PURIFICATION AND CHARACTERIZATION OF A CYCLOOXYGENASE-LIKE ENZYME FROM *Trypanosoma brucei*

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

I hereby declare that this research thesis is my original work and has not been submitted wholly or in part for any award in any other institution of learning

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We confirm that this research thesis was done under our supervision and has our approval to be presented for examination as per the Egerton University regulations

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DEDICATION

I dedicate this thesis to my parents, Jean Chrysostome Mudogo Virima and Agnes Vumilia Nsigayehe for the sacrifices they made towards my education.

To my family members and friends for being there for me and giving me moral support that enabled me to successfully carry out the study.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AP	Alkaline phosphatase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BecANet	Biosciences eastern and central Africa Network
Bp	Base pair
CGA	Citrate glucose anticoagulant
COX	Cyclooxygenase
DEAE	Diethylaminoethyl
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EC	Enzyme commission
EDTA	Ethylene diamine tetra acetic acid
FPLC	Fast protein liquid chromatography
HAT	Human African trypanosomiasis
HDL	High density lipoprotein
IFIB	Interfaculty Institute of Biochemistry
IPTG	Isopropyl-beta-D-thiogalactopyranoside
MITat	Molteno institute trypanozoon antigenic type
NEPAD	New partnerships for Africa development
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Procyclic form
PCD	Programmed cell death
PGs	Prostaglandin
PGHS	Prostaglandin H_2 synthase
PGI_2	Prostacyclin
PI	Protease inhibitors
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis

TAE	Tris acetate EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered-Tween 20
TDB	Trypanosome dilution buffer
Tris	Tris (hydroxymethyl) aminomethane
TXA_2	Thromboxane
VSG	Variant surface glycoprotein

ABSTRACT

The protozoan hemoflagellate *Trypanosoma brucei* is the causative agent of African trypanosomosis in humans and nagana in domestic animals. The human disease is a health concern in many African countries. The infection of mammalian-host by African trypanosomes is characterised by an up-regulation of prostaglandin (PG) production in the plasma and cerebrospinal fluid (CSF), which modulates the host responses and causes symptoms of the infection. It has been shown that the protozoan parasite *T. brucei* is involved in PG production and that it produces PGs enzymatically from arachidonic acid (AA) and its metabolite, prostaglandin 2 alpha ($PGH_{2\alpha}$). In view of the AA cascade metabolism, the cyclooxygenase (COX) or PGH synthase (PGHS) is the enzyme that catalyses the rate-limiting step in the biosynthesis of the PGs. This work involved the purification and characterization of a COX-like enzyme from trypanosomes using chromatographic techniques such as Gel filtration and Ion exchange chromatography under the control of AKTA-FPLC system, running an SDS polyacrylamide gel and Western blot analysis for immunodetection of a COX-like enzyme from trypanosomes and cloning the COX- like gene in bacteria and/or insect cell system. A probable COX-like enzyme from the trypanosomes lysate was identified specifically in the cytosolic fraction by western blot analysis using a polyclonal anti-ovine-COX antibody. Proteome data analysis from the 10% SDS-PAGE and silver stain of the complete trypanosomes lysate, gel-digested at range of 50-95KDa generated 23 proteins probably putative COX-like protein candidates. The cytosolic fraction from trypanosomes lysate was used in an attempt to design purification strategies of the COX-like enzyme from *T. brucei* using gel chromatography. Ion exchange chromatography was done as an intermediate step and the fraction from the strong cation exchange gave absorbance in the COX-fluorometric assay, indicating the presence of a COX-like enzyme from trypanosomes. This suggested that trypanosome COX is positively charged. These observations together with the data from chromatographic steps, western blotting and COX-fluorometric assay, suggest that a COX-like enzyme exists in *T. brucei* as a cytosolic protein. Further experiments need to be done to optimize the purification, SDS-PAGE and Western blot analysis, and biochemical characterization of the recombinant COX-like enzyme. Functional and structural biology studies and phylogenetic analysis should be done to determine whether TbCOX is completely distinct from mammalian COXs. The finding that PG-producing enzymes are distinct from their mammalian counterparts makes them suitable drug targets.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The protozoan hemoflagellate *Trypanosoma brucei* is the causative agent of sleeping sickness and nagana in human and domestic animals respectively. The human disease is a health concern in many African countries. The infection of mammalian host by African trypanosomes is characterized by an-up regulation of prostaglandin (PGs) production in the plasma and cerebrospinal fluid (CSF), which modulates the host responses and causes symptoms of the infection (Pentreath *et al.,* 1990).

In the search for secreted molecules of trypanosome origin, it was discovered that trypanosomes produce PGD_2, PGE_2 and $PGF_2α$ from arachidonic acid (Kubata *et al*., 2002; Kubata *et al*., 2007). Identification and characterization of $PGF_{2α}$ synthase led to structural analysis by X-ray crystallography (Kubata *et al*., 2000).

PGD_2 and its metabolites have been demonstrated to induce programmed cell death (PCD) in *T. brucei* bloodstream forms (Figarella *et al.,* 2005; Duszenko *et al*., 2006). In addition it was also found that the stumpy but not the slender trypanosomes undergo programmed cell death through the formation of reactive oxygen species (Figarella *et al.,* 2006).

The discovery that the parasites produce PGs, just like their mammalian hosts, and that they possesses the enzymatic machineries for PGs biosynthesis has led to the identification and characterization of PG-catabolizing enzyme system in the parasites (Kubata *et al*., 2007). Cyclooxygenase (COX) or prostaglandin H synthase (PGHS) is the enzyme that catalyzes the first step in the biosynthesis of the prostaglandins.

In this pathway, following the stimulation of cells, AA is released from the endoplasmic reticulum (ER) and nuclear membranes by phospholipase A_2. AA is then converted to PGH_2, the common substrate for prostanoid biosynthesis by cyclooxygenases, namely COX-1, COX-2 or both. The action of COX can be inhibited by nonsteroidal anti-inflammatory drugs such as indomethacin and aspirin. Subsequently, the resulting PGH_2 is converted *in vivo* and *in vitro* into PGD_2, PGE_2, $PGF_2α$, prostacyclin (PGI2), and thromboxane A_2 (TXA2) by the action of their respective synthases in trypanosomes.

In trypanosomes, eicosanoids are believed to play a role in life cycle control, growth-stage specific transformation, sexual maturation and communication with the host on a cellular basis. The biochemical pathways of parasite eicosanoid metabolism are poorly characterized. However, accumulating data suggests that control of phase change and

differentiation in these organisms is controlled by oxylipins, including prostaglandins and lipoxygenase products (Noverr *et al*., 2003).

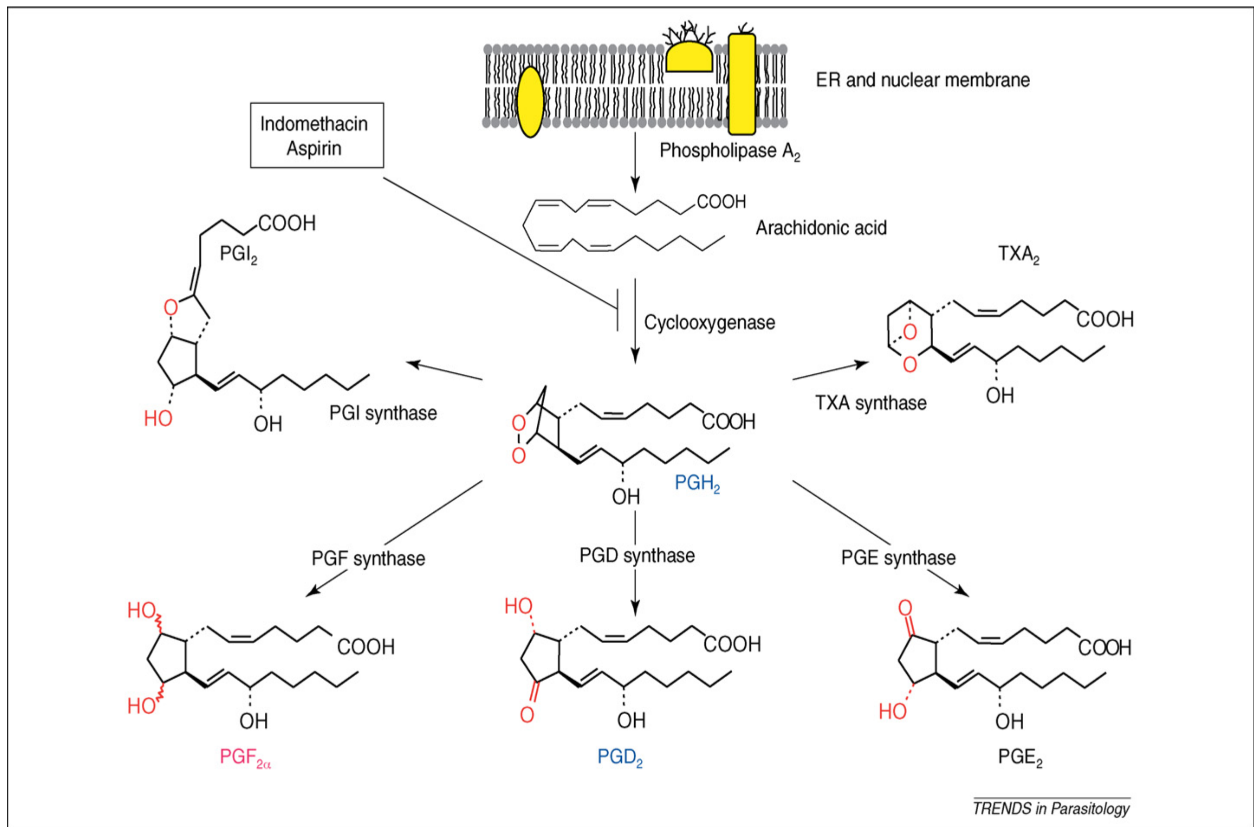

Figure 1. Arachidonic acid (AA) cascade and prostanoid biosynthesis pathway.

1.2 Statement of the Problem

The discovery of PG-synthesizing enzymes in the pathogenic parasites has generated new interest in the roles of PGs during parasitic infections. *Trypanosoma brucei* produces PGs including PGE_2, PGD_2 and $PGF_{2\alpha}$ from arachidonic acid and $PGF_2\alpha$ synthase has been identified and characterized in trypanosomes in membrane fractions. However, in view of the PGs metabolism, the structure and function of a COX-like enzyme in *T. brucei* is still poorly known. Experiments done on the enzymes involved in PG metabolism have shown that the production of PG-like compounds was not inhibitable by the classical inhibitors of PG synthesis including non-steroidal anti-inflammatory drugs (NSAID) such as aspirin and indomethacin. This finding suggested the existence of a new and possibly unique system of PG synthesis in trypanosomes with a non-classical COX-dependent first step and a non-classical synthase-dependent second step.

1.3 Objectives

1.3.1 Main objective

To determine the possible existence of an alternative system of prostaglandin synthesis in trypanosomes, clone and express its genes in bacteria and/or baculovirus expression system.

1.3.2 Specific objectives

1. To purify a cyclooxygenase-like enzyme from *Trypanosoma brucei* using chromatographic techniques.
2. To identify a cyclooxygenase-like enzyme from *Trypanosoma brucei* using SDS polyacrylamide gel electrophoresis and Western blot analysis.
3. To clone gene for cyclooxygenase in insect vector.

1.4 Hypotheses

1. Cyclooxygenase-like enzyme cannot be purified from *Trypanosoma brucei*.
2. A cyclooxygenase-like enzyme from *Trypanosoma brucei* cannot be immunologically identified by Western blotting.
3. A cyclooxygenase genes cannot be cloned in insect vector.

