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Characterization of *Ld*LIP3 Enzyme Activity from *Leishmania donovani*

Christine Ganim[1], Jessica Barowski[1], Alison Shakarian[1,2]
[1]*Salve Regina University, Newport, RI, USA*
[2]*Advisor*
shakaria@salve.edu

ABSTRACT

Lipases catalyze the hydrolysis of fats to form glycerol and fatty acids. Secreted lipases have been implicated as virulence factors in some pathogens. Previously a gene encoding a secretory lipase from the human protozoan pathogen *Leishmania donovani* was identified and characterized however, experimental evidence to support the biological role of secreted lipases in *Leishmania sp*. remains to be elucidated. The goal of this project was to express the secretory lipase gene, *LdLIP3*, from *L. donovani* and characterize its lipolytic activity. Culture supernatants of *L. donovani* over-expressing the secretory LdLIP3 enzyme were used to characterize lipolytic activity at several different pHs and temperatures that are biologically relevant in the life cycle of the parasite. At 26°C the optimal lipolytic activity was determined to be pH5.0 with palmitate as the substrate. Similarly, at 37°C the optimal activity was at pH 6.5 with palmitate, and at 42°C, pH 6.0 using stearate as substrate. These results indicate that LdLIP3 has lypolytic activity that could be significant throughout the parasite life cycle. Characterization of the optimal lipolytic activity for the LdLIP3 enzyme should allow further analysis of the physical and biochemical properties of the secreted lipase and to define its possible role in the life cycle of *Leishmania sp*.

INTRODUCTION

There are several species of *Leishmania* which are known to infect humans (Courret et al 2002). *Leishmania donovani* causes the most lethal form of disease, visceral leishmaniasis, which is also known as kala azar (Bhattarai et al 2010). Although the mechanism of pathogenesis is not understood, visceral leishmaniasis is fatal if left untreated (Bhattarai et al 2010) as it attacks the liver, spleen, and bone marrow, and causes fever and weight loss (Saha et al 2007). Moreover, drugs such as pentavalent antimony compounds that are used in the treatment of visceral leishmaniasis are nephrotoxic (Shakarian et al 2002). *L. donovani* is endemic in many subtropical and tropical regions such as Africa, the Middle East, and India as these geographical areas are where the insect vector that transmits *L. donovani* is found (Chappuis et al 2007).

Leishmania donovani has two distinct life cycle stages, one in the human and the other in the *Phlebotomus sp*. sandfly vector (Courret et al 2002). The two major parasite developmental stages are: 1) extracellular flagellated promastigote forms that reside and multiply within the alimentary tract of their sandfly vector and 2) obligate intracellular nonflagellated amastigote forms which reside and multiply within the phago-lysosomes of infected human macrophages (Handman 2001). When found in the sandfly vector the parasite is at 26°C and presumably exposed to a range of pH between the midgut and the proboscis of the sandfly (Courret et al 2002). When the parasite is first in the human host, it is at a temperature of 37°C, but as the human becomes infected and spikes a fever, the temperature can reach 42°C (Courret et al

2002). When in the human host, the parasite can be found in the macrophages (pH 4.5-5.0) (Daleke 1990), and in the blood (pH 7.4) (Rosenthal 1948).

Lipases are ubiquitous enzymes, either secreted or membrane-bound, that cleave the ester bonds of fats to release free fatty acids and glycerol (Gupta et al 2003). Lipases are a type of esterase which display a high activity towards insoluble longer chain substrates (Fojan et al 2000). The role of lipases is not currently known in the biology of *Leishmania sp.*, but lipases, especially when extracellular, are a known virulence factor in many other organisms (Stehr et al 2003) such as *Candida albicans* where lipases induced cytotoxicity and contributed to the deposit of lipids in the cytoplasm of macrophages (Paraje et al 2008).

Leishmania are typically facultative lipid scavengers. As obligate parasites they must salvage these macromolecules from their hosts. For example, during their life cycle these organisms undergo changes in physiology and architectural membrane remodeling (Tetley et al 1986; Wassef et al 1985; Berman 1987). In addition, previous studies have shown that amastigotes have elevated fatty acid metabolism compared to promastigotes (McConville et al 2007; Rosenzweig et al 2008; Naderer and McConville 2008; Tielens and van Hellemond 2009; Opperdoes and Coombs 2007; Hart and Coombs 2007). While precedence exists for such biological activities (i.e. beta oxidation and membrane remodeling) to date, no reports exist concerning the involvement of parasite derived lipases in these processes. The focus of this study was to further characterize the enzymatic properties of the secreted lipase encoded by the *LdLIP3* gene from *L. donovani* by testing various temperature profiles, pH range with 4-Methylumbelliferone palmitate and 4-Methylumbelliferone stearate as fatty acid substrates.

METHODS

Lipase and Control Samples:

The leishmanial expression plasmid, *pKSNEO* (Zhang et al 1996), has been used to express a variety of genes in several species of *Leishmania* (Charest et al 1996; Shakarian et al 2010; Debrabant et al 2000). The *pKSNEO* vector previously transfected into *L. donovani* parasites by Shakarian et al (2010) were used as a background control for this study. To express the leishmanial lipase enzyme *pKSNEO* was modified by Shakarian et al (2010) to contain the *LdLIP3* gene and subsequently this plasmid construct (*pKSNEO::LdLIP3*) was transfected into *L. donovani* parasites (Shakarian et al 2010). The parasites bearing the *LdLIP3* expression construct were generously supplied by Shakarian et al (2010) and were used to characterize the secretory lipase activity encoded by the *LdLIP3* gene. For these studies, the cell-free culture supernatants of the two sample groups (*pKSNEO* and *pKSNEO::LdLIP3*) were harvested by centrifugation at 2100 X *g* at 4°C for 20 minutes and stored at -20°C until use.

Lipase Assay Stock Solutions:

4- Methylumbelliferone (4-MU) is a highly fluorescent molecule which provides a sensitive and convenient method of determining the activity of enzymes (Roberts 1985). In this study, a molecule of 4-MU is attached to fatty substrates of different chain lengths (i.e. palmitate, 16C chain length and stearate, 18C chain length) via an ester bond. In the presence of a lipase the ester bond is cleaved and the released 4-MU molecule will fluoresce. One benefit of using 4-MU is the ability of the fluorometer to detect very small amounts of fluorescent output, which correlates to the amount of lipolytic activity in the experiment (Roberts 1985).

A 20mM 4-MU stock solution was made in dimethylformamide (DMF) and stored at -20°C. This stock solution was diluted to make twelve "known" concentrations of 4-

MU standards which ranged from 0μM – 250μM. Standards were run on every 96-well plate experiment to generate independent 4-MU standard curves. Stock solutions of the substrates 4-MU palmitate (16C) and 4-MU stearate (18C) were made using DMF as a solvent at a concentration of 6mM and stored at -20°C.

At the end of the enzyme incubation period, stop buffers were added to the assays to bring the pH up to 10.5, the optimal fluorescent condition for 4-MU, before being read by the fluorometer (McCreath and Gooday 1992). Stop buffers consisted of 1.0M glycine/1.0M NaOH and 0.75M glycine/1.0M NaOH and were stored at room temperature. A mixture of the two stop buffers were added to each of the assay samples to a final pH of 10.5.

Lipase Assay Conditions:

McIlvaine's buffer stock solutions of 0.1M citric acid and 0.2M Na_2HPO_4 were used to produce nine buffers ranging from pH 4.0- 8.0 (Shakarian et al 2010). All buffers were filter sterilized and stored at room temperature.

Lipase activity assays were carried out in 96-well plates. Each assay plate tested one pH at one temperature with one substrate, and was set up according to Table 1.

	Blank	Positive Control	*pKSNEO* Sample	*pKSNEO* Negative	*LdLIP3* Sample	*LdLIP3* Negative	Standard Curve
Buffer	855 μL	823 μL	832 μL	832 μL	832 μL	832 μL	830 μL
Substrate	–	23 μL	23 μL	23 μL	23 μL	23 μL	–
Unconditioned Media	45 μL	45 μL	–	–	–	–	45 μL
Supernatant	–	–	45 μL *pKSNEO*	45 μL *pKSNEO* Boiled	45 μL *LdLIP3*	45 μL *LdLIP3* Boiled	–
4-MU Standard Concentation	–	–	–	–	–	–	25 μL
Lipase Control	–	9 μL	–	–	–	–	–

Table 1: Set up of the components of the lipase assays. Lipase assays were carried out in 96-well microtiter plates at three different temperatures (26°C, 37°C 42°C) with either 4MU palmitate or 4-MU stearate as substrates across a pH range of 4.0-8.0. Each plate contained a blank (background control) a positive control with a commercially available lipase (Sigma), a pKSNEO sample as a background lipase activity control, a boiled pKSNEO sample to serve as the negative control for this background sample, a LdLIP3 test sample and a boiled LdLIP3 as a negative control for the test sample. In addition several wells were used to generate the 4-MU standard curve.

Substrate either 150μM 4-MU palmitate (16C) or 4-MU stearate (18C) was added to the buffer. For each plate assayed, samples included: 1) supernatant from *pKSNEO:: LdLIP3* cultures (*LdLIP3)*; 2) *LdLIP3* cultures which were boiled to destroy any enzyme activity (*LdLIP3* negative); 3) *pKSNEO* control (*pKSNEO*) and 4) boiled *pKSNEO* (*pKSNEO* negative). Additionally, a commercially available lipase (from *Chromobacterium viscosum*, Sigma) was used as a positive control. Each plate also had a standard curve with various known concentrations of 4-MU as described above, and a blank consisting of buffer and unconditioned media. Quadruplicates of each sample were added to a black, flat bottomed, 96-well plate and incubated for 30 minutes at 26°C, 37°C, or 42°C. After incubation, the appropriate stop buffers were added and the fluorescence was read by a SPECTRAmax Gemini XS fluorometer, with SoftMax Pro Software (Molecular Devices). The plates were read at an excitement value of 365nm and an emission value of 460nm, and were blanked. The raw fluorescence units of the samples were compared to the standard curve to obtain the 4-MUμM concentration for the unknown samples.

Data Analysis:

The 4-MU concentration generated in the assay samples were converted from μM 4-MU released to pmol/min/mL of activity using the standard curve. To normalize the samples, the *LdLIP3* negative was subtracted from the *LdLIP3* sample. Similarly, the *pKSNEO* negative was subtracted from the *pKSNEO* sample. The net activity was then calculated by subtracting the net activity of *pKSNEO* from the net activity of *LdLIP3* [i.e. Net activity = (*LdLIP3- LdLIP3* negative) - (*pKSNEO* - *pKSNEO* negative)]. The results were

graphed by temperature and the standard deviation was calculated.

RESULTS

Lipases are a type of esterase which prefers long chain fatty acids as substrates. While lipases and especially secretory lipases are known virulence factors in other organisms (Stehr et al 2003), its role in the biology of *Leishmania sp*. is not currently understood. Previously, the gene *LdLIP3* was shown to encode a secretory lipase (Shakarian et al 2010*)*. In the current study we further characterized the activity of an episomally expressed secretory lipase, *pKSNEO:: LdLIP3*, from the human pathogen *Leishmania donovani* to determine the substrate preference and conditions for the optimal activity of this parasite derived enzyme.

In lipase assays carried out at 26°C (Figure 1) several biologically relevant pHs showed an overall net increase in lipolytic activity over controls when palmitate and/or stearate were used as substrates. For example a net increase in activity was observed with both palmitate (35 pmol/min/mL) and stearate (42 pmol/min/mL) at pH 4.5. Increased activity was seen at pH 5.0 (213 pmol/min/mL), pH 5.5 (83 pmol/min/mL), pH 6.0 (184 pmol/min/mL), pH 7.0 (67 pmol/min/mL), and pH 8.0 (106 pmol/min/mL) with palmitate and at pH 6.5 (50 pmol/min/mL) with stearate (Figure 1).

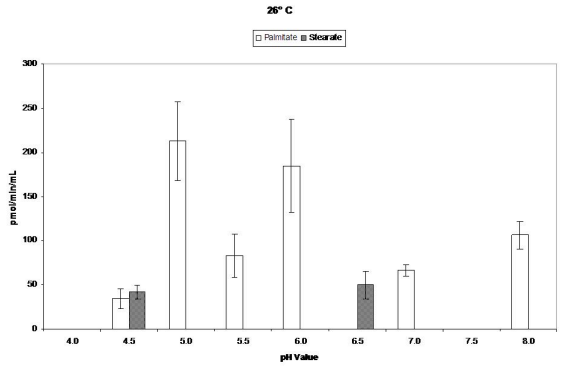

Figure 1: *LdLIP3* at 26°C with palmitate and stearate as substrate. Lipolytic activity assays of *LdLIP3* vs *pKSNEO* control were carried out at 26°C with Palmitate (open boxes) and Stearate (gray boxes) at various pH points. Standard calculations were used to determine the net change of activity (*LdLIP3* – *pKSNEO*) and is recorded as pmol/min/mL. Standard error bars are indicated by solid black lines (n=10).

Similarly a net increase of lipolytic activity was observed with several pHs at 37°C (Figure 2). For example, an increase in activity was observed in assays that used palmitate as substrate at pH 6.5 (114 pmol/min/mL) and pH 7.5 (18 pmol/min/mL). Increased activity was also seen with stearate at pH 5.0 (57 pmol/min/mL) and at pH 7.0 (14 pmol/min/mL).

A net increase in lipolitic activity at 42°C (Figure 3) was seen at pH 4.0 (97 pmol/min/mL), pH 4.5 (133 pmol/min/mL), and pH 5.5 (103 pmol/min/mL) when palmitate was used as substrate. Palmitate and stearate both showed an increase in activity at several pHs tested at this temperature. For example, pH 5.0 with palmitate (34 pmol/min/mL) and stearate (51 pmol/min/mL), at pH 6.0 with palmitate (14 pmol/min/mL) and stearate (64 pmol/min/mL), at pH 7.5 with palmitate (147 pmol/min/mL) and stearate (1.2 pmol/min/mL), and at pH 8.0 with palmitate (94 pmol/min/mL) and stearate (23 pmol/min/mL).

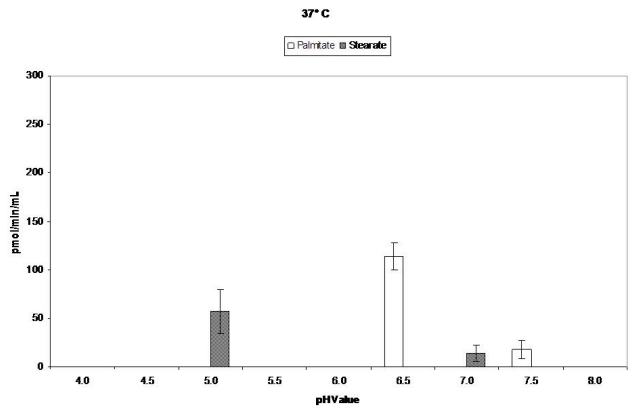

Figure 2: *LdLIP3* at 37°C with palmitate and stearate as substrate. Lipolytic activity assays of *LdLIP3* transfectants vs *pKSNEO* control transfecant were

carried out at 37°C with Palmitate (open boxes) and Stearate (gray boxes) at various pH points. Standard calculations were used to determine the net change of activity (*LdLIP3 – pKSNEO*) and is recorded as pmol/min/mL. Standard error bars are indicated by solid black lines (n=10)

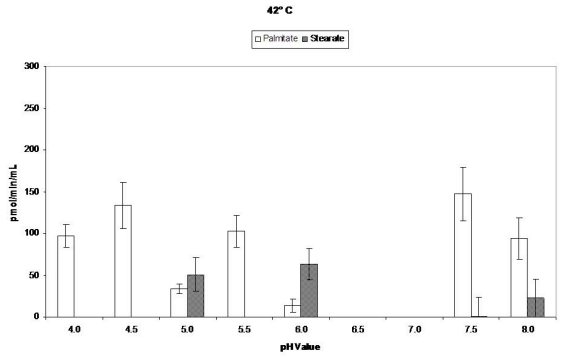

Figure 3: *LdLIP3* at 42°C with palmitate and stearate as substrate. Lipolytic activity assays of *LdLIP3* transfectants vs *pKSNEO* control transfecant were carried out at 42°C with Palmitate (open boxes) and Stearate (gray boxes) at various pH points. Standard calculations were used to determine the net change of activity (*LdLIP3 – pKSNEO*) and is recorded as pmol/min/mL. Standard error bars are indicated by solid black lines (n=10)

Taken together, these results demonstrate that there is *LdLIP3* lipolytic activity at various biologically relevant conditions using palmitate and stearate as substrates. This indicates that the previously sequenced *LdLIP3* gene encodes a lipase enzyme with lipolytic activity under a variety of experimental conditions. Overall, palmitate appears to be the preferred substrate under the various assay conditions tested. The optimal conditions for activity were observed at 26°C, pH 5.0 with palmitate (213 pmol/min/mL) as substrate.

DISCUSSION

Leishmania donovani is a parasitic protozoan which causes visceral leishmaniasis in humans and can be fetal if left untreated (Courret et al 2002). Previously the *LdLIP3* gene of *L. donovani* was shown to encode a secretory lipase (Shakarian et al 2010). Lipases are a known virulence factor in many organisms however its role in *Leishmania sp.* is currently unknown. It is possible that the secretory lipases of *L. donovani* are partly responsible for causing the devastating outcome of this disease. The current study was conducted to further characterize the lipolytic activity of the LdLIP3 enzyme.

When in the sandfly vector, *L. donovani* parasites are most likely subjected to a variable extracellular pH range that presumably occurs between the sandfly midgut and its mouth parts. Therefore, the parasite and its secretory enzymes must have the ability to adapt to a wide range of pHs while being exposed to 26°C, the ambient temperature of the sandfly. The highest lipolytic activity at 26°C was seen with palmitate at pH 5.0 (213 pmol/min/mL) and pH 6.0 (184 pmol/min/mL). The highest activity using stearate was seen at pH 4.5 (42 pmol/min/mL) and pH 6.5 (50 pmol/min/mL). We hypothesize that the secretory lipase could allow these parasites the ability to salvage fatty acids from both their insect vector and human host. These fatty acids could then be used to synthesize complex lipids required for the growth and development of the parasite throughout its life cycle. For example, these organisms are known to remodel their membrane lipid components as they transition between the amastigote and promastigote developmental forms (Tetley et al 1986; Wassef et al 1985).

At 37°C using stearate as substrate, the largest increase of activity was observed at pH 5.0 (57 pmol/min/mL). This corresponds to the approximate pH of the macrophage (pH 4.5-5.0) (Daleke 1990), one of the resident host cells for *L. donovani*. Interestingly, previous studies showed that amastigotes have an elevated fatty acid metabolism when compared to promastigotes which typically prefer to metabolize glucose and proline (Berman 1987; Tielens and van Hellemond 2009; Hart and Coombs 1982). This indicates that the amastigotes preferentially use fatty acids in energy metabolism via beta oxidation (Berman 1987; McConville et al 2007;

Rosenzweig 2008; Naderer and McConville 2008; Tielens and van Hellemond 2009; Opperdoes and Coombs 2007; Hart and Coombs 1982). Additionally, activity observed at 37°C using palmitate as substrate at pH 7.5 (18 pmol/min/mL) is also biologically relevant because this is the approximate pH of blood (pH 7.4) (Rosenthal 1948) the compartment the parasites are exposed to when they first enter the human host.

Results of the assays at 42°C show activity across the pH range. Despite this wide array of activity, there were some biologically relevant peaks of activity that were observed. For example, at 42°C there are peaks of activity with both palmitate and stearate between pH 4.5 and 5.5 which correlates to the pH in the macrophage where the parasite is found in the human host and relates to the high temperature that occurs during the course of infection. Additionally, a peak of activity (147 pmol/min/mL) was seen with palmitate at pH 7.5, the approximate pH of blood (Rosenthal 1948).

This study was carried out to better understand the lipolytic activity of the enzyme encoded by the gene *LdLIP3* of the human pathogen *L. donovani*. The activity of the LdLIP3 enzyme was studied for the first time at three biologically relevant temperatures and a wide range of pHs. The results of this study showed that increased activity using the substrates 4-MU palmitate and 4-MU stearate, was seen under biologically relevant conditions. We hypothesize this enzyme plays a role in acquiring lipids from the insect vector and human host to satisfy the parasite's need for energy metabolism (i.e. beta oxidation). Further, it is hypothesized that this enzyme has important functions in the synthesis of complex lipids and in the structural remodeling of membrane lipids during parasite development. The functional activities of this parasite enzyme suggest that it could play a significant role in the pathophysiology associated with this human disease. For example, release of this parasite secretory lipase into host extracellular spaces could account in part for the extensive tissue damage associated with leishmaniasis. Being able to further understand the activity of this secretory lipase should lead to a better understanding of *L. donovani* and the role of the LdLIP3 enzyme as a potential virulence factor during the course of human disease.

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