SUB) has also been identified and characterized. The gene encoding *L. donovani* SUB was cloned and sequenced. SUB is a large 1,744 amino acid protein; however, the catalytic domain is contained in only the N-terminal 400 amino acids. The potential function of the remaining C-terminal portion of the protein remains unknown. Bioinformatic searches did not reveal any potential functional domains in this portion of the protease, although there is a potential transmembrane helix at the C-terminus. The SUB sequence from *L. infantum* (GeneDB) has a C-terminal truncation that removes this potential transmembrane helix. If the *L. infantum* SUB sequence is correct, that may indicate that the helix is not functional or is not required for SUB to function properly. Future research should address potential functions of the C-terminal portion of this enzyme. In addition, the *L. infantum* gene should be cloned and sequenced to verify the C-terminal truncation.

We have found that subtilisin from the *Leishmania donovani* complex is a unique Clan SB enzyme that has a non-canonical catalytic triad. This enzyme uses a Glu-His-Ser triad instead of the Asp-His-Ser triad used by all other known Clan SB proteases, including *L. major* and *L. braziliensis* SUB. We have shown that *L. donovani* SUB is an active enzyme. The enzymatic characteristics of *L. donovani* and *L. major* SUBs should be investigated in detail to determine what effects the non-canonical catalytic triad has on enzyme function. *L. major* SUB and *L. donovani* SUB with the Glu-to-Asp mutation

should be used as add-back constructs for SUB *-/- L. donovani* to determine if these enzymes can rescue the knockout phenotype.

Subtilisins and site-1 proteases are known to undergo autocatalytic cleavage in order to activate themselves. This autocatalytic cleavage site should be determined for *Leishmania* SUB. Pileups of the amino acid sequences of site-1 proteases from *Homo sapiens* and *Apis mellifera* (the western honey bee) reveal that the known site-1 cleavage sites align with an Arg-An-Leu-Thr motif in *Leishmania* SUB. This motif is also a predicted site-1 cleavage site (Rawlings, Morton et al. 2008) and could therefore represent the cleavage site for *Leishmania* SUB. This enzyme should be tested for activity against a synthetic peptide with this sequence.

Our research has uncovered that *Leishmania* SUB regulated the trypanothione reductase system. This system has been identified as a key target for anti-trypanosomal drugs (Tetaud, Giroud et al. 2001), thus the identification of SUB as a player in this pathway has implications for chemotherapeutics research. Additional research needs to be performed to determine how SUB affects the trypanothione reductase system. We predict that SUB is cleaving the C-terminus of these enzymes. Recombinant TXNPx can be used as a SUB substrate *in vitro* and digestion products can be N-terminally sequenced. TXNPx has putative cleavage sites, Arg-Leu-Leu-Glu and Lys-Gly-Ala/Asp-Pro, at position 161 and 182, respectively.

Previous studies, along with our research, have shown that serine protease inhibitors reduce *Leishmania* replication and viability *in vitro*. We have investigated two of the twenty-one distinct serine proteases of *Leishmania*. Several of these remaining

proteases should be studied as potential virulence factors and chemotherapeutic targets. Enzymes of particular interest are the two remaining Clan SC subfamily S8A enzymes, the prolyl oligopeptidase and an unusual additional enzyme putatively categorized as a second oligopeptidase B (GeneDB). Activity of this latter enzyme has not yet been detected; however, our OPB *-/-* parasites represent a potential system for uncovering the activity of this enzyme since the major proteolytic activity has been abolished. Additional serine proteases that should be investigated are the two rhomboid proteases. These unusual proteases cleave proteins within membranes. Recent research on rhomboid proteases has implicated them in adhesion and invasion of *Plasmodium* (Baker, Wijetilaka et al. 2006; O'Donnell, Hackett et al. 2006).

Over the past fifteen years, serine protease inhibitors and serine protease-blocking antibodies have been shown to function as anti-trypanosomal reagents in *in vitro* and *in vivo* studies. Prolyl oligopeptidase inhibitors have been found to block the invasion of mammalian cells by *T. cruzi* *in vitro* (Grellier, Vendeville et al. 2001). Similarly, anti-OPB antibodies prevent *T. cruzi* from initiating Ca<sup>+2</sup>-signalling in normal rat kidney cells, thereby preventing cell invasion (Burleigh, Caler et al. 1997). Anti-OPB antibodies have also been found to reduce the degradation of host atrial natriuretic factor in rat plasma following *T. evansi* infection. Hydrolysis of this peptide hormone by OPB is believed to contribute to disease pathology (Morty, Pelle et al. 2005). In addition, serine protease inhibitors exhibit antiparasitic activity against the bloodstream form of *T. brucei*. In an *in vivo* mouse model of *T. brucei* infection, a peptidyl phosphonate diphenyl ester was able to cure infected mice (Morty, Troeberg et al. 2000). These studies indicate that inhibition

or blocking of parasite-specific serine proteases has the potential to directly reduce parasite viability, to prevent host infection, and to alleviate disease pathology.

Several trypanosomatid serine proteases are attractive chemotherapeutic targets. Humans do not have an endogenous OPB, and the trypanosomatid SUB and prolyl oligopeptidase are quite divergent from the human enzymes; thus inhibitors of these proteases may not have significant off-target effects on the host. Interestingly, three of the most commonly used anti-trypanosomal drugs (pentamidine, diminazene, and suramin) are reversible inhibitors of *T. brucei* OPB (Morty, Troeberg et al. 1998). Furthermore, suramin and pentamidine are known to reach intracellular concentrations sufficient for inhibition to occur (Berger, Carter et al. 1995; Hanau, Rippa et al. 1996). This research suggests that the effectiveness of these drugs may be due, at least in part, to their function as serine protease inhibitors. Future research should evaluate if the addition of serine protease inhibitors to suramin- or pentamidine-based therapeutic regimes can overcome or prevent drug resistance. Serine protease inhibitors may also be useful as part of combination therapies with antileishmanial drugs, many of which are quite toxic. This could potentially shorten the length of treatment, thereby reducing both the cost and the total amount of drug administered. Initial investigations into the efficacy of serine protease inhibitors as anti-trypanosomal compounds have been promising; however, more research is needed to identify lead compounds and to validate specific serine proteases as drug targets.

