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Cloning of the Gene, Purification as Recombinant Protein and Functional Characterization of Leishmania mexicana Cytochrome b5 Reductase

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Cloning of the Gene, Purification as Recombinant Protein and Functional Characterization of *Leishmania mexicana* Cytochrome b5 Reductase

by

Ala Azhari

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Public Health Department of Global Health College of Public Health University of South Florida

Major Professor: Andreas Seyfang, Ph.D. Azliyati Azizan, Ph.D. Boo Kwa, Ph.D. Dennis Kyle, Ph.D.

Date of Approval: November 9, 2012

Keywords: Leishmaniasis, protein expression, drug resistance, NADH, detoxification of xenobiotics

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DEDICATION

This thesis is dedicated to my parents Ahmed and Zain, my husband Ashraf, my brothers Ali, Amr, Abdullah, and Ady, and my parents-in-law Shafek and Wedad for their endless support, love and encouragement.
ACKNOWLEDGMENTS

This work was only possible due to the collaboration of many individuals, to which I desire to express my gratitude.

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Finally, I must particularly acknowledge my dear husband Ashraf. His love, support, encouragement and patience gave me the strength to complete this work.
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## LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbols and Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Cb5r</td>
<td>Cytochrome b5 reductase</td>
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</table>
ABSTRACT

Leishmania are protozoan parasites that are transmitted by a sand fly vector. These parasites affect not only humans but also wild animals including domestic dogs and rodents, which form an additional challenge and public health problem to control the disease. Leishmaniasis is an important disease with worldwide distribution, including Saudi Arabia, the Middle East, and other tropical and subtropical areas around the world. Due to the expansion of irrigation and agricultural activities, more exposure to sand fly occurs, which leads to the expansion of leishmaniasis infections as newly emerging disease.

Emerging drug resistance in leishmaniasis is an additional problem, contributed by enzymes involved in the detoxification of pharmacological agents and other xenobiotics. Cytochrome b5 reductase (Cb5r) has a high pharmacological significance because of its essential role in fatty acid elongation, biosynthesis of cholesterol (humans) or ergosterol (Leishmania, fungi), and cytochrome P450-mediated detoxification of xenobiotics. Leishmania Cb5r has seven different isoforms whereas human has only one. Cb5r-7 isoform in Leishmania has the closest homology to the human Cb5r.

The following three aims of this project are focusing on cloning the Cb5r-7 isoform from Leishmania mexicana, its purification as recombinant protein from E.coli and its functional characterization as potential pharmacological target against Leishmania.
**Aim 1:**

Cloning of the gene for LmexCb5r-7 isoform from *Leishmania mexicana*.

1. Genetic engineering of LmexCb5r-7 to truncate and delete the predicted transmembrane domain in order to produce soluble recombinant protein.
2. Add a 6-His tag before the gene at the amino terminus of LmexCb5r-7 for subsequent nickel column purification.
3. Amplification of the gene by PCR and cloning into the pET23b expression vector.

**Aim 2:**

Recombinant expression and purification of LmexCb5r-7 protein in *E.coli*. Large scale bacterial expression and His$_6$-tag protein purification by Nickel column chromatography.

**Aim 3:**

Functional characterization of recombinant LmexCb5r-7 enzyme. Enzyme assays as basis for kinetic biological characterization and subsequent drug assays.
INTRODUCTION

Leishmania Life Cycle and Disease

Leishmania are protozoan parasites that are transmitted by a sand fly vector of the genus Phlebotomus in the old world and Lutzomyia in the new world. These parasites affect not only humans but also wild animals including domestic dogs and rodents, which form an additional challenge and public health problem to control the disease. The life cycle of this parasite has two parts, vector cycle and men cycle. In the vector cycle, the female sand fly is infected by a blood meal containing round, non-motile amastigotes that change into motile, elongated flagellated promastigotes in its mid gut. Promastigotes then multiply by binary vision and migrate to the salivary glands ready to be inoculated with the next blood meal. In the human cycle, promastigotes inoculated into the bite wound are engulfed by skin macrophages and transformed into amastigotes that multiply intracellularly (CDC, 2012a) (Fig. 1).

There are different forms of Leishmania infection depends on the type of Leishmania. Cutaneous leishmaniasis is the most common form of leishmaniasis, usually infects the skin causing localized or diffused cutaneous leishmaniasis. The infection is characterized by a red papule appears at the bite site after about 2-8 weeks and form a skin lesion (oriental sore) which increases in size gradually and filled with amastigotes. The lesion can be dry or weeping and it is painless unless a secondary bacterial infection occurs at the lesion. The lesion usually heals by itself with in a year. Diffused cutaneous
leishmaniasis is a rare form and occurs when the parasites spread through lymphatic causing a secondary lesion on the skin with no ulceration, and can be incurable in some cases (an anergic response). The patient may initiate a hypersensitivity response if the ulcerated nodules decreased in size and heals leaving a scar. In this case the patient produces immunity. Cutaneous leishmaniasis is very difficult to cure in HIV individuals.

Figure 1: *Leishmania* Life Cycle. (CDC, 2012a).

Visceral leishmaniasis is the systematic form of leishmaniasis and can be mild or sever. It affects the human internal organs and can be fatal if not treated. This form may also affects the kidneys leading to kidney failure. Patients can be asymptomatic or symptomatic. Symptoms appear within weeks or months after the sand fly bite. Major symptoms are: weight loss, decreased blood count causing anemia, hepatomegaly, splenomegaly, increased level of immunoglobulin in blood, fever, vomiting, and diarrhea. Darkness of the skin is also a symptom of visceral leishmaniasis known as Kala-azar.
Mucocutaneous leishmaniasis is a rare form of the disease. This form appears as nodules inside the nose, mouth and larynx and can occur after months or year after healing from cutaneous leishmaniasis (Markell et al, 2006; CDC, 2012a).

**Leishmania Species and Geographical Distribution**

There have been over 30 species described in *Leishmania* and at least 20 of them are pathogenic to mammals. *Leishmania* disease depends on the species, host immune response and geographical region (Tab. 1).

Table 1: Clinical Disease, Causative Organism and Geographical Distribution of Leishmaniasis.

<table>
<thead>
<tr>
<th>Clinical disease</th>
<th>Causative organism</th>
<th>Geographical distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral leishmaniasis</td>
<td><em>L. donovani</em></td>
<td>China, India, Iran, Sudan, Kenya, Ethiopia</td>
</tr>
<tr>
<td></td>
<td><em>L. infantum</em></td>
<td>Mediterranean basin</td>
</tr>
<tr>
<td></td>
<td><em>L. chagasi</em></td>
<td>Brazil, Colombia, Venezuela, Argentina</td>
</tr>
<tr>
<td></td>
<td><em>L. amazonensis</em></td>
<td>New World</td>
</tr>
<tr>
<td>Cutaneous leishmaniasis</td>
<td><em>L. tropica</em></td>
<td>Mediterranean basin, Afghanistan</td>
</tr>
<tr>
<td></td>
<td><em>L. major</em></td>
<td>Middle East, W. and N. Africa, Kenya</td>
</tr>
<tr>
<td></td>
<td><em>L. aethiopica</em></td>
<td>Ethiopia</td>
</tr>
<tr>
<td></td>
<td><em>L. mexicana</em></td>
<td>Central America and Amazon basin</td>
</tr>
<tr>
<td>Mucocutaneous leishmaniasis</td>
<td><em>L. braziliensis complex</em></td>
<td>Brazil, Peru, Ecuador, Columbia, Venezuela</td>
</tr>
</tbody>
</table>
morphological structures for all species. Isoenzyme analysis, monoclonal antibodies and DNA sequencing are used to differentiate between *Leishmania* species (Markell et al, 2006; CDC, 2012b) (Tab. 1).

