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Lana Hoertz

Salve Regina University, lana.hoertz@salve.edu

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Further characterization of a secreted lipase from the human pathogen *Leishmania donovani* by determining the effect of various metal ions on its enzymatic activity

Lana C. Hoertz
Alison M. Shakarian, PhD

BIO471 Dr. Zuccarelli
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Salve Regina University
Department of Biology and Biomedical Sciences
Abstract

*Leishmania donovani*, a protozoan parasite, is the causative agent of the often fatal disease visceral leishmaniasis. The current treatments available are minimal and toxic to the patient. It has been shown that these organisms exhibit lipolytic activity during their growth *in vitro*. Lipases are enzymes that are known to aid in the development and virulence of several pathogenic organisms such as *Candida albicans* and *Staphylococcus warneri*. Little information is known, however, about the role of lipases in *Leishmania* species. We hypothesize that lipase may play a part in *Leishmania*’s ability to survive within the human host as well as its pathogenesis. In past studies, the *L. donovani* secretory lipase gene (*LdLip3*) was episomally expressed with an HA tag by transfected promastigotes. The purified protein was tested for enzyme activity by performing assays with McIlvaine’s buffer pH 4-8 at 26°C, 37°C, and 42°C using 4MU-palmitate as a substrate. The results showed that there was an average thirtyfold increase in specific activity when comparing the purified protein to the unpurified supernatant samples. Furthermore, metal ions are known to be cofactors of a variety of enzymes, greatly affecting their activity, hence, in the current study a panel of metal ions was tested to determine their effect on the activity of purified LdLip3. Optimal conditions for this enzyme were established at pH 8, 42°C, with the addition of Zn^{2+}, whereas the addition of Mn^{2+} consistently produced a strong inhibitory effect. We are currently in the process of validating this previously collected data via the same experimentation protocol to determine reproducibility. Chelating agent studies with EDTA, also underway, will determine if the effect of metal ions on the enzyme activity could be reversed. Thus far, EDTA has countered the addition of the cofactors. Future studies include inhibitory studies with organophosphates that have been shown to inhibit lipases, in the hope to develop new drug targets for this parasite.
Introduction

*Leishmania donovani* is a unicellular parasite that takes up residence in its host’s macrophages causing visceral damage. Like all other eukaryotes, *Leishmania* shares the same basic morphological features. The pathogen has a nucleus, which stores DNA, and compartmentalized organelles. Its genome is rich in guanine-cytosine base pairs and contains no introns, an uncommon aspect in the Eukaryota domain. Also within the cell, unique to the class Kinetoplastida, is a kinetoplast, a network of mini- and maxicircular DNAs inside a large mitochondrion which encode the substrates for RNA editing. This is the organism’s only mitochondrion (Roy *et al* 2008). This kDNA system most likely evolved from a highly supercoiled plasmid “harbored within the mitochondrion of an ancient flagellate” (Lukes *et al* 2002). Additionally, leishmanial species possess a lipophosphoglycan coat that sheaths the entire cell’s outside surface, which is involved in modulating the host’s immune response, promoting the parasite’s survival (Turco and Descoteaux 1992). This layer atop the surface membrane is a mechanism used by a number of other parasites such as *Trypanosoma brucei* and *Plasmodium falciparum*.

*Leishmania donovani* primarily causes visceral leishmaniasis, also known as kala-azar, black fever, or dumdum fever, which is classified as the most severe form of leishmaniasis. For those people who do not harbor a silent infection, the patient clinically presents with symptoms including, but not limited to, fever, hepatosplenomegaly, fatigue, and leukopenia. Fatal if left untreated, the infection is evident from a distended abdomen, caused by the enlargement of the liver and spleen. This swelling is due to an acute infection of these major organs as well as the bone marrow, ultimately destroying the patient’s immune system. The disease has a mortality rate as high as 100% within just two years (“Leishmaniasis”). Other forms of leishmaniasis
consist of wet cutaneous lesions, sometimes resembling leprosy, on the skin or within the host’s mucous membranes. In these forms, only the macrophages within the reticuloendothelial system are infected. Not typically fatal, yet still horrific to the patient, the cutaneous forms heal within a few months leaving scars.