*Leishmania spp.*, along with a number of other protozoan and helminthic parasites, cause a major global disease burden. These are largely diseases of the developing world and are therefore unknown to much of the population that lives in

developed countries. For these reasons research into many of these diseases is severely neglected. As globalization increases, population densities rise, global travel becomes easier, climate change unfolds, and civil unrest continues in parts of the developing world the toll of these neglected diseases is likely to rise. Drugs are available for several of these parasitic diseases; however, drug resistance is inevitable and is already occurring in some endemic regions. Research into neglected diseases must therefore increase, and global awareness and education must become priorities.



**Chapter Five:**  
**Materials and Methods**

### **5.1.1. Animals and parasite strains for OPB experiments.**

Commercially bred, 6-8 week-old, female BALB/c mice (*Mus musculus*) were used for the murine footpad infection model. Microarray analysis was performed bone marrow-derived macrophages from female C57BL/6 mice (Charles River Laboratories International, Inc., Davis, CA). *Leishmania donovani donovani* MHOMISD/OO/IS-2D (Ldd IS C12) was used for enzyme isolation and knockout studies. *Leishmania major* LV39 MRHO/SU/59/P was also used for knockout studies as well as animal infections. Serine protease activity assays were also performed on lysates from *Leishmania mexicana* MNYC/BZ/62/M379, and *Trypanosoma cruzi* M/HOM/AR/74/CA-I CL72. *Leishmania* promastigotes were cultured at 27°C in M199 (Sigma, USA) liquid medium as previously described (Wallis and McMaster 1987). Axenic amastigotes of *L. donovani* were cultured at 37°C in 100% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) as previously described (Doyle, Engel et al. 1991).

### **5.1.2. Parasite lysates and protease determination.**

Cultures of *Leishmania* promastigotes, axenic amastigotes, and *T. cruzi* epimastigotes were pelleted and resuspended three times with phosphate buffered saline. The resultant washed pellets were then lysed in 50 mM Tris-HCl, pH 8.0 with 0.25% Triton X-100 (Sigma, USA) at 4°C for 1 hour. These solutions were then centrifuged and the supernatants were isolated and sterilized with 0.2 µm syringe filters (Nalge Nunc, Rochester, NY). Protein concentrations were determined by NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA). Protease activity was determined by measuring the hydrolysis of the fluorogenic peptide substrate benzyloxycarbonyl-prolyl-arginyl-7-

amino-4-methylcoumarin (Z-PR-AMC) (Peptides International, Inc., Louisville, KY) in 50 mM Tris-HCl, pH 8.0 and 5.5, with and without 10 $\mu$ M E-64 (*N*-[*N*-(*L*-3-*trans*-carboxyoxirane-2-carbonyl)-*L*-leucyl]-agmatine) or 2 mM PEFABLOC (AEBSF, 4-(2-Aminoethyl)benzenesulfonylfluoride, HCl) (EMD Chemicals, Gibbstown, NJ) (see protease activity assay procedures below).

### **5.1.3. Purification and identification of serine protease activity (OPB) from *Leishmania* lysate by mass spectrometry.**

Filtered lysate from 10<sup>9</sup> *L. donovani* promastigotes, prepared described above, was loaded onto a HiTrap Q Sepharose Fast Flow column using an ÄKTApurifier (GE Healthcare, USA). A 50 mM Tris-HCl, pH 8.0 buffer was used for column equilibration, sample loading, and eluting at a 1mL/min flow rate. Protein was eluted using a linear gradient of 0-1M NaCl over 20 column volumes while collecting 0.25 mL fractions. OPB-containing fractions were identified by Z-PR-AMC hydrolysis at pH 8.0. Peak OPB-containing fractions were run on a Criterion 10-20% Tris-HCl SDS-PAGE gel and stained by Coomassie (Bio-Rad Laboratories, Inc., Hercules, CA). Protein bands were excised and tryptic digests were performed. Samples were analyzed by liquid chromatography-mass spectrometry/mass-spectrometry with a QStar XL (Applied Biosystems, Foster City, CA) equipped with a Protana nanospray ion source (Protana, Inc., Toronto, Ontario). A peptide mass accuracy tolerance of 100 ppm and a fragment ions mass accuracy tolerance of 0.15 Da was used. A database search was conducted using Mascot (Matrix Science Inc., Boston, MA) on the Sanger Institute *Leishmania major* GeneDB.