**Pathology of Leishmaniasis**

**Cutaneous Leishmaniasis:**

After the bite of an infected sand fly, promastigotes enter the skin and transform to amastigotes in the macrophages. Amastigotes disruption activates the macrophages by sensitized lymphocytes causing a granulomatous reaction forming a localized nodule. The nodules get ulcerated when the parasite damage the infected area. Healing of the ulcer depends on the immune system. Stronger immune system results in a faster healing (Markell et al, 2006).

**Visceral Leishmaniasis:**

The main issue with visceral leishmaniasis is the suppression of cell-mediated immunity resulting in an uncontrolled spread and multiplication of the parasite leading to serious outcome if untreated. Reticulo-endothelial cell hyperplasia occur when the mononuclear phagocytic cell accumulate in the invaded tissue. Anemia and granulocytopenia occur when the white and red blood cells life span is reduced. Splenomegaly and hepatomegaly resulted from the rapid increase in the number of reticulo-endothelial cells of the spleen and liver. Splenomegaly can increase the destruction of white and red blood cells. Repeated infection with pneumonia, tuberculosis and dysentery are frequent and are the main cause of death in advanced stage of visceral leishmaniasis (Malla & Mahajan, 2006).
Mucocutaneous Leishmaniasis:

Ulcerated lesions occur the same as oriental sore but larger and more frequent and seem to develop metastasis. Secondary infection is responsible for the persistence of the ulcer and its size (Markell et al, 2006).

Treatment

Treatment of leishmaniasis depends on its form. The main drug used to treat leishmaniasis is antimony-based drug. Pentavalent antimony is important in inhibiting glycolytic enzymes and fatty acid oxidation in leishmania amastigotes.

Usually, cutaneous leishmaniasis is not treated. Oral Ketoconazole, Stibogluconate (Pentostam), intravenous liposomal Amphotericin B or Pentamidine can be used to treat cutaneous leishmaniasis. In some cases, plastic surgery is used to treat the scars. Interferon gamma can be injected intradermally around the lesion to enhance the healing process. Clotrimazole (1%) is a topical cream and was effective in Saudi Arabia (Markell et al, 2006).

Treatment of mucocutaneous leishmaniasis is similar to the cutaneous form but differ in the length of treatment. Cycloguanil pamoate is more effective when given intramuscularly as it inhibits folic acid (Markell et al, 2006).

Visceral leishmaniasis can be treated by intravenous liposomal Amphotericin B, Miltefosine (Miltex) or Paromomycin (Humatin); the last two are not available in the US. Immunocompromised patients can be treated with Allopurinol. A combination of interferon gamma and antimony can also be used in treating visceral leishmaniasis (Cecil et al, 2011; CDC, 2012c).
These drugs are toxic and have strong side effects, which leads to the urgent need to develop new drugs.

**Epidemiology**

Leishmaniasis is an important disease that affects the populations of 88 countries worldwide, including the Middle East, Central and South America and other tropical and subtropical areas around the world. Due to the expansion of irrigation and agricultural activities, more exposure to sand fly occurs, which leads to the expansion of leishmaniasis infections as newly emerging disease. Sand fly is known to be the natural vector for all types of leishmaniasis transmission but infection by contact transmission is also possible in cutaneous leishmaniasis. Infection caused by *L. tropica* can be transmitted from person to person by contact transmission while all other forms of cutaneous leishmaniasis are zoonoses. Mechanical transmission by some types of flies bite like *Stomoxys* has also been reported. In some cases blood transfusion can also transmit the disease if the monocytes were infected with leishmaniasis. In visceral leishmaniasis, two forms of transmissions were reported, human-to-human transmission was seen in urban areas but the zoonotic form was seen in rural areas. Humans are an accidental host. The African Kala-azar forms are different from all other Kala-azar forms seen anywhere else. It is characterized by a lesion in the legs and shows a healed ulceration at the infection site (Markell et al, 2006).
Leishmaniasis in Saudi Arabia

Cutaneous leishmaniasis is the common form of leishmaniasis in Saudi Arabia. It was recognized as a medical condition in the kingdom since 1950. An epidemic of cutaneous leishmaniasis occurred in 1973 and reached its plateau in the mid 80’s. In general, male adults represent 75% of the cases. There are two main species responsible for cutaneous leishmaniasis in Saudi Arabia depending on the region. *Leishmania major* is the causative agent in the Eastern and central regions and is transmitted by *P. papatasi* while *Leishmania tropica* is the causative agent in the Southwest and Western regions and is transmitted by *P. sergenti* (Al-Aboud, 2004).

The majority of cases were reported in the Qasim province, which is located in the central part of the kingdom bordering the Riyadh province (WHO, 2008). The highest number of cases occurs between August and February while the lowest number of cases occurs in May and June (Al-Aboud, 2004). Cutaneous leishmaniasis has local names known as Okhet (sister) (اختت), Nafra (the rash) (نفرة), Domal (boil) (دمل), and El-Mohtafura (the digger) (المحتافرة) (Al-Tawfiq & AbuKhamis, 2004). The percentage of cases of cutaneous leishmaniasis in 2004 was 20.9% in the Eastern province, 26.6% in the Qaseem (Qasim) province, 4.1% in the Riyadh province, 4.1% in the Aseer province, 9.1% in the Hail province and 18.5% in the Medina province (Al-Aboud, 2004) (Fig. 2).

In a study conducted between 1983 and 2004, the highest number of reported cases of cutaneous leishmaniasis was in 1983 (18,318 cases) and the lowest number of reported cases was in 2003 (3,842 cases) (Fig. 3).
Male represent the highest number of reported cases of cutaneous leishmaniasis compared to female patients (Al-Aboud, 2004).

Visceral leishmaniasis (VL) is rare but there are some reported cases in the Southwestern region mostly in the Jezan province (Fig. 4). Male children represent 69% of the VL cases (WHO, 2008). *L. donovani* is the causative agent for visceral leishmaniasis, which has been found with lower prevalence in the Eastern province of Saudi Arabia. Between 1990 and 1992, eight soldiers who were veterans of Operation
Dessert Storm in the Western region of Saudi Arabia were evaluated for visceral leishmaniasis, which nevertheless did not show the classic signs and symptoms of visceral leishmaniasis (Kala-azar). After several diagnostic tests, \textit{L.tropica} was found to be the causative agent for the visceral leishmaniasis in these 8 soldiers. Until then, \textit{L.tropica} has been known to cause cutaneous leishmaniasis but not systemic illness. The strain of \textit{L.tropica} that was isolated from the soldiers shared some features with \textit{L.donovani} suggesting that the \textit{L.tropica} strain that causes visceral leishmaniasis is different from the strain that causes cutaneous leishmaniasis (Magill et al, 1993).