According to Dr. Robert Killick-Kendrick with the World Health Organization (WHO), as of 2010, there are on average 500,000 new cases of visceral leishmaniasis worldwide each year and of these, ten-percent of them result in death. These numbers are not known with certainty however. He continues by explaining that “leishmaniasis is not a notified disease in many countries and therefore these figures are underestimates,” (Kendrick 2010). The devastating disease has a parasitic fatality rate that comes in second only to malaria (Chappuis et al 2007) and the future holds no reassurance. Both incidence and mortality rates have steadily increased in the past five years and the parasite threatens new areas as it spreads across Middle Eastern and South American regions, exposing more people to its severity (Bhattarai et al 2010). This dispersion could possibly be due to climate change and its effect on the sandfly vector (González et al 2010). A more recent study conducted by the WHO Leishmaniasis Control Team suggests that this neglected tropical disease remains just as prevalent today, if not more so, and remains a deep concern for people in endemic countries (Alvar et al 2012).

Visceral leishmaniasis is found in both Old World (eastern hemisphere) and New World (western hemisphere) countries, where climates are mild to temperate. Tropical and subtropical regions, as well as in southern Europe along the Mediterranean are hotspots for the disease. Its epidemiology is far reaching, existing in a total of ninety eight countries across almost every continent, being endemic in highly populated, yet underdeveloped nations such as Sudan, India, Iraq, Brazil, and Bangladesh (“Parasites-Leishmaniasis”). Current numbers put more than
twenty five-percent of the entire world’s population at risk for contracting visceral leishmaniasis (Alvar et al 2012). Furthermore, United States soldiers are being deployed to places considered high threat areas to fight during military Operations Iraqi and Enduring Freedom. Over a two year span, four U.S. soldiers returned from tours in Iraq and Afghanistan, infected with visceral leishmaniasis, while over 200,000 soldiers came back infected with cutaneous leishmaniasis (Myles et al 2007; Pehoushek et al 2004).

*Leishmania donovani* is transmitted to humans and other mammalian reservoir hosts, such as canines and rodents, via an insect vector. The bite of an infected gravid female sandfly will deposit the parasite into the definitive host’s bloodstream. *Phlebotomus* sandfly species typically transport the parasite in the Old World and *Lutzomyia* sandflies are the carriers in the New World (Lewis 1971). *Leishmania* species have two main phases in their life cycle, each characterized by distinct morphological and perhaps physiological differences. To begin, the organism starts as a flagellated, extracellular promastigote found within the midgut of its intermediate host, the sandfly. Promastigotes are teardrop shaped and their flagellum is located at the anterior end of the cell body. When the sandfly takes a blood meal, the metacyclic, or infectious, parasites thriving in the foregut pass through the insect’s proboscis and are injected into the new host’s circulatory system. Host macrophages then phagocytize the foreign free swimming promastigotes. The ingested parasites transform into the amastigote stage within the phagolysosome. Amastigotes are circular, non-flagellated obligate intracellular cell bodies and are among the smallest known eukaryotic cells. They continuously multiply via mitosis until the actual macrophage bursts, releasing thousands of parasites back into the bloodstream where they subsequently find refuge in various other tissues. The cycle repeats itself when another sandfly feeds on the infected host, taking up the amastigotes, where they again transform into the
promastigote stage. Amastigotes are the causative agents of disease. Throughout the entire *Leishmania* genus the same dichotomic life cycle is observed (Shakarian *et al* 2010).

Treating visceral leishmaniasis poses a twofold problem. *Leishmania* cells hide and thrive within the host’s immune system, specifically the phagolysosome, in conditions that were meant to destroy them. This makes targeting the actual parasite, rather the symptoms, difficult. Secondly, due to the parasite’s intracellular nature for the majority of its life cycle, killing it results in the destruction of the host’s cells as well, both normal and infected, making drugs that successfully combat the disease extremely toxic.