#### 5.1.4. OPB cloning and sequencing.

Genomic DNA from *L. donovani* was isolated as previously described (Medina-Acosta and Cross 1993). The *OPB* gene was then amplified from this genomic DNA by PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN). The forward (5'-CTC **AGA TCT** CCA CCC CCA CCA GCC CGG-3') and reverse (5'-CTC **AGA TCT** CCT AGA GTT AAT GAT CAC GAC TC-3') primers (BglII sites, in bold, were included on the primers for cloning) were designed based on the non-coding sequences flanking the *L. infantum* *OPB* open reading frame (*Leishmania infantum* GeneDB: LinJ09\_V3.0820). The resultant 2.2 kb PCR product was cloned into pCR2.1-TOPO using TOPO TA Cloning and sequenced using the included sequencing primers (Invitrogen, Carlsbad, CA). The amino acid sequence of *L. donovani* *OPB* was aligned with homologous sequences from *L. infantum*, *L. major*, *L. braziliensis*, *T. cruzi*, *T. brucei*, *L. amazonensis*, and *T. evansi* (respectively, GeneDB: LinJ09\_V3.0820, LmjF09.0770, LbrM09\_V2.0850, Tc00.1047053511557.10, and Tb11.52.0003; GenBank: ABQ23350 and AAS55050) using the ClustalW algorithm from MegAlign (DNASTAR, Madison, WI). The *Leishmania* *OPB* add-back transfection construct was generated by cutting the cloned *OPB* from pCR2.1 with the BglII enzyme (New England Biolabs, Ipswich, MA) and cloning the resultant fragment into the BamHI site of pXG (Ha, Schwarz et al. 1996). The *Pichia pastoris* transformation construct was generated by PCR amplification of *OPB*, adding a 5' XhoI site (bold) followed by a Kex2 cleavage site (underlined) and a 3' XbaI site (underlined bold) using forward (5'-CTC **CTC GAG** AAA AGA ATG TTG TCG GGC AAC ACC ATC G-3') and reverse (5'-CTC **TCT**

**AGA** TTA CCT GCG AAC CAG CAG GCG-3') primers, and then cloned into pPICZ $\alpha$  A (Invitrogen).

#### **5.1.5. Expression and purification of recombinant OPB from *Pichia pastoris*.**

The pPICZ $\alpha$ -OPB construct was electroporated into X-33 *P. pastoris* and expression clones were isolated and induced for 48 hr as per the manufacturer's protocol. Supernatant from induced cultures was harvested by centrifugation at 3,000 g for 10 min, followed by 0.2  $\mu$ m filtration (Nalge Nunc) and lyophilization. The crude lyophilized protein was resuspended in 20% of its original volume using 10 mM Tris-HCl, pH 8.0 with 1 M ammonium sulfate (AS) and loaded onto a HiTrap Butyl Sepharose 4FF hydrophobic interaction column (GE Healthcare). A 10 mM Tris-HCl, pH 8.0 buffer with 1 M AS was used for column equilibration, sample loading, and washing at a 1 mL/min flow rate. The AS concentration was decreased to 0.75 M for 20 column volumes to elute the OPB protein. The eluate was desalted by buffer exchanging with 10 mM Tris-HCl, pH 8.0 and then concentrated on an Amicon Ultra-4 30,000 NMWL filter device (Millipore, Billerica, MA). The concentrated sample was loaded onto a MonoQ HR 5/5 anion exchange column (GE Healthcare) on an ÄKTApurifier and fractionated as described above. OPB containing fractions were identified by Z-PR-AMC hydrolysis. Peak fractions were pooled, desalted, and then re-concentrated as before. OPB protein concentration was measured by NanoDrop 1000.

#### **5.1.6 Protease activity assays and OPB enzymatic characterization.**

Protease activity was measured using peptide substrates containing C-terminal AMC or ACC (7-amino-4-carbamoyl-methylcoumarin) as the fluorogenic leaving group. Reactions contained 20  $\mu$ M substrate unless otherwise noted. Enzyme samples were mixed with substrate in 50 mM Tris-HCl, pH 8.0 with 0.2% DMSO in 150  $\mu$ L total volume. Hydrolysis of the substrates was measured at 25°C using a FlexStation microplate spectrofluorimeter (Molecular Devices, Sunnyvale, CA). Excitation/emission for AMC and ACC were 355/460 nm and 380/460 nm, respectively.  $V_{\max}$  values were calculated using the accompanying SoftMax Pro v4.8 software. An inhibitor or salt was pre-incubated with OPB for 10 min prior to the addition of substrate and then read on a FlexStation. To determine the pH preference of OPB, protease activity assays were performed in triplicate using the Z-PR-AMC substrate at pH 3.0-10.5 with a 0.5 pH unit step size in 100 mM Citrate-Phosphate buffer (pH 3.0-7.0), Sodium-Phosphate buffer (pH 6.5-8.0), Tris-HCl buffer (pH 7.5-9.0), and Glycine-NaOH buffer (pH 8.5-10.5).

#### **5.1.7. Positional Scanning Synthetic Combinatorial Library profiling and validation.**

The P1-P4 substrate specificity profile for OPB was determined using a completely diverse positional scanning synthetic combinatorial library (PS-SCL) as has been previously described (Choe, Leonetti et al. 2006). This library is composed of substrates that are N-terminally acetylated and have an ACC fluorogenic leaving group. Protease activity assays were performed using 1  $\mu$ L of substrate in a 150  $\mu$ L reaction with 50 mM Tris-HCl, pH 8.0 and 0.02 ng of enzyme. Assays were performed in duplicate. To test the predictive capability of the PS-SCL substrate specificity profile, fluorogenic peptides that were either predicted to be good or bad substrates of OPB were selected for

screening in protease activity assays. The substrates were used at 20  $\mu\text{M}$ : Glut-GR-AMC, Z-LR-AMC, Ac-VFRSLK-AMC, H-A-AMC, Ac-DEVD-AMC, Ac-WEHD-AMC, Z-LLE-AMC, H-L-AMC, Suc-GPLGP-AMC (Peptides International, Inc.), Ac-KQKLR-AMC (MP Biomedicals, USA), Z-GGR-AMC, NtBoc-LGR-AMC, Z-GPR-AMC, Z-PR-AMC, Z-VVR-AMC, Z-RR-AMC, Boc-LRR-AMC, Ac-FR-AMC, Z-FR-AMC, Boc-RVRR-AMC, Z-R-AMC, Z-VAD-AMC, Suc-AAF-AMC, H-GF-AMC, Z-GGL-AMC, Suc-LY-AMC, Suc-LLVY-AMC (Bachem California Inc., Torrance, CA), Z-AARR-AMC, Suc-PSPF-AMC, Z-AEN-AMC, Ac-ASN-AMC, Ac-AVN-AMC. To test the PS-SCL prediction that Glycine is the preferred P2 amino acid, two substrates that differ only at the P2 position, Z-GGR-AMC and Z-GPR-AMC, were compared. Protease activity assays were performed on these substrates at concentrations ranging from 80-0.2  $\mu\text{M}$  and the  $K_{\text{cat}}$  and  $K_{\text{m}}$  values were calculated.