![Geographical Distribution of Visceral Leishmaniasis in Saudi Arabia in 2004.](image)

A retrospective study was conducted between 1989 and 1994 in Tabuk (located at the Northwest province of Saudi Arabia) for the existence of visceral leishmaniasis in that area. The results suggested that \textit{L.tropica} has changed its pathogenic role and caused visceral leishmaniasis in the area (Hanly et al, 1998). In 1994, visceral leishmaniasis cases in Jezan (Gizan) province was observed more in late spring and summer but fewer cases in the winter season. Hepatosplenomegaly was rare in patients infected with \textit{L.donovani} compared to the African Kala-azar form (Al-Orainey et al, 1994). Reported
cases of visceral leishmaniasis between 1984 and 2004 showed the highest number in 1989 with 305 cases while the lowest number was in 2003 with 9 cases (Al-Aboud, 2004) (Fig. 5).

![Reported Cases of Visceral Leishmaniasis 1984-2004](image)

**Figure 5: Reported Cases of Visceral Leishmaniasis in Saudi Arabia between 1984-2004.**

There are 20 different species of sand flies found in Saudi Arabia, *Phlebotomus* and *Sergentomyia* represent the two major genera with 35% and 65% respectively (Al-Aboud, 2004; Doha & Samy, 2010). The Ministry of Health provides vector and reservoir control programs in the infected areas to control the disease (WHO, 2008).

![Map of Saudi Arabian Provinces](image)

**Figure 6: Map of Saudi Arabian Provinces.**
Cytochrome b5 Reductase and Drug Resistance

Emerging drug resistance in leishmaniasis is an additional problem, contributed by enzymes (involved in the detoxification of pharmacological agents and other xenobiotics). The patient’s immune status plays an important role in leishmaniasis drug efficacy. The biochemical and molecular differences between *Leishmania* species is responsible for the variation in the sensitivity of *Leishmania* species to several drugs. Pharmacokinetics is also different from one species to the other. These variations can complicate the treatment of leishmaniasis. Drug combination and drug monitoring play an important role in avoiding the emergence of resistance to new drugs (Croft et al., 2006).

Figure 7: Electron Transfer by Cytochrome b5 Reductase.
Cytochrome b5 reductase enzyme transfers two electrons from NADH (electron donor) to two molecules of cytochrome b5 (electron acceptor). These electrons then passed on to cytochrome P450, a protein with high significance in *Leishmania* drug resistance and xenobiotics detoxifications. Cytochrome P450 reductase enzyme may transfer electrons from NADPH (electron donor) to either cytochrome P450 or cytochrome b5 (donor acceptor).

Cytochrome b5 reductase (Cb5r) is a member of the ferredoxin NADP⁺ reductase (FNR) family. Two NADH binding motifs, a flavin-binding motif, and a FMN/FAD selectivity motif are the four conserved motifs in the FNR family. Cytochrome b5 reductase has a high pharmacological significance because of its essential role in fatty
acid elongation, biosynthesis of cholesterol (humans) or ergosterol (*Leishmania*, fungi), and cytochrome P450-mediated detoxification of xenobiotics. Cb5r is also important in NADH-mediated electron transfer with cytochrome P450 as the target and electron acceptor. It also plays an important role in erythrocyte function (Bewley et al, 2001), (Fig. 7).

**Phylogram of Cytochrome b5 Reductase**

*Leishmania mexicana* has 7 isoforms. The ClustalW2 Multiple-Sequence Alignment program can be used to produce a phylogram of all seven *Leishmania* isoforms. As shown in the resulting phylogram below (Fig. 8), LmexCb5r-7 isoform (red star) is the closest isoform in homology compared to the human Cb5r and fungal CBR1, while LmexCb5r isoforms 1 and 2 (blue star) are most distant to the mammalian isoform. Three major subclades were found: one clade containing isoforms 1, 2 and 3, a second clades containing isoforms 4, 5 and 6, and the clade containing LmexCb5r isoform 7 together with the human and fungal CBR1 isoforms. (Fig. 8; ClustalW2, 2012).

**Nicotinamide Adenine Dinucleotide (NAD^+)**

NAD^+ is made up of two nucleotides, adenine base and nicotinamide joined by two phosphate groups. NAD^+ is a coenzyme and has the ability to accept electrons from other molecules and forms NADH a reducing agent to donate electrons. NAD^+ can act as a substrate of enzymes that add or remove chemical groups from proteins, which makes NADH utilizing enzyme a target for drug discovery. (Fig.9 and 10)
Figure 8: Phylogram of Cytochrome b5 Reductase.
ClustalW2 Multiple-Sequence Alignment program used to produce a phylogram of cytochrome b5 reductase showing that isoform 7 in LmexCb5r is the closest in homology to human Cb5r and fungal CBR1.
Figure 9: Nicotinamide Adenine Dinucleotide (NAD\(^+\)).

$$
\text{NAD}^+ + H^+ + 2e^- \rightarrow \text{NADH}
$$

Figure 10: Oxidation of NAD\(^+\).
**Isopropyl β-D-1-thiogalactopyranoside (IPTG) in Protein Expression**

IPTG transcribed T7 RNA polymerase that activates T7 promoter in the expression system. IPTG is a drug used to activate the expression of T7 RNA polymerase located in the chromosome of the *E.coli* BL21 strain. It is a more useful inducer than the lactose because it is not included in any metabolic pathways, which will prevent it from the consumption by the cell. This will insure that the IPTG concentration added remains constant. (Fig. 11)

![BL(DE3) Host Chromosome](image)

**Figure 11: Schematic Representation of E.coli BL21 Host Strain.**
The lac promoter ($P_{lac}$, red), the lac operator ($loc_O$, cyan), the T7 RNA polymerase-encoding gene (T7 RNA Pol, pink), and the lac inducer ($lacI$, blue) are highlighted. A black line depicts the bacterial host chromosome in this engineered *E.coli* strain.
MATERIALS AND METHODS

Gene Identification of Cytochrome b5 Reductase Isoform 7 in the *L. mexicana* Genome

The gene of Cb5r isoform 7 was identified in the *L. mexicana* genome using the database TriTrypDB.org (Tab. 2).

Table 2: Gene LmxM13.1060. 927bp Gene, 308 amino acid protein of LmexCb5r-7 isoform.

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Theoretical pI/Mw</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVAVLVIIAVSI</td>
<td>8.6/34,819 Da</td>
</tr>
</tbody>
</table>

Transmembrane Domain Analysis

In the LmexCb5r-7 protein sequence, only one transmembrane domain was identified using the transmembrane analysis program TMpred at [www.ch.EMBnet.org](http://www.ch.EMBnet.org) (Hofmann & Stoffel, 1993). The LmexCb5r-7 transmembrane domain was spanning from
Met1 to Phe20 that needed to be deleted to produce soluble recombinant protein. Therefore, a start position at threonine 21 of the LmexCb5r-7 gene was chosen as part of the gene engineering of the gene for recombinant LmexCb5r-7 (Fig. 12).