1.5 Justification

Efficient management of African trypanosomosis requires proper understanding of the interactions between the host and the parasite. There is evidence that the production of PG in trypanosome may play a significant role in a host "cross-talk" that contributes to chronic infection. Compared to mammalian PG production, very little is known about the biochemistry of the oxylipins production in eukaryotic microbes. Comparative genomic searches for the enzymes responsible for PG production in protozoa, helminths and fungi have not yielded a single enzyme with homology to the mammalian enzymes. The search for homologs of mammalian cyclooxygenase in protozoans has proven to be difficult. This project will bring better understanding of a COX-like enzyme and prostaglandin (PG) biosynthesis that might be responsible for programmed cell death in trypanosomes using molecular tools. The identification of the enzymes involved in prostaglandin metabolism therefore could be of great interest because they might have a key role during parasitic infections. The finding that PG-producing enzymes are distinct from their mammalian counterparts makes them suitable drug targets. As the enzymes involved in PG synthesis are

discovered, genetic manipulation might allow definitive understanding of the roles of non-mammalian eicosanoids in metabolism and pathogenesis. The possible identification and purification of a COX-like enzyme from *T. brucei* would therefore be of great interest because it would bring new insight in the understanding of its role during parasitic infections. On the other hand, as more crystal structures of these enzymatic systems are determined, it will be possible rationally to design specific and better drugs against sleeping sickness.

1.6 Expected Output

1. Characterization of a cyclooxygenase like enzyme will be done.
2. Cloning and expression of a putative COX-like gene in bacteria and/or insect cell system.
3. Better understanding of a COX-like enzyme, prostaglandins (PG) biosynthesis/arachidonic acid pathway in trypanosomes.
4. Thesis for a Master of Science in Biochemistry
5. Publications in peer – reviewed journals.

CHAPTER TWO

LITERATURE REVIEW

2.1 Background of Human African Trypanosomosis

Human African trypanosomosis (HAT), also known as sleeping sickness, is one of the "neglected diseases" (Kennedy, 2007). HAT is a vector-borne parasitic disease which primarily affects the poorest rural populations in some of the least developed countries of East and Central Africa (Berriman *et al*., 2005). Moreover, HAT is the world's third most important parasitic disease affecting human health after malaria and schistosomiasis as defined by the global burden of parasitic disease, calculated as the disability- adjusted life years lost (Kennedy, 2007).

Trypanosomes are extracellular, eukaryotic flagellated protozoa belonging to the order kinetoplatida. Some species are responsible for HAT in tropical Africa and for a similar disease called 'nagana' in livestock. HAT is caused by protozoan parasites of the genus *Trypanosoma* which undergo a complex life cycle through the bloodstream of their mammalian host and the blood-feeding insect vector, the tsetse fly (*Glossina* spp.) (Hellemond and Tielens, 2006).

About 30 species and subspecies of tsetse flies exist, and these are separated into three groups that prefer different habitats (Jordan, 1993) and show different abilities to transmit *T. brucei gambiense* or *T. brucei rhodesiense* sleeping sickness. Infection with trypanosomes starts with a blood meal on a mammalian host (Figure 2).

In man the bloodstream form show polymorphism; this includes (a) dividing (black arrows) slender forms, (b) intermediate forms, and (c) stumpy forms. In tsetse fly vector, bloodstream forms transform to (d) dividing midgut forms, then to (e) the migrating epimastigote forms, which develop in the salivary glands to (f) the infective metacyclic forms, which are injected during the next blood meal into the mammalian host (Barrett *et al.,* 2007).

There are two forms of the human disease, the East African variant caused by *Trypanosoma brucei rhodesiense* and the West African form caused by *Trypanosoma brucei gambiense*. The third subspecies, known as *Trypanosoma brucei brucei* is not pathogenic to humans, because this parasite is lysed by a component of the human serum haptoglobin in high density lipoprotein (HDL) (Vanhollebeke *et al*., 2008).

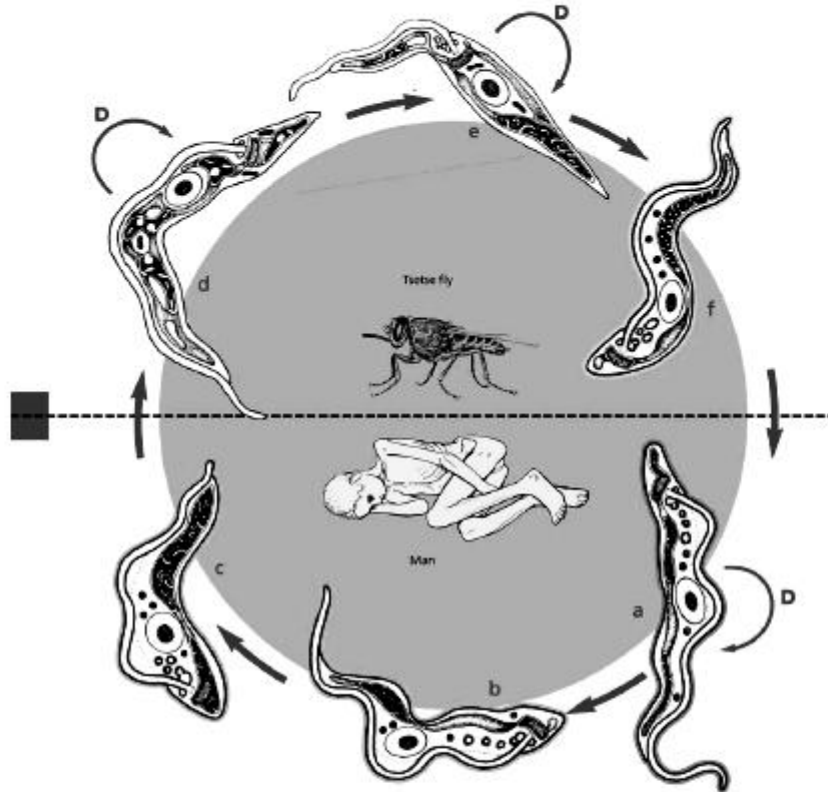

Figure 2. Life cycle of African trypanosome

Infection with African trypanosomes can result in disease manifestations ranging from asymptomatic or mild to a severe fulminating disease. *T. b. rhodesiense* is more likely to cause a rapidly progressing and fulminating disease than *T. b. gambiense*. *T. b. gambiense* tends to cause a slow progressing disease which may either be self-limiting or develop into a chronic disease involving the lymphatics and the central nervous system (CNS).

Disease progression is characterized by two stages known as the haemolymphatic stage and the meningo-encephalitic stage. Symptoms during the lymphatic stage include enlargement of lymph nodes, weight loss, weakness, rash, itching and edema as well as the continued intermittent febrile attacks. Higher parasitaemia are often associated with the disease symptoms (WHO, 2003).

A hallmark feature of African trypanosomosis is the invasion of the CNS and thus nervous system impairment. Trypanosomes crossing the blood-brain barrier result in a generalized meningoencephalitis characterized by progressively worsening symptoms. Indications of nervous impairment include apathy, fatigue, confusion, somnolence and motor changes such as tics, slurred speech and incoordination. Disease caused by either of the two parasites leads to coma and death if left untreated (WHO, 2003).

Infection of mammals by African trypanosomes is characterized by an up-regulation of prostaglandin production in the plasma and cerebrospinal fluid (Pentreath *et al*., 1990). These metabolites of AA may, in part, be responsible for symptoms such as fever, headache, immunosuppression, deep muscle hyperaesthesia, miscarriage, ovarian dysfunction, sleepiness, inflammation, bronchoconstriction and other symptoms observed in patients with chronic African trypanosomosis (Mathe *et al.,* 1977; Samuelsson, 1979; Oliw *et al.,* 1983; Glew, 1992; Dubois, 1998; Hayaishi, 2000). Disturbance of sleep cycle, which gives the disease its name, is the most important feature of the late stage disease. If the patient does not receive treatment before the onset of the second phase, neurological damage is irreversible even after treatment. If the disease is diagnosed early, the chances of cure are high. Suramin and pentamidine are used as first phase drugs and melarsoprol and eflornithine are used as treatment in the second phase of infection. However, most drugs used for treatment are old, toxic and drug resistance has developed to most of the drugs (WHO, 2003).

2.2 Cyclooxygenases

Cyclooxygenases (COXs), also known as prostaglandin H synthases (PGHS) or prostaglandin endoperoxide synthases are fatty-acid oxygenases of the myeloperoxidase superfamily that are most closely related to the pathogen-inducible oxidases and linoleate diol synthases of plants and fungi (Daiyasu, 2000). PGHS is a key enzyme in prostanoid biosynthesis and mammals have two distinct PGHS isozymes sharing about 60% sequence identity (Smith *et al*., 2000; Simmons, 2004). The constitutive isozyme, PGHS-1, is thought to function usually as a housekeeping enzyme, whereas the inducible isoenzyme, PGHS-2 is associated with cytokine and mitogen- dependent processes such as inflammation and cell proliferation (Tsai and Kulmacz, 2010). Both PGHS isozymes catalyze the same two reactions namely dioxygenation of AA to yield prostaglandin G_2 (PGG_2), containing both a 9-11 endoperoxide and a 15-peroxide group and a peroxidase reaction, which converts PGG_2 to PGH_2 where the 15-peroxide is reduced to an alcohol (Tsai and Kulmacz, 2010). The reason for the existence of the two PGHS isozymes is unknown. However, PGHS-1 and PGHS-2 are interesting in the context of both structural biology and enzymology in that they are homodimeric, heme- containing, and glycosylated proteins with two catalytic sites. Moreover, the enzymes have a novel mechanism for membrane attachment where they are anchored to one leaflet of the lipid bilayer through the hydrophobic surfaces of amphipathic helices rather than through transmembrane motifs typical of many integral membrane proteins (Smith *et al.,* 1996).

The isoenzymes are also pharmacologically important as targets of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (Marfat, 1996). Aspirin acts via PGHS-1 to inhibit platelet thromboxane A_2 and as a clinical consequence lowers the relative risk for mortality from cardiovascular disease (Patrono, 1994). PGHS-2 is the relevant target of NSAIDs acting to inhibit inflammation, fever, pain and probably colon cancer (Smith *et al.,* 1996).

In the past, very specific inhibitors of various mammalian cyclooxygenases and lipoxygenases have been discovered (Chandrasekharan *et al.,* 2004; Simmons *et al*., 2004). In the study of mammalian systems, the classical inhibitor that is employed can identify specific subtypes of these enzymes. However, the same rules do not apply to other organisms. In trypanosomes, it has been shown that the production of prostaglandins was not inhibitable by the classical COX-1/COX-2 inhibitors-NSAIDs such as aspirin and indomethacin (Kubata *et al*., 2000). This finding suggests the existence of a new and possibly unique system of PGs synthesis in trypanosomes and a non classical COX-dependent first step in PG synthesis and a non classical synthase-dependent second step. Therefore, the fact that different cyclooxygenase inhibitors have different degrees of effectiveness on nonclassical eicosanoid production is related more to the structure of the inhibitor and less to the enzyme it was originally described as inhibiting.