Diagnosis of visceral leishmaniasis should be absolutely certain before treatment begins to ensure no unnecessary damage to the patient. Also, every case should be taken on an individualized basis where factors such as species and location of contraction are noted. The first line of treatment for the disease is pentavalent antimonial (Sb\(^V\)) compounds. These drugs have been the staple for leishmaniasis treatment since the 1940s, but are not licensed for commercial use in the U.S., rather are strictly available through the Centers for Disease Control and Prevention (CDC). Sodium stibogluconate (Pentostam\(^\text{®}\)) can be delivered intravenously or intramuscularly at a high volume per dose, 20 mg Sb/kg/day for about twenty days. The drug works by reducing fructose diphosphate and inhibiting glycolysis (Frezard *et al* 2009). Sodium antimony gluconate (SAG) is another common drug used to treat visceral leishmaniasis. For twenty-eight days 20 mg/kg is pumped throughout the body (Abdo *et al* 2003). The mechanism of action for SAG involves the induction of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation. This is imperative information recently discovered by Basu *et al* (2006) which “has provided the first evidence that SAG treatment induces activation of some important components of the intracellular signaling
pathway.” Another drug used is meglumine antimoniate (Glucantime®). Some of the side effects for antimony containing drugs include arthralgia, myalgia, pancreatitis, acute renal failure, and cardiotoxicity. Relatively effective, these antimonial compounds are the first drugs given to infected patients.

Recently, however, traditional chemotherapies are not working due to drug resistance. *Leishmania donovani* isolates have increasingly shown resistance to SAG and as of 2003 it was estimated that ten-percent, a rising statistic, of all *Leishmania* parasites are resistant to this treatment (*Sundar et al* 2000; *Abdo et al*). Resistance has skyrocketed in India; rates of resistance as high as sixty-percent have been seen in Bihar (*Thakur et al* 2004). In fact, antimony resistance is so common in India today that patients do not even receive this initial treatment line.

This rapid appearing non-responsiveness to antimonial compounds has pushed for the discovery of a second line of treatment, amphotericin B (ampB), an antiprotozoal medication available in intravenous, liposomal preparations (*Kumar et al* 2009). Considered a drug of last resort due to its greater toxicity and potency, ampB can be given to patients at recommended dosages no higher than 10 mg/kg over several days (*Kafetzisa et al* 2005), although there are a variety of different treatment regiments. Its side effects are severe and potentially lethal, including high fever, hypertension, anorexia, dyspnea and tachypnea, and nephrotoxicity (*Lahiado-Laborin et al* 2009). Initial doses should be low and increase every day to prevent a serious acute reaction to the compound. According to a recent study, the effect of ampB treatment is due to sterol complexation, both with ergosterol of the *Leishmania* membrane and cholesterol of host macrophages, inhibiting the binding and entry of promastigotes (*Paila et al* 2010). Resistance to ampB has yet to be detected in wild type parasites, however it has emerged
in *L. donovani* clinical isolates in the laboratory (Purkait *et al* 2012). The mechanism by which these parasites are producing resistant strains so quickly is unclear and could stem from several factors.

There is a third alternate treatment for visceral leishmaniasis approved for use in many endemic countries such as Brazil, India, Pakistan, and Afghanistan, called miltefosine. The phospholipid drug acts as an Akt (protein kinase B) inhibitor and was named by a 2005 survey the most effective oral treatment for both forms of leishmaniasis (Dorlo *et al* 2012; Berman). “Up to date, more than 2500 patients have been treated with miltefosine in India obtaining cure rates over 91% when a dose of 2.5 mg/kg/day during twenty-eight days was prescribed,” (Soto *et al* 2006), but the long-term safety of the drug is still in question; sufficient evidence is lacking in terms of its pharmacology and toxicology.

Due to minimal treatment options, *Leishmania* research is continually performed, especially in regards to genomics and proteomics. The pathogenicity of *Leishmania donovani* has yet to be attributed to a specific factor. However, it is known that these parasites secrete a lipase (LdLip3) that may contribute to its survival within the human host, development, or virulence. Infectious organisms such as *Mycobacterium tuberculosis*, *Candida albicans*, and *Staphylococcus warneri* all produce lipases that aid in their pathogenicity or survival (Cotes *et al* 2008; Schaller *et al* 2005; van Kampen *et al* 2001), thus we suspect that LdLip3 may mimic these lipases in their functions. It is hypothesized that LdLip3 may disrupt cell signaling, play a role in the catabolism of lipids via $\beta$-oxidation for nutrients since they are lipid scavengers, or directly destroy host tissues. Shakarian *et al* (2010) proved that LdLip3 mRNA is constitutively expressed by both the promastigote and amastigote. Lipases are enzymes which hydrolyze, or
cleave, the ester bonds in fats releasing glycerol and free fatty acid chains. Metal ions often are cofactors for enzymes like LdLip3, increasing their activity.