**Figure 12: Trans-membrane Domain Analysis.**

**Cloning of *Leishmania mexicana* Cb5r-7 Gene**

LmexCb5r-7 gene was cloned from *L. mexicana* genomic DNA using PCR. Primer design for PCR cloning of LmexCb5r-7 gene (DNA) was conducted using genomic DNA as template for PCR. Restriction enzyme analysis of LmexCb5r-7 gene was performed to find restriction enzymes that do not cut in the LmexCb5r-7 gene. *Bam*H1 and *Hind*3 were the restriction enzymes used in this case. Lists of the enzymes that are not found in
LmexCb5r-7 gene are shown in (Tab. 3). Then six-histidine tag was added to the DNA sequence at the beginning of the gene (Tab. 4).

**Table 3: Non-cutting Restriction Enzymes in LmexCb5r-7.**
Underlined are enzymes found in the pET23b multiple cloning site.

<table>
<thead>
<tr>
<th>Enzyme</th>
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<tbody>
<tr>
<td>Aat2</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>Psil</td>
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<td>PspOM</td>
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</table>
| Fwd strand: 5’-ACACCGCCCCTATCTCATCTCAAGTATTAAAATGTTATCGCGGGTGTGT-3’

**Table 4: PCR Primer Design for LmexCb5r-7.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>LmexCb5r7-T21-His6-BamH1-Fwd</td>
<td>5’-TAGCATGGATCCATGCACCATCACCATCACCATATGACGCGCACGACGAAGGTGG-CGATG-3’</td>
<td>915bp</td>
</tr>
<tr>
<td>60-mer, 24-mer, 16G/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BamH1= GGATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LmexCb5r7-Hind3-Rev</td>
<td>5’-ACTGATAAGCTTTTAGAAGTAAAGTATCGGCGGGTGTGT-3’</td>
<td>915bp</td>
</tr>
<tr>
<td>41-mer, 29-mer, 11G/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Fwd strand: 5’-ACACCGCCCCTATCTCATCTCAAGTATTAAAATGTTATCGCGGGTGTGT-3’)</td>
<td></td>
<td></td>
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<tr>
<td>Hind3= AAGCTT</td>
<td></td>
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</table>

The end of each primer contained a specific restriction enzyme (BamH1 or Hind3, respectively) for ligation into the multiple cloning sites of the pET23b vector. The pET23b vector was used for protein expression. PCR primers also included the His6-tag for protein purification. PCR product (915bp bands) is first concentrated by Na acetate method 20μl volume, then restriction enzyme digested with BamH1 and Hind 3, and finally purified by agarose gel electrophoresis and gel extraction (QIAquick gel extraction kit). LmexCb5r-7 PCR band (Hind3-BamH1 digested) was inserted into pET 23b plasmid vector (Hind3-BamH1 digested) by ligation then transformed into E.coli bacteria XL10-Gold strain for DNA work. Screening for positive colonies that contain
LmexCb5r-7.pET 23b (our gene in the pET 23b expression vector) was then performed followed by restriction enzyme digest with BamH1 and Hind3. Agarose gel electrophoresis was used to identify positive clones and confirmed by DNA sequencing. After the confirmation, ligation product was transformed into E.coli BL 21 strain (ideal strain for protein expression, NOT so good for DNA work). Positive clones were digested and confirmed by sequencing (Fig. 13).

**Purification of Recombinant LmexCb5r-7 Protein**

One L of E.coli BL21 with positive clone was grown and protein expression was induced with IPTG (drug to start protein expression in E.coli of our protein in pET 23b vector) followed by overnight expression and the next day, the cell pellet was collected and stored at -20 °C until further use for purification.

Nickel column chromatography is a common method used in protein purification. The cell pellet was lysed using the French press technique. His6-tag was included in the recombinant protein that allows affinity binding to Ni²⁺, which is part of the Ni-NTA matrix in the column. When we loaded our recombinant protein to the column, 2 of the 6 histidines bind with one Ni²⁺ ion, trapping the tagged protein in the column while unbound protein was washed out. After the washing step, we added 250mM imidazole. This drug competes with the histidines for the Ni²⁺ binding interaction. This process effectively released the recombinant protein from nickel resin and resulted in elution of the purified recombinant protein. (Fig.14)

Size exclusion chromatography was used for additional purification of LmexCb5r-7 (purification based on the size of our protein = 34,819 Da).
Figure 13: pET23b Map for Cloning of LmexCb5r-7.
Measurement of Enzymatic Activity of Recombinant \textit{L.mexicana} Cb5r-7

Enzyme activity of recombinant \textit{L.mexicana} Cb5r-7 was performed by using NADH:ferricyanide assay. NADH acts as a substrate and ferricyanide as an artificial electron acceptor. The loss of NADH and conversion to NAD$^+$ was measured by spectrophotometry at 340nm. Substrate affinity ($K_m^{NADH}$) and catalytic rate ($K_{cat}$) of recombinant \textit{L.mexicana} Cb5r-7 was determined by the same assay.

Bradford Protein Assay

Bradford assay is a colorimetric assay. Coomassie blue dye converts from red color (465nm) to blue (595nm) when it binds to the protein of the sample. Protein concentration is directly proportionate to the darkness of the blue color, which can be measured by a spectrophotometer at 595nm (Bio-Rad, 2012).
Substrate Specificity of *L. mexicana* Cb5r-7

This assay used to test the inhibitor potential of the enzyme that helps in drug discovery. ADP-Ribose, ADP, GDP, AMP, adenosine and nicotinamide (NAD analogues competitor) and the antibiotic Novobiocin were used in the lab. The assay was performed using the NADH:ferricyanide assay. One μl of 2.5μM protein/assay and wavelength of 340nm were used for the control. Two μl of 2.5μM protein/assay was used for all substrate analogues. A wavelength of 370nm was used for Novobiocin and 340nm for all other substrates.

Adenosine Diphosphate (ADP):

ADP is made up of adenine base, pentose sugar ribose and pyrophosphoric acid. ATP synthase can convert ADP back to ATP. This conversion is important in supplying energy (Fig. 15).

![Figure 15: Adenosine Diphosphate (ADP).](image)

Adenosine Diphosphate Ribose (ADP-Ribose):

ADP-ribose is composed of adenine base, pentose sugar, two phosphate groups and ribose sugar molecule (Fig. 16).
Adenosine Diphosphate Ribose (ADP-Ribose):

Figure 16: Adenosine Diphosphate Ribose (ADP-Ribose).

Adenosine Monophosphate (AMP):

AMP is made up of adenine, ribose sugar, and phosphate group. Adenylate kinase enzyme can produce AMP during ATP synthesis by combining 2 ADP molecules. AMP is used as a monomer in RNA (Fig. 17).

Figure 17: Adenosine Monophosphate (AMP).

Adenosine:

Adenosine is composed of adenine and ribose sugar molecule. It plays an important role in energy transfer. It also acts as an inhibitory neurotransmitter (Fig. 18).

Figure 18: Adenosine.
Nicotinamide:

Nicotinamide is a vitamin belongs to the vitamin B group (Fig. 19).

![Nicotinamide](image)

Figure 19: Nicotinamide.

Novobiocin:

Novobiocin is an aromatic ether compound composed of a benzoic acid derivative, a coumarin residue and the sugar novobiose. Novobiocin behaves as a competitive inhibitor of the ATPase reaction that is part of the GyrB subunit of the topoisomerase-2 enzyme. But may also interact with Cb5r (NADH shares similar structure with ATP) (Fig. 20).

![Novobiocin](image)

Figure 20: Novobiocin.
RESULTS AND DISCUSSION

PCR Amplification and Cloning of LmexCb5r-7

DNA gel electrophoresis of our PCR product resulted in a band size of 915bp, which confirmed that we amplified the LmexCb5r-7 gene (Fig. 21).

Figure 21: Gel Electrophoresis of PCR Product LmexCb5r-7.

After running the gel for 1 hour and 30 minutes, the LmexCb5r-7 PCR band and the pET 23b vector band were excised from the gel and weighted to determine their mass.
for subsequent gel purification by QIAquick gel extraction kit (0.470g = 470µl for LmexCb5r-7 band gel piece; 0.400g = 400µl for pET 23b band gel piece.) (Fig. 22A, 22B).

Spectrophotometric analysis of quality and quantity of the purified extracted DNA of LmexCb5r-7 and pET23b was performed according to manufacturer’s protocol for the QIAquick gel extraction kit and showed an absorbance A260/A280 (quality check; ideally higher than 1.8) and concentration of LmexCb5r-7 as 1.8 and 24.6 ng/µl respectively, while the absorbance and concentration for pET23b were 1.96 and 10.6 ng/µl respectively.

Ligation was an important step for the insertion of the digested LmexCb5r-7 (Hind3 and BamH1) into the digested pET 23b plasmid vector (pET23b: 3641bp, 3641 bp).
LmexCb5r-7: 891bp). The ligation product was then transformed into the *E.coli* XL10-Gold strain (best suited for DNA work).

Fourteen colonies were picked after the transformation of Z-competent cells using *E.coli* XL10-Gold and subsequently digested with *Xho*I restriction enzyme. Expected bands are 3753bp for pET23b (with insert) and 779bp for LmexCb5r-7 (or 3641bp for pET23b without successful cloning of LmexCb5r-7 insert). Four out of 14 colonies showed positive bands after running the gel for one and a half hours and staining with ethidium bromide (Fig. 23).

**Figure 23: Gel Electrophoresis for the Digested Colonies.**
Screening was performed by *Xho*I digest. Expected bands are 3753bp for pET23b (with insert) and 779bp for LmexCb5r-7 insert (or 3641bp for pET23b without successful cloning of LmexCb5r-7 insert). Four positive colonies were identified that contained the expected LmexCb5r-7 band of 779bp (colonies 1, 3, 4 and 12).
Measurement of Enzymatic Activity of Recombinant LmexCb5r-7 Protein

To test the activity of Cb5r enzyme, NADH was used as a substrate and its conversion to NAD\(^+\) was measured at 340nm wavelength by a spectrophotometer. A substrate range of 3-100 µM NADH was used for kinetic analysis. Michaelis-Menten kinetics analysis was then used to determine the affinity of the substrate (K\(_m\)\(^{NADH}\)) and the catalytic rate (K\(_{cat}\)) of the recombinant LmexCb5r-7 enzyme. The plot was formed using the Michaelis-Menten rate equation. It shows the quantitative relationship between the initial velocity (V\(_0\)), the maximum velocity (V\(_{max}\)), and the initial substrate concentration [S]. All these points are related through Michaelis constant K\(_m\) which is equal to V\(_0\) = \(\frac{1}{2}\) V\(_{max}\). A large K\(_m\) means that a high concentration of substrate was needed to achieve V\(_{max}\) and a small one required a small amount of substrate. K\(_{cat}\) is the maximum number of substrate molecules converted to product on a single enzyme molecule per second (“turnover number”). The K\(_{cat}\)/K\(_m\) ratio describes the overall enzyme efficiency. Low K\(_{cat}\)/K\(_m\) ratio indicates that the product turnover rate is lower than the substrate concentration, which means that the enzyme and substrate has a high affinity for each other.

Recombinant LmexCb5r-7 enzyme had a V\(_{max}\) = 38.2 ± 0.5 µmol/min/nmol enzyme, K\(_m\) = 19.3 ± 0.8 µM with a regression coefficient r = 0.9995, K\(_{cat}\) = 636.4 ± 8.3 sec\(^{-1}\) and K\(_{cat}\)/K\(_m\) = 33.0 x 10\(^6\) sec\(^{-1}\) M\(^{-1}\) (Fig. 24).

The comparison of the initial-rate enzyme kinetics between LmexCb5r-7 and human Cb5r enzyme showed that they have similar affinity, twice the catalytic rate but about the same catalytic efficiency (Tab.5).
Table 5: Comparison of Initial-rate Enzyme Kinetics of LmexCb5r-7 with Human Cb5r Enzyme.

<table>
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<tr>
<th></th>
<th>$K_{\text{cat}}$</th>
<th>$K_m$</th>
<th>$K_{\text{cat}}/K_m$</th>
<th>$V_{\text{max}}$</th>
</tr>
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<tr>
<td></td>
<td>(sec$^{-1}$)</td>
<td>($\mu$M)</td>
<td>(sec$^{-1}$ M$^{-1}$)</td>
<td>($\mu$mol/min/nmol)</td>
</tr>
<tr>
<td>LmexCb5r-7</td>
<td>636 ± 8.3</td>
<td>19.3 ± 0.8</td>
<td>$33.0 \times 10^6$</td>
<td>38.2 ± 0.5</td>
</tr>
<tr>
<td>Human Cb5r</td>
<td>328 ± 7</td>
<td>13 ± 0.9</td>
<td>$25.3 \times 10^6$</td>
<td>19.7 ± 0.4</td>
</tr>
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</table>

Recombinant LmexCb5r enzyme provides a promising target in rational drug design and drug screening against *Leishmania*.

Figure 24: LmexCb5r-7 Kinetics with the NADH:Ferricyanide Assay.
Michaelis-Menten Kinetics showed a $V_{\text{max}} = 38.2 \pm 0.5 \mu$mol/min/nmol enzyme, a $K_m = 19.3 \pm 0.8 \mu$M with a regression coefficient $r = 0.9995$, a $K_{\text{cat}} = 636.4 \pm 8.3 \text{ sec}^{-1}$ and a $K_{\text{cat}}/K_m = 33.0 \times 10^6 \text{ sec}^{-1} \text{ M}^{-1}$.
**Bradford Protein Assay**

The Bradford assay was performed to determine the protein concentration in our samples. His₆-Thr21-LmexCb5r-7 protein concentration was 38.1 mg/ml using the Bradford assay vs. 22.8 mg/ml using the spectrophotometer assay. The spectrophotometer assay measures Cb5r flavin, which makes this assay more specific and accurate for Cb5r determination compared to the Bradford assay that uses bovine serum albumin (BSA) as protein standard. From this experiment we developed a conversion equation (Spectrophotometer Cb5r protein concentration by [FAD] x 1.70 = Bradford Cb5r protein concentration) (**Fig. 25**).

![Bradford Protein Assay](image)

**Figure 25: Bradford Protein Assay.**

**LmexCb5r-7 Substrate Analogues**

This experiment was testing the inhibition potential on the enzyme that will help in drug discovery. Six NAD analogues and the antibiotic Novobiocin were tested for
LmexCb5r-7 using the NADH:ferricyanide assay. These six NAD substrate analogues are used as lead drugs for subsequent design.

ADP and Novobiocin showed the highest inhibition effect with 80% (LmexCb5r-7) compared to 82% (human Cb5r) inhibition by ADP and 77% (LmexCb5r-7) compared to 89% (human Cb5r) inhibition by Novobiocin. Human Cb5r and LmexCb5r-7 showed almost the same inhibition profile, therefore we would need to test their inhibition in live Leishmania cells (macrophages or in-vitro testing for Leishmania parasite) to better characterize the individual drug inhibition effects on the parasite compared with the mammalian host (Fig. 26).

Figure 26: L. mexicana Cb5r-7 and Human Cb5r Substrate Specificity.
LIGPLOT Analysis of Cb5r-NAD Enzyme-Substrate Interaction

LIGPLOT for rat Cb5r interaction with NAD was generated from the crystal structure of enzyme that forms 5 hydrogen bonds and 12 hydrophobic interactions between protein and ligand NAD (Wallace et al., 1995; Wallace & Laskowski, 2005), (Fig. 27).

LmexCb5r-7 has the same LIGPLOT analysis results of rat Cb5r-NAD enzyme-substrate interactions except for alanine 208 in rat that is changed to glycine 203 in LmexCb5r-7 at the nicotinamide moiety interaction of Cb5r-NAD enzyme-substrate and phenylalanine 251 in rat that is changed to tyrosine 246 in LmexCb5r-7 interacting with the adenine purine ring of the NAD substrate. The interaction between protein and ligand NAD is particularly important in drug design when using substrate analogues as potential drugs.

Figure 27: LIGPLOT Analysis of Cb5r-NAD Interaction.
SUMMARY

Leishmaniasis is an important disease with worldwide distribution. This disease can be fatal if untreated. The existing drugs are very toxic and strong side effects lead to the urgent need to develop new drugs. Cytochrome b5 reductase is an enzyme involved in fatty acid elongation and ergosterol biosynthesis in *Leishmania*. Cb5r is also important in NADH-mediated electron transfer with cytochrome P450 as the target and electron acceptor that can neutralize drugs and xenobiotics. A phylogram was generated using the ClustalW2 program to align the amino acid sequences of *Leishmania*, human and fungal Cb5r enzymes. From the seven isoforms of LmexCb5r, LmexCb5r-7 showed the closest homology to humanCb5r and fungal CBR1. The Cb5r isoform 7 gene was identified in the *Leishmania mexicana* genome using the TriTrypDB.org database. Subsequently, a single transmembrane domain was identified in LmexCb5r-7 using a transmembrane domain analysis program, spanning 20 amino acids from Met1 to Phe20 that had to be removed for the cloning approach to produce a soluble recombinant protein. Genomic DNA was used as template for PCR cloning of the LmexCb5r-7 gene since this gene does not contain any introns. As part of the cloning strategy, restriction enzyme analysis of LmexCb5r-7 gene was performed and *Bam*H1 and *Hind*3 were selected for cloning. Additionally, a 6-histidine tag was added to the beginning of the gene for nickel column purification. The digested LmexCb5r-7 PCR band (*Hind*3-*Bam*H1) was then inserted into the digested pET23b plasmid vector (*Hind*3-*Bam*H1) by ligation followed by
transformation into the *E.coli* XL10-Gold strain. Screening, identification and confirmation of positive clones was performed and confirmed by DNA sequencing. Recombinant LmexCb5r-7 protein expression was conducted by transforming the positive clone into the *E.coli* BL21 strain optimized for IPTG-inducible protein expression for subsequent purification by nickel column chromatography. Enzyme activity of recombinant LmexCb5r-7 protein was measured by using the NADH:ferricyanide assay, where NADH acts as a substrate and ferricyanide is used as an artificial electron acceptor. Loss of NADH was measured by spectrophotometry at 340nm. Enzyme kinetics were performed that resulted in a substrate affinity ($K_m$) of 19.3 ± 0.8 µM, catalytic rate ($K_{cat}$) 636.4 ± 8.3 sec$^{-1}$, maximum velocity ($V_{max}$) 38.2 ± 0.5 µmol/min/nmol enzyme, and $K_{cat}/K_m = 33.0 \times 10^6$ sec$^{-1}$ M$^{-1}$ of recombinant LmexCb5r-7. The initial rate enzyme kinetics of LmexCb5r-7 enzyme was compared to the human Cb5r enzyme and the results showed that they have similar affinity, twice the catalytic rate, but about the same catalytic efficiency. Six NAD analogues (ADP-Ribose, ADP, GDP, AMP, adenosine and nicotinamide which act as substrate competitors) and the antibiotic Novobiocin were tested for LmexCb5r-7 and compared to the human Cb5r enzyme. These inhibitor assays were used as a first step to characterize LmexCb5r isoforms as a potential drug target. ADP and Novobiocin showed the highest inhibition effect with 80% (LmexCb5r-7) compared to 82% (human Cb5r) inhibition by ADP and 77% (LmexCb5r-7) compared to 89% (human Cb5r) inhibition by Novobiocin. ADP and Novobiocin showed similar activity on both human Cb5r and LmexCb5r-7. The next step would be to test their activity in live cells (in macrophage infectivity assays or *in-vitro* for *Leishmania* parasites) to characterize their inhibitory effects in more detail. A LIGPLOT...
analysis for Cb5r was generated in silico that showed the interactions between protein and ligand NAD at the atomic level. The comparison of the amino acid interaction with the substrate NAD in LmexCb5r-7 LIGPLOT and rat Cb5r LIGPLOT were similar except for two amino acid changes: Alanine 208 to glycine 203 substitution in LmexCb5r-7 at the nicotinamide binding moiety and phenylalanine 251 to tyrosine 246 substitution in LmexCb5r-7 at the adenine purine ring binding moiety of Cb5r-NAD enzyme-substrate interaction. The protein:NAD ligand interactions play an important role in subsequent drug design. While LmexCb5r-7 is the isoform with closest homology to human Cb5r, the LmexCb5r isoforms 1 and 2 are most distant from the human enzyme, which makes LmexCb5r-1 and 2 enzymes the best target for future rational drug design and drug screening against Leishmania Cb5r.
FUTURE DIRECTIONS

Cloning and characterization of all seven *Leishmania mexicana* Cb5r isoforms and comparison of the different isoforms with respect to the enzyme activity (kinetics, substrate affinity), pharmacology and differential expression in insect or mammalian stage of the parasite. Another step would be to generate LmexCb5r knockout cells for each isoform in *Leishmania* parasites and test them for their effect on survival in macrophage infectivity assays. Furthermore, performing structure-function analysis of Cb5r isoforms would be another strategy for rational drug design and pharmacological characterization of *Leishmania* Cb5r.
REFERENCES


http://www.cdc.gov/parasites/leishmaniasis/treatment.html


APPENDICES

Appendix A: Protocols

Protocol 1:

Large-Scale Protein Purification of Recombinant Proteins

A. Growth of Bacterial Cultures and Induction with IPTG

Day 1: Set up an overnight broth in 4mL Terrific broth-Amp (completed by 5:00 p.m. for appropriate overnight growth).