#### **5.1.8. Constructs for targeted gene deletion of OPB.**

Two knockout cassettes (one for each allele) were created to delete the *L. donovani* and *L. major* *OPB* genes. These cassettes each contained an antibiotic resistance gene, *hyg*<sup>r</sup> (conferring hygromycin B resistance) (Gritz and Davies 1983) or *pac*<sup>r</sup> (conferring puromycin resistance) (Lacalle, Pulido et al. 1989), followed by 1.5 kb of the 3' untranslated region of the *L. major dhfr-ts* gene (Joshi, Sacks et al. 1998). This untranslated region ensures high-level expression during *Leishmania*'s life cycle. To target these knockout cassettes to the *OPB* locus, 5' and 3' targeting flanks were created and ligated into their respective sides of the cassettes. These targeting flanks were generated by PCR amplification of the untranslated regions directly 5' and 3' of the *OPB*

open reading frame. PCR primers were designed based on the published *L. infantum* and *L. major* sequences (GeneDB): 5' flank forward (5'-CTC **ACT AGT** ATC TAC GAC CAT GTG ACA CGG-3') and reverse (5'-CTC TCT AGA CCG GGC TGG TGG GGG TGG-3') (1.4 kb fragment) primers; 3' flank forward (5'-CTC **ACT AGT** GAG TCG TGA TCA TTA ACT CTA GG-3') and reverse (5'-CTC TCT AGA ACG CTT GTA AGC GAG CGA GG-3') (0.9 kb fragment) primers (SpeI sites, in bold, and XbaI sites, underlined, were included for cloning). These constructs were maintained and amplified in the pGEM-9Zf(-) vector (Promega, Madison, WI). For targeted gene deletion, 50 µg of the targeting constructs were excised from their vectors using the flanking restriction endonucleases SpeI and XbaI (New England Biolabs) and purified by electrophoresis on 0.8% agarose gels (Maniatis, Fritsch et al. 1982) then purified using the QIAEX II Gel Extraction Kit (QIAGEN Inc., Valencia, CA).

#### **5.1.9. *Leishmania* transfections, clone isolation, and Southern blot analyses.**

Purified transfection constructs described above were used to transfect log-phase *Leishmania* promastigotes by electroporation (2.25 kV/cm, 500µF) as previously described (Joshi, Sacks et al. 1998). After electroporation, the cells were grown and selected using hygromycin B and puromycin (for gene deletions) or G418 (for the add-back) on both plates and in liquid media and clones were isolated as previously described (Joshi, Kelly et al. 2002). For Southern blot analyses, genomic DNA was isolated from knockout clones and digested with NotI (New England Biolabs) and fragments were separated by electrophoresis on 0.6% agarose gels. These were then transferred to Hybond-N+ (GE Healthcare) nylon filters by the manufacturer's instructions for alkali



transfer. A knockout probe and an OPB core probe for the Southern blots were amplified by PCR and gel purified: knockout probe forward (5'-TAT GCG CTG GTG ACG ATG CCG-3') and reverse (5'-CAC GCT CAG CGG CTG CCA TG-3') primers (1.5 kb fragment upstream of the OPB deletion locus); OPB core probe forward (5'-CGAGGTCGGCGGTGGCTTCGAC-3') and reverse (5'-TTA CCT GCG AAC CAG CAG GCG C-3') primers (0.9 kb fragment containing the predicted OPB catalytic core). The probes were <sup>32</sup>P-labeled using Rediprime II Random Prime Labeling System (GE Healthcare) as per the manufacturer's instructions. Hybridization and washing conditions were performed as previously described (Button, Russell et al. 1989). Southern blots were scanned on a Typhoon Trio Variable Mode Imager (GE Healthcare).

#### **5.1.10. Protease knockout replication rates and differentiation experiments.**

Day 4 OPB or SUB knockout and wildtype parasites were split in triplicate into new M199 (for promastigote replication rates) and into 37°C fetal bovine serum (for axenic amastigote replication). Parasite culture densities were determined on days 1-4 post-split by cell counting on a Multisizer 3 COULTER COUNTER (Beckman Coulter, Inc., Fullerton, CA). Axenic amastigote differentiation was observed by microscopy.

#### **5.1.11. Protease knockout mouse infections.**

Metacyclic *L. major* promastigotes were purified from day 4 OPB +/-, SUB +/-, and wildtype cultures using negative selection by binding to peanut agglutinin as has been previously described (Sacks, Hieny et al. 1985). BALB/c mice (groups of 5) were anesthetized by isoflurane inhalation and infected subcutaneously in the left hind footpad

with  $5 \times 10^6$  metacyclic promastigotes in 50  $\mu$ L Hanks' balanced salt solution. Footpad swelling was measured weekly after inoculation using a Mitutoyo caliper. Parasites were recovered from infected mice by resection of the left popliteal lymph node.