2.3 Cyclooxygenase Activity in Parasites

The search for homologs of mammalian COX in parasitic microbes has not been fruitful, although proteins that are similar to mammalian COX have been detected (Kubata *et al.,* 2007). More conclusive evidence of a COX activity in pathogenic parasites was obtained from the intestinal protozoan parasite *Entamoeba histolytica*. A cyclooxygenase like enzyme was isolated, characterized and cloned by reverse transcriptase PCR (RT PCR) (Dey *et al*., 2003). It possesses neither the arachidonate-binding domain nor the heme-coordinating and catalytic sites, which are conserved in other species and showed little homology with COX-1 and COX-2 enzymes from mammals and others species at the nucleotide and amino acid levels (Kubata *et al.,* 2007; Varvas *et al*., 2009). Amoeba COX expressed in *Escherichia coli* demonstrated COX-like enzyme activity *in vitro* by converting AA into PGH_2 but not into PGD_2 or $PDF_{2\alpha}$. PGH_2 production by amoeba was constitutive but greatly dependent on exogenous AA substrate. The COX-like enzyme was localized to nuclear fraction and was inhibited by 1mM aspirin but not indomethacin or other COX-1 and COX-2 inhibitors (Sanchez-Ramirez *et al*., 2004).

A $PGF_{2\alpha}$ -synthase from *Trypanosoma brucei* has been purified, cloned and expressed in *E. coli* (Kubata *et al.,* 2000). Both live *Trypanosoma brucei* and lysates of the organism are readily capable of producing a number of oxylipin products that were identified as prostaglandins (PGs). The production of these molecules was eliminated when the lysates were boiled, indicating enzymatic activity. However, prostaglandin production was not inhibitable by either aspirin or indomethacin. Taken together, these data suggest the existence of a completely different system of prostaglandin (PG) synthesis in trypanosomes, with a nonclassical cyclooxygenase-dependent first step and a nonclassical synthase-dependent second step.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Isolation of Bloodstream Forms from Rat Blood

The research work was carried at the Interfaculty Institute of Biochemistry (IFIB)/Germany where the trypanosome strain BF-221 is kept frozen and used for the propagation of the parasites in rat.

Anaesthetized rats were infected by intraperitoneally injecting 5 x 10^7 trypanosomes/ml. After 3 days of infection at peak parasitaemia, the rat was sacrificed using carbon dioxide as anaesthetic. The thorax of the animal was cut open and washed with 2 ml citrate glucose anticoagulant. The inferior vena cava was cut open and the blood collected in a pasteur pipette before being transferred into fresh falcon tube. The rest of the blood was collected by puncturing the left ventricle and blood similarly collected into falcon tubes. The blood was centrifuged at 3000rpm at 4°C for 10-15 minutes. The buffy coat containing white blood cells and trypanosomes was removed and loaded onto diethylaminoethyl-cellulose column pre-equilibrated with separation buffer ($Na_2HPO_4.H_2O$ 57mM, KH_2PO_4 3mM, NaCl 44mM and Glucose-monohydrate 55mM, pH 8.0) (Lanham and Godfrey, 1970).

The flow through the column containing parasites was collected and thereafter centrifuged at 3000rpm at 4°C for 5 minutes. The supernatant was discarded and the parasites pellet re-suspended in 5ml trypanosome dilution buffer. The parasites were then washed twice by centrifuging at 3000rpm at 4°C for 5 minutes before use.

3.2 Lysis of Trypanosomes

Trypanosomes were lysed with phosphate buffer ($Na_2HPO_4.H_2O$ 10mM, KH_2PO_4 10mM, pH 7.4) containing protease inhibitor mix (complete Roche free-EDTA) for 15 minutes, and then centrifuged at 10,000xg for 10 minutes to obtain the supernatant and the pellet fractions. The supernatant was immediately treated with phosphate buffer ($Na_2HPO_4.H_2O$ 10mM, KH_2PO_4 10mM, pH 7.4) and protease inhibitor mix. Into the pellet fraction with phosphate buffer and proteases inhibitors was added 0.1% Triton X-100 and then stored at 4°C. The lysis of the parasites was confirmed by microscopic examination.

The protein concentration of the lysate was determined by the method of Bradford (1976) using the Bio-rad protein assay with bovine serum albumin (BSA) as a standard. The protein preparation was diluted with water to the final volume of 800µl and mixed with 200µl Bradford reagents. After 15 minutes of incubation at room temperature the absorbance was determined at wavelength of 595nm using 800µl H_2O with 200µl Bradford reagent as a blank.

The protein concentration was obtained from the absorbance using a calibration curve with BSA as standard. Subsequently, all fractions were stored at 4°C till further use.

3.3 COX-like Enzyme Purification Strategies

The main goal was to purify an unknown COX-like enzyme from trypanosomes using gel filtration and ion exchange chromatography under the control of AKTA-Fast protein liquid chromatography system.

3.3.1 Gel filtration and size exclusion chromatography

Gel filtration is an important preparative technique used as a chromatographic step in the purification of proteins, polysaccharides and nucleic acids (GE Healthcare). Gel filtration separates proteins solely on the basis of size. Purification of the native COX-like enzyme from the cytosolic fraction was performed by gel filtration chromatography on a Superdex 200pg HR 10/30 pre-packed column which has optimal resolution in the molecular weight range 10,000–600,000 daltons and greatly extends the separation range of high performance gel filtration (Amersham Pharmacia Biotech). The sample consisting of cytosolic fraction from trypanosomes lysates was loaded onto the column pre-equilibrated with 10mM phosphate buffer and O.1M NaCl. The column was eluted with buffer at 0.25ml/minute to collect 1ml fraction under AKTA-Fast protein liquid chromatography system controller. Each eluted fraction was concentrated with speed vacuum. Bradford protein determination and sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting analysis were done to identify the active fraction/target fraction after gel filtration run to be used for next chromatography steps.

3.3.2 Ion exchange chromatography

Ion exchange chromatography separates biomolecules on the basis of charge (GE Healthcare). This second chromatographic step was used as an intermediate or polishing stage of the COX-like enzyme purification from trypanosomes. The strategic planning was based on the selection of an ion-exchange medium and a suitable buffer system depending on the type of ion exchange to be performed, the pH stability of the sample, the pH range to be used and the required buffering capacity. As the isoelectric point (pI) of the COX target protein was unknown, the process was begun by selecting either anion or cation exchange and optimized the pH range for each case following the pI of COX-amoeba (Dey *et al.,* 2003).

Following the SDS-PAGE and western blot analysis of each gel filtration fraction pre-concentrated with speed vacuum, the fractions which gave a cyclooxygenase antibody response were pooled and then loaded onto a strong cation SP Fast Flow HiTrap 1ml column volume (GE Healthcare manufacturer) using binding buffer (A) (50mM acetic acid, pH 5.0) and elution buffer (B) (a mixture of binding buffer and 1M NaCl). The pooled fraction sample mixed with the binding buffer was loaded onto the column at 1ml/minute flow rate as recommended by the providers with a linear gradient elution 0-1M NaCl.

On the other hand, the pooled target fraction from the gel filtration step was loaded onto a strong anion Q Fast Flow HiTrap 1ml column volume (GE Healthcare provider) with binding buffer (20mM Tris-HCl, pH 8.0) and elution buffer (20mM Tris-HCl plus 1mM NaCl) with the same flow rate, linear gradient elution and length column volume.

3.4 SDS-PAGE and Western Blotting Analysis

SDS polyacrylamide gels were used to determine the molecular weight of denaturated proteins and to check the efficiency of the protein purification. In the majority of cases 10% acrylamide was used for SDS-PAGE as described by Laemmli (1970) using the minigel-system. The entire sample was diluted 1:2 with double concentration loading buffer and denaturated at 95°C for 10 minutes. Electrophoresis was performed at 60V, for collecting the samples in the stacking gel and was increased to 140V, for separating the proteins in the running gel. A broad-range molecular weight marker (Fermentas SM1811) was loaded next to the protein samples for evaluating the molecular weight of the denaturated proteins. The cytosolic and membrane fractions were prepared and loaded on 10% SDS-PAGE, the SDS-PAGE was done in the gel chambers according to the method of Laemmli (1970).

The western blotting was used as an analytical method for immunological detection of proteins on nitrocellulose membranes using a semi-dry-apparatus as described by Towbin *et al.* (1979). Three filter papers were laid on the anode plate. All the materials were soaked with transfer buffer before use. The electro-transfer was carried out at a constant electric current of 5-10mA/cm^2 for 30-45 minutes. The blotting membrane was blocked for non specific sites by incubating it in 10% milk powder overnight on a shaker in a cold room.

The polyclonal anti-ovine-COX-1 was used as the primary antibody (1:1000) and anti-rabbit IgG alkaline phosphatase (AP) as secondary antibody (1:15000) using Tris buffered saline–Tween 20 (TBS-T).

The nitrocellulose membrane was then incubated with the presence of the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP-7.35mg in 250µl 99% dimethylformaldehyde (DMF) and

nitroblue tetrazolium (NBT- 16.35mg in 500µl 70% DMF) as substrates. The 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and NBT solution were dissolved in 50ml alkaline phosphatase buffer (Tris 20mM, NaCl 150mM, $MgCl_2$ 10mM, pH 8.9) for each blot.

3.4.1 Gel staining

Following electrophoresis the gels were stained in 1mg/ml Commassie blue R for at least 4 hours. The gels were destained in destaining solution (10% acetic acid, 40% ethanol) for about 2 hours.

Silver staining is 30-50 fold more sensitive than the staining by Coomassie blue dye (Sasse and Gallagher, 2009). During the whole procedure of silver staining, the gels were shaken continuously. The gels were fixed in fixative (24ml acetic acid, 80ml ethanol, 100µl 37% formaldehyde and 200ml distilled water) for 10 minutes, washed with ethanol (50%) 3 times for 10 minutes each and then incubated for 1 minute in incubation buffer (400µl of 10% $Na_2S_2O_3.5H_2O$, 200ml distilled water). The gels were washed three times in water for 20 seconds. The gels were then incubated in silver stain solution (0.4g Silver nitrate, 150µl 37% formaldehyde and 200ml distilled water) for 20 minutes and then further washed for 2 minutes with water. Thereafter the gels were soaked in developing solution until bands or protein spots appeared. The gels were treated with stop solution (40% ethanol and 12% acetic acid) and were washed with 50% ethanol for 20 minutes. Later the gels were preserved by "sandwiching" the gels between two sheets of wet cellophane in gel storage solution (25% ethanol and 2% glycerol). Excess solution and air bubbles between the cellophane sheets and the gels were expelled by applying pressure using a ruler.

3.4.2 Gel digestion for mass spectrometry analysis

The gels were placed on a clean glass plate or plastic foil and rinsed with water. The band of interest was cut with a clean scalpel and the excised bands chopped into pieces of approximately 1x1mm and the cubes transferred to a clean micro-centrifuge tube. The gels were hydrated, dehydrated and washed with digestion buffer. The gels were then dried in a speed-vac after addition of trypsin solution followed by the spectrometric analysis of the digested peptides by method of Rosenfeld (1992).

3.4.3 Fluorometric assay

The COX isozymes both have sequential cyclooxygenase and peroxidase activities within the same holoenzyme. When oxidized by COX-1 or COX-2 in the presence of arachidonic acid, the colorless and nonfluorescent amplex red reagent (10-acetyl-3, 7-dihydroxyphenoxazine) is converted to the highly fluorescent dye resorufin (7-hydroxyphenoxazone) (Batchelor *et al.,* 2003). The microplates assay provides a high-throughput method for identification of COX-1 and COX-2 inhibitors.

3.5 Cloning and Expression of the COX-like Gene in Bacteria and Insect Cells

3.5.1 Cloning

DNA sequence of Cox-like gene was selected from GeneDB for cloning experiments. Both primers for cloning (sense and anti-sense) were designed according to the gene sequence data and plasmids multiple cloning restriction sites.

3.5.2 PCR using phusion DNA polymerase

The PCR reactions were performed using phusion high-fidelity DNA polymerase kit (BioLabs) according to the manufacturer's instructions. The reaction mixture was made of a reaction mixture consisting of distilled water (50µl), 5x Phusion HF buffer (10µl), 10mM dNTPs (0.2µl), sense primer (1:10 diluted) (1µl), antisense primer (1:10 diluted) (1µl), template DNA (1µl) and phusion DNA polymerase (0.5µl).

The mixture was run in PCR reaction programme cycle of an initial denaturation at 98°C for 30 seconds, denaturation at 98°C for 5-10 seconds. The primer annealing step depended Tm of primer pairs and extension was performed at 72°C for 15 or 30 seconds per 1kb.

The PCR product consisting of 25µl of the PCR product, 5µl of bromophenol and 4µl of 1kb DNA ladder gene ruler (MBI Fermentas, Germany) was subjected to electrophoresis in 1% agarose gel at 80V, 200mA for 45 minutes. The gel was stained with ethidium bromide, analyzed and visualized under ultraviolet light.

3.5.3 DNA digestion with restriction enzyme

The restriction enzymes and respective buffers were used to digest plasmid DNA and constructs. According to required analysis, the plasmid and plasmid constructs with gene of interest were checked for suitable restriction enzyme using DNAMAN software. The reaction mixture consisted of DNA, respective buffer for each enzyme, restriction enzyme and elution

buffer to make up a volume of 25 µl. The reaction mixture was incubated at 37°C and for different time span depending on enzyme and DNA concentration.

3.5.4 Agarose gel electrophoresis for DNA

After running the PCR with Phusion DNA polymerase, the reaction product was verified on agarose gel electrophoresis. The product was then cloned into sub-cloning PJET 1.2/Blunt as described in section 3.5.6.1.

The gel was polymerized by weighing agarose (1% agarose gel) dissolved in 1X Tris acetate EDTA (TAE) buffer by heating in microwave oven until the agarose was completely solubilized and solution turned clear. The solution was left to cool and immediately ethidium bromide was added. The sample was prepared by adding loading buffer to the calculated amount of DNA and then the sample and also 1kb DNA ladder (Fermentas) loaded into gel.

3.5.5 DNA extraction

The DNA was resolved in agarose gel, the gel was placed on a UV transilluminator and the desired DNA band immediately cut out from the gel with a clean scalpel. The gel slice was placed in a pre-weighed sterile eppendorf tube. The gel mass was determined in a weighing balance. QIAquick gel extraction kit (Qiagen) was used as per manufacturer's instruction to extract the DNA from the agarose slice. The DNA concentration and quality were measured at wavelength of 260nm, 280nm and 320nm using a spectrophotometer.

3.5.6 Ligation of PCR products and bacterial transformation

3.5.6.1 *Blunt-end cloning protocol*

A volume of 10µl of 2X reaction buffer, 2µl of purified PCR products, 6µl water nuclease-free, DNA blunting enzyme, 1µl of pJET1.2/blunt cloning vector (50ng/µl) and 1µl T4 DNA ligase was added, vortexed briefly and centrifuged for 3-5 seconds. The ligation mixture was incubated at room temperature (22°C) for 5 minutes and further extended for 60 minutes. The ligation mixture was used directly for bacterial transformation. The ratio of 1:3 was used for the ligation reaction.

3.5.6.2 *Transformation using one shot top10 competent cells protocol*

The ligation reaction mixture to transform competent *E. coli* cells was prepared and then placed on ice for 30 minutes and incubated for exactly 45 seconds at 42°C in a water bath. The vial was then placed on ice. Super optimal broth (SOB medium) with catabolite

repression (SOC) medium was added to the ligation reaction mixture and competent *E. coli* cells prepared then the vial shaken at 37°C for exactly 1 hour at 225rpm in a shaking incubator. The content of the vial was then poured into two different LB-ampicillin agar plates pre-warmed at 37°C for at least 20 minutes. The plates were incubated at 37°C overnight for at least 17 hours for blue-white color screening.

After transformation, the selected colonies were immediately picked and put on an overnight culture at 37°C to give them time to multiply into a solution composed of LB, sterile water and ampicillin (50mg/ml). A negative control solution was added and then cultures were analyzed by PCR, plasmid isolation and sequencing.

3.5.7 Bacterial culture stabilates

The fresh starter culture of tested clones and sterile 50% glycerol were added in volumes of 700µl and 300µl, respectively in cryo-vials. The tubes were immediately frozen in liquid nitrogen.

3.5.8 DNA purification

3.5.8.1 *MiniPreparation (MiniPrep)*

After transformation, the colonies were selected and inoculated into respective liquid LB medium with ampicillin (50mg/ml). The culture was propagated at 37°C with shaking at 225-250rpm overnight for 17 hours. All the buffers used were from QIAprep spin Miniprep test kit (Fermentas). The starter culture was harvested by centrifugation at 13,000rpm for 2 minutes. The supernatant was discarded and the pellet was re-suspended in P1 buffer at room temperature. The re-suspended cells were lysed by adding alkaline P2 buffer, gently inverting the solution 4-6 times and then let to stand for 2 minutes. The solution was neutralized with 350µl N3 buffer and then inverted 4-6 times. The solution was then centrifuged for 10 minutes at 13,000rpm and the supernatant pipetted out into a new eppendorf tube and then applied to the QIAprep spin column. The solution was centrifuged again for 60 seconds and the flow-through discarded. The QIAprep spin column was washed by adding PE buffer and then centrifuged for 60 seconds. The flow-through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column was placed in a clean microcentrifuge tube. The DNA was eluted by adding EB buffer (10mM Tris-Cl, pH 8.5) or water to each QIAprep spin column. The content was left to stand for 1 minute and then centrifuged for 1 minute.

If necessary, the MidiPrep was performed following the NucleoBond AX (MidiPrep) handbook procedures.

3.5.8.2 *Bacterial colony PCR*

The PCR was performed using HotStar *Taq* master mix kit from Qiagen. The PCR reaction was performed initially by activation at 95°C for 15 minutes, denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute followed by 35 cycles to step 2 and final extension 72°C for 10 minutes with an end stage at 4°C for 9 hours. After PCR reaction, 10µl from each sample was loaded and run in agarose gel with DNA ladder in order to identify correct clones.

3.5.9 DNA sequencing

The isolated plasmid constructs after verification on agarose gel electrophoresis and DNA quantification were sent for sequencing to GATC Biotech using single Run 24 supreme reaction in concentration of 30-100ng/µl. The results of sequenced DNA were analyzed with DNAMAN software by alignment with plasmid construct.

3.5.10 Cloning of COX-like gene from *Trypanosoma brucei*

The primer design of both sense and anti-sense primers of the COX-like gene was synthesized using DNA sequence from GeneDB, hosted by Sanger Institute (http: //www.genedb.org/).

The Cox-like enzymes were synthesized by plasmid-directed expression in *E. coli* and baculovirus system. Plasmid carrying resistance genes to particular antibiotics were used as vectors so that treatment with antibiotics was used as an indicator of plasmid/vector maintenance in *E. coli* cultures. Appropriate restriction enzymes were used to cut both the flanking ends of the amplified genes and the plasmid vector. Amplified genes cut with particular restriction enzymes were then introduced into the multiple cloning site of a plasmid vector which had been cut with the same restriction enzymes. The sticky ends of the gene and the vector, annealed to each other by their complementary base pairing as they were generated by the same restriction enzyme. The gene was then ligated into the vector by the use of a ligation kit which exploits the action of DNA ligase. All products were verified by DNA sequencing. The generated recombinant plasmid was then used to transform the appropriate competent *E. coli* by heat shock treatment. The transformed *E. coli* was then grown in the most suitable growth medium and isopropyl-beta-D-thiogalactopyranoside

(IPTG) induction of expression of the recombinant genes was carried out at optimized levels during the growth of the *E. coli*.

The gene of COX was also cloned into baculovirus expression system. The construct of COX with Pjet 1.2 subcloning/Blunt vector and baculovirus vector pFastBacHTa was digested with restriction enzymes simultaneously to cleave out the insert DNA with these specific sites. Afterwards, pFastBacHTa and insert DNA were purified from agarose gel using QIAquick gel extraction kit (Qiagen). The purified insert DNA and pFastBacHTa were used to transform into One Shot Top 10 competent cells. The colonies were verified by bacterial colony PCR and starter culture was cultivated for Miniprep. The correct DNA construct was used to transform into MAX Efficiency®DH10Bac competent cells by using transformation protocol on selective LB-agar plates containing kanamycin, gentamycin and tetracycline. The white colonies were picked and analyzed to verify successful transposition to the Bacmid. The recombinant Bacmid DNA with insert were analyzed by PCR using M13 sense and anti-sense primers with HotStar *Taq* master mix kit from Qiagen and run on agarose gel electrophoresis. Finally, correct clones were picked and starter culture cultivated for Miniprep. The DNA from Miniprep was solubilized in a specific elution buffer and another starter culture from the same clone was used for stabilates.

3.5.11 Heterologous expression of COX-like gene in *E. coli* and Sf 9

3.5.11.1 *Expression in bacteria*

The transformants of TbCOX with pProExHTa in BL-21 (DE3) expression competent cells were cultivated overnight for 17 hours at 37°C with shaking at 225rpm. The logarithmic phase growing bacterial cells from starter culture was inoculated into LB liquid medium with ampicilin for BL 21-DE3 in the ratio of 1:50. The cells were cultivated at 37°C with shaking at 225rpm till the absorbance of culture reached 0.4 at 600nm, which took 2 to 3 hours. Subsequently, the culture was divided into portions. One portion of the culture was designated as control culture without induction and other cultures were induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) to final concentrations ranging from 0.1mM to 1mM. All the cultures were additionally grown for different experiments at different temperatures at 16°C, 27°C or 37°C for a time span of 9 hours. The cells were subsequently harvested by centrifugation at 3000xg for 15 minutes at 4°C. The medium was discarded from control and induced cells. The cell pellets were frozen at -20°C. The pellets were then re-suspended in 5ml of column buffer and the suspensions sonicated 5 times using short pulses of 10 seconds on ice in a Sonifer cell disparaptor B-30.

The lysates were centrifuged at 5000rpm for 20 minutes at 4°C. The supernatant were separated and protease inhibitor mix (complete Roche–free EDTA) immediately added after centrifugation. The pellet was re-suspended in column buffer with protease inhibitor mix. Supernatant and pellet protein content were estimated with Bradford method and were analyzed on SDS-PAGE under reducing conditions.

3.5.11.2 ***Expression in Sf9 insect cells***

After confirming that the recombinant Bacmid contains the gene of interest, the material was used to transfect insect cells and produce recombinant baculovirus. Cellfectin reagent was used for transfection of the recombinant Bacmid into the *Spodoptera frugiperda* Sf9 insect cells as the host for the baculovirus transfer vector following the baculovirus expression system (Invitrogen). The insect cells were cultured under serum-free medium (Invitrogen). The serum-free medium was optimized for the growth and maintenance of Sf9 cells and for large-scale production of recombinant proteins expressed using Bac-to-Bac system (Invitrogen). After a period of 72 hours following transfection, the cells were daily visualized for signs of infection using an inverted microscope.

The transfected cells from well plate with signs of late stage infection were collected with the medium containing virus in a volume of approximately 2ml and were transferred to sterile falcon tubes. The suspension was centrifuged at 1700rpm for 5 minutes at 4°C to remove cells and large debris. The clarified supernatant containing viruses was transferred to another sterile falcon tube and this was labeled as the P1 stock with low viral titer, protected from light and stored at 4°C. The amplification of the PI viral stock and cell lysis was then done following the baculovirus expression system (Invitrogen).

CHAPTER FOUR
RESULTS

4.1 Protein Analysis

The results for the protein levels for the various proteins fractions are presented in table 1. The cytosolic fraction had protein levels of 1.96µg/µl while the membrane fraction had protein level of 16.8µg/µl. The membrane fraction was 8 fold more concentrated than the cytosolic fraction and did not show a COX antibody response in the western blot analysis. The cytosolic fraction had a lower abundance of protein and the intensity of the COX band on the blot was variable depending on the number of washing steps.

Table 1: Protein quantification using the Bradford reagent at OD 595nm using the standard curve A= 0.06C + 0.079 where A represent the absorbance and C represent the concentration

Sample	Absorbance (A)	Concentration (C= A/0.06)
Cytosolic fraction	0.235	1.96
Membrane fraction	2.016	16.8

4.2 Western Blot Analysis

Western blot analysis of cytosolic fraction showed a possible cyclooxygenase with a molecular weight of approximately 72 KDa. As shown in figure 1, the antibody detected COX in the cytosolic fraction of the trypanosomes lysate and protein of approximate of 72KDa. Lanes 2 and 6 show a strong cyclooxygenase signal from the first step of subcellular fractionation after sample concentration under speed vacuum. The COX Immunodetection signal was stronger after sample concentration under the speed vacuum while those signals was weak after differential centrifugation and with more wash steps of the trypanosomes lysates.

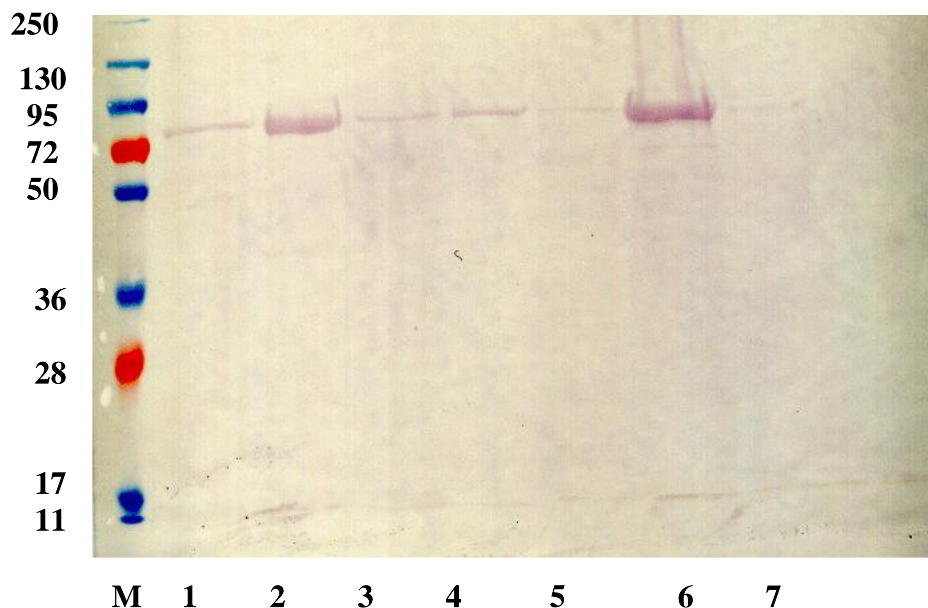

Figure 3: Western blotting analysis of the cytosolic α membrane fractions from the trypanosomes lysates (10 µg per lane) showing where the possible Cyclooxygenase protein (72KDa) located in the cytosolic fraction of the trypanosomes lysate.

Lane 1, 3, 4, 5 show a weakness cyclooxygenase signal protein (72KDa) after 1, 2, 3 and 4 washing steps respectively, Lane 2, 6 shows a strong cyclooxygenase signal after sample concentration under speed vacuum. Lane 7 indicates no immunoreactive COX-protein from the membrane fraction and Lane M represents the molecular weight protein marker (Fermentas SM 1811-KDa).

4.3 SDS-PAGE and Silver Stained of the Complete Trypanosomes Lysate

The complete trypanosomes lysate was passed through 10% SDS-PAGE and silver stained for protein detection and gel digestion at range of 50-95KDa. As shown in figure 4 the gel was cut off at approximate 72-95KDa and sent for proteome analysis. The proteome generated proteins data base of 23 proteins as shown in table 2 which could be possible candidate proteins and also putative COX-like proteins found in complete trypanosomes lysate. The variant surface glycoprotein (50 KDa) was also visible. In this data 5 out of 23 proteins are hypothetical proteins by means proteins whose existence have been predicted, but for where there is no experimental evidence that it is expressed *in vivo*. The 5 hypothetical proteins might be the one related to the COX-like protein from *Trypanosoma*

brucei and need to undergo structure and function prediction by the application of computational methods and Bioinformatics.

Figure 4 : Silver stained SDS-PAGE gel of complete trypanosomes lysate. All the lanes were loaded with 10 µg of protein. Also shown is an excision of the gel band between 72 and 95 KDa. The variant surface glycoprotein (VSG) of trypanosome was visualized at around 50KDa.

Table 2: Proteins data base identification of silver stained bands cut off range 50-95KDa

Protein number	Protein description	Protein mass (Da)
1	Beta prime COP protein [*Trypanosoma brucei*]	95022
2	Prolyl-tRNA synthetase [*Trypanosoma brucei* TREU927]	92328
3	C-terminal kinesin KIFC1 [*Trypanosoma brucei*]	91457
4	Hypothetical protein [*Trypanosoma brucei* TREU927]	88849
5	Methionyl-tRNA synthetase [*Trypanosoma brucei* TREU927]	87484
6	Asparaginyl-tRNA synthetase [*Trypanosoma brucei* TREU927]	85386
7	Oligopeptidase A [*Trypanosoma brucei brucei*]	84770
8	Heat shock protein 83[*Trypanosoma brucei* TREU927]	81169
9	Hypothetical protein [*Trypanosoma brucei* TREU927]	80142
10	Arginyl-tRNA synthetase [*Trypanosoma brucei* TREU927]	79124
11	Hypothetical protein [*Trypanosoma brucei* TREU927]	78735
12	Prolyl oligopeptidase [*Trypanosoma brucei*]	78005
13	Tyrosyl-tRNA synthetase [*Trypanosoma brucei* TREU927]	77635
14	Adaptin complex 1 subunit [*Trypanosoma brucei* TREU927]	76688
15	Hypothetical protein [*Trypanosoma brucei* TREU927]	75312
16	Acetyl-CoA synthetase [*Trypanosoma brucei* TREU927]	75246
17	Intraflagellar transport protein component [*Trypanosoma brucei* TREU927]	72675
18	Ubiquitin carboxyl-terminal hydrolase [*Trypanosoma brucei* TREU927]	71904
19	Phenylalanyl-tRNA synthetase [*Trypanosoma brucei* TREU927]	71293
20	Transmembrane glycoprotein [*Trypanosoma brucei brucei*]	68271
21	ABC transporter [*Trypanosoma brucei* TREU927]	67993
22	Hypothetical protein [*Trypanosoma brucei* TREU927]	62133
23	Variant surface glycoprotein MITat 1.8 [*Trypanosoma brucei*]	50790

4.4 COX-like Enzyme Purification Strategies

4.4.1 Size exclusion chromatography

The size exclusion chromatography fulfilled a dual role in being the first purification step and also allowing the determination of the COX approximate molecular weight at 72KDa. The size exclusion chromatography was used to fractionate the cytosolic protein fractions. The eluted profile of COX-like enzyme on HR 10/30 Superdex 200 pg column for the first run is show in figure 5.

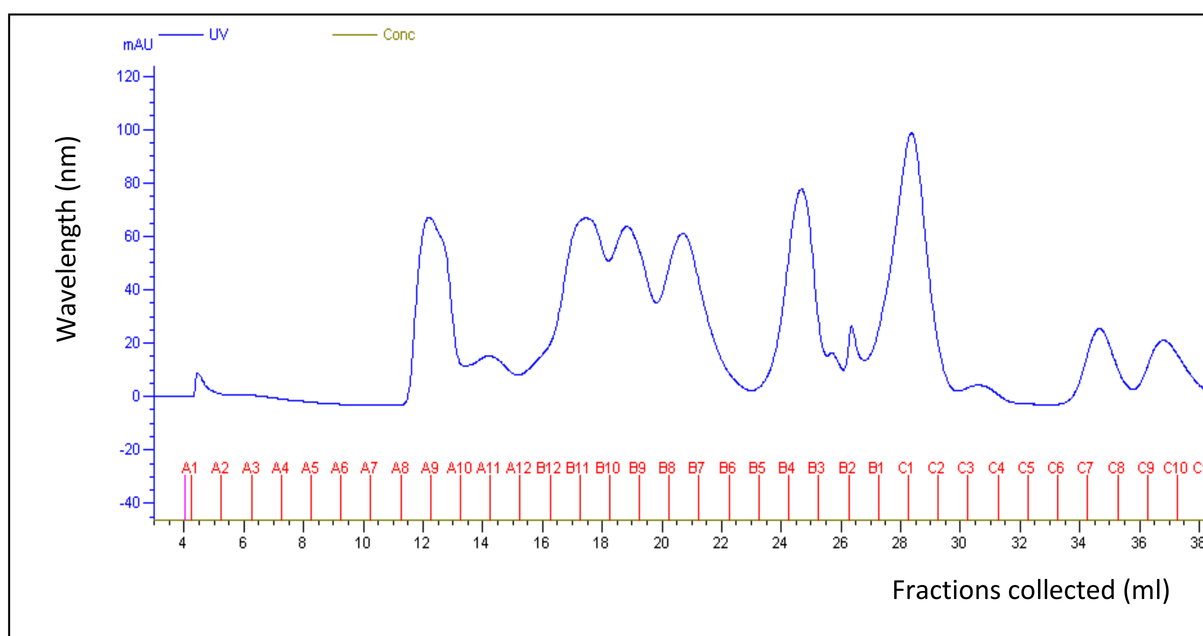

Figure 5: Chromatogram illustrating protein fractionation. A Superdex 200pg HR 10/30 column was used. The sample consists of cytosolic fraction from trypanosome lysate (1.96µg/µl).

This figure showing (X-axis) different fractions collected and (Y-axis) absorbance wavelength (nm). The size exclusion chromatography gave several peaks that were different in height absorbance. The first run was unsatisfactory suffering from poor resolution and low recovery. However, other attempt were done to reproduce another gel filtration run under the same condition from different isolates to determine if there will be a better resolution, recovering and reproducibility.

Better resolution was obtained and the chromatogram showed several peaks of different fractions with better resolution and an increase of the absorbance for each fraction of the eluted COX-like enzyme from *T. brucei* is as shown in figure 6.