1. Setting up the large broths requires these components in set amounts:
   - 50.8 g/L of Terrific Broth powder medium
   - 4 mL of glycerol
   - 38 mg/L of riboflavin
   - 1L of de-ionized water

2. Once all broth has been mixed, it is ready to be placed in the autoclave (liquid setting, 20 min. @ autoclaving pressure & temperature, 15 min. total)
   - after autoclaving and cooling of broth to room temperature, add: Amp
   - 5 mL/L Ampicillin stock (15 mg/mL stock in 50% EtOH, -20°C)

3. Sterile procedure: The 4mL broth can be found in the 4°C refrigerator with the appropriate labeling (Terrific broth with Amp, in a 100mL rectangular bottle).
   In the sterile tissue culture hood, aspirate 4 mL from this stock broth and aliquot 2mL each to two sterile test tubes.

4. Inoculate the 4 mL overnight broth with your stabilate of choice (stored in -80°C freezer in hallway). To do this, take a 1mL sterile pipette and stab the top of the frozen stabilate to procure a few cells in and around the pipette tip (use about as much force as you would tapping your fingers on a desk). Immerse the tip, now harboring cells, into each of the 2mL broth-containing test tubes. Leave these test tubes overnight in the incubator around the other side of the hallway (windows facing Busch Gardens). The incubator should be left shaking 220 RPM @ 37 °C.

5. Leave overnight shaking

Day 2: (10:00-11:00 a.m.) In the hood: Add all 4mL of o/n broth to 36mL pre-warmed Terrific broth-Amp combined in a small, sterile, baffled flask – the ones on the shelf over the PCR and scales. Return this 40mL total starter culture to the same incubator where the test tubes were placed overnight. Allow the culture to grow for ~2-3 hours.

1. (~12:30 p.m.) One hour before inoculating larger broths, put broths at 37°C with shaking to pre-warm the media (incubator down the hall in autoclave/rotor room).
2. (~1:30 p.m.) Before inoculating, take 1mL of broth from a 1L broth for OD\textsubscript{600} blank. Then, in the hood, inoculate 1000mL Terrific broth-Amp\textsuperscript{75}-100\textmu M riboflavin with 10mL starter culture/each (usually grow 4L total) and grow until OD\textsubscript{600} = 0.5-0.8. (doubling time 45-50 min)
   - OD = cell density @ 600 nm
   - Use 1 mL of broth for blank

3. (~4:00 p.m.) Check the OD. If it has reached the appropriate density, begin the induction: Add 0.2mM IPTG (2mL 100mM IPTG stock per 1000mL).
   - Put cultures back at 37°C for 20 minutes. Then,

4. Incubate at room temperature overnight ~220 RPM.
   - Leave overnight: Overnight expression at room temperature is better than 3 hours at 37°C.

**Day 3:** (10:00 a.m.) Take OD\textsubscript{600} of overnight broth (make a 1:10 dilution [900 \textmu L pure broth (can be from blank) + 100 \textmu L overnight broth culture], to bring the solution into a measurable range: OD <1). This is to monitor density and possibly calculate doubling times.

1. Transfer each 1000mL broth into large bottles (caramel colored screw-on caps on shelf over the scales) for centrifugation. Centrifuge 30 minutes at 3,500 RPM at 4°C in a JLA8.1000 rotor (Beckman Avanti J-20, 3\textsuperscript{rd} floor common room: it has a foot pedal to open it. The various rotors are on the shelf by the cold room. This process requires the largest rotor which has silver rimmed wells - the only ones big enough to fit the large bottles).

2. After centrifugation, the supernatant should be almost clear. It will be the honey/tea color of the original broth with no colloidal appearance (cells should be aggregated at the bottom). Decant the supernatant in the corner sink of our lab, by the Eppendorf centrifuge.

3. Resuspend pellet by:
   - Adding 10mL COLD 1x PBS and resuspending with a transfer pipet to a 50mL conical tube (best performed with PBS on ice; keep the samples on ice as well – three ice buckets are ideal but two buckets with alternation of the samples will suffice).
   - Rinse bottles with another 10mL PBS and transfer to same 50mL conical tube

4. Centrifuge 3,500 RPM (~2,500 x g) 20 minutes at 4°C (Eppendorf centrifuge).

5. Decant supernatant.

6. Freeze the pellet (about 5-10mL per 1 L culture) at -20°C until further use.

**CANNOT FREEZE PROTEIN AFTER THIS POINT, once cells are lysed**

**B. Use French Press to Obtain Bacterial Cell Lysate**

1. Add 25mL lysis buffer to each pellet (make sure to do this individually).
2. Add 25\textmu L 50mM PMSF to each sample (50mM PMSF as 1000x fold stock, will give 50\textmu M)
3. Use French Press in Ferreira Lab on second floor according to instructions. Run each 30mL sample twice through French Press.
4. Spin 12,000 x g (~10,000 RPM) 15 minutes at 4°C in JA-20 rotor (autoclave room, 3rd floor). Use round-bottom bicarbonate tubes.

C. **Nickel Resin Binding**

NOTE: Use 1/5 volume nickel resin to supernatant sample (25mL supernatant will need 10mL resin (5mL gel bed)

1. Spin down nickel resin at 500 x g (1576 RPM) for 2 minutes at 4°C to obtain gel bed. Decant liquid portion (retain resin).
2. Wash nickel resin (if using new batch from Qiagen bottle) to equilibrate with lysis buffer by adding 5mL lysis buffer and spin as before. Repeat 2x (3x total). Decant liquid (should be 5mL gel bed).
3. Resuspend in 5mL lysis buffer (10mL total because gel bed=5mL)
4. Add each 25mL cell lysate sample to 5mL resin in 50 mL conical tube
5. Add 50μM PMSF to each sample (50μL from 50mM stock, 25μL/sample)
6. Rotate for 1 hour at 4°C. (on belly dancer in the fridge)
7. Spin down 2,000 x g (~3000 RPM) for 5 minutes at 4°C
8. Decant (pour out supernatant) liquid portion, retaining resin with bound protein at bottom of tube.
9. Wash by adding 5mL lysis buffer to each sample, capping conical tube, and inverting several times to mix (or flick, agitate the bottom of the conical). Centrifuge 500 x g for 2 minutes at 4°C. Decant liquid portion. Repeat for a total of 10 washes.