LdLip3 appears to be vitally important, still its particular function has not been found. The structure of LdLip3 has also yet to be assembled. Such information can lend great aid to discovering its role in *Leishmania*’s life cycle. Over the past several years, we have managed to characterize LdLip3 by identifying the protein’s optimal conditions, purifying the enzyme, and showing that the addition of metal ions greatly effects enzymatic activity, suggesting possible cofactors. Information about the protein will eventually lead to potential drug targets and better methods in diagnosing, treating, and preventing visceral leishmaniasis.

**Materials and Methods**

LdLip3 Activity with the Addition of Various Salts

The leishmanial vector pKS NEO is a plasmid, or a circular piece of DNA, which has been previously used a multitude of times to express genes associated with different *Leishmania* species (Charest *et al* 1996; Debrabant *et al* 2000; Shakarian *et al* 2010). In order to study and characterize LdLip3 it has to be specifically selected for and isolated. This is done by first genetically modifying pKS NEO to include both the lipase gene (*LdLip3*) and also a human influenza hemagglutinin (*HA*) tag. An *HA* tag codes for a glycoprotein that is generally utilized as an epitope tag in expression vectors, as we demonstrate here, facilitating the detection, isolation, and purification of recombinant proteins. Once *LdLip3::HA* was ligated into pKS NEO by Shakarian *et al* (2010), the plasmid was then transfected into wild type *L. donovani* promastigotes. The new transfectants were subsequently cultured in chemically defined M199+ medium with ten-percent fetal bovine serum and 100 µg/mL of the selective agent G418.
(Geneticin). G418 is an antibiotic that blocks polypeptide synthesis in eukaryotic cells (Eustice and Wilhelm 1984). Resistance is conferred by the NEO gene, hence its inclusion within pKSNEO. The cells that took up and contain the plasmid will be resistant to the dissolved G418 and be able to grow in its presence, while the ones that do not will die. Growth indicates that those cells can overexpress the LdLip3 gene. Another set of cells were transfected with a non-chimeric pKSNEO, which acted as a control since these cells would not overexpress LdLip3. The cultures were maintained at 26°C in ventilated culture flasks and passaged weekly.

When the parasitic cultures were mid-log phase (about 4.7x10^7 cells/mL) the supernatants were harvested by centrifugation at 2400×g and 4°C for fifteen minutes. These samples were then used for experimentation. The supernatant, or the surrounding media, is where the lipase is located because its encoding gene contains a signal sequence that directs the protein into the cell’s secretory pathway, thus LdLip3 is found outside the cell.

4-Methylumbelliferone (4-MU) is a fluorescent molecule which provides an effective and accurate method in measuring enzymatic activity in biological assays. Known concentrations of 4-MU dissolved in dimethylformamide (DMF) were used to generate a standard curve so as to compare the actual samples. The lipid substrate used was 4-MU palmitate, a sixteen-carbon chained fatty acid. The 4-MU in this molecule replaces the glycerol portion of the fat and when cleaved by a lipase, 4-MU fluoresces. From this, fluorescence is directly proportional to the amount of enzymatic activity there is in a sample. In earlier studies performed by Ganim et al (2011), more enzymatic activity was apparent with longer chained substrates, hence the use of 4-MU palmitate in the detection of lipolytic activity.

To ensure that LdLip3 is the only esterase acting on 4-MU palmitate, it was necessary to purify the original crude supernatant samples, which consist of a mixture of proteins. Anti-HA
protein G affinity matrix columns from Roche were used to purify LdLip3::HA. The HA tag initially attached to LdLip3 and ligated into the plasmid is probed for by the antibody in the affinity resin which identifies the amino acid sequence YPYDVPDYA ("Anti-HA Affinity Matrix from rat IgG_1"). This sequence was recognized in our fusion protein. The supernatants from both sets of transfectants, pKSNEO and pKSNEO::LdLip3::HA, were run on the columns. The columns were first equilibrated and then loaded with the 1x supernatants. The purified samples were collected in three separate elution fractions which were later pooled together for use in the enzyme activity assays.

The eluted samples were subjected to a bicinchoninic acid (BCA) assay to determine purified protein concentrations. After incubation for thirty minutes at 37°C, a SPECTRAmax M2 spectrophotometer was used to read a clear ninety-six well flat bottom microplate to measure the amount of protein (mg) in each sample by comparing the raw data to a standard curve constructed by known BSA protein standards. Visually, the total protein concentration is exhibited by a color change from green to purple. In the BCA assay, the peptide bonds within the purified LdLip3 reduce Cu^{2+} ions to Cu^{+}. Next, bicinchoninic acid chelates each Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562 nm (Wiechelman et al 1988).

Enzymatic activity assays were prepared with 89% McIlvaine’s buffer pH 4-8, 5% purified sample, 3% 4-MU palmitate, and 3% 1M salt solution. A sample containing only the purified LdLip3, without the addition of any salt, was also loaded on each microplate to establish the percent difference in lipase activity when comparing it with the samples with salt. It has been shown that metal ions often act as cofactors for enzymes, increasing their activity (Kumar et al 2005). Here, we test an entire panel of ions, including Zn^{2+}, K^{+}, Na^{+}, Cu^{2+}, Ca^{2+}, Mg^{2+},
Co$^{2+}$, Fe$^{2+}$, and Mn$^{2+}$, to assess their effect on LdLip3. Analyzing a variety of metal ions will yield more information on the enzyme’s preferable conditions. The *Leishmania* cell is constantly encountering significantly different pHs during its life cycle, therefore a range of pHs must be tested to discover LdLip3’s optimal pH where it functions at $V_{\text{max}}$. The unit of $V_{\text{max}}$ is defined as the maximum initial velocity or rate of a catalytic reaction, or in other words, the rate at which an enzyme is operating most efficiently. Sandflies have an acidic thoracic midgut at a pH of 6.8, while the human bloodstream is fairly neutral at pH 7.4 (Nestor Añez *et al* 2003; Waugh 22). The pH in the phagolysosomal system is much lower at 5.7 (Cech and Lehrer 1984). Assays were incubated for thirty minutes at one of three biologically relevant temperatures. The temperature within the midgut of the sandfly is around 26°C. Normal human body temperature is 37°C and the feverish human body temperature is 42°C. Following incubations, stop buffers (1M NaOH/1M glycine, and 0.75M NaOH/1M glycine) were added to the wells in order to terminate the reactions. A SPECTRAmax M2 fluorometer read the black ninety-six well flat bottom microplates at emission and excitation wavelengths 365 nm and 460 nm, respectively, to determine the amount of lipase activity (nmol) in each well. The samples were assayed in quadruplicate for reproducibility measurements. A 4-MU standard curve was formed with standards at increasing concentrations from 0.25µM to 500µM and was used to deduce the amount of fluorescent product present. Results from the BCA assay and salted activity assays were used to calculate the specific activity (pmol/min/mg) of LdLip3 along with the percent difference in net lipase activity.
EDTA as a Chelating Agent

To ensure the activity changes observed with LdLip3 were in fact due to the addition of various metal ions, EDTA, a chelating agent, was added to the samples. Chelators bind metal atoms to ligands within the compound to form a heterocyclic ring containing the metal atom, thus the metal ions are unable to interact with the enzyme. EDTA has previously been used to negate the effects of the metal ions in other studies (Park et al. 2008). Activity assays were prepared as previously described with the addition of salt and 1M EDTA. Specific activity was again calculated and compared to the activity of purified LdLip3.

Results

A BCA assay was performed before the actual activity assays, but its results were later combined with the data collected from said activity assays in order to generate the specific activity of both the 1x and purified protein. Results indicated an average thirty fold increase in activity in comparing the purified samples to the 1x supernatants as displayed below (Figure 1). This demonstrates that even though there is less protein present in each sample, purified lipase produces more activity than unpurified lipase.
Figure 1. Specific Activity of 1x Supernatant in Comparison to Purified LdLip3

Comparing specific activity of 1x supernatants to the purified LdLip3 at three biologically relevant temperatures. Even though there is less protein, purified lipase produces more activity than unpurified lipase. There was an approximate thirty-fold increase in activity.

Results from initial assays without the addition of salt conducted by Ganim et al (2011) showed optimal conditions for peak LdLip3 activity with 4-MU palmitate at pH 5.0 after incubation at 26°C. From this information, 4-MU palmitate was verified as a good substrate to exploit in the continuation of the project; hence it was utilized in the succeeding salted enzymatic activity assays. Nine different metal ions (Zn$^{2+}$, K$^+$, Na$^+$, Cu$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Co$^{2+}$, Fe$^{2+}$, and Mn$^{2+}$) were added to the activity assays in the form of salt to determine whether there would be any activity change. Specific activity was calculated and then individually compared to the purified LdLip3 activity. Percent difference of net lipase activity was determined using a basic equation [(Pure LdLip3 with Salt − Pure LdLip3 without Salt) x 100]. Depending on the assay conditions, ZnSO$_4$, KCl, NaCl, MgCl$_2$, CoCl$_2$, FeSO$_4$, CuSO$_4$, and CaCl$_2$ all had variable effects on enzymatic activity whereas MnCl$_2$ consistently showed strong inhibitory effects for all three...
temperatures at every pH. Optimal conditions for LdLip3 were established at pH 8, 42°C, with the addition of Zn+2, when using 4-MU palmitate as the substrate, indicated by the black arrow (Figure 2). These results are in the process of being validated through the same procedures as outlined above. Thus far, data appears to be over eighty percent reproducible. A summary graph shows the data from every assay performed and further highlights which metal ions activated LdLip3 and which inhibited the enzyme (Figure 3).

**Figure 2. Percent Difference of LdLip3 Activity with the Addition of Various Metal Ions**

Percent difference in net LdLip3 activity with the addition of various metal ions when compared to LdLip3 assays without salts at pH 8. The baseline set at 0 represents 100% of purified LdLip3 activity. Assays were carried out with final salt concentrations of 1 mM at three different temperatures at different pHs. Peak activity was seen at 42°C with the addition of Zn^{2+} when using 4-MU palmitate as the substrate as indicated by the black arrow.
Figure 3. Summary of Percent Difference in Net LdLip3 Activity with Various Cofactors
Summary graph detailing the results for every assay performed, displaying peak enzyme activity for each pH, with the addition of various salts. Individual points indicate the temperature at which the peak activity was observed. Red specifies an activating effect while blue represents an inhibitory effect. Peak activity was seen at 42°C with the addition of Zn^{2+} when using 4-MU palmitate as the substrate as indicated by the red circle.
With the addition of EDTA, initial results from the salted activity assays were reversed. The original strong decline in enzymatic activity with the addition of Mn$^{2+}$ was lessened by about eight-percent when EDTA was added to the samples at pH 7. Although the chelating agent did not completely return the specific activity of LdLip3 with added Mn$^{2+}$ to the same value as the purified activity with no additional salts, it did mildly decrease the metal ion’s effect on the enzyme by about eight-percent, showing that metal ions are indeed affecting LdLip3 activity. Our chelator studies are still in progress, but thus far have been somewhat successful in reversing the effects of added metal ions.

![Figure 4. Activity Assays with the Addition of Mn$^{2+}$ and EDTA at pH 7](image)

EDTA appears to lessen the inhibitory effect of the manganese ion on LdLip3 by approximately eight-percent. Assay was carried out in the presence of a final 1mM EDTA concentration.
Discussion

As mentioned earlier, *Leishmania donovani* is a deadly pathogen which causes a dangerous disease that puts at risk over twenty five-percent of the entire world’s population. With this being said, it is imperative to be able to treat such a severe illness effectively and quickly at an affordable price, seeing that the majority of cases take place in under developed nations where medical supplies are limited. Unfortunately, however, as we’ve seen, current treatments are detrimental to the patient, costly, and already are experiencing parasite resistance. Hence, a quest to find new drug targets and develop novel medications is imminent. Since it has been previously proven that lipases can sometimes act as virulence factors in other pathogenic organisms, we hypothesize that LdLip3 may play a significant role in *Leishmania*’s life cycle either as a virulence factor, a means of nutrient breakdown, or a step in the cell signaling process. From this, it is necessary to collect more information on the essential enzyme. As of today, LdLip3’s actual function has yet to be fully determined. Shakarian *et al* (2010) speculates that the protein’s potential functions include “the acquisition of host resources for energy metabolism and as simple building blocks for the synthesis of complex parasite lipids important for membrane remodeling and structural purposes.” Thus, here we further characterize LdLip3 by establishing the enzyme’s optimal conditions after purification and identifying possible cofactors and in turn possible drug targets.

Optimal conditions for LdLip3 were found out by performing many assays of which provided a lot of information concerning the enzyme. From this data it was concluded that LdLip3 operates most efficiently under specific environmental conditions, at pH 8, 42°C, with the addition of Zn+2, using 4-MU palmitate as the substrate. These experimental circumstances are related to the natural conditions that exist within the bloodstream of a feverish human being.
with alkalosis, or basic blood. A study conducted by Verde et al (2011) discovered that in a group of fifty-five kala-azar patients 75.5% were described with having chronic mixed alkalosis. This corresponds to an individual who has had a parasitic infection for a year or longer and has spiked a fever in parallel the waves of parasitemia. People with visceral leishmaniasis usually become sick within months, or sometimes as long as years, of when they were initially bitten by an infected sandfly (“Parasites – Leishmaniasis”).

Given the resemblance of these conditions to the genuine biological conditions in Leishmania’s life cycle, there is serious evidence that suggests LdLip3 may in fact be a key enzyme in nutrient scavenging and salvaging for energy metabolism, specifically fatty acids. These fatty acids will be used for the parasite’s growth, attesting that LdLip3 should be targeted in drug development. Enzyme inhibition would be an ideal solution in deactivating LdLip3. The fact that Mn$^{2+}$ consistently decreased LdLip3 activity offers another direction that drug engineering could take. Instead of blocking the enzyme’s active site with an antagonist similar in structure to a fat through competitive inhibition, an allosteric inhibitor could be used to deactivate the enzyme. According to a recent study, LdLip3 inhibitors are in the process of being investigated. The molecule 3,4-dichloro-N-(3-nitrophenyl)benzenesulfonamide might be a non-covalent inhibitor based on the Structure Based Drug Design approach using the NCI diversity set and ZINC database (Parameswaran et al 2011).

Upon protein purification with Anti-HA affinity columns we were able to isolate the chimeric protein LdLip3::HA. Purified specific enzymatic activity increased thirtyfold over the unpurified 1x supernatant sample. This large increase in activity shows that we isolated a functional protein that retained enzymatic activity after being genetically modified and overexpressed. Furthermore, we proved that metal ions do have an influence on enzymatic
activity, under certain conditions, severely affecting the enzyme’s $V_{\text{max}}$. The addition of Zn$^{2+}$ increased LdLip3 activity by approximately twenty one-percent, demonstrating that it might be an important cofactor.

This research has yielded large amounts of information on the secretory enzyme LdLip3 and although we have yet to give the protein a definite function in *Leishmania donovani*’s life cycle, we do know that it is produced in great amounts, thus it must serve a critical purpose. We have accomplished many goals in investigating LdLip3, being successful in several biological techniques and learning the important of persistence. In the future, now that we know the optimal conditions for LdLip3, inhibitor studies with organophosphates that have been show to inhibit lipases (Quistad *et al* 2006; Quistad, Liang *et al* 2006) can be conducted using said conditions. The larger objective for this project was to characterize the activity of LdLip3 and contribute to the mission in developing new drugs in treating or preventing visceral leishmaniasis. We have triumphed in achieving this long-term goal and hopefully future information will arise concerning the enzyme’s function in the deadly human pathogen *Leishmania donovani*.

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