#### **5.1.12. OPB two-dimensional gel electrophoresis.**

Lysates from OPB knockout and wildtype *L. donovani* promastigotes (405  $\mu$ g each) were prepared with the ReadyPrep 2-D Cleanup Kit and dissolved in 300  $\mu$ L Rehydration/Sample buffer. Using the ReadyPrep 2-D Starter Kit, samples were absorbed into 17 cm pH 7-4 IEF strips, isoelectrically focused, and then separated on 8-16% Tris-HCl PROTREAN II Ready Gels using the manufacturer's protocols (Bio-Rad Laboratories, Inc.). Gels were stained using SimplyBlue SafeStain (Invitrogen) and imaged on a Typhoon Trio using the red laser (633 nm) in the fluorescence acquisition mode with no emission filter. Images taken of the gels with wildtype and OPB knockout lysates were compared. Spots that had significantly different intensities or that had shifted were excised from the gels, trypsin-digested, and analyzed by mass-spectrometry as described above.

#### **5.1.13. Microarray analysis of *Leishmania*-infected bone marrow-derived macrophages.**

Bone marrow-derived macrophages were isolated from the femora and tibiae of five female C57BL/6 mice. The cells were pooled and cultured in DMEM with 0.5% FBS and macrophage colony-stimulating factor (M-CSF) on 10 cm non-tissue culture treated plates at  $5 \times 10^6$  cells per plate. The cells were fed an additional 10 mL of media on day 3.

On day 6, macrophages were harvested from the plate using ice-cold PBS and frozen at  $10^7$  cells/mL in 90% FBS with 10% DMSO. The day prior to infecting with *Leishmania*, macrophages were thawed and plated on T25 flasks at  $5 \times 10^6$  cells per flask in media containing M-CSF. On the day of infection, macrophages were washed once with PBS, and their media was replaced with DMEM with 0.5% FBS containing day 5 post-split *L. donovani* at an MOI of 10. Uninfected control cells received media without parasites. The flasks were spun down at 1000 rpm for 5 min to synchronize the infections. RNA was collected at 0-, 2-, 6-, 12-, and 24-hour time points by removing the media and lysing cells with ice-cold TRIzol (Invitrogen). RNA was isolated from the lysates using the RNeasy Mini Kit (QIAGEN Inc.) and then amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Applied Biosystems). This amplification incorporates aaUTP into the aRNA during *in vitro* transcription, which facilitates coupling to NHS ester dyes.

Four micrograms of amplified RNA from each sample were labeled with Cy5 dye. A pooled reference (consisting of an equal mass of RNA from all of the time points within a particular infection time course) was labeled with Cy3 dye and hybridized against the Cy5-labeled samples on Mouse Exonic Evidence-Based Oligonucleotide (MEEBO) Arrays. These arrays were hybridized in a 63°C water bath overnight. After hybridization, the arrays were sequentially washed in three solutions: 0.6X SSC with 0.01% SDS, 0.6X SSC, and 0.06X SSC. Arrays were scanned using a GenePix 4000B scanner with GenePix PRO v4.1 software (Molecular Devices). The SpotReader program (Niles Scientific, Portola Valley, CA) was used for array gridding and image analysis. The resultant data files were uploaded to Acuity v4.0 (Molecular Devices) where the raw

data was log transformed, filtered for “good quality spots” (((‘RgnR<sup>2</sup>(635/532)’>0.6) AND (‘Flags’>=0))AND((‘F532Mean-B532’>200) OR (‘F635Median-B635’>200))), and normalized to the 0-hour control. The non-mouse control genes were removed from the dataset as well as genes lacking data in at least 70% of the arrays. This data was then analyzed for statistically significant genes using the Statistical Analysis of Microarray (SAM) software v2.23A, which works with the open-source R software package (available at <http://www-stat.stanford.edu/~tibs/SAM/>).

Three approaches were used in data analysis. First, pairwise comparisons were performed between cells infected with *Leishmania* and the uninfected control cells in order to determine the number of genes significantly affected by the infection and the relative fold changes of these genes. To do this, two-class unpaired SAM analysis was employed using a false discovery rate (FDR) cutoff of <1% and the condition that genes must have at least a two-fold change. Each time point within a time course was treated as a replicate for the purposes of statistical analysis. Second, multiclass comparisons were performed between cells infected with wildtype *Leishmania*, OPB knockout *Leishmania*, and uninfected cells to determine the number of genes significantly different between these groups. This was accomplished through multiclass analysis in SAM with an FDR of <0.1%. Cluster software v2.11 (available at <http://rana.lbl.gov/EisenSoftware.htm>) was then utilized to make self-organizing maps and to perform average linkage clustering of both genes and arrays. Third, gene ontology and pathway analyses were performed using Database for Annotation Visualization and Integrated Discovery (DAVID) (available at <http://david.abcc.ncifcrf.gov/home.jsp>). The list of upregulated genes identified by the pairwise SAM analysis were inputted into DAVID along with a background gene list

representing all of the genes used in the pairwise analysis. The pathways and molecular functions that were over-represented in the upregulated genes list were identified and sorted based on fold change and p-value.

#### **5.1.14. Polyclonal OPB antibody production.**

A polyclonal antiserum against gel purified recombinant OPB was raised in a set of 5 female Swiss-Webster mice (Simonsen Laboratories, Inc., Gilroy, CA). Mice were injected subcutaneously with 50µg protein in incomplete Freund's adjuvant (1:1). The immune response was boosted 3 times at two-week intervals. Mice were bled via tail bleeds and sera were stored at -20°C. Specificity of the antiserum was verified by Western blot analysis by probing whole *L. donovani* lysate with serum at a 1:2 dilution in PBS. HRP-conjugated sheep anti-mouse IgG (GE Healthcare) was used as a secondary antibody at a 1:10,000 dilution.

#### **5.1.15. Animals and parasite strains for SUB experiments.**

Commercially bred, 6-8 week-old, female BALB/c mice (*Mus musculus*) were used for the murine footpad infection model (Charles River Laboratories International, Inc., Davis, CA). Commercially bred, 4-5 week-old, male Golden Syrian hamsters (*Mesocricetus auratus*) were used for the visceral infection model (Simonsen Laboratories, Inc., Gilroy, CA). *Leishmania donovani donovani* MHOM/ET/67/HU3 cloned stock and *Leishmania major* LV39 MRHO/SU/59/P were used for knockout studies and for animal infections. *Leishmania* promastigotes were cultured at 27°C in M199 (Sigma, USA) liquid medium as previously described (Wallis and McMaster

1987). Axenic amastigotes of *L. donovani* were cultured at 37°C in 100% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) as previously described (Doyle, Engel et al. 1991).

#### **5.1.16. Subtilisin cloning and sequencing.**

Genomic DNA from *L. donovani* was isolated as previously described (Medina-Acosta and Cross 1993). The *subtilisin* gene was then amplified from this genomic DNA by PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN) in two overlapping pieces, termed the 5' and 3' halves. For each half, an external primer from the non-coding flanks of the gene and an internal primer were designed based on regions of identity between the known *L. infantum* and *L. major* sequences (Sanger Institute GeneDB: LinJ13\_V3.0940 and LmjF13.1040). Both halves were cloned into the pGEM-7Zf(-) vector (Promega, Madison, WI) and then spliced together using an internal HindIII site. The open reading frame was sequenced using the primers listed in Table 5.1. The amino acid sequence of *L. donovani* subtilisin catalytic core was identified using Pfam (Accession number PF00082, Subtilase family) and was aligned with homologous sequences (Table 5.2) using the ClustalW algorithm from MegAlign (DNASTAR, Madison, WI).

#### **5.1.17. Constructs for targeted gene deletion of *SUB*.**

Two knockout cassettes (one for each allele) were created to delete the *L. donovani* and *L. major SUB* genes. These cassettes each contained an antibiotic resistance gene, *hyg<sup>r</sup>* (conferring hygromycin B resistance) (Gritz and Davies 1983), *pac<sup>r</sup>*

(conferring puromycin resistance, used for *L. donovani* only) (Lacalle, Pulido et al. 1989), or *sat'* (conferring nourseothricin resistance, *L. major* only) (Joshi, Webb et al. 1995), followed by 1.5 kb of the 3' untranslated region of the *L. major dhfr-ts* gene (Joshi, Sacks et al. 1998). This untranslated region ensures high-level expression during *Leishmania's* life cycle. To target these knockout cassettes to the *SUB* locus, 5' and 3' targeting flanks were created and ligated into their respective sides of the cassettes (Table 5.3). These targeting flanks were generated by PCR amplification of the untranslated regions directly 5' of the *SUB* ORF and 3' of the *SUB* catalytic core (*L. donovani*) or 3' of the ORF (*L. major*). PCR primers were designed based on the published *L. infantum* and *L. major* sequences (GeneDB). These constructs were maintained and amplified in the pGEM-9Zf(-) vector (Promega). For targeted gene deletion, 50 µg of the targeting constructs were excised from their vectors using the flanking restriction endonucleases SpeI and XbaI (New England Biolabs) and purified by electrophoresis on 0.8% agarose gels then purified using the QIAEX II Gel Extraction Kit (QIAGEN Inc., Valencia, CA).

#### **5.1.18. Transmission electron microscopy.**

Approximately 10<sup>8</sup> day 4 *L. donovani* axenic amastigotes from wildtype or *SUB* -/- cultures were pelleted and washed 3x in PBS. The parasites were processed for conventional EM by freeze-substitution in 1% OsO<sub>4</sub>/0.1% uranyl acetate in acetone and embedded in epon resin. Sections were cut with a Leica Ultracut UCT Ultramicrotome (Leica Microsystems, Bannockburn, IL) and viewed on a Tecnai T20 electron microscope (FEI Company, Hillsboro, OR) with a 4k x 4k UltraScan CCD camera (Gatan Inc., Pleasanton, CA).

#### **5.1.19. SUB knockout hamster infections.**

Hamsters were infected intraperitoneally with  $10^9$  day 4 SUB knockout or wildtype *L. donovani* promastigotes (groups of three) (Wyllie and Fairlamb 2006). Animals were weighed weekly over the length of the experiment. Hamsters were culled 200 days post-infection by CO<sub>2</sub> inhalation followed by thoracotomy. Pieces of each spleen and liver were fixed in 10% formalin in PBS and then embedded in paraffin for histology. Sections were cut at 5  $\mu$ m and stained with either Wright-Giemsa or hematoxylin and eosin by the UCSF Morphology Core using standard protocols. Psammoma bodies were identified by microscopy and counted.

#### **5.1.20. SUB two-dimensional gel electrophoresis.**

Three experimental replicates were prepared from separately cultured samples of both wildtype and SUB knockout *L. donovani*. Approximately  $10^9$  cells were pelleted, washed 3x with PBS, and stored at -80° C. Lysates were prepared by resuspending the cell pellets in 2 mL of native lysis buffer containing 20 mM HEPES pH 7.5, 250 mM sucrose, 3 mM MgCl<sub>2</sub>, 0.5% NP40, 1 mM DTT, and 1x Halt EDTA-free protease inhibitor cocktail (Pierce, Rockford, IL). Cells were then broken by mechanical lysis using 70 strokes of a Dounce homogenizer. The lysates were centrifuged at 12,000 g for 20 min at 4° C and the clarified supernatants were dialyzed overnight against 50 mM Tris-HCl pH 7.5, 100 mM NaCl using 8 kDa MWCO dialysis membranes. The following day the protein samples were concentrated and washed by precipitation using the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Approximately