Preparation of nickel resin column:
10. Add 1mL lysis buffer to empty blue column to equilibrate column.
11. Add all resin-sample mixture to column and let it flow through by gravity (waste). This is the blue column. Pour slowly down the side to not disturb the resin
12. Elute with ~10-15mL of elution buffer (imidazole elution buffer) by gravity.

D. **Concentration of Samples**

1. Use Amicon Ultra-4 Ultracel-10K (proteins less than 10 kDa flow through, including imidazole).
2. Equilibrate Amicon membrane by adding 1mL lysis buffer and spinning at 3400 RPM 4°C for 10 minutes.
3. Add samples to Amicon concentrator. For a 5mL sample per Amicon tube, centrifuge 10 minutes at 3400 RPM 4°C. There should be ~500μL left. If there is more liquid, spin an additional few minutes.
4. Use 100μL of lysis buffer to get remaining protein from the inside of the membrane with a small pipette

E. **Size Exclusion Chromatography**

1. Make sure the Superdex 75 column has been equilibrated with the corresponding buffer (stock TBS)!
2. Yellow skinny column holding only 7 mL
3. Add buffer to the reservoir chamber and turn on peristaltic pump to 1mL/min setting to run fresh buffer through the column. Do this for ~ 5 minutes.
4. Add all of the nickel column elution (concentrated to ~0.5mL) to the column apparatus.
5. Use a small pipette to slowly add the elution, so as not to disturb the SE column
6. Watch for yellow elution of cb5r running through the column. Collect samples ~5 minutes before and after yellow color is seen.
7. Concentrate samples as previously described in Section D. The sample volume can range from 250-500µL, depending on how concentrated you want it. Centrifuge for 20 minutes (for 2 mL) x 3500 rpm to get the 0.5 mL of the flavin containing protein
8. At this point, you can perform spectral analysis to see if bound-flavin peak at 461nm and characteristic shoulder at 480nm is visible for cb5r samples. (Set up a 1:100 dilution using TBS buffer and see protocol on Spectral Analysis).

Freeze sample in liquid nitrogen by pipetting drop wise into a liquid nitrogen bath. Store at -70°C.
- Use 100 µL per pipet to make the protein beads (each drop will be about 10-15 µL)
- Freeze immediately in the -70°C freezer (in hallway, top shelf, in recombinant protein box)

TBS Buffer (For Equilibration and Lysis Buffers)
\[
\begin{align*}
50\text{mM Tris} & \quad 6.057\text{g Trizma base} \\
200\text{mM NaCl} & \quad 11.688\text{g NaCl}
\end{align*}
\]
Dissolve in 950mL water and pH to 8.0 with HCl. Bring up to 1000mL and filter sterilize.

2X Stock TBS Buffer (For Elution Buffer)
\[
\begin{align*}
100\text{mM Tris} & \quad 1.2114\text{g} \\
400\text{mM NaCl} & \quad 2.3376\text{g}
\end{align*}
\]
Add to 100mL water and pH to 8.0 with HCl. Filter sterilize.

Lysis Buffer (Makes 100mL)
\[
\begin{align*}
50\text{mM Tris} \\
200\text{mM NaCl} \\
20\text{mM Imidazole} & \quad (4\text{mL of 500mM stock}) \\
0.2\text{mM EDTA} & \quad (40\mu\text{L of 0.5M stock}) \\
0.1\% \text{ Triton-X 100} & \quad (1\text{mL of 10% stock})
\end{align*}
\]
Protocol 2:

Kinetics and Biochemical Assays

A. Spectral Analysis Using UV Absorbance

1. Start up computer connected to HP8453 UV-Vis Spectrometer
2. Use wavelengths: 272nm and 461nm
3. Background correction: ‘substrate average over range’ 800-900nm
4. Data type: Absorbance
5. Display spectrum: from 250 to 750nm
6. Turn on UV and Tungsten lamps
7. Make a blank of absorbance with 200 μl of 10 mM KPi buffer in quartz cuvette labeled 6Q
8. Prepare 1:8 dilution of protein sample (25 μl sample + 175 μl 10mM KPi buffer: 10 mM KH₂PO₄, 0.1 mM EDTA, pH 7.0)
9. Mix sample in cuvette and place it in the spectrophotometer, then click ‘sample’
10. Record Net 272nm, Net 461nm

\[
\text{Purity} = \frac{\text{Net 272nm}}{\text{Net 461nm}} \quad \text{Concentration} = \frac{\text{Net 461nm}}{10.6}
\]

B. Kinetics and Activity Assay (UV-2101PC Spectrophotometer)

1. Turn UV Spectrophotometer on first, then turn on the computer
2. Set up spectrophotometer parameters as followed
   Acquire Mode - Time Course
   Configure – Parameters:
   - Low: -0.100
   - High: 1.300
   - Wavelength: 340 nm
   - Slit width: 20nm
   - Timing mode: auto
   - Reaction time: 60 sec

C. For ACTIVITY assay, follow this pipette order:

- 974 μL MOPS
- Insert stir bar
- 15 μL K₃Fe(CN)₆
- Click ‘Auto Zero’ – 340nm absorbance should be ~0.000
- 10 μL NADH (10 mM stock) – 340nm absorbance should be ~0.622
- Add 1 μL 5μM protein stock and immediately click START
- Record 40 seconds of activity
- Save the data with a suitable 3 character name
- Click Manipulate at Data Print and select your data file
- Highlight from 8 seconds until 38 seconds and click Edit and Copy
D. For INHIBITOR assay, follow this pipette order:

- 924 µL MOPS
- Insert stir bar
- 15 µL K₃Fe(CN)₆
- 50 µL Inhibitor
- Click ‘Auto Zero’ – 340nm absorbance should be ~0.000
- 10 µL NADH (10 mM stock) – 340nm absorbance should be ~0.622
- Add 1 µL 5µM protein stock and immediately click START
- Record 40 seconds of activity
- Save the data with a suitable 3 character name
- Click Manipulate at Data Print and select your data file
- Highlight from 8 seconds until 38 seconds and click Edit and Copy
- Paste into the Excel Template and record column **G (activity rate)** and the **b intercept** from the graph (initial NADH absorption).
Appendix B: Permissions

CDC Permission:

RE: Permission to Use a Figure [ ref: _00DU0YCBU__500U05dxuO:ref ]
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To: Ashley, Al

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Thank you.

DAH/AK 5457

------------- Original Message -------------
From: Ashari, Ala (aalashari@health.usf.edu)
Sent: 11/3/2012 8:17 AM
To: cdcinfo@cdc.gov
Subject: Permission to Use a Figure

To whom it may concern,

My name is Ala Ashari, I am a master student at the college of public health, University of South Florida, Tampa FL. I am writing my thesis and I would like to include the CDC Leishmania life cycle figure on it. Do I need a permission from CDC to do so? This is the link for the CDC leishmania life cycle figure. http://www.cdc.gov/parasites/leishmaniasis/biology.html

Kind Regards,
Ala Ashari

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