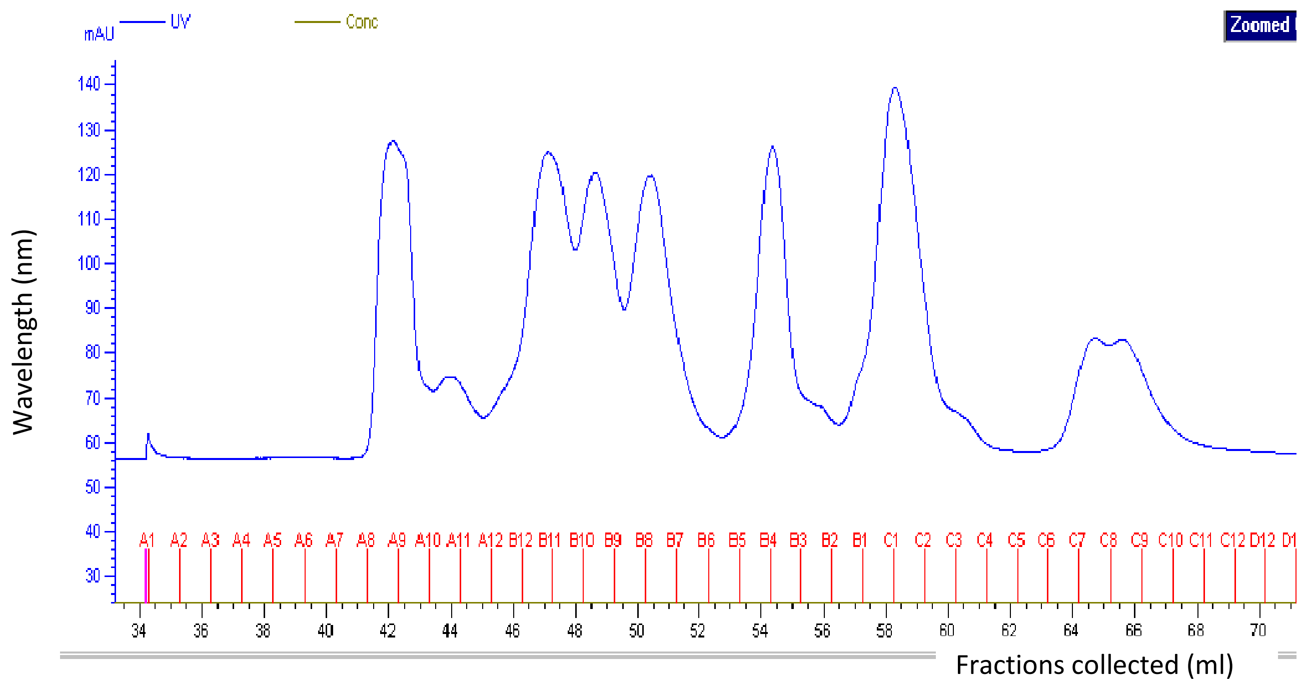

Figure 6. Chromatogram illustrating protein fractionation. A Superdex 200pg HR 10/30 column was used. The sample consisted of cytosolic fraction from trypanosome lysate. This figure shows (X-axis) different fractions collected and (Y-Axis) Absorbance wavelength.

4.4.2 Gel silver staining of different fractions after gel filtration

All the fractions collected after size exclusion chromatography step, were very diluted that even the spectrophotometry protein quantification using Bradford methods was unsuccessful because of the sample dilution and low abundance protein concentration in the starting cytosolic fraction materials. The gel silver staining of different fractions volume after gel filtration was done to visualize the pattern of those fractions in term of proteins contents as shown in figure 7. The two silver stained gels showed different protein pattern in term the gel filtration principle where the interaction of solute with the matrix is determined by pore size and larger molecules are excluded and thus migrate faster than smaller molecules. The expectation was to get a single band at around 70-72KDa molecular size at the first flow path fraction collection by means A8, A10, A11, A12, B12, B11, B10 and B9 (Figure 7a) but no single band appeared just the variant surface glycoprotein (VSG) (50KDa) was visualized in the figure 7a.

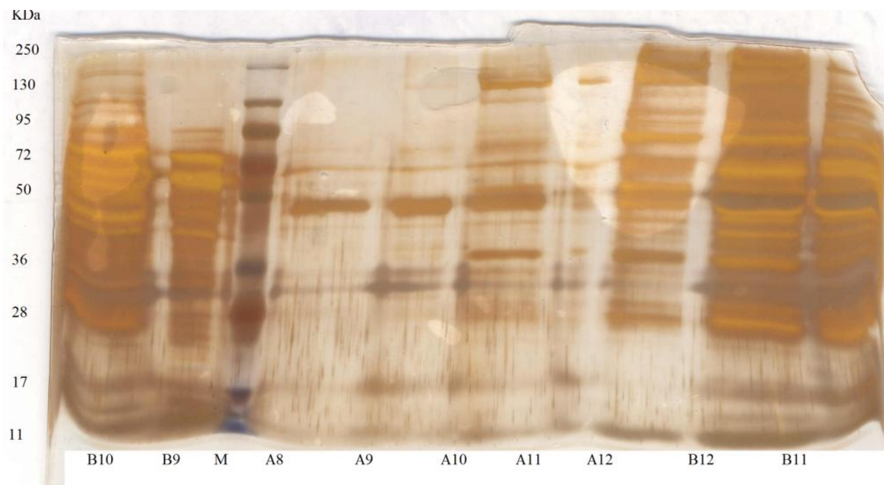

Figure 7a: The silver stained gels showing proteins patterns of different fractions collected after size exclusion chromatography. Fractions samples A8, A10, A11, A12, B12, B11, B10 and B9.

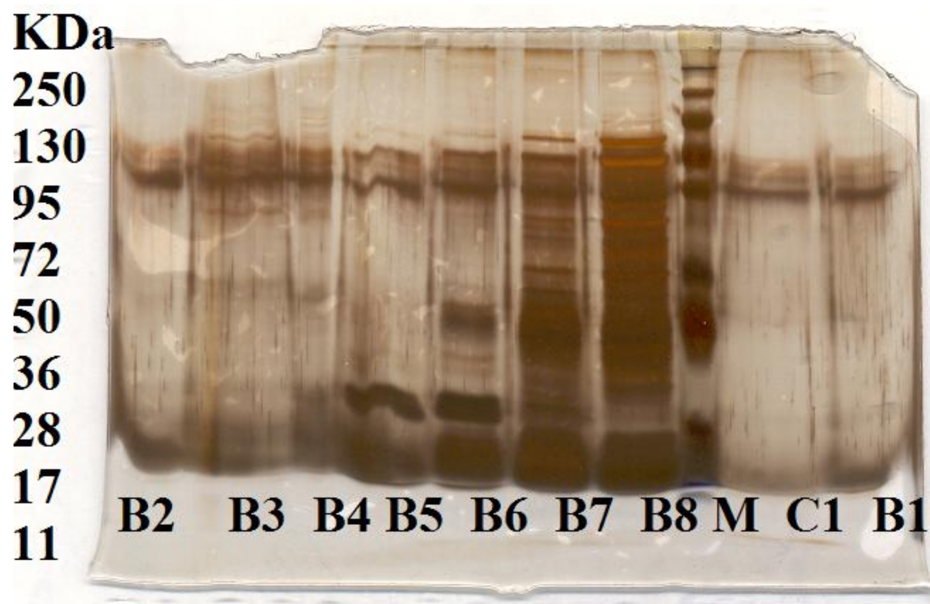

Figure 7b: The silver stained gels showing proteins patterns of different fractions collected after size exclusion chromatography. Fractions samples B8, B7, B6, B5, B4, B3, B2, B1 and C1

4.4.3 Western blot analysis

Following size exclusion chromatography the entire fractions sample collected were subjected to 10% SDS-PAGE and western blot analysis. The fractions sample was concentrated under speedvacuum for 30 minutes or 1 hour to circumvent the problems of lack of specificity and low recovery encountered with chromatography techniques.

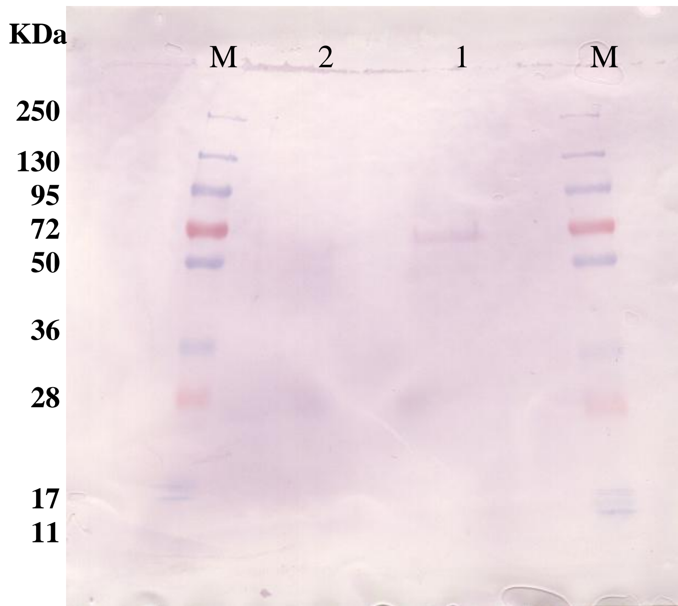

Figure 8: The western blotting analysis of the pooling fractions speed vac and TCA precipitation. M: Molecular weight protein Marker,

Lane 1: Represents the elute fractions **(A8, A9, A10, A11, A12, B12, B11, B10, B9)** from the gel chromatography step and the first flow path fraction collection. Those 9 fractions were pooling-concentrated under speedvac-TCA precipitated showed a weak band anti-COX response within that range fraction.

Lane 2: Represents the elute fractions **(B8, B7, B6, B5, B4, B3, B2, B1, C1)** from the gel chromatography step and the second flow path fraction collection. No band was visualized.

Those fractions were concentrated in the speed vacuum for 30 min or 1 hour, and then precipitated using trichloroacetone (TCA-precipitation protocol) treated each pooling samples with SDS-buffer and loaded through 10% SDS-PAGE.

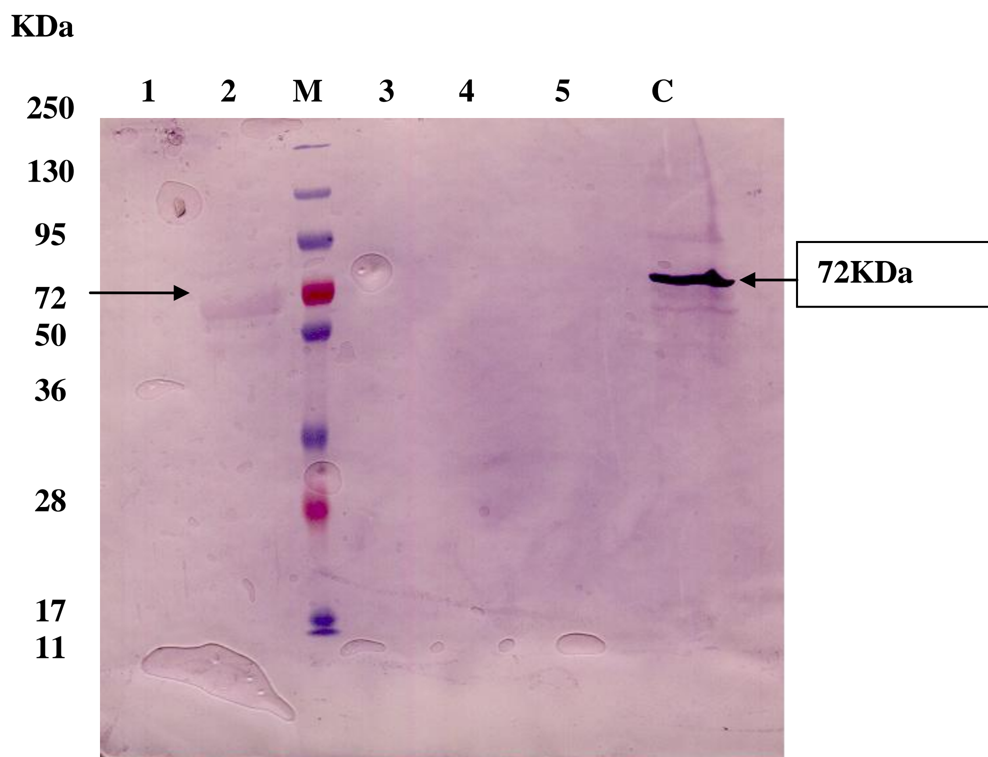

Figure 9a: The western blot analysis showing the COX-like protein with a molecular weight of 72 KDa.

M: Molecular weight protein markers

Lane 1: Represents the 4 elute fractions (**A8, A9, A10, A11)** from the gel filtration chromatography step and the first flow path fraction collection**.** The 4 fractions were pooled and concentrated under speedvac-TCA precipitated showed no band to anti-COX antibody within that fraction range.

Lane 2: Represents the 5 elute fractions (**A12, B12, B11, B10, B9)** from the gel filtration chromatography step and the first flow path fraction collection. Those 5 fractions were pooled and concentrated under speedvac-TCA precipitated showed weak band to anti-COX antibody inside that fraction range.

Lane 3: Represents the 3 elute fractions (**B8, B7, B6)** from the gel filtration chromatography step and the second flow path fraction collection. Those 3 fractions were pooled and concentrated under speedvac-TCA precipitated showed no band to anti-COX antibody inside that fraction range.

Lane 4: Represents the 3 elute fractions (**B5, B4, B3)** from the gel filtration chromatography step and the second flow path fraction collection. Those 3 fractions were pooled and concentrated under speedvac-TCA precipitated showed no band to anti-COX antibody inside that fraction range.

Lane 5: Represents the 3 elute fractions (**B2, B1, C1)** from the gel filtration chromatography step and the second flow path fraction collection. Those 3 fractions were pooled and concentrated under speedvac-TCA precipitated showed no band to anti-COX antibody inside that fraction range.

C: Control/COX–insect cell medium represent a positive control showed a strong band of a COX-like protein from the COX-like gene expression in baculovirus insect cells medium system (expression of the COX recombinant in insect cells system).

The gel filtration chromatography step was replicated and another western blot analysis done to partition the fraction A12, B12, B11, B10, B9 obtained two samples contained each A11, A12, B12 and B11, B10, B9 fractions pooled-concentrated-TCA precipitated shown in figure 9b

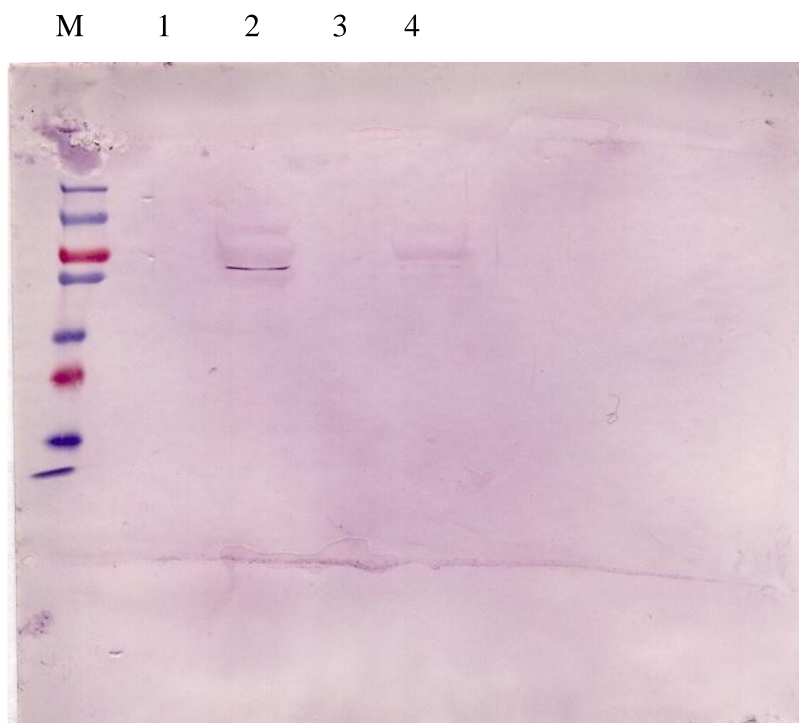

Figure 9b: The western blot analysis showing the COX-like protein with a molecular weight of 72 KDa.

Lane M: Molecular weight proteins markers

Lane 1: Represents the 4 elute fractions (**A8, A9, A10, A11)** from the gel filtration chromatography step and the first flow path fraction collection**.** The 4 fractions were pooled

and concentrated under speedvac-TCA precipitated showed no band to anti-COX antibody inside that fraction range.

Lane 2: Represents the 5 elute fractions (**A12, B12, B11, B10, B9**) from the gel filtration chromatography step and the first flow path fraction collection. The 5 fractions were pooled and concentrated under speedvac-TCA precipitated showed one band to anti-COX antibody within that fraction range.

Lane 3: Represents the 3 elute fractions (**A11, A12, B12**) from the gel filtration chromatography step and the first flow path fraction partitioned in two. The 3 fractions were pooled and concentrated under speedvac-TCA precipitated showed no band to anti-COX antibody inside that fraction range.

Lane 4: Represents the 3 elute fractions (**B11, B10, B9**) from the gel filtration chromatography step and the first flow path fraction partitioned in two. The 3 fractions were pooled and concentrated under speedvac-TCA precipitated showed a single weak band to anti-COX antibody inside that fraction range.

The figure 9b showed the COX-like protein with a molecular weight of 72 KDa. Lane 2 showed a stronger signal than lane 4. This blot demonstrated the three fractions B11, B10, B9 interest from the gel filtration chromatography step where to find out a COX-like enzyme from trypanosome cytosolic fraction.

4.4.4 Ion exchange chromatography

The three fractions **B11, B10, B9** which gave a cyclooxygenase antibody response were pooled and loaded onto a strong cation SP Fast Flow Hi Trap 1ml column volume obtained the eluted profile showed in figure 10a and then also loaded onto a strong anion Q Fast Flow HiTrap 1ml column volume obtained the eluted profile showed in figure 10b.

The results showed two different eluted profiles which looked very different in terms of the peak heights. The attempts to carry out a COX-fluorometric assay suggested the presence of a COX-like enzyme from trypanosomes in fraction A8, A9 and A 11 (figure 10a) from the strong cation exchange column, suggesting that the TbCOX is positively charge.

The main goal was to use the ion exchange chromatography as an intermediate or polish step and to move from the three target fractions from the gel chromatography first step to a single fraction after ion exchange chromatography run. The western blot analysis of the fractions from the two ion exchange chromatography step was done but no band was detected.

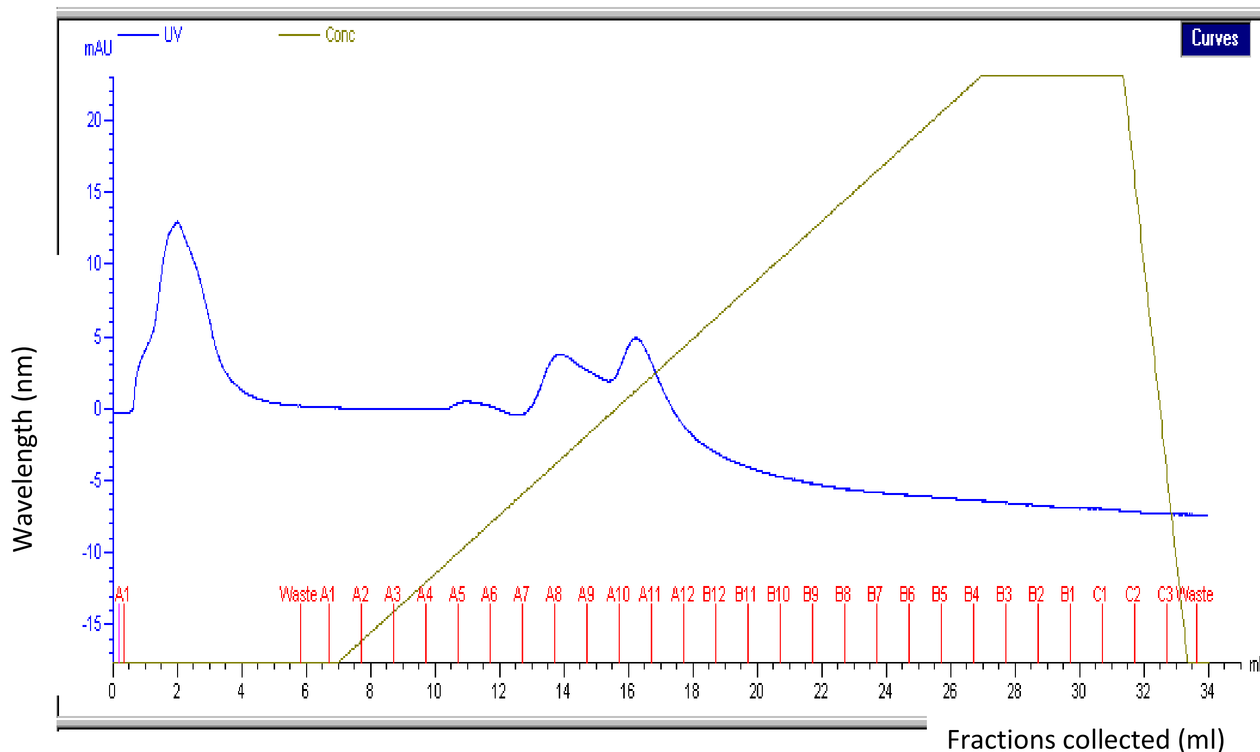

Figure 10a: Ion exchange chromatography eluted profiles, IEX profile using strong cation exchange SP Fast flow HiTrap 1ml column under AKTA FPLC controller system.

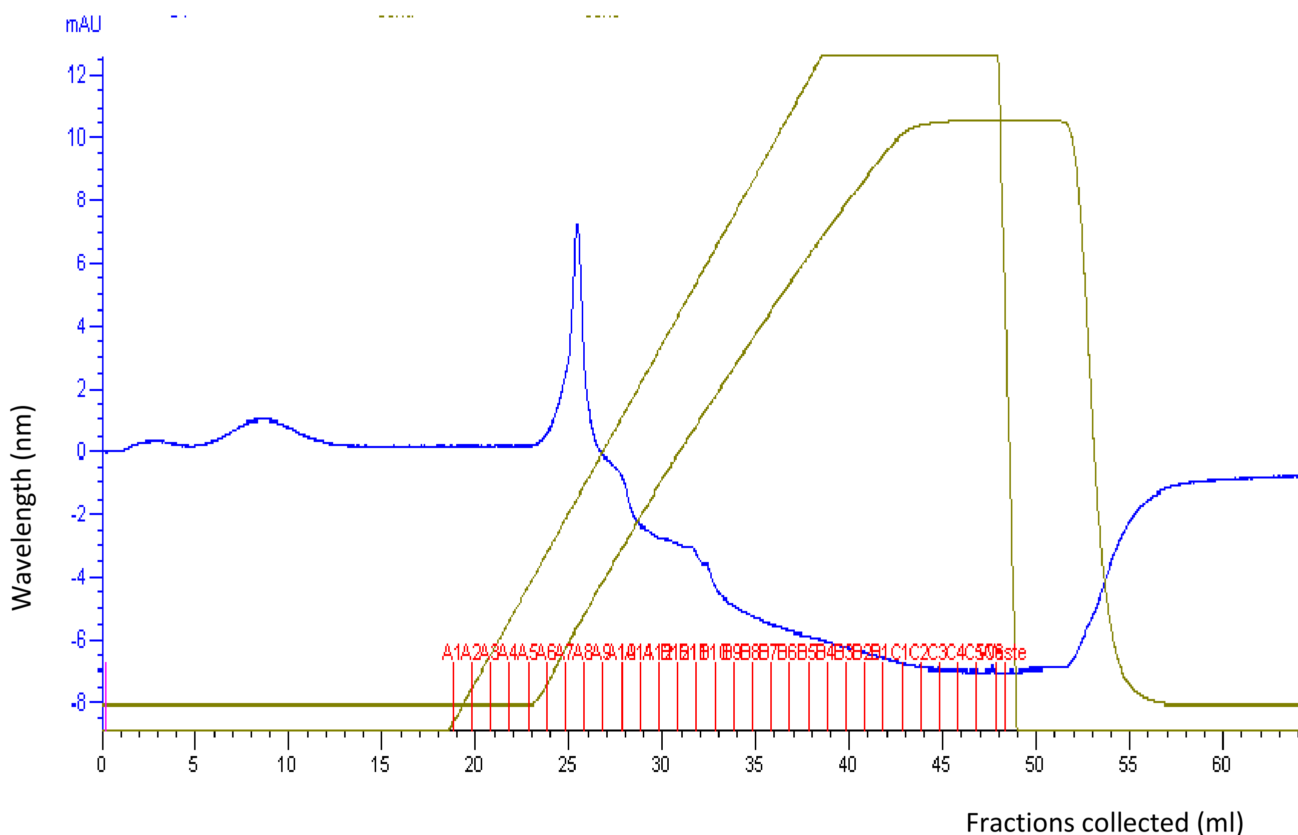

Figure 10b: Ion exchange chromatography eluted profiles, IEX profile using strong anion exchange Q Fast flow HiTrap 1ml column under AKTA FPLC controller system.

CHAPTER FIVE
DISCUSSION AND CONCLUSION

5.1 Discussion

Human African trypanosomiasis or sleeping sickness is caused by infection with the tsetse-fly transmitted protozoan *Trypanosoma brucei* (Stich *et al.,* 2002). This protozoan hemoflagellate lives exclusively as an extra-cellular parasite unlike other Trypanosomatidae. The sub-species of this parasite include *T. b. rhodesiense* and *T. b. gambiense* which cause the human disease and are a health concern in many African countries (Berrang Ford, 2007).

The infection of mammalian hosts by African trypanosomes is characterized by an up - regulation of prostaglandins (PGs) production in the plasma and cerebrospinal fluid (CSF), which modulates the host responses and causes symptoms of the infection (Noverr *et al,* 2003). Studies on mice infected with *Trypanosoma brucei brucei* have shown that the immune suppression that normally occurs during trypanosomiasis correlates with change in AA metabolism of macrophages (Alafiatayo *et al.*, 1994). This hypothesis of PG-mediated immunosuppression is supported by the findings that *in vitro* disrupted *Trypanosoma brucei* induces increased secretion of PGE_2 and PGD_2 by fibroblast and astrocytes, similar to endotoxin stimulation (Alafiatayo *et al.*, 1994). In addition to immunosuppression, somnolence is another typical clinical feature of HAT and both clinical signs have been suspected to result from increased PGD_2 synthesis following trypanosome infection (Walker *et al.,* 2006). Patients suffering from HAT caused by *Trypanosoma brucei gambiense* have been shown to have three-fold higher levels of PGD_2 in their cerebrospinal fluid as compared to non-infected patients, while the PGE_2 levels are similar (Pentreath *et al.*, 1990). Kubata *et al.*, (2000) and Kubata *et al.*, (2002) showed that trypanosomes produce PGD_2, PGE_2 and PGF_2 alpha from arachidonic acid. These PGs led to a broad of variety of different physiological effects in higher eukaryotes and their accumulation in serum coincides remarkably with symptoms observed during trypanosomosis, such as fever, pain, immunosuppression, and dysregulation of sleep/wake cycles among other signs (Figarella *et al.*, 2005). So far, it is not clear why protozoa produce PGs, but it is tempting to speculate that these parasites may have adopted the formation of PGs to modify host reactions for their own benefit. In addition, Kubata *et al.*, (2000) found that PGF_2 alpha was mainly produced in fast dividing forms of the parasite such as the slender bloodstream form, in the procyclic insect form and that the PGF_2 alpha was scarcely secreted into media. In contrast PGD_2, another derivative of the PGs was mainly produced by the non-dividing stumpy bloodstream forms and primarily secreted into media (Figarella *et al.*, 2005).

COX or PGHS is the enzyme that catalyses the rate-limiting step in the biosynthesis of PGs. The COX-like enzyme from *T. brucei* involved is markedly different from its mammalian counterpart because the non-steroidal anti-inflammatory drug (NSAIDs) such as aspirin and indomethacin does not affect PG synthesis (Kubata *et al.*, 2000).

Although our current knowledge of the metabolism and function of the parasite derived eicosanoid is still scarce, it evident from the available data that these mediators play an essential role in the physiology of various protozoan and metazoan parasites. The wide variety of parasites that are able to synthesize eicosanoid makes it highly probable that eicosanoid production and excretion is a common feature in parasitic protozoa, helminths and arthropods (Daugschies and Joachim, 2000). These relevant biochemical pathways are highly conserved and are also present in non-parasitic invertebrates, indicating that eicosanoid production by parasites was originally evolved for intrinsinc physiological purposes (Kubata *et al.*, 2007).

The present study was carried out to purify the possible cyclooxygenase like enzyme and to determine the possible existence of a cyclooxygenase like enzyme in trypanosomes and in addition, to clone and express the cyclooxygenase like gene in bacteria and insect system.

Results from the current study demonstrated that *Trypanosoma brucei* produces PGs through a COX-like enzyme in the presence of exogenous or endogenous AA but the COX-like enzyme from *T. brucei* seems to be different from the mammalian COX.

The difference is also supported by previous studies by Kubata *et al.,* (2002) who demonstrated that the production of PG-like compounds by trypanosome system was not inhibited by NSAIDs such as aspirin and indomethacin which are well established inhibitors of mammalian COX-1 and COX-2 (Vane, 1971). This suggests that the trypanosomal enzyme system involved in PG synthesis from AA is markedly different from its mammalian counterpart. Furthermore, the results from the current study demonstrated that the COX-like enzyme from *T. brucei* was localized in the cytosolic fraction, unlike mammalian COXs, which are membrane-bound and present on the luminal surfaces of the endoplasmic reticulum and the inner and outer membranes of the nuclear envelope (O'Neill *et al.,* 1994; Spencer *et al.,* 1999).

Multiple molecular species of COX-2 can be readily observed with SDS-PAGE with a molecular mass of 68-72KDa (Otto *et al.,* 1993). Results from the current study using an immunoblot detection of COX using a polyclonal anti-ovine COX indicate the presence of a COX-like enzyme with a molecular size of about 72KDa. This COX-like molecule is a single

band reproducible from the cytosolic fraction of trypanosome lysate. The single band on the blot suggests that the COX-like enzyme from *T. brucei* is different from that found in other species of parasites. Dey *et al.* (2003) demonstrated the presence of two distinct bands with molecular weights of 66KDa and 72KDa as opposed to the single band obtained in *Typanosoma* study. It was thus evident that the two bands are variants of the same protein, as polyclonal antibodies against COX-like protein detected both bands in *Entamoeba histolytica* nuclear envelope and recombinant COX-like protein. This could be due to differences in the different degrees of glycosylation of the same COX-like protein *in vivo* (Percival *et al.,* 1997), analogous to mammalian COX-2. The multiple alignment of COX-like protein and DNA sequence of *Amoeba* COX with COX sequences from various other species also showed less homology (Dey *et al.,* 2003).

Proteins purification using gel filtration chromatography as a first step resulted in some interesting elution profiles with different fractions volume samples which were at the beginning concentrated using a speed vacuum and the protein quantified by Bradford protein assay. The protein quantification by spectrophotometry was not successfully due to the dilution effect of the eluted buffer during gel filtration run as indicated by the lower protein concentration of the cytosolic fraction as the starting materials. The gel run using the different elution fraction however, revealed the presence of the various protein content pattern of each fraction after running the gels and the silver staining.

Afterwards the various fractions were passed through 10% SDS-PAGE and western blot analysis. The first attempt of western blotting after chromatography-gel filtration did not produce a strong band in the blot, suggesting the weakness of the band might have been due to the dilution of the protein by the buffer used during the chromatography run or may be due to the low level of the COX-like target enzyme in that the cytosolic fraction. Other causes of the weak band could be due to the low amount of starting materials resulting from low number of rat. At the same time the weak band could be due to the method used for the lysing of the trypanosomes which would have had an impact in lowering the protein concentration in the cytosolic fraction of the starting material.

In spite of the drawbacks, from the purification strategy, the COX from *T. brucei* showed that the eluted gel filtration was reproducible as running the gel filtration at the same condition resulted in reproducible results with the resolution being the only unique parameter changed. However the low recovery still remained a challenge because the gel filtration even when repeated twice, then concentrated using the same fraction volume and also same

fractions pooled for use in the SDS-PAGE and western blotting analysis still produced a weak band of molecular weight size of 72KDa.

An attempt using the same approach was carried out after concentrating the samples using the speed vacuum of the pooled fractions of interest, followed by precipitation of samples using trichloroacetone (TCA). This resulted in a band of expected COX-like with a molecular size of 72KDa from trypanosomes cells in the western blotting analysis. The respective blots showed that the COX-like enzyme is present at around three interesting fractions after gel filtration chromatography. Of interest was the fact that the TCA precipitation step did not inactivate the enzyme since there was a positive antibody response.

In the second chromatography step, where three fractions of interest were from gel filtration chromatography step followed by ion exchange using a strong cation and anion exchange chromatography, two different eluted profiles were obtained which looked very different in terms of the peak height. Attempts to carry out a COX-fluorometric assay results indicating the presence of a COX-like enzyme from trypanosomes in fractions from the strong cation exchange, suggesting that trypanosome COX is positively charged.

On the other hand, a silver stained gel was made with the trypanosome lysates. The gel was then cut off at around 72-95KDa range where the new COX was expected to be found. This section was sent to Proteome Analysis Centre at Tuebingen University. At least 23 proteins with different molecular weights were identified.

From these observations together with the data from chromatography steps, western blotting and COX-Fluorometric assay it can be postulated that COX-like enzyme exists in *T. brucei* as a cytosolic protein. Further experiments need to be done to optimize the purification, SDS-PAGE, western analysis and biochemical characterization of the recombinant COX-like enzyme.

5.2 Conclusion and Recommendation

The current work describes the purification and characterization of a COX-like enzyme from *Trypanosoma brucei* using a classical approach using gel filtration and ion exchange chromatography under the control of AKTA-FPLC system, running an SDS-PAGE for Western blot analysis and cloning the COX-like gene in bacteria and insect cell system. This study established a partial data identified a probably COX-like enzyme from the trypanosomes lysate specifically in the cytosolic fraction by western blotting analysis using a polyclonal anti-ovine-COX. This finding indicates the location of our COX-like enzyme target inside the cell. Future research based on this dissertation should mainly focused on

optimization of the chromatography steps, monitored the recovery of the COX- like enzyme targeting after each chromatography steps, SDS-PAGE, Western blot analysis, the probable function prediction need to be done by using Bioinformatics web tools by searching sequence data bases for the presence of orthologous enzymatic conserved domains in the hypothetical sequences and biochemical characterization of the recombinant COX-like enzyme for supporting functional and structural biology efforts.

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