300 µg of protein per gel was brought up to 300 µL in Bio-Rad Rehydration/Sample buffer and was passively loaded onto 17 cm, 3-10 pH IPG IEF strips. Isoelectric focusing was performed using slow increases in voltage over multiple steps up to 10 kV for a total of 60-65 kVh focusing time. Next, the strips were reduced and alkylated using sequential 10 min incubations in 2% DTT then 2.5% iodoacetamide and dissolved in Sample Equilibration Buffer. The IEF strips were run in the second dimension on 17 x 17 cm, 12.5% acrylamide tris-glycine, SDS PAGE gels. Gels were stained with SYPRO Ruby and imaged using a Typhoon Trio Variable Mode Imager (GE Healthcare). These images were utilized for spot intensity analysis using Bio-Rad PDQuest software (v. 7.4). For proteomic analysis, gels were silver stained (Shevchenko, Wilm et al. 1996), and selected protein spots were excised and in gel digested with trypsin (Rosenfeld, Capdevielle et al. 1992; Hellman, Wernstedt et al. 1995). The resulting peptides were extracted and analyzed by on-line liquid chromatography/mass spectrometry using an Eksigent nanoflow pump (Dublin, CA) coupled to a QStar Pulsar quadrupole-orthogonal-acceleration-time-of-flight hybrid instrument (Applied Biosystems, Foster City, CA). The reversed-phase chromatographic column was controlled with a Famos autoinjector (Sunnyvale, CA) and Eksigent software to run at a 5-50% acetonitrile gradient in 0.1% formic acid with a 350 nL/min flow rate. Data were analyzed in Analyst 2.0 software (Applied Biosystems) with the Mascot script 1.6b20 (Matrix Science, London, UK). Analyst processing options for peak finding in spectrum were 0.5% default threshold, 400 Gaussian filter, and a Gaussian filter limit of 10; for TOF auto-centroiding: 20 ppm merge distance, 10 ppm minimum width, 50% percentage height, and 100 ppm maximum width. Default parameters were used except that “no deisotoping” was selected and

precursor mass tolerance for grouping was set to 0.2. Database searches were performed using ProteinProspector v. 5.3.0 (<http://prospector.ucsf.edu>) using the Batch-Tag and Search Compare modules (Chalkley, Baker et al. 2008). Searches were performed on the SwissProt databank (December 16, 2008) to evaluate sample purity followed by searching TriTrypDB v. 1.0 beta (<http://www.tritrypdb.org>, downloaded January, 2009).

#### **5.1.21. Peroxide sensitivity assay.**

To analyze the growth inhibitory effect of hydroperoxides on wildtype and SUB-deficient *Leishmania* a peroxide sensitivity assay was performed as described by Castro *et al.* (Castro, Sousa et al. 2002). Promastigotes were split to  $10^6$  cells/mL in 2 mL M199 in a 24-well tissue culture plate. To each well *tert*-butylhydroperoxide (*t*-bOOH, Sigma, USA) was added with final concentrations of 50, 40, 30, 20, 10, or 0  $\mu$ M. After four to five days, parasites densities were determined. Relative densities were calculated by dividing the culture densities by the corresponding wildtype culture density.

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**Table 5.1: Primers used for *L. donovani* SUB Sequencing.**

The sequence of *L. donovani* SUB was determined by cloning the gene from genomic DNA. The gene was then sequenced using the following primers. Primers were designed that were 17 bp in length with a 3' G or C. For complete coverage, with at least 2x overlap in sequencing, 23 primers were used.

**Primers Used for *L. donovani* SUB Sequencing**

1. 5'-CAT GCA TCA GCC GGT AC-3'
  2. 5'-GCG GCA TGG TCA TCT AC-3'
  3. 5'-TAC TCA CAA TCT CTA CG-3'
  4. 5'-CAC CAG TAA GAG TGC GG-3'
  5. 5'-GAA GAG CCG CCA CCG TG-3'
  6. 5'-CGT GCT GGC AGG ACA GC-3'
  7. 5'-TCC TCT TTG AGG GTG CG-3'
  8. 5'-TAG CCA TAG CCC ACC GC-3'
  9. 5'-CTC CTC TTT GAG GGT GC-3'
  10. 5'-CGC TCT GTC TCG AGG CG-3'
  11. 5'-GTG TGG GGC AGC GGC AG-3'
  12. 5'-ACC GTT GGC TGT CAG AG-3'
  13. 5'-CGT TAG GAG ACG CCG CA-3'
  14. 5'-CGT CGT CAG CAC AAG AG-3'
  15. 5'-ACC CAC CTT CCG CTT CG-3'
  16. 5'-GTG CCA GCA GAC CAC GG-3'
  17. 5'-TAG GCA GCG GTG CCG AC-3'
  18. 5'-AAC GGC AGC AGG CTC TC-3'
  19. 5'-GTC GGC ACC GCT GCC TA-3'
  20. 5'-ATC GGC TAT AGG ATT CC-3'
  21. 5'-CAG TAG CCC GCA GGT GC-3'
  22. 5'-CGT TGT CTG TGC CGA CC-3'
  23. 5'-ACT GAT CAG CCA AGG CG-3.'
-

**Table 5.2: Sequences used for generating the subtilisin phylogenetic tree.**

The *L. donovani* SUB sequence was used to BLAST the GenBank protein database and the GeneDB protein database. Proteins from related species, model organisms, and those that had high degrees of similarity were chosen. In addition, prototypical Clan SB, family S8 proteases were also included. The proteins listed for the *Leishmania* and *Trypanosoma* species are from GeneDB. The other sequences are from GenBank.

**Sequences Used for Generating the Subtilisin Phylogenetic Tree**

Species:	Accession/ID:	Protein:
<i>Leishmania infantum</i>	LinJ13_V3.0940	Subtilisin-like serine peptidase
<i>Leishmania major</i>	LmjF13.1040	Subtilisin-like serine peptidase
<i>Leishmania braziliensis</i>	LbrM13_V2.0860	Subtilisin-like serine peptidase
<i>Trypanosoma cruzi</i>	Tc00.1047053511045.40	Subtilisin-like serine peptidase, SUB1
<i>Trypanosoma cruzi</i>	Tc00.1047053511859.60	Subtilisin-like serine peptidase, SUB2
<i>Trypanosoma brucei</i>	Tb11.02.1280	Subtilisin-like serine peptidase, SUB1
<i>Trypanosoma brucei</i>	Tb927.3.4230	Subtilisin-like serine peptidase, SUB2
<i>Plasmodium falciparum</i>	CAD51440	Subtilisin-like protease 1, SUB1
<i>Plasmodium falciparum</i>	XP_001348051	Subtilisin-like protease 2, SUB2
<i>Plasmodium falciparum</i>	CAD51437	Serine protease 3, SUB3
<i>Toxoplasma gondii</i>	XP_002370002	Subtilisin-like serine protease, SUB1a
<i>Toxoplasma gondii</i>	XP_002368971	Subtilisin-like protease, SUB1b
<i>Toxoplasma gondii</i>	XP_002364650	Subtilisin-like protease, SUB2
<i>Bacillus licheniformis</i>	P00780	Subtilisin Carlsberg, SUBA
<i>Bacillus amyloliquefaciens</i>	P00782	Subtilisin BPN', SUBB
<i>Bacillus subtilis</i>	P04189	Subtilisin E, SUBE
<i>Homo sapiens</i>	P09958	Furin
<i>Mus musculus</i>	P23188	Furin
<i>Saccharomyces cerevisiae</i>	P13134	kexin
<i>Schizosaccharomyces pombe</i>	Q09175	kexin
<i>Ciona intestinalis</i>	XP_002122807	Membrane-bound transcription factor peptidase, Site 1
<i>Apis mellifera</i>	XP_395754	Membrane-bound transcription factor peptidase, Site 1
<i>Xenopus laevis</i>	NP_001087381	MGC80830 protein, Site 1
<i>Homo sapiens</i>	EAW95506	Membrane-bound transcription factor peptidase, Site 1
<i>Danio rerio</i>	CAK04389	Membrane-bound transcription factor protease, Site 1

**Table 5.3: Primers used for subtilisin gene targeting.**

Targeting flanks were generated by PCR amplification of the untranslated regions directly 5' of the *SUB* ORF and 3' of the *SUB* catalytic core (*L. donovani*) or 3' of the ORF (*L. major*). PCR primers were designed based on the published *L. infantum* and *L. major* sequences (GeneDB). The 5' Targeting Flanks were designed to be 1.3-1.5 kb in length, while the 3' Targeting Flanks were designed to be 0.7-1.0 kb in length. SpeI sites, in bold, and XbaI sites, underlined, were included for cloning. The 5' end of each primer contains an extra CTC sequence to aid in restriction digestion reactions.

**Primers Used for Subtilisin Gene Targeting**

*L. donovani* 5' Targeting Flank

Forward: 5'-CTC **ACT AGT** CGC CTC CTC GTC GTC GCA CTC-3'

Reverse: 5'-CTC TCT AGA CAC CAC TAC CTC AAT CGG AGC G-3'

1.3 kb fragment

*L. donovani* 3' Targeting Flank

Forward: 5'-CTC **ACT AGT** TGG TCA TCT ACG TCC GCT GTA GC-3'

Reverse: 5'-CTC TCT AGA CGT GCC CTG ATC TGC GGC AGC-3'

0.7 kb fragment

*L. major* 5' Targeting Flank

Forward: 5'-CTC **ACT AGT** TGC GCA ACC ACA GCG GTC ATC-3'

Reverse: 5'-CTC TCT AGA TAC CTC AAT GGG AGC GTG CTT G-3'

1.5 kb fragment

*L. major* 3' Targeting Flank

Forward: 5'-CTC **ACT AGT** TCG TTG GAG AGG CCA ACG CGC-3'

Reverse: 5'-CTC TCT AGA CGA GTA GGA AGA GGT GAC CGT C-3'

0.8 kb fragment

**Chapter Six:**  
**Abbreviations and References**

## 6.1. Abbreviations Used

OPB: oligopeptidase B

SUB: subtilisin

DAPI: 4,6-diamidino-2-phenylindole

DMSO: dimethyl sulfoxide

AMC: 7-amino-4-methylcoumarin

ACC: 7-amino-4-carbamoyl-methylcoumarin

Z: benzyloxycarbonyl

PS-SCL: positional scanning synthetic combinatorial library

AEBSF: 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride

PMSF: phenylmethanesulphonyl fluoride

PPACK: biotin-X-D-Phe-Pro-Arg-CMK

TLCK: N-p-tosyl-L-lysine chloromethyl ketone

TPCK: N-tosyl-L-phenylalanine chloromethyl ketone

ShPI-I: Kunitz-type inhibitor from the sea anemone *Stichodactyla helianthus*

SBTI: soybean trypsin inhibitor

SAM: Statistical Analysis of Microarray

DAVID: Database for Annotation Visualization and Integrated Discovery

MEEBO: Mouse Exonic Evidence-Based Oligonucleotide

GeneDB: The Sanger Institute Pathogen Sequencing Unit's Genome Database

Site-1: Membrane-bound transcription factor peptidase

SREBP: sterol regulatory element binding protein

NADPH: reduced nicotinamide adenine dinucleotide phosphate



TR: trypanothione reductase

TS<sub>2</sub> & T(SH)<sub>2</sub>: oxidized and reduced trypanothione

TXN: tryparedoxin

TXNPx: tryparedoxin peroxidase

Prx: peroxidoxin

*t*-bOOH: *tert*-butylhydroperoxide

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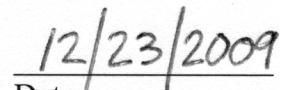
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