Identification and characterization of phospholipase A_2 from *Trypanosoma brucei*

Identifizierung und Charakterisierung von Phospholipase A_2 aus *Trypanosoma brucei*

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I dedicate this thesis to all human kind

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Abbreviations

AA	Arachidonic acid
AP	Alkaline phosphatase
APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BCS	Bathocuproine disulfonic acid
BF	Bloodstream form
bp	Base pair
BSA	Bovine serum albumin
CGA	Citrate glucose anticoagulant
CNS	Central nervous system
COX	Cycloxygenase
DEAE	Diethylaminoethyl
DG	Diacylglycerol
DL-PC	Dilinolenoyl phosphatidylcholine
DMSO	Dimethylsulfoxide
DMG	Dimyristoylglycerol
DTT	Dithiothreitol
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FFA	Free fatty acid
GPCho	Glycerophosphocholines
GPI	Glycosylphosphatidylinositol
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HETE	Hydroxyeicosatetraenoic acid
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kDa	kilo dalton
LO	Lipoxygenase
LT	Leukotrienes
MEM	Minimum essential medium
MITat	Molteno institute trypanozoon antigenic type

PAF-AH	Platelet activating factor-acetyl hydrolase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Procyclic form
PG	Prostaglandin
PI	Protease inhibitors
PL	Phospholipid
PLC	Phospholipase C
RIPA	Radio-immunoprecipitation assay
SAP	Signal activated phospholipases
SDS	Sodium dodecyl sulfate
TAE	Tris acetate EDTA
$TbPLA_1$	*T.brucei* phospholipase A_1
$TbPLA_2$	*T.brucei* phospholipase A_2
TBS	Tris buffered saline
TDB	Trypanosoma dilution buffer
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
VSG	Variant surface glycoprotein
WHO	World health organization

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1. Introduction

I. *Trypanosoma brucei*

1.1 *Trypanosoma brucei*, the cause of African sleeping sickness

Human African Trypanosomiasis (HAT), also known as sleeping sickness, is a vector-borne parasitic disease which primarily affects the poorest rural populations in some of the least developed countries of Central Africa. (Berriman *et al.*, 2005). *Trypanosoma brucei* is a unicellular eukaryote that causes sleeping sickness in humans and nagana in livestock. It lives exclusively as an extra-cellular parasite unlike other members of this family of Trypanosomatidae, such as *Trypanosoma cruzi* and *Leishmania* species, which possess intra-cellular stages. *T. brucei* parasites 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).

1.2 Epidemiology

Sleeping sickness threatens millions of people in 36 countries of sub-Saharan Africa. The number of new cases is currently between 50,000 and 70,000 per year. The parasites concerned are extracellular protozoa belonging to the *Trypanosoma* Genus. They are transmitted to humans by tsetse fly (*Glossina* Genus) bites which have acquired their infection from human beings or from the animals' reservoir. Tsetse flies are found in Central Africa and only certain species transmit the disease. Different species have different habitats. They are mainly found in vegetation by rivers and lakes, in gallery-forests and in vast stretches of wooded savannah. Sleeping sickness occurs only in those regions where there are tsetse flies that can transmit the disease (Figure 1.1) (http://www.eanett.org/whatis/).

For reasons that are so far unexplained, there are many regions where tsetse flies are found, but sleeping sickness is not. HAT takes two forms, depending on the parasite involved. *T. brucei gambiense* is found in West and Central Africa. This form represents more than 90% of reported cases of sleeping sickness and causes a chronic infection. A person can be infected for months or even years without major signs or symptoms of the disease. When symptoms do emerge, the patient is often already in an advanced disease stage when the central nervous system is affected. *T. brucei rhodesiense* is found in Eastern and Southern Africa. This form represents less than 10% of reported cases and causes an acute infection. First signs and symptoms are observed after a few months or weeks. The disease develops rapidly and invades the central nervous system. (http://www.who.int/mediacentre/factsheets/fs259/en/)

Other parasite species and sub-species of the *Trypanosoma* Genus are pathogenic to animals and cause animal Trypanosomiasis in many wild and domestic animal species (in cattle the disease is called *Nagana*, a Zulu word meaning "to be depressed"). Animals can host the human pathogen parasites, especially *T.b. rhodesiense*; thus domestic and wild animals are an important parasite reservoir. Animals can also be infected with *T.b. gambiense*, however the precise epidemiological role of this reservoir is not yet well known. This disease kills live stock and is a major obstacle to the economic development of the rural areas affected (WHO report, 2006).

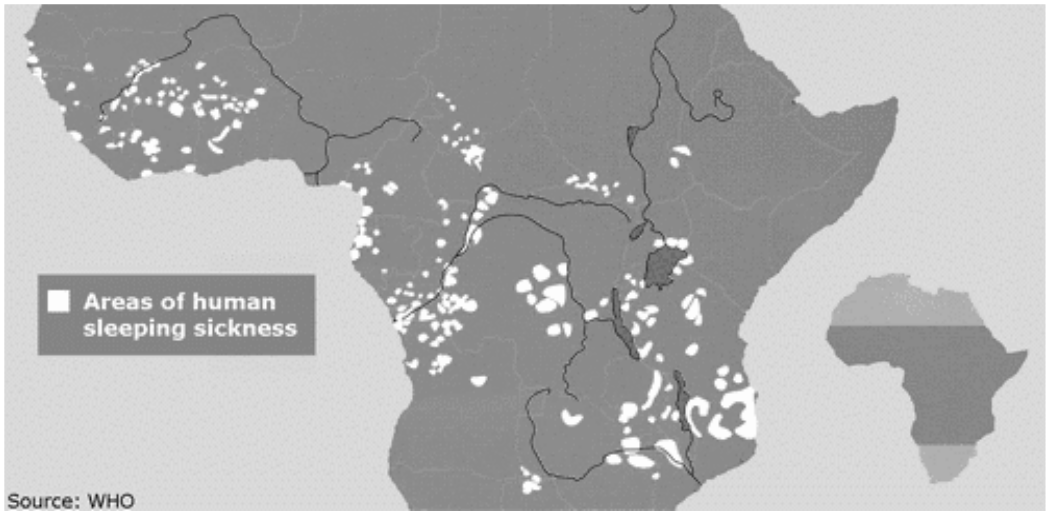


Figure 1.1 Distribution of Trypanosomiasis in countries considered endemic in Africa (W.H.O.).

1.3 Classification

Trypanosomes are protozoans which belong to the order of Kinetoplastida. They contain a range of ubiquitous free-living species which are pathogens of invertebrates, of vertebrates and even of some plants. *Trypanosoma* species cause sleeping sickness and Chagas disease, whereas the leishmaniases kill and debilitate hundreds of thousands of people worldwide each year. The taxon Kinetoplastida was created by uniting two groups: Bodonidae and Trypanosomatidae (Simpson *et al.*, 2006). Organisms of the former group are characterized by the presence of two flagella and a quite large kinetoplast. This group is formed by both free living organisms and parasites of fish and snails. Trypanosomatidae have only a single flagellum and a smaller kinetoplast than Bodonidae. The former comprise, Trypanosomatidae, of which all members are parasites of invertebrates, all classes of vertebrates or plants. The genus *Trypanosoma* is further divided into two sections, according to the place of where the

parasite develops in the insect host. Salivaria develop in the anterior portion of the digestive tract and within the salivary gland, while the stercoraria develop in the hindgut. *T. brucei* belongs to the salivaria sub-genus (Levine *et al.*, 1980).

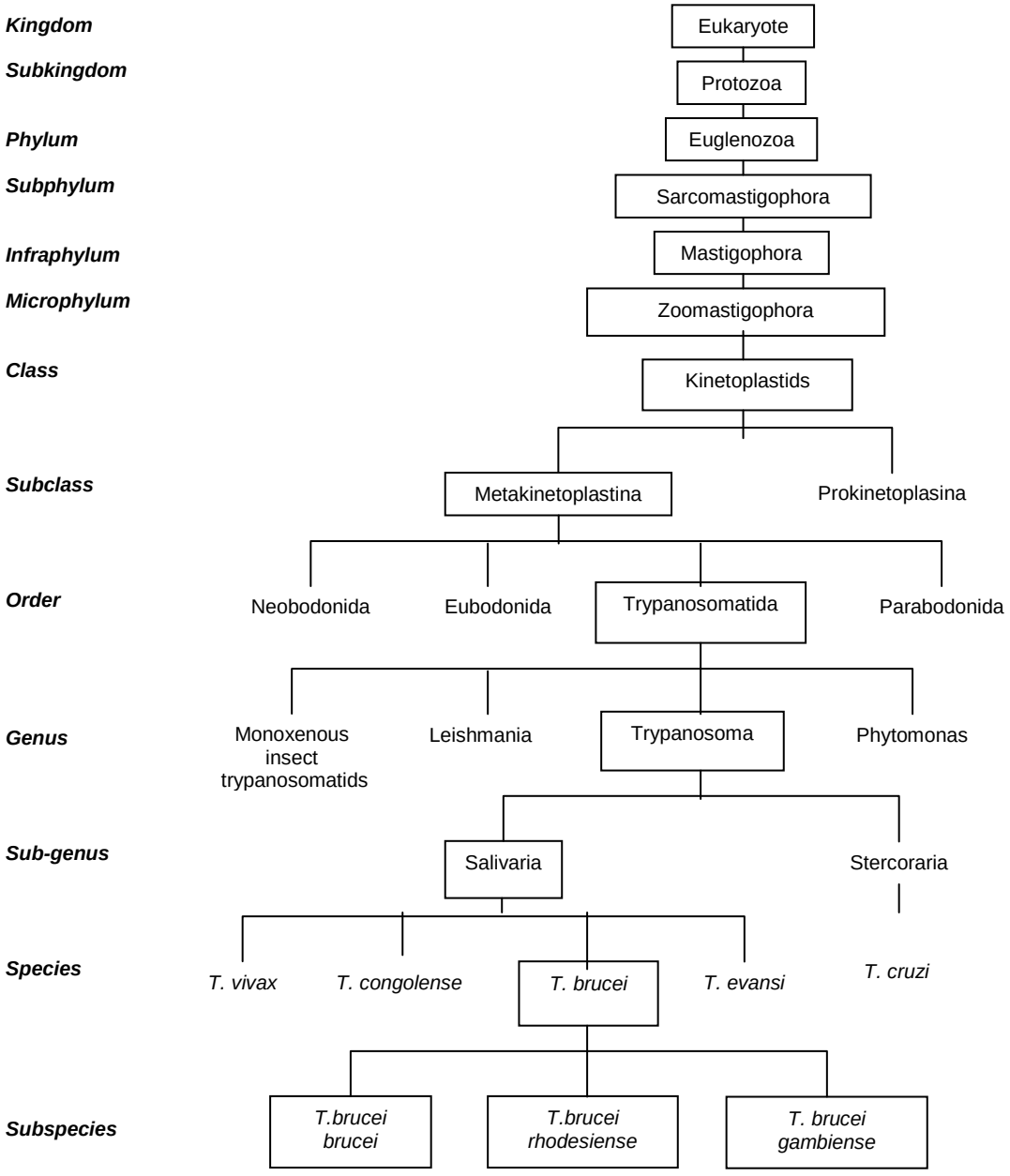


Figure 1.2 Phylogenetic tree of *T.brucei* according to Simpson *et al.*, 2006

1.4 Morphology

T. brucei live in the blood and tissue fluid of a mammalian host; the bloodstream form trypomastigotes are relatively long (about 20 µm), with a flagellum that emerges from the posterior end (defined relative to the direction of motion) and is attached along the body of the parasite. The cell surface is protected by a thick layer of variant surface glycoprotein (VSG) which is changed at frequent intervals: this antigenic switching enables the parasite to survive indefinitely in the face of recurrent immune responses. Stumpy, nondividing forms may survive preferentially when the parasites are taken up by a feeding tsetse fly. The parasites in the fly midgut differentiate into the procyclic form which is morphologically quite similar to bloodstream forms, although the kinetoplast is relatively nearer to the nucleus. Before entering the mammalian bloodstream, procyclic trypanosomes differentiate into epimastigotes and then into VSG- bearing metacyclic forms in the tsetse fly salivary glands (Figure 1.3). The major cell surface protein (VSG) in African trypanosomes are linked to the plasma membrane via glycosyl phosphatidylinositol (GPI) anchors whose composition varies according to the antigenic variant and life cycle stage (Clayton *et al.*, 1995).

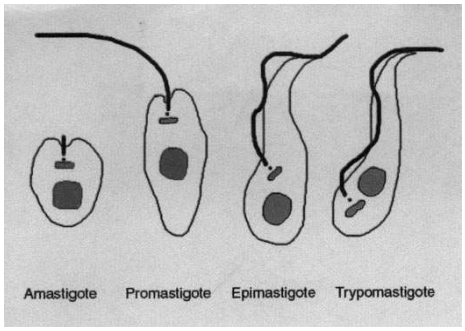


Figure 1.3 Major cellular forms of trypanosomatids defined by cell shape, flagella presence and position of the basal body, kinetoplast and nucleus (Gull, 1999).

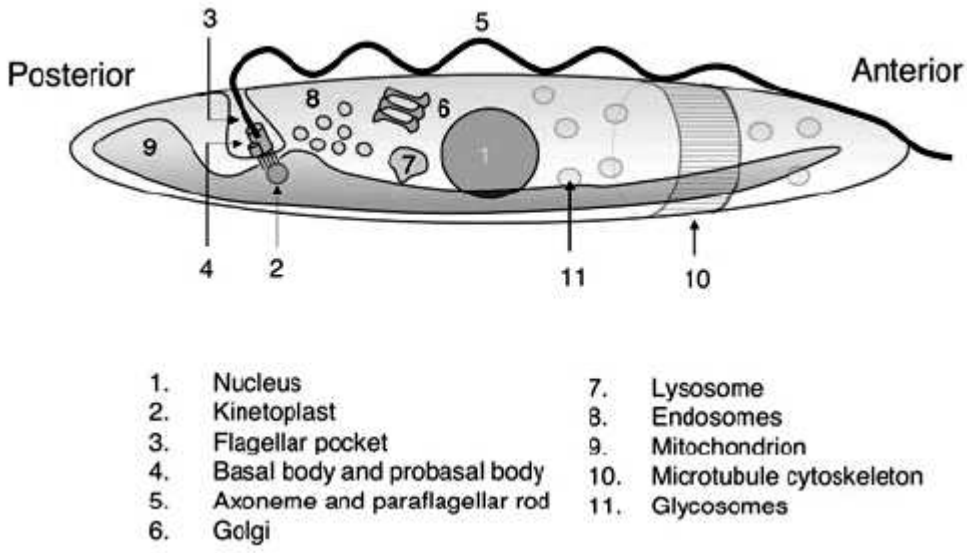


Figure 1.4 Trypanosome cell architecture with locations of the major features of the cell (Matthews, 2005).

Membrane transport functions are important both in terms of interaction with insect vectors and mammalian hosts as well as recycling VSGs. Trypanosomes have a polarized endomembrane system which restricts both exocytosis and endocytosis, which occur exclusively through the flagellar pocket (Overath *et al.*, 2004; Field *et al.*, 2004). They appear to have a reduced dependence on the acto-myosin network balanced by an elaboration of the tubulin-based cytoskeleton. These organisms have six members of the tubulin superfamily especially δ- and ε- tubulin presence is characteristic for basal bodies and flagella. There is no actin-based motility known so far and acto-myosin may even not be necessary for cytokinesis (Berriman *et al.*, 2005). The following organelles of the trypanosomes i.e. kinetoplast and nucleus are precisely positioned between the posterior end and the centre of the cell (Figure 1.4). The motility of the parasite is dependent upon its single flagellum, which has a conventional axonemal structure (9 X 2 + 2) plus an associated paraflagellar rod. The mitochondrion is a single elongated structure that runs from the posterior to the anterior of the cell. Bloodstream form mitochondrion is simple tubular structure largely devoid of cristae and does not contain a functional respiratory chain. Thus energy generation is dependent on glycolytic reactions compartmentalized within glycosomes. The kinetoplast (i.e. the mtDNA) consists of mass of catenated DNA molecules. The positioning of kinetoplast is different in bloodstream form and procyclic form. The kinetoplast has many other unusual features and is composed of two classes of circular DNA molecules: maxicircles, containing genes that

encode mitochondrial proteins and minicircles which encode short guide RNAs (Matthews, 2005). The Golgi complex is more extensive in the bloodstream form as compared to the procyclic form, the lysosome and the endosomes are all located in the posterior region of the cell. The endoplasmic reticulum (ER), the mitochondria, acidocalcisomes and the glycosomes are all dispersed throughout the cell volume (Field *et al.*, 2004)

1.5 Life cycle

The *T.brucei* has exquisite co-ordination of events occurring as the parasites proceed through their life cycle. They make a series of transitions between three major environments: mammalian host, tsetse midgut and tsetse salivary gland. The life cycle is characterized by changes in cell shape, cell cycle, metabolism, surface coat, etc. At two of these transition points, bloodstream to tsetse midgut and tsetse salivary gland to mammalian bloodstream, there is a specific pattern of events. In each case, the transmitted parasite (stumpy form and metacyclic, respectively) exhibits particular differentiated attributes useful to its survival in the next environment. On reaching that environment, in each situation, the cell enters a proliferative cell cycle and colonizes the midgut or bloodstream, respectively. In one of the other transitions, tsetse midgut to the salivary gland, the parasite differentiates to an epimastigote form (Gull, 2001).

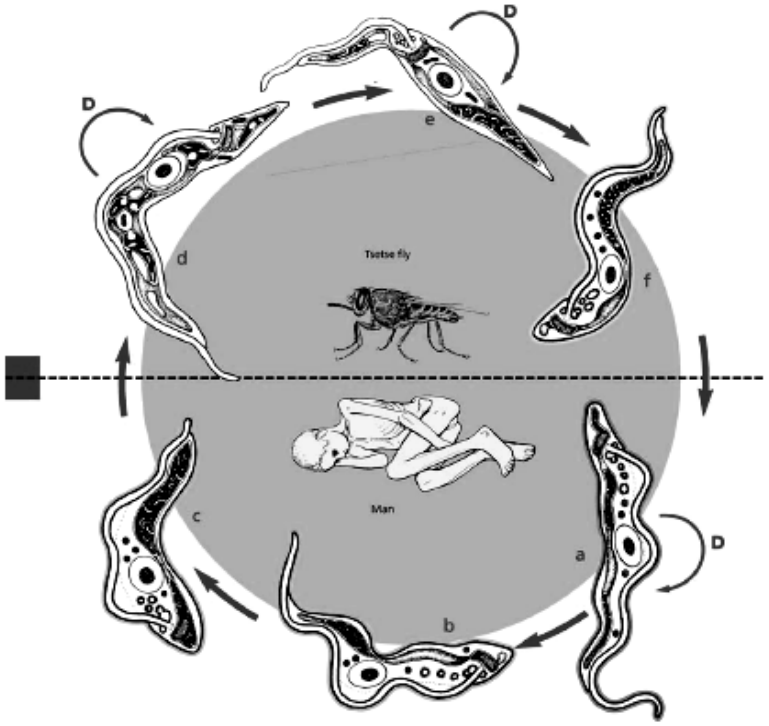


Figure 1.5 Life cycle stages of *T.brucei* in insect vector and mammalian host. Arrowheads represent differentiation events in the trypanosome life cycle (Barrett *et al.*, 2007)

The trypanosome is transmitted between mammalian hosts by the tsetse fly, *Glossina* spp, in which it initially establishes in the midgut after a blood meal but then migrates to the salivary glands in preparation for transmission to a new mammalian host (Figure 1.5). In mammals, the parasite survives free in the bloodstream, being able to evade antibody responses through antigenic variation (McCulloch, 2004; Pays *et al.*, 2004). Trypanosomes proliferate in the mammalian system as morphological slender form, these being replaced by the non-proliferative stumpy form as parasite numbers increase (Matthews *et al.*, 2004).

The accumulation of division-arrested form limits the increase in parasite numbers and thereby prolongs host survival and disease transmission. The uniform arrest of stumpy form in G1 phase of the cell cycle ensures that the morphological changes that occur upon transmission to the tsetse fly can be coordinated with re-entry into the cell cycle. This is important because correct organelle positioning is crucial for successful completion of the cell cycle of tsetse midgut procyclic forms. Upon uptake by the tsetse, bloodstream trypanosomes replace the VSG coat with a less-dense surface coat composed of procyclin, which is also GPI anchored (Roditi and Linger, 2002). Energy generation also switches from being exclusively based on glycolysis to a mitochondrion-based respiratory system, which requires structural elaboration and metabolic activation of the organelle. After proliferation in the tsetse midgut, the parasite migrates to the salivary gland. The epimastigote forms generated there attach to the gland membrane through elaborations of the flagellar membrane. After further multiplication, the parasite undergoes division arrest, reacquires a VSG coat and is released into the salivary gland lumen, in preparation for inoculation into a new mammalian host (Matthews, 2005).

1.6. Metabolism of parasite

An adaptive evolution shapes the metabolism of parasites which results in the development of novel pathways that are integral for parasite subversion of host defenses. Yet the availability within the host of an assortment of metabolites which includes small organic molecules for energy generation or building blocks for assembling macromolecular structures. The parasites lack various core pathways of metabolism that are present in many other organisms (Borza *et al.*, 2005).

T.brucei resides in the bloodstream within an aerobic environment, but use glycolysis (i.e. without oxidative phosphorylation) for energy generation (Figure 1.6). In bloodstream forms, glucose catabolism is aerobic, requiring an oxygen-dependent alternative terminal oxidase for

regeneration of the NAD^+ reduced during glycolysis, and pyruvate is the end-product of metabolism. These parasites compartmentalize many of their glycolytic enzymes behind the peroxisomal membrane, giving rise to glycosomes. In bloodstream form, approximately 90 % of the peroxisomal or glycosomal, matrix proteins are glycolytic enzymes (Moyersoen *et al.*, 2004).

Bloodstream form contains cytoplasmic phosphoglycerate kinase which means it needs multiple pathways in order to maintain a balance of ATP and ADP concentrations inside the glycosomes (Misset and Opperdoes, 1987). The glycerol-3-phosphate shuttle, which is essential in bloodstream form, is again operative but there is a second route for maintaining glycosomal NAD^+/NADH homeostasis. This pathway utilizes NADH-dependent fumarate reductase to generate succinate from the phosphoenol pyruvate intermediate of glycolysis. This fumarate reductase activity is present on a modular multifunctional protein, which also has the NADH-dependent cytochrome c reductase activity (Besteiro *et al.*, 2002). There is also pyruvate phosphate dikinase, a glycosomal protein, for which the functional role is not known (Bringaud *et al.*, 1998; Coustou *et al.*, 2003).

The sugars are not thought to be readily available in the digestive tract of the tsetse fly. The glucose content of the blood meal is depleted rapidly following ingestion. The tsetse flies unlike many other invertebrate vectors do not acquire nutrients by feeding on sugar-rich plant sap (Tetaud *et al.*, 1997; Vedrenne *et al.*, 2000). The shift towards mitochondrial energy generation in procyclic form can therefore be considered to reflect a predominance of peptides and amino acids. These are liberated by digestion of the blood meal, as the primary carbon source available for ATP production. In the presence of glucose, the end-products of proline catabolism are equimolar amounts of CO_2 and succinate. The enzymes required for this catabolism result in the generation of ATP through substrate-level phosphorylation, and NADH and $FADH_2$ which both provide electrons for the respiratory chain (van Weelden *et al.*, 2003, 2005).

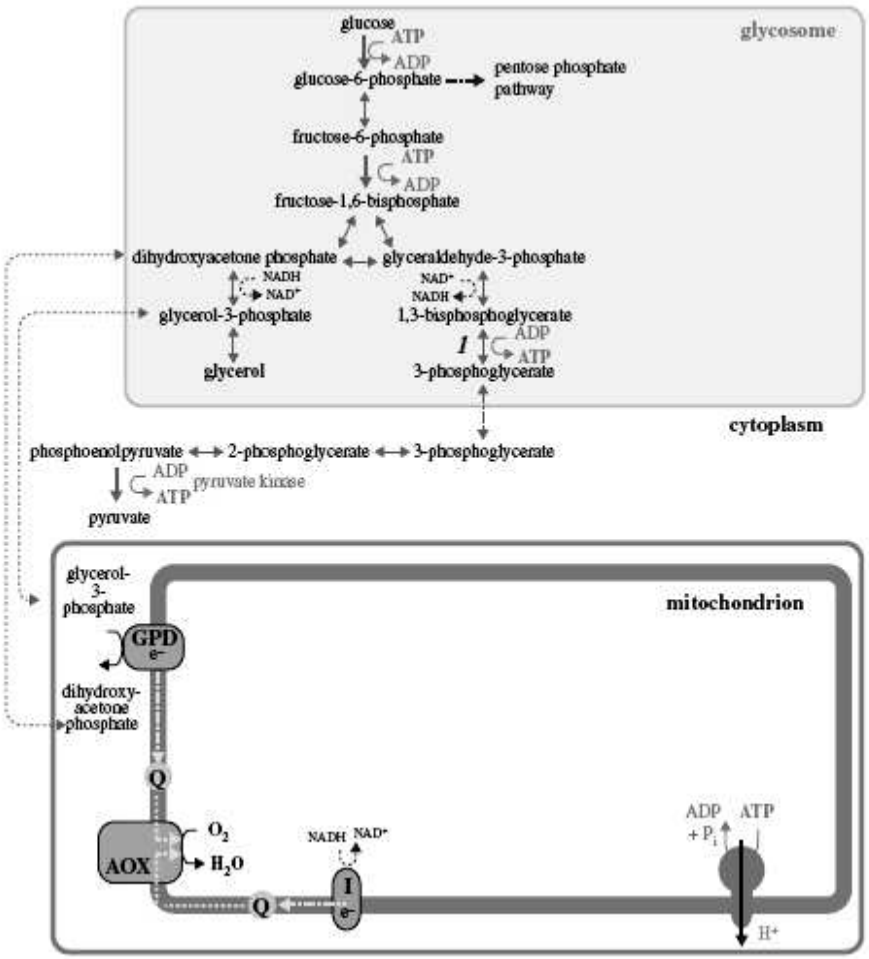


Figure 1.6 Energy metabolism in bloodstream *T.brucei*

Procyclic form relies on mitochondrial energy generation (Figure 1.7) for its viability (Bochud-Allemann and Scheneider 2002; van Weelden *et al.*, 2003). These form express a cohort of unusual enzymes, some of which are more characteristic of anaerobic metabolism (Riviere *et al.*, 2004; Coustou *et al.*, 2005). At least in procyclic form acetate is an end-product of energy metabolism because; despite the presence of a complete cohort of Krebs cycle enzymes. This observation does not of course preclude a role for the Krebs cycle in these parasites energy generation at some point during the transmission cycle through tsetse (van Weelden *et al.*, 2003, 2005). In the absence of glucose, the procyclic forms are critically dependent upon oxidative phosphorylation for energy generation (Lamour *et al.*, 2005).

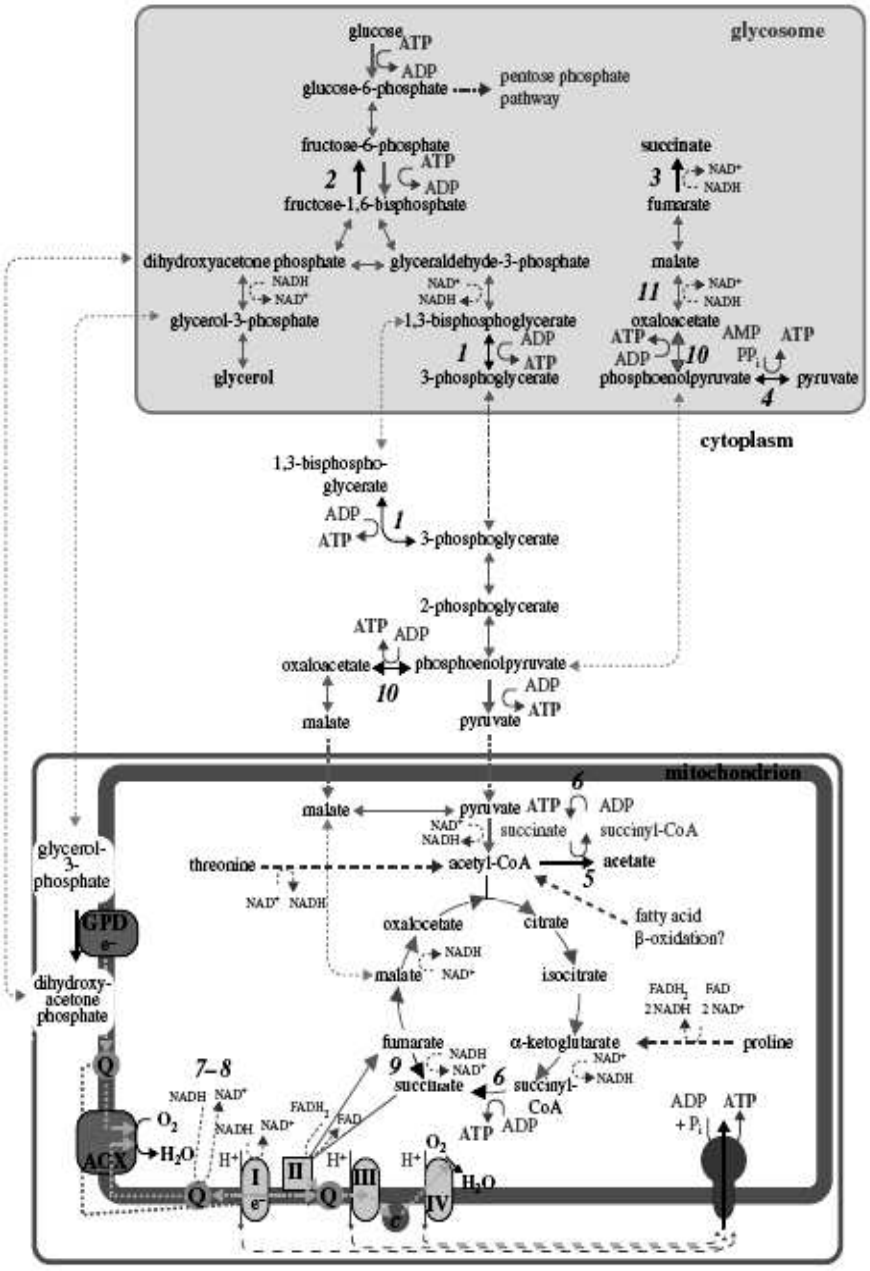


Figure 1.7 Energy metabolism in procyclic form

T.brucei also retains an ability to synthesize carbohydrates as evidenced by the presence in the genome of the key gluconeogenic enzyme fructose-1, 6-bisphosphatase (Morris *et al.*, 2002; Hannaert *et al.,* 2003). It is known that many of the enzymes found in *T.brucei,* including components of the branched respiratory chain acetate:succinate CoA-transferase, NADH-dependent fumarate reductase and pyruvate phosphate dikinase, are expressed (Bringaud *et al.*, 1998; Van Hellemond *et al.*, 1998 A,B; Besteiro *et al.*,2002). The evidence indicates that differences in the expression of these enzymes, between different forms are always likely to reflect the environment in which that cell type lives (Ginger, 2006).

1.7 Pathogenesis

The infection is initiated after the bite of a trypanosome-infected tsetse-fly, the metacyclic trypanosomes in saliva of fly, differentiate to the bloodstream stage and spread via the local draining lymph node into the vascular system. In some but not all infections, a local skin reaction or chancre occurs at the site of inoculation, which is caused by a local inflammatory response to the parasites, and subsides after 4 weeks. The early (or hemolymphatic stage) commences 1-3 weeks after an infective fly bite and presents with periods of fever, lasting 1-7 days and generalized lymphadenopathy. During this period, the parasites proliferate within the blood and lymphatic system. Symptoms include general malaise, anemia, headache, pyrexia, weight loss and weakness. Immune-activation is evident from lymph node enlargement, hepatomegaly, and splenomagaly. The late (meningoencephalitic) stage of infection coincides with the invasion of the CNS by parasites and is associated with psychiatric, motor and sensory disorders, along with sleep abnormalities. If untreated, late-stage patients progress to a final stage involving seizures, somnolence, coma and death.

Both *T.b. rhodesiense* and *T.b. gambiense* infections follow this sequence of infection stages, but with a marked difference rate of progression. *T.b. gambiense* presents as a chronic infection, in which progression to late stage may take several months or longer, and late-stage CNS infection may last several years. On the other hand, *T.b. rhodesiense* is generally regarded as an acute infection, with progression to late stage occurring in a matter of weeks and the late-stage CNS infection leading to death within 3 months, although this may not always be the case.

Trypanosomes primary immune-evasion strategy is antigenic variation. The parasites are covered with a coat of 10^7 VSG molecules attached to the trypanosome cell membrane via GPI anchor (Magez *et al.*, 2002). VSG is an immunodominant antigen, capable of eliciting both T-cell dependent and independent B-cell responses, depending on its conformation. Antibody opsonized trypanosomes are effectively cleared by the host lymphoreticular system (Macaskill *et al.*, 1981). Thereafter the parasite undergoes antigenic variation. For this purpose the VSG occupy 10 % of the trypanosomes genome and has a repertoire of more than 1000 transcriptionally inactive VSG genes but only a single active transcription site. VSG molecule plays a pivotal role in host responses as it elicits polyclonal B-cell activation, and in human infection this manifests in the generation of auto-antibodies and immune complex disease. Phospholipase C is responsible for the cleavage of the GPI anchor through release of VSG molecule in a soluble form. Given the central role of inflammatory immune responses in

sleeping sickness pathogenesis, it is possible that immunogenetic variation in host populations may influence disease progression and outcome (Sternberg, 2004).

The changes in the cell of the parasite as it traverses from the mammalian bloodstream to the tsetse fly and back again must be highly regulated and interconnected. Mapping of the events that occur during synchronous transition from bloodstream stumpy forms to procyclic form has revealed that the developmental program of the parasite is temporally ordered (Matthews, 1999). Inappropriate migration of the kinetoplast during or after segregation of that organelle would be likely to disrupt the highly orchestrated events required for successful cell division. The mitochondrion and the mitochondrial genome clearly have a central role in the development of the cell; this is evident from the importance of mitochondrial metabolism in surface antigen regulation (Vassella *et al.*, 2004).

1.8. Diagnosis

Trypanosomes are variable and inconstant characters and the clinical symptoms are insufficient for diagnosis of sleeping sickness. Through detection of trypanosome specific antibodies by agglutination assays, immunoflourescence or ELISA, diagnosis of mainly *T.b. gambiense* sleeping sickness is facilitated. The card agglutination test for trypanosomiasis, CATT/*T.b. gambiense* (Magnus *et al.*, 1978) is used in *T.b. gambiense* endemic areas for mass screening of the population at risk. Due to treatment cost and risks, definite diagnosis should be obtained by demonstration of the parasite in body fluids. Unfortunately, simple parasitological techniques such as microscopic examination of a lymph node aspirate, or of a wet or thick blood film are insensitive, especially for diagnosis of *T.b. gambiense* infection, where the number of parasites in the blood can be low. Due to the workload, parasitological examination is in practice limited to clinical or serological suspects.

Due to the selective permeability of the blood-brain barrier and the high toxicity of second stage drugs, accurate determination of the respective disease stage is important for optimal treatment. There are no specific clinical signs except in very advanced cases or any clear changes at blood level indicating the evolution from the hemolymphatic to the meningoencephalitic stage (Bisser *et al.*, 1997). According to the W.H.O., stage determination should be performed by examination of the cerebrospinal fluid (CSF) for the presence of trypanosomes, the white blood cell count and the total protein concentration (W.H.O., 1998).

For follow-up after treatment, the blood and the CSF of the patient is re-examined on several occasions (ideally 3, 6, 12, 18 and 24 months after the end of treatment). A patient is considered cured when during this 2 year follow-up period no trypanosomes are detected in

the blood, lymph or CSF (W.H.O., 1998). Thus, CSF examination plays a key role in diagnosis, selection of treatment and post-treatment follow-up of sleeping sickness patients (Van Meirvenne, 1999; Buscher and Lejon, 2004; Kennedy, 2004).

1.9. Treatment

Therapy is complicated by the different disease stages. In the meningoencephalitic stage, the selective permeability of the blood-brain barrier protects trypanosome in the brain against the action of several drugs, effective in the hemolymphatic compartment. Treatment of meningoencephalitic stage sleeping sickness requires toxic drugs and hospitalization (Van Nieuwenhove, 1999; Legros *et al.*, 2002; Burri and Brun, 2003). First stage *T.b. rhodesiense* infection is treated with suramin, *gambiense* infection with pentamidine. Pentamidine is usually well tolerated, whereas suramin may have severe adverse effects (Lejon and Buscher, 2005).

Treatment of the meningoencephalitic stage relies almost exclusively on melarsoprol, an arsenicum derivative. It is administered intravenously and requires hospitalization for at least 10 days for the short, accepted standard course (Burri *et al.*, 2000; Schmid *et al.*, 2004), or 35-36 days for the old treatment schedule. The worst adverse effect is treatment associated encephalopathy, which occurs in 5-10 % of patients and is fatal in 50-100 % of them (Pepin *et al.*, 1994). Alternatives for late stage *T.b.gambiense* sickness treatment are difluoromethylornithine (eflornithine) and nifurtimox. Both drugs are used for treatment of melarsoprol refractory cases, but difluoromethylornithine has been introduced as first-line drug by some organizations. In view of the observed increase in melarsoprol resistance, clinical trials on combination therapy are ongoing (Lejon *et al.*, 2005).

Eflornithine was developed over 20 years ago, and was registered for the treatment of *gambiense* disease in 1990. While the drug is safer than melarsoprol, eflornithine does have side effects; fever, unusual bleeding, weakness, diarrhea, nausea, stomach pain, and vomiting are common, while rarer side effects such as convulsions, loss of hearing, hair loss, headache, anemia, leucopenia and thrombocytopenia have also been observed. The administration of eflornithine, which requires multiple daily infusions, limits its use in the context of rural Africa, despite the determination of some program to use it as a first-line drug.

With the development of parasite resistance to some of the available drugs, a number of studies have attempted to combine existing drugs to overcome treatment failures. The major challenge in developing a new drug that can ensure sustainable disease control will be to find

a safe and affordable, orally administered drug that is effective against both forms of the disease, in both disease stages, and that does not require any particular skills or care to administer. The ideal regimen should not last more than a few days, thus making it manageable by peripheral health staff in an out-patient context (Simarro *et al.*, 2008).

II. Phospholipases A_2

2.1. Lipases

2.1.1 General Feature

Lipases [EC 3.1.1.3] are a family of hydrolase enzymes cleaving glycerol esters of long chain fatty acids (water insoluble). They are different from esterases [EC 3.1.1.1], which hydrolyze esters of short chain fatty acids (water soluble) (Shimada *et al.*, 1989). Lipases are serine hydrolases which act at the lipid-water interface. The catalytic triad is composed of Ser-Asp/Glu-His and usually also a consensus sequence (Gly-X-Ser-X-Gly) is found around the active site. The lipases are a versatile group of enzymes and often co-express other activities like phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase and other esterases. Generally, lipases have preference for the substrate type, whether it is a triacylglyceride or a diacylglyceride, and therefore have diacylglyceride and monoacylglyceride as products, rather than glycerol and fatty acids. The regioselectivity is often rather high for the positions *sn*-1 and *sn*-3 and less frequently for *sn*-2. The lipases belong to the α/β hydrolase family (Svendsen, 2000). Interfacial activation of lipases can be traced to the unique structural characteristics of this class of enzymes. Lipases contain a helical oligopeptide unit that shields the active site. The movement of the oligopeptide flap probably occurs when the enzyme docks to the phospholipid surface which facilitates translocation of the substrate to the cleavage site. Lipases have biological functions in bacteria, fungi, plants, higher animals in addition they function as biocatalysts in numerous industrial processes including such areas as oils and fats, detergents, baking, cheese making, leather and paper processing. Moreover, lipases are the most used enzymes in synthetic organic chemistry, catalyzing the chemo-, region- and/or stereoselective hydrolysis of carboxylic acid esters or the reverse reaction in organic solvents (Reetz, 2002).

2.1.2 Classification

The lipase enzymes consist of a large family showing the same overall structural fold but which has versatility of loop structures in contact with the substrate, and exhibits versatile substrate specificities. The lipases can be grouped into subfamilies by analysis based on sequence homology, thus dividing them into two main families.

The mammalian lipase family includes pancreatic lipase, hepatic lipase, lipoprotein lipase and pancreatic lipase –related protein. Within microbial lipases, several families have now been found, the bacterial lipases, containing the Staphylococcus lipase family, Pseudomonas lipase

family, Bacillus lipases and others, and the fungal lipases with the *Rhizomucor miehei* lipase family, *Candida rugosa* lipase family and other subfamilies (Svendsen, 2000).

2.1.3 Substrate specificity

Lipases may be divided into three categories depending on substrate specificity like nonspecific, regiospecific and fatty acid-specific. Nonspecific lipases act at random on the triacylglyceride molecule and result in the complete breakdown of triacylglyceride to fatty acid and glycerol. Regiospecific lipases are 1,3-specific lipases which hydrolyze only primary ester bonds (i.e. ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyze triacylglyceride to give free fatty acids, 1,2 (2,3)-diacylglyceride and 2-monoacylglyceride. The fatty acid-specific lipases exhibit a pronounced fatty acid preference ranging from triacylglycerides with long-chain fatty acids, small or medium-chain fatty acids and unsaturated fatty acids.

Another important property of lipases is their enantio-/stereoselective nature, wherein they possess the ability to discriminate between the enantiomers of a racemic pair. Such enantiomerically pure or enriched organic compounds are steadily gaining importance in the chemistry of pharmaceutical, agricultural, synthetic organic and natural products. The stereospecificity of a lipase depends largely on the structure of the substrate, interactions at the active site and the reaction conditions (Gupta *et al.*, 2004).

2.2 Phospholipases

2.2.1 General characteristics of phospholipases

Phospholipids are present in all living organisms and are a major component of all biological membranes along with glycolipids and cholesterol. Enzymes aimed at modifying phospholipids, namely phospholipases, are consequently widespread in nature, playing very diverse roles from one of common component in snake venom to signal transduction and digestion in humans. The phospholipases are a complex and crucially important group of enzymes that hydrolyze phospholipids (PLs) releasing a variety of products, like for example lysophospholipids, free fatty acids (FFAs), diacylglycerols (DGs), choline phosphate and phosphatidates, depending on the site of hydrolysis. They play crucial roles in many biochemical processes related to, among others, digestion and inflammation. They are used for industrial applications like bread making, emulsification for different applications and

degumming (De Maria *et al.*, 2007). Apart from these roles, phospholipases play a critical role in generating lipid-derived second messengers. Because their products are often second messengers, they are highly regulated by the cell. For a given ester bond, there are separate secreted as well as cytoplasmic phospholipases with different substrate specificities and modes of regulation. Phospholipases also have a wide and complex array of regulatory mechanisms involving cytoplasmic proteins, as well as different effector lipids (e.g., phosphatidylinositol 4,5-biphosphate, or PIP_2) or Ca^{2+} . Many of these enzymes are water soluble while their substrates are insoluble and organized in a 2-dimensional matrix. They have thus evolved unique strategies for carrying out and regulating catalysis at an interface. They have a large increase in activity toward a substrate organized in an aggregate (e.g. micelle) compared to the same substrate presented as a monomer in solution which is known as interfacial activation (Roberts, 1996).

2.2.2 Family of phospholipases (PLA, PLB, PLC, PLD)

Phospholipases are classified according to the bond cleaved in a phospholipid. The IUBMB enzyme nomenclature for respective phospholipase is stated below and with substrate specificity for each (Figure 1.8)

Phospholipase A_1 (PLA_1) 3.1.1.32
Phospholipase A_2 (PLA_2) 3.1.1.4
Phospholipase C (PLC) 3.1.4.3
Phospholipase D (PLD) 3.1.4.4

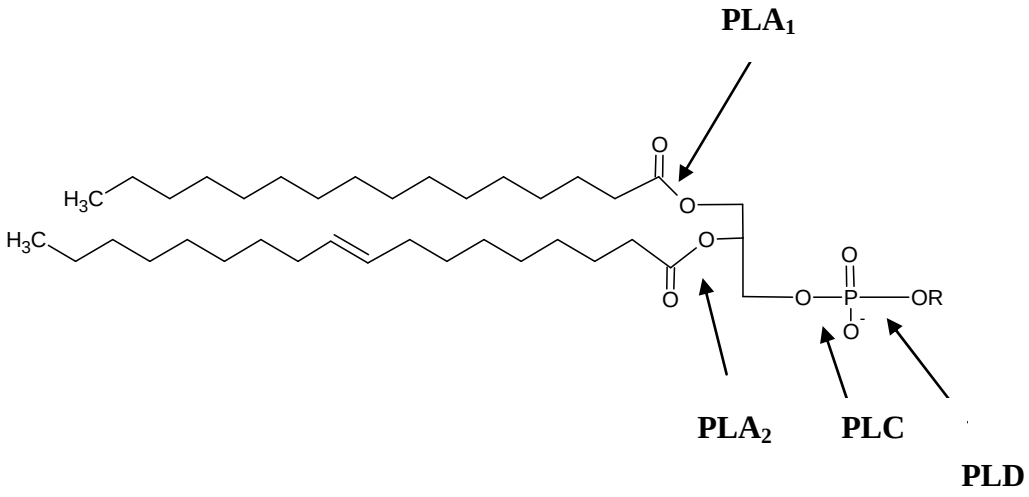


Figure 1.8 Reactions catalyzed by PLA_1, PLA_2, PLC and PLD

Phospholipase A_1 (PLA_1)

PLA_1 activities have been detected, by measuring hydrolysis of phosphatidyl choline (PC) to lysophosphatidyl choline, in many cells and tissues from various organisms. PLA_1 from different sources (Figure 1.9 a-e) show considerable sequence similarity to human hepatic and pancreatic lipases (HHL and HPL) and the pancreatic lipase-related protein 2. All these enzymes have the typical Ser-His-Asp catalytic triad. The activity of most of these lipases increases in the presence of Ca^{2+}, while with EDTA correspondingly acts as an inhibitor.

Phospholipase A_2 (PLA_2)

PLA_2s (Figure 1.9 f-j) are the most widely studied phospholipases which are subdivided into four categories:

1. Secreted PLA_2s ($sPLA_2$s)
2. Cytosolic PLA_2s ($cPLA_2$s)
3. Ca^{2+}-independent PLA_2s ($iPLA_2$s)
4. Platelet-activating factor acetyl hydrolase (PAF-AH)

These overall categories are further subdivided into 15 different groups.

$sPLA_2$s have a large number of disulphide bridges (5-7) which is consistent with working in an extracellular environment and require millimolar concentrations of Ca^{2+} ions for optimum catalytic activity. $cPLA_2$s preferentially hydrolyze phospholipids containing arachidonic acid and play a key role in the biosynthesis of eicosanoids. Full activation of these enzymes requires Ca^{2+} binding to an N-terminal C2 domain and phosphorylation on serine residues. $iPLA_2$s contain 7 to 8 ankyrin repeats (one of the most common sequence motif), contain the consensus lipase motif GXSXG. PAF-AH acts on platelet-activating factor (PAF) and not on long chain phospholipids. PAF-AH has implications in infammation and atherosclerosis.

Phospholipase C (PLC)

PLCs (Figure 1.9 k) belong to a large superfamily of enzymes called phosphoinositide-specific phospholipases (PI-PLCs). The EC number 3.1.4.11 is associated with phosphatidylinositol phospholipase C activity. PI-PLCs molecules were found in a broad spectrum of organisms including bacteria, simple eukaryotes, plants and animals. Whereas the eukaryotic PI-PLCs play a central role in most signal transduction cascades, the prokaryotic enzymes act as virulence factors in some pathogenic bacteria. There is large structural similarity between them. Both the eukaryotic and the prokaryotic enzymes need cofactors, calcium and zinc, respectively, for optimum catalytic activity.

Phospholipase D (PLD)

PLD is a ubiquitous enzyme found in bacteria, fungi, plants and mammals. The PLD superfamily (Figure 1.9 l) includes enzymes that are involved in phospholipid metabolism, nucleases, toxins and virus envelope proteins of unknown function. Phosphatidic acid (PA), one of the reaction products is involved in signal transduction (Leiros *et al.*, 2000). The PLD enzymes have a characteristic modular structure in which a common catalytic domain is flanked by regulatory sequences. These include lipid-binding PX and PH domains and motifs that are unique to the PLD enzymes. The active site region of the PLD superfamily consist of a conserved sequence motif, the $HXK(X)_4D$ motif (or the HKD motif), members of this superfamily share two copies of such a motif (De Maria *et al.*, 2007).

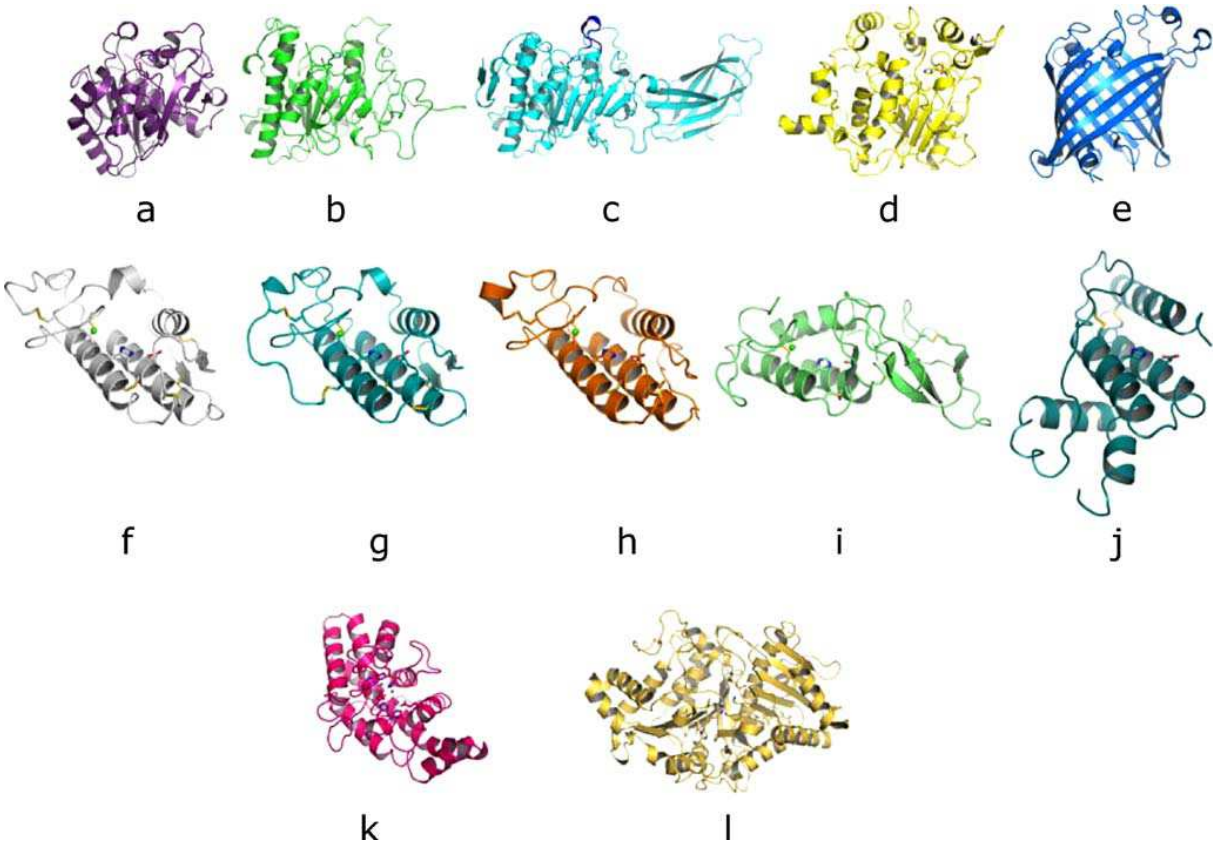


Figure 1.9 Phospholipase structural diversity. Cartoon representation of the structures of the enzymes **a**. *T. lanuginosus* phospholipase A_1. **b** Homology model of the vespid venom PLA_1. **c** Guinea pig pancreatic lipase-related protein 2. **d** *S. hyicus* P1 lipase. **e** Outer membrane PLA. *$sPLA_2$s from different origins*, **f** Pig pancreatic. **g** Human synovial fluid. **h** Taiwan cobra venom. **i** *A. mellifera*. **j** *S.violaceoruber*. **k** Phospholipase C from *Bacilllus cereus*. **l** Phospholipase D from *Streptomyces* sp. Strain PMF (De Maria *et al.*, 2007).

2.3 Phospholipase A_2 (PLA_2)

The phospholipase A_2 (PLA_2) superfamily consists of a broad range of enzymes defined by their ability to catalyze specifically the hydrolysis of the ester bond (*sn*-2) of substrate phospholipids. The products of the PLA_2 reaction are free fatty acid and lysophospholipid. The fatty acids released are arachidonic acid (AA) and oleic acid (OA) which are important stores of energy but more importantly AA can also function as a second messenger and as the precursor of eicosanoids, which include prostaglandins and thromboxanes generated through the cyclooxygenase reaction, and the leukotrienes, generated through the lipoxygenase reaction. The other product of PLA_2 action, lysophospholipid, is important in cell signaling, phospholipid remodeling, and membrane perturbation. The actions of these enzymes can be

important for down-regulating cell signals, as seen in case of the PLA_2 -catalyzed hydrolysis of the bioactive phospholipid, platelet activating factor (PAF), to its inactive lysophospholipid form. The progress in genomics has observed an expansion in the number of PLA_2 subgroups. This has lead to the characterization of new subgroups with the expectation of even more in the future (Six and Dennis, 2000).

2.4 Classification of PLA_2

PLA_2 have been systematically classified on the basis of their nucleotide and amino acid sequence. The diverse PLA_2 enzymes have been classified into Groups from I to XV thus far (Table 1 and 2 according to Six and Dennis, 2000; Schaloske and Dennis, 2006). A broader classification of the PLA_2 that has historically been used to describe PLA_2 activities for which sequence data are not available.

Table 1

PLA_2 groups utilizing a catalytic histidine

Group		Initial/common sources	Size (kDa)	Disulfide bridges
I	A	Cobra, krait venom	13-15	7
	B	Mammal pancreas	13-15	7
II	A	Human synovial fluid, platelets, rattlesnake, viper venom	13-15	7
	B	Gaboon viper venom	13-15	6
	C	Rat/mouse testis	15	8
	D	Human/mouse pancreas/spleen	14-15	7
	E	Human/mouse brain/heart/uterus	14-15	7
	F	Human/mouse testis/skin	16-17	7
III		Bee/lizard/scorpion/human	15-18	5
V		Mammal heart/lung/macrophage	14	6
X		Human spleen/thymus/leukocyte	14	8
IX		Snail venom (Conodipine-M)	14	6
XI	A	Green rice shoots (PLA_2 -I)	12.4	6
	B	Green rice shoots (PLA_2-II)	12.9	6
XII		Mammal heart/kidney/skin, Muscle	18.7	7
XIII		Parvovirus	< 10	0
XIV		Symbiotic fungus/*Streptomyces*	13-19	2

This classification has numerous caveats, e.g. the Group IV-C PLA_2 is generally referred to as cPLA$_2$-γ, despite its being a Ca^{2+}-independent enzyme. However, the system remains useful

for making generalizations when describing properties of multiple PLA_2 groups or when the specific identity of a PLA_2 is unknown. With the expansion of the superfamily of these enzymes, it has become increasingly difficult to generalize the properties of the PLA_2s. However, in general, the mammalian $sPLA_2$s (Groups I-B, II-A, C-F, III, V, X, XII) have low molecular masses (13-19 kDa) and lack specificity for arachidonate-containing phospholipids. The $cPLA_2$s (Group IV, comprising three subgroups) have higher molecular masses (> 60 kDa), and preferentially hydrolyze arachidonate-containing phospholipids (although Group IV-C PLA_2 exhibits only a marginal preference). Finally, the $iPLA_2$ (Group VI) have high molecular masses (about 85 kDa) but are not selective for arachidonate-containing phospholipids. The Ca^{2+} requirements of the PLA_2s do not distribute within this classification system as most of them require millimolar concentrations of Ca^{2+} for enzymatic activity while others require µM levels. Some only on the other hand, require Ca^{2+} for translocation to membranes but not for activity. Except Group IV-C which requires no Ca^{2+} similar to the $iPLA_2$s (Balsinde *et al.*, 2002).

Table 2

PLA_2 groups utilizing a catalytic serine

Group		Initial/common sources	Alternate names employed	Size (kDa)	Ca^{2+} effects
IV	A	Human U937 cells/platelets, RAW 264.7/rat kidney	$cPLA_2\alpha$	85	< mM
	B	Human pancreas/liver/heart/brain	$cPLA_2\beta$	114	< mM
	C	Human heart/skeletal muscle	$cPLA_2\gamma$	61	None
VI	A-1	P388D1 macrophages, CHO	$iPLA_2$ or $iPLA_2$-A	84-85	None
	A-2	Human B-lymphocytes, testis	$iPLA_2$-B	88-90	None
	B	Human heart/skeletal muscle	$iPLA_2\gamma$ or $iPLA_2$-2	88	None
VII	A	Human/mouse/porcine/bovine plasma	PAF-AH	45	None
	B	Human/bovine liver/kidney	PAF-AH(II)	40	None
VIII	A	Human brain	PAF-AH Ib α_1 (subunit of trimer)	26	None
	B	Human brain	PAF-AH Ib α_2 (subunit of trimer)	26	None
XV		Human,murine,bovine	ACS,lysosomal PLA_2 ($LPLA_2$), LLPL	42	None

2.5 Catalytic mechanism of PLA_2s

Mechanistic studies have shown that catalysis by $sPLA_2$s does not take place via the formation of the classical acyl-enzyme intermediate of serine esterases. Instead, the $sPLA_2$s use a His residue, assisted by an Asp to polarize a bound H_2O which then attacks the carbonyl group. The Ca^{2+} ion bound to the conserved Ca^{2+} loop is required to stabilize the tetrahedral transition state intermediate, which for this class of PLA_2s plays an active role in catalysis.
On the other hand, the catalytic mechanism of the Group IV $cPLA_2$ is completely independent of Ca^{2+} and interacts with the membrane where its substrate is localized. The $cPLA_2$ appears to function as a serine hydrolase, acting via an acyl-enzyme intermediate. Although its catalytic mechanism has not been fully clarified, they can be defined as serine hydrolases because a role for His, as in the classical Ser/His/Asp triad has not been demonstrated so far.
The $iPLA_2$ also appears to function as a serine hydrolase, with the active Ser residue located in the middle of the consensus sequence GXSXG, which is common to many other lipases as well. These enzymes function via an acyl-enzyme intermediate (Balsinde *et al.*, 1999).

2.6 Interfacial catalysis of PLA_2s

The phospholipase A_2 acts on the aggregated form of its substrate phospholipids and is probably one of the best characterized examples of interfacial catalysis. The catalysis occurs on the substrate interface and the enzyme remains bound to the interface during several catalytic turnover cycles. It is proposed that binding of PLA_2 to its substrate is part of a lipid-water interface such as a micelle or membrane. The enzyme (E) first binds to the interface itself but not to any individual phospholipid molecules. This produces an interfacially activated enzyme (E*), which subsequently binds a phospholipid substrate (S) as shown in equation:

$$E \rightleftharpoons E^* \rightleftharpoons E^*S \longrightarrow E^*P \rightleftharpoons E^* + P$$

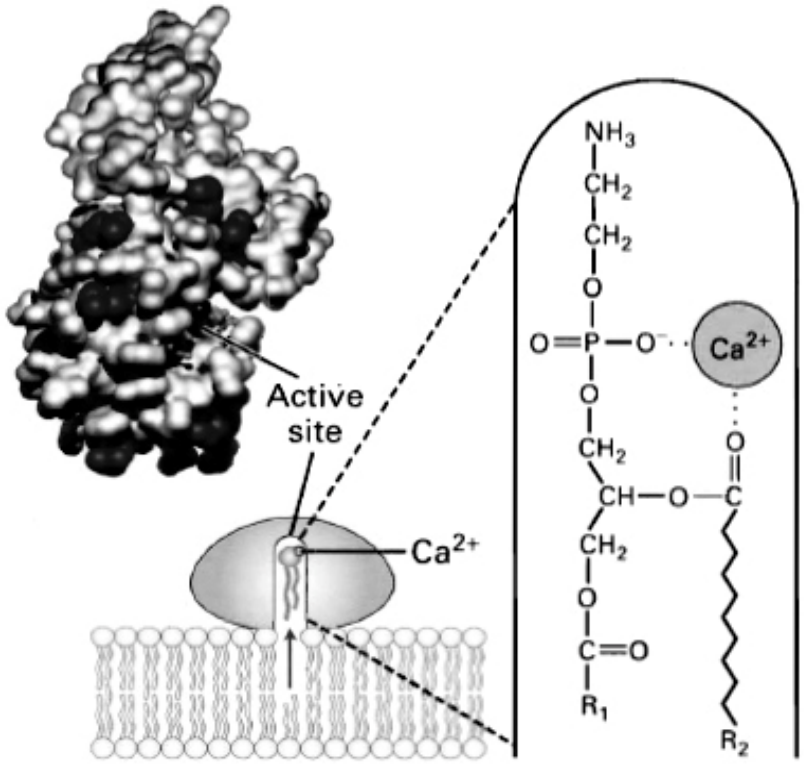


Figure 1.10 $sPLA_2$ catalytic mechanism on phospholipid substrate (Haas *et al.*, 1974)

To work on a bilayer vesicle, PLA_2 (Figure 1.10) stays associated with the interface while it hydrolyzes successive phospholipid substrate molecules (scooting mode). Alternatively, it dissociates from the interface after each catalytic event (hopping mode). Use of anionic vesicle substrates forces the equilibrium toward the scooting mode. Only about 9-10 carbons of the *sn*-2 acyl chain interact with the enzyme. The remainder of the chains presumably remains buried in the interface (Dennis, 1994). Apart from this mechanism, this process is regulated by diverse factors including the membrane charge, fluidity, mode of membrane binding (insertion, orientation) and allosteric conformational effects (Ray *et al.*, 2007).

2.7 Role of PLA_2s in arachidonic acid metabolism

In general, not one but several PLA_2s are involved in cellular regulation and lipid messenger formation. This mechanism includes participation of at least two different PLA_2s namely, $cPLA_2$ and $sPLA_2$, for generation of arachidonic acid (AA) in cells. Activation of the $cPLA_2$ is the foremost event and may be mediated by several signals, such as phosphorylation

cascades, intracellular Ca^{2+} elevations and perhaps phosphatidylinositol 4,5-bisphosphate levels. The synchronous coupling between these signals may converge to produce a prolonged activation of the $cPLA_2$. In cells not expressing $sPLA_2$, the $cPLA_2$ probably contributes most of AA mobilized during cellular activation. However, in those cells that contain $sPLA_2$, the bulk of AA release appears to be mediated by the $sPLA_2$, not the $cPLA_2$. Many types of eicosanoid-producing cells (e.g. phagocytes, mast cells, platelets) synthesize and secrete $sPLA_2$. Once secreted, it associates with the other outer surface of the surrounding cells and releases AA which can be captured by these cells to produce eicosanoids. Despite its lack of AA specificity, the $sPLA_2$ releases AA in preference to other fatty acids. $sPLA_2$ action appears to be dependent on $cPLA_2$. Thus, $cPLA_2$ is key for AA signaling even in those settings where the $sPLA_2$ is the major effector of the response.

Although $iPLA_2$ does not appear to be directly involved in effecting stimulated AA release, it is important for the AA metabolism, in particular for phospholipid fatty acid remodeling. Thus, the $iPLA_2$ participates in the main pathway through which the cells incorporate AA and other fatty acids into membrane phospholipids. The AA-releasing PLA_2s use different AA pools for the release. Thus, by regulating fatty acid remodeling reactions, the $iPLA_2$ may influence the subcellular distribution of AA among different compartments and the relative amount of fatty acid present in each compartment (Balsinde, 1999).

Free arachidonic acid (Figure 1.11) is then converted into potent bioactive mediators by the action of various cyclooxygenases (COX), lipoxygenases (LO), and cytochrome P450s. The eicosanoid production cascade often works in a nonlinear fashion, with multiple enzymes creating a single product and multiple products acting as a substrate for a single enzyme. COX activity produces prostaglandins (PG) and hydroxyeicosatetraenoic acids (HETEs) whereas lipoxygenases can create leukotrienes (LT), HETEs, and lipoxins. Cytochrome P450s catalyze the production of HETEs and epoxy-eicosatetraenoic acids, as well as ω-oxidation of various eicosanoids. Other enzymes can further act on eicosanoids by catalyzing hydration, dehydration, and β-oxidation reactions. Eicosanoid-producing enzymes and their biological receptors are differentially expressed among various cell and tissue types, enhancing signalling specificity (Buczynski, 2007).

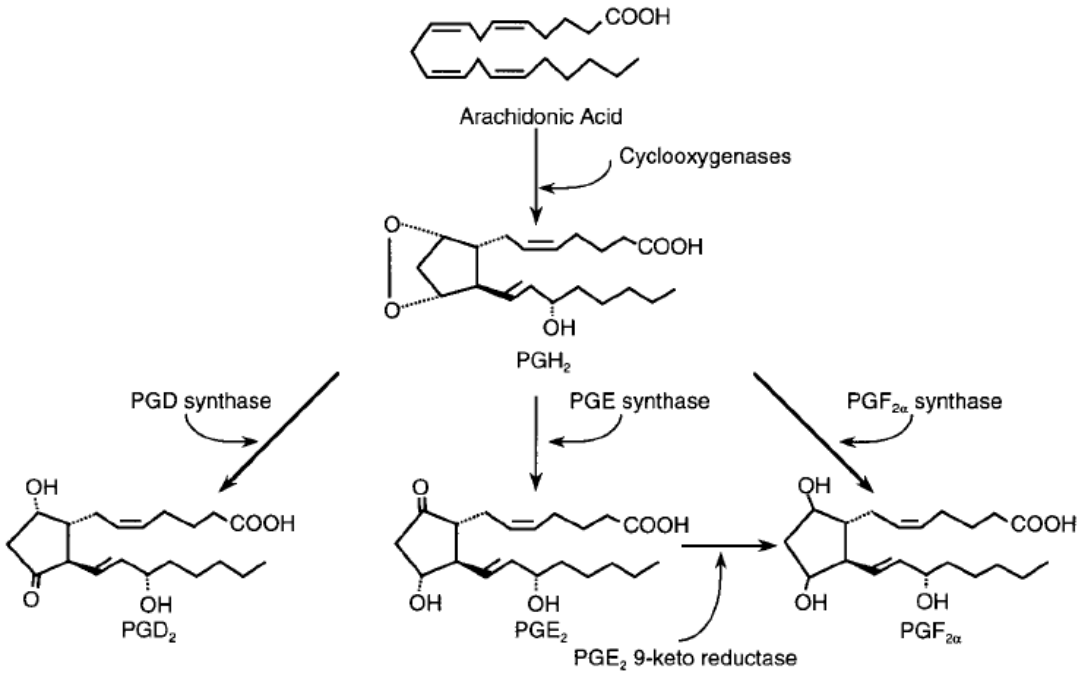


Figure 1.11 Arachidonic acid metabolism and biosynthesis of prostaglandins (Kubata *et al.*, 2000)

In resting cells, arachidonic acid is stored within the membranes usually esterified to glycerol in phospholipids. A receptor-dependent event, requiring a transducing G protein, initiates phospholipid hydrolysis and releases the fatty acid into the intracellular medium. PLA_2 catalyzes the hydrolysis of phospholipids at *sn* (stereospecific numbering)-2 position. Therefore, this enzyme can release arachidonate in a single-step reaction. On the other hand, PLC and PLD do not release free arachidonic acid directly. Rather, they generate lipid products containing arachidonate (diacylglycerol and phosphatidic acid). After they are released, free arachidonate has possible fates like reincorporation into phospholipids and metabolism by distinct enzyme pathways like COX, LO and cytochrome P450. The end products of these pathways modulate different activities in the cell like ion channels, protein kinases and ion pumps. The eicosanoids may also exit the cell of origin and the action may be terminated by uptake into phospholipids, or enyzmatic degradation (Piomelli, 2001).

2.8 Importance of phospholipase A_2 in eukaryotes

Phospholipase A_2s presence in pancreatic juices and in cobra venom was discovered around the beginning of the twentieth century. The role of these secreted PLA_2s in digestion and

snake venom has since been well documented. Evidence began to appear in the last several decades that PLA_2s are also present in most types of cells and that they are involved in many different cellular functions including general lipid metabolism and membrane homeostasis. During the same period, the view about PLA_2's substrate, phospholipids and their metabolites which are involved in a large number of cellular control systems including signal transduction and eicosanoid production has also been changed. Due to PLA_2s actions, which affect a wide range of human physiological functions and diseases including asthma and allergy, the initiation and maintenance of parturition, blood clotting, atherosclerosis, sepsis, inflammatory bowel disease, arthritis and other inflammatory diseases (Dennis, 2000).

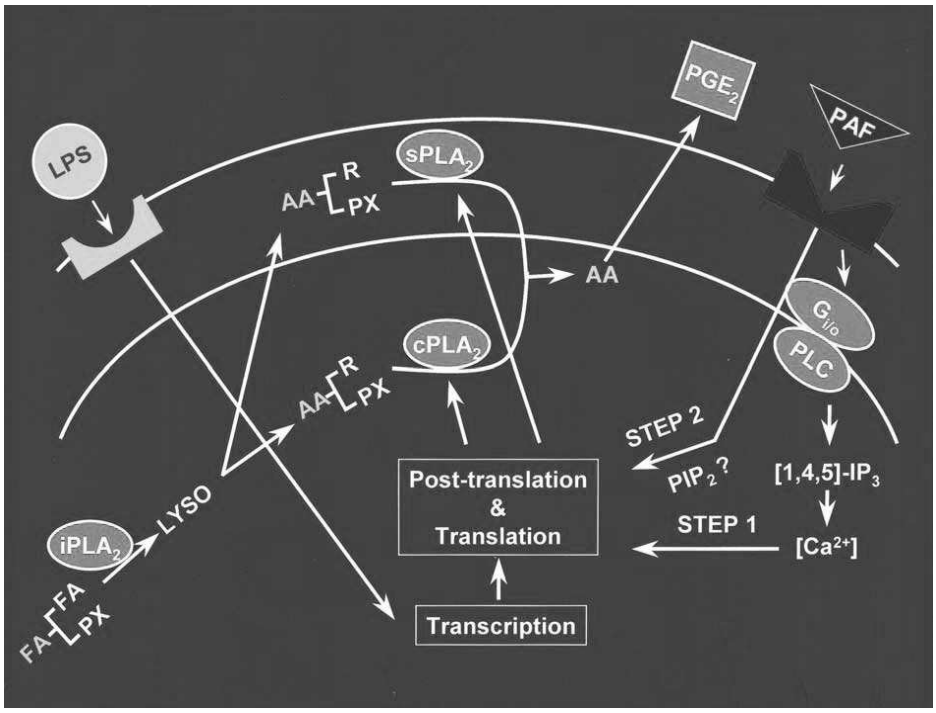


Figure 1.12 Signal transduction mechanism of phospholipases.$iPLA_2$ which regulates phospholipid reacylation, $cPLA_2$ is activated by external stimuli, precedes with activation and/or secretion of $sPLA_2$ .LPS (lipopolysaccharide) (Balsinde *et al.*, 2002).

Signal transduction (Figure 1.12) mediates changes in cellular metabolism and function. Phospholipases are required for transduction of extracellular signals across the cellular plasma membrane. Several signal activated phospholipases (SAPs) may be activated by a given agonist in a given cell. This signalling cascade begins with one or more phospholipases that are directly coupled to the receptor. These phospholipases may be PtIns-PLC, PLC, PLD, PLA_2 or a combination of all depending on cell type and agonist. Some phospholipases e.g.

PLCs are induced and provide very long-term signals for processes such as cell proliferation and differentiation. The exact sequence in which these SAPs are activated appear to be highly cell-type specific. The activated hydrolysis of phosphatidylinositol-4,5-bisphosphate [PtIns(4,5)P_2] by a phosphoinositide-specific phospholipase C (PtIns-PLC) is involved in signal transduction pathway. The products inositol-1,4,5-triphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) intracellular targets are Ca^{+2}-storage organelles and protein kinase C (PKC) respectively. The activated protein kinase C phosphorylates the lipocortin moiety of the lipoprotein-PLA_2 complex and PLA_2 is liberated. In the presence of elevated cytosolic Ca^{+2} ions, PLA_2 is activated and splits phospholipids into lysophopholipid and arachidonic acid. Different cells express a different complement of enzymes for metabolizing lipid-derived messengers which indicates that this signalling pathway is considerably complex (Liscovitch, 1992).

2.9 Phospholipases in trypanosomes

Phospholipids constitute the major proportion of total lipids in African trypanosomes. Both procyclic and bloodstream form of *T.brucei* contain phospholipids of all classes present in mammalian cells, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), cardiolipin and sphingomyelin. Trypanosomes do not obtain intact phospholipids from their hosts, but instead synthesize their own phospholipids using headgroups (such as choline, ethanolamine, and inositol) acquired from the host and fatty acids which are then often modified. Fatty acid composition of these lipids of the parasite differs during life cycle (van Hellemond and Tielens, 2006). Both elongation and desaturation of fatty acids in trypanosomes has been observed. *T.brucei* uses three of its elongases (elongases 1-3) in bloodstream form and procyclic form to elongate pre-existing fatty acids to myristate (C14:0) and stearate (C18:0) respectively; therefore, elongase 4 shown to elongate polyunsaturated fatty acids may be the only elongase which may play a role in arachidonic acid synthesis from unsaturated fatty acids (Lee *et al.*, 2006)

Eukaryote cells experience various environmental challenges (e.g. thermal, osmotic, oxidative, glucose deprivation, and extremes of pH) in normal processes, some of which may be associated with cell differentiation. To these transient changes in the extracellular environment, cells have developed several response pathways including the activation of phospholipases after osmotic stress. In the trypanosomatid *Leishmania donovani*, a

phospholipase D is activated during hypotonic shock. With *T.brucei*, the life cycle of which involves alternating sojourns in vertebrates and tsetse flies, environmental differences encountered by the cells include temperature variation, glucose deprivation, and osmotic differences (Subramanya and Mensa-Wilmot, 2006). Phospholipases play important roles in generating lipid second messengers. One of the best characterized is phosphatidylinositol (PI)-specific phospholipase (PL) C (PI-PLC). Phosphatidylcholine (PC)-degrading activities have been the subject of a number of studies in African trypanosomes (Bertello *et al.*, 2000).

Glycosylphosphatidylinositols (GPIs) are glycolipids in eukaryotes. *T.brucei* has a surface coat which comprises predominantly , the GPI-anchored variant surface glycoprotein (VSG). Free GPIs that are not linked to proteins exist in large amounts in the protozoan parasites, *T.brucei*, *Leishmania major* and in vertebrates (McConville and Menon, 2000). The free glycolipids, glycolipid A (also termed P2; EtN-phospho-Man3-GlcN-PtdIns) and glycolipid C (or P3; EtN-phospho-Man3-GlcN-(2-acyl)-PtIns) are produced in bloodstream form *T.brucei* (Masterson *et al.*, 1989; Mayor *et al.*, 1990). Some free GPIs (e.g. glycolipid A) are precursors of protein-linked GPIs (McConville and Menon, 2000). *T.brucei* expresses a GPI-specific phospholipase C (GPI-PLC). The enzyme is not secreted but associated with intracellular vesicles (Bulow *et al.*, 1989). There are two possible substrates for GPI-PLC *in vivo*: GPI-anchored proteins (e.g. VSG) and free intracellular GPIs. There is no evidence that free GPIs are cleaved in *T.brucei*. In differentiating parasites, GPI-PLC contributes to the release of VSG from the plasma membrane (Gruszynski *et al.*, 2003). The biological functions of GPI-PLC in non-differentiating bloodstream form *T.brucei* is not resolved. In addition it is not clear how the enzyme is prevented from digesting intracellular GPIs. They accumulate at the endoplasmic reticulum (ER) whereas GPI-PLC associates with glycosomes (Zheng *et al.*, 2004). GPI-PLC is a membrane protein, which lacks peptide signals that direct soluble proteins to glycosomes (Parsons, 2004; Subramanya and Mensa-Wilmot, 2006).

In four different species, calcium-independent phospholipase A_1 was the predominant PC-degrading activity. The levels of PLA_1 varied widely, with very high activity in *T. brucei* and relatively low activity in *T.lewisi*. *T. brucei* PLA_1 appears to bear lysophospholipase activity and as such it participates in the supply of fatty-acyl and polar-head moieties for the synthesis of cellular phospholipids. In *T. cruzi*, due to mechanism of membrane-lipid adaptation suggests the action of phospholipase A activity as part of a deacylation-reacylation cycle (Wainszelbaum *et al.*, 2001). It was observed that *T. congolense* possessed a mixture of free fatty acids and lysolecithin produced by the action of phospholipases and phospholipase A_2 on phospholipids (Nok *et al.*, 1993). In trypanosomes, the process of Ca^{2+} influx is controlled

with products of PLA_2 and is not initiated by depletion of intracellular pools. Arachidonic acid stimulates Ca^{2+} influx in a process that cannot be mimicked by short chain saturated fatty acids. Metabolic processing of arachidonic acid to prostaglandin D contributes to growth arrest. *T. brucei* bloodstream forms produce prostaglandins (Kubata *et al.*, 2000) and therefore, phospholipases may play a pivotal role in its regulation (Ridgley and Ruben, 2001). The fate of unsaturated fatty acids liberated from lipids in *T.brucei* is not certain, but free fatty acid has been implicated on regulating calcium mobilization in the cells (Eintracht *et al.*, 1998: Catisti *et al.*,2000). The fatty acids have been shown to serve as a precursor for prostaglandin biosynthesis in *T.brucei*. Presence of PLA_1 activity in *T.brucei* was observed after cleavage of fatty acid at *sn-1* of phosphatidylcholine (GPCho, after new nomenclature) (Fahy *et al.*, 2005). $TbPLA_1$ has been cloned, identified and characterized in *T.brucei*, which is intracellularly localized within the cytosol and whose preferred substrate is GPCho which are the major phospholipids in *T.bruei*. $TbPLA_1$ is constitutively expressed in both procyclic and bloodstream forms trypanosomes. Lysoglycerophosphatidylcholine (lysoGPCho) synthesis is mediated by $TbPLA_1$, whose level is 3-fold higher in bloodstream form as compared to procyclic form. This enzyme does not require metal co-factors for activity, but it does require interfacial activation prior to catalysis. Furthermore, this enzyme appears not to be essential for cell viability or for virulence in a mammalian host (Richmond and Smith, 2007 A, B).

2.10 GPI-phospholipase C (GPI-PLC) and variable surface glycoprotein (VSG) in *T. brucei*

During the parasitemia, the majority of the dividing parasites (e.g. long slender form) belong to the same antigen type, called the homotype, expressing each 10^7 densely packed identical VSG molecules on their surface. GPI-linked VSG represents 10 % of the total trypanosome protein content, and on every peak of infection, the host is confronted with the release of an enormous quantity of these molecules (Paulnock *et al.*, 2001).The molecular structure of trypanosome GPI show the presence of a trypanosome-specific galactose modification on the molecule (Ferguson *et al.*, 1985; 1988). Based on the VSG N-linked carbohydrate, VSGs has been categorized into three groups (Ferguson *et al.*, 1989). Group I VSGs encompass a collection of molecules carrying a single conserved glycosylation site, about 50 amino acid residues from the C-terminus (Zamze *et al.*, 1990). In contrast, group II VSGs have a conserved glycosylation site five or six residues from the C-terminus as well as an additional, second less conserved glycosylation site (Zamze *et al.*, 1991). Finally, group III, thus far, is

represented only by one single trypanosome variant, i.e. *T.brucei* MITat 1.5. While this VSG variant is characterized by the presence of three glycosylation sites, it is so far the only VSG known that lacks the trypanosome-specific galactose modification of the VSG-GPI anchor (Magez *et al.*, 2002).

The parasite has a repertoire of about 1000 different VSG genes, can switch expression from one VSG to another that has a different amino acid sequence. It is this mechanism of antigenic variation that enables trypanosomes to evade the host's immune response (Cross, 1990). The trypanosome VSG anchor is unique in that its fatty acids are strictly myristate, a 14 –carbon saturated fatty acid. The VSG anchor is constructed as free GPI, which is subsequently attached to the newly synthesized VSG in the endoplasmic reticulum (ER). Glycolipid A itself is synthesized in a pathway in which glucosamine, mannose, and phosphoethanolamine are sequentially added to phosphatidylinositol (Englund, 1993). This undergoes a myristate-specific fatty acid remodelling reaction to replace its longer chain fatty acids with myristate (Masterson *et al.*, 1990). The myristate exchange involves attachment of myristate at both *sn-1* and *sn-2* positions of GPI. Remodelling generates the dimyristoylated GPI which is then added to newly synthesized VSG. Fatty acid remodelling reaction involving deacylation and reacylation, which seemed to provide an adequate explanation for the exclusive presence of myristate on the VSG anchor. The myrsitates are added in the final stages of GPI biosynthesis in a remodeling reaction. (Morita *et al.*, 2000). Myristate exchange and fatty acid remodelling appear to occur in different subcellular compartment. Myristate exchange may be a proofreading or a repair mechanism for VSG, ensuring that VSG are fully myristoylated (Buxbaum *et al.*, 1996).

These parasites express up to 10^7 copies of GPI anchor and/or GPI-related glycolipids per cell (Ferguson *et al.*, 1994). VSG is liberated as a soluble molecule by means of a GPI-PLC (Fox *et al.*, 1986). When VSG is cleaved by a GPI-PLC, the dimyristoylglycerol (DMG) of the GPI anchor is left in the membrane while VSG is released in a soluble form (Zamze *et al.*, 1988). Cleavage of the VSG-GPI anchor correlates with the induction of an enhanced inflammatory response during chronic trypanosome infections due to which the role of GPI-PLC activation on the interaction between trypanosomes and the host immune system is established (Magez *et al.*, 2002).

Procyclic trypanosomes express a different cell surface coat, made up of 10^6 procylcin glycoproteins (Roditi *et al.*,1987; Mowatt *et al.*, 1987; Richardson *et al.*, 1988) and a smaller number (10^6) of poly-N-acetyllactosamine containing free GPIs (Lillico *et al.*, 2003; Vassella *et al.*, 2003; Nagamune *et al.*, 2004; Roper *et al.*, 2005). The procyclins are polyanionic, rod-

like proteins encoded by procyclin genes (Roditi and Clayton, 1999). *T.brucei* contains (per diploid genome) two copies of the GPEET1 gene encoding 6 GPEET repeats, one copy each of the *EP-1* and *EP-2* genes, encoding EP1 procyclins with 30 and 25 consensus repeats, respectively, two copies of the *EP2-1* gene, encoding EP2 procyclin with 25 EP repeats, two copies of the *EP3-1* gene, encoding EP3 procyclin with 22 EP repeats (Acosta-Serrano *et al.*, 1999). The EP1 and EP3 procyclins contain a single N-glycosylation site, occupied exclusively by a conventional $Man_5GlcNAc_2$ oligosaccharide, at the N-terminal side of the EP repeat domain (Treumann *et al.*, 1997; Acosta-Serrano *et al.*, 1999). Whereas neither EP2 nor GPEET procyclin is N-glycosylated. GPEET1 procyclin is phosphorylated on six of seven T residues (Butikofer *et al.*, 1999; Mehlert *et al.*, 1999; Schlaeppi *et al.*, 2003). GPEET and EP procyclins contain similar GPI membrane anchors (Guther *et al.*, 2006) . Procyclic form express an abundant stage-specific GPI anchored glycoprotein, the procyclic acidic repetitive protein (PARP) which contains palmitate esterified to inositol, and stearate at *sn-1*, in a monoacylglycerol moiety. Addition of GPI anchor moiety to PARP is dependent on *de novo* protein synthesis, excluding the possibility that incorporation of fatty acids into PARP can occur by a remodelling of pre-existing GPI anchors (Field *et al.*, 1991).

III. Aim of this study

The parasites lack the ability to synthesize arachidonic acid (AA) from acetate (Frayha and Smyth, 1983). Nonetheless, they acquire fatty acid from their environment and utilize it into their AA and prostaglandin (PG) synthesis. *T.brucei*, *T.cruzi*, leishmania species and *Crithidia fasciculata* convert AA and PGH_2 to PGs (Kubata *et al.*, 2007). Trypanosomes produce different prostaglandins like PGD_2, PGE_2 and PGF_2 which possibly interfere with the host's immune response (Kubata *et al.*, 2000). In *T.brucei*, a $PGF_2\alpha$ synthase activity has been identified which is capable of specifically converting PGH_2 to PGF_2 (Kubata *et al.*, 2000). Figarella *et al.* (2005) reported the effect of PGD_2 on the population density regulation of the *T.brucei* bloodstream form. The PGD_2 induced a programmed cell death (PCD) with characteristic features of apoptosis which is also known to be induced by PGs in higher eukaryotes.

It has been reported by Eintracht *et al.* (1998) that free AA can regulate Ca^{2+} flux across the plasma membrane and inhibits cell growth in a process that appears to involve metabolic processing of AA into prostaglandins. One part of AA is stored within membrane phospholipids of both bloodstream and procyclic forms. Richmond and Smith (2007) reported the identification and characterization of cytosolic phospholipase A_1 in *T.brucei*. This enzyme deacylates GPCho containing long polyunsaturated and highly unsaturated fatty acids.

T. brucei bloodstream forms produce prostaglandins (Kubata *et al.*, 2000) and therefore, phospholipases may play a pivotal role in its regulation (Ridgley and Ruben, 2001). So, several attempts had been made to identify and characterize phospholipase A_2 activity in *T.brucei*. Until now, there is no success on proposed search for phospholipase A_2 activity instead PLA_1/LysoPLA activity has been reported. As reported by our group (Figarella *et al.*, 2005; 2008), the PGs induces apoptosis and cell density regulation in *T. brucei*. So, it was imperative to search for PLA_2 activity in these parasites.

The object of this study was to identify, clone and characterize PLA_2 from *T.brucei*. A search of *T.brucei* genome database revealed a putative phospholipase A_2 –like gene which urged to identify and characterize this protein. The cloning and biochemical characterization of this protein was carried out by heterologous expression in *E. coli* and Sf9 insect cells. The localization of $TbPLA_2$ in *T.brucei* bloodstream form and procyclic form was also attempted.

The objective achieved in this study was the cloning and expression of this protein. On the other hand, localization and functionality of this protein was attempted. In addition, the

detection of PLA_2 in *T. brucei* can be utilized as potential target in inhibition studies and for understanding the metabolic pathway of arachidonic acid and prostaglandins in *T. brucei*.

2. Materials and methods

I. Materials

1. General

1. Cloning

1.1 DNA sequence of T. brucei phospholipase A_2 ($TbPLA_2$)

The following gene sequence of $TbPLA_2$ was selected from GeneDB for cloning experiments

Tb 09.211.3650, phospholipase A_2-like protein, putative, *T. brucei*, chr 9

5´ATGGTAACGT GGGCGCTGAA GTATTTTGTT CGCGTAGTCC GATGGTCGAC AGAAGCATTC
CTAATTTGGC CCACACGGCC ACTTTTTGAC TATGCCACCT CATTGCATTG TGTTCCCATA
AGCGGCACAT TTATTACTTC GGTTCTGCTC TGCTTCTACG GGTTCCCACT TTTCTGGTCT
ACCGCCATGG TTGCTGTCGG GTTTATCATC TCCGGTGTGT TATTTCTTGT GAGCCCTCTT
CCACTATTGA AACCCATTGG CGGTCGCTAT AGCGTGGGCC TTGTGCATAT GAACGGCTGC
CGTTCCCAAT CCATACCACC AGTAGCTGTG TTTTATCCTA CTAATATGGT CCCAGAGAAA
AAGGGACTAC CGTATGTCCC GTTTGGAGAT GACCGTTTTC TTCGGGGCGT GGCGGCGTAT
GCAAACGTGC CATTTTTCTT CATAAGGGAT TTCTCCTTTG TTCGCATCAG TGCGTCCCGA
AACGCGGTGC CCGCCGCTTT GCTCAACCAA TATGAGAGAG TGCCACCTAT TGTTGTGTTC
AGTCACGGTC TTGCCGGATA CCATTTGTTC TACAGTTGCT TTGCGTTGGA TCTTGCGGCG
CGGGGTGCAA TCGTGATTTG TCTTGGCCAT TGCGACAATA GTGCTTCTTT CATGCGTGAC
AGTAGCGGTA AGGAAAGTGA AGTTCCGCTC AAGGACTATG GATGGGAGGT ACCCGCACGT
GAAGCCCAAG TTGCACAAAG GGTAAGCGAG GTGAGGGGAA CACTTCAACG CCTAACGGAA
AAGGACTTTT GGACAACTTT GGGCTACATT AATTCAGATA TTGACAAGTT TCTTAGCAAA
CCGTTGCAGG TACATCTTGC GGGTCATTCA TTTGGCGGTG CCACTGTACT CGCGGCTGCA
TTAGAAGAGA ACCAAAATCC CGTGAAGGGA GTCAGCGTAA AGAGCGTGTA TACGTTTGAC
CCATGGATGG TACCAATACA AAATGAACAT TTTTGCAACC CGCTTTCTGA TGGCCGTAAA
TCCTATACTG TTCCAACGGT TACTGTGCAT TCAGACGACT GGGTAAAAGA TTCTGAGAGT
TGGGAATTCT TTAAAAGGAT GAAAGCGCTG GTGTTAGAAC AATCTGCATA TGCTTCGCTC
AATGAAGTGG AGAAACAAGC GCTCTTTGGT ATTGTGGTCA CGAAGAATAC GAACCACCTT
TCTCTTGTAG ATGTCTCTGT ACTCAGTCCC GTCATGCATG GAAATATCTG GGCCACAGTG
TCACCGCGAG TACAAATTAT GGAGTGGTGT AATGCACTTC TTCGCTTCGC AAAGCAAAAT
ACCGAAGTGT GTTCAACGTG TTAG ´3

1.2 Primers

Primers for cloning (Invitrogen)

Both primers were chosen according to the gene sequence above and plasmids multiple cloning restriction sites. The sense primer has a restriction site for *BamH1* only and the anti-sense primer has a *Pst1* restriction site only which are indicated below by inverted arrows. The primers were designed to include the signal peptide, transmembrane sequence and termination codon of protein using the OligoPerfect^{TM} designer from Invitrogen. The following was the primer sequences for both sense and anti-sense respectively:

Sense primer 5’ -TAT G$^{\downarrow}$GA TCC ATG GTA ACG TGG GCG CTG A- 3’

Primer length: 28

Tm 58°C

Anti-sense primer 5’ -ATA ACT GCA $^{\downarrow}$GCT AAC ACG TTG AAC ACA CTT CGG T-3’

Primer length: 34

Tm 58°C

Primers for Northern blotting (Operon)

Sense primer 5’-ATGGTAACGT GGGCGCTGAA GTAT- 3’

Primer length: 24

Tm 64.57°C

Anti-sense primer 5’-CTAACACGTTGAACACACTTCGGTA- 3’

Primer length: 25

Tm 62.94°C

2. Protein sequence of $TbPLA_2$ from databank

The following protein sequence of $TbPLA_2$ was chosen from GeneDB for peptide synthesis

Tb 09.211.3650, phospholipase A_2-like protein, putative, *T. brucei*, chr 9

MVTWALKYFV RVVRWSTEAF LIWPTRPLFD YATSLHCVPI SGTFITSVLL CFYGFPLFWS
TAMVAVGFII SGVLFLVSPL PLLKPIGGRY SVGLVHMNGC RSQSIPPVAV FYPTNMVPEK
KGLPYVPFGD DRFLRGVAAY ANVPFFFIRD FSFVRISASR NAVPAALLNQ YERVPPIVVF
SHGLAGYHLF YSCFALDLAA RGAIVICLGH CDNSASFMRD SSGKESEVPL KDYGWEVPAR
EAQVAQRVSE VRGTLQRLTE KDFWTTLGYI NSDIDKFLSK PLQVHLAGHS FGGATVLAAA
LEENQNPVKG VSVKSVYTFD PWMVPIQNEH FCNPLSDGRK SYTVPTVTVH SDDWVKDSES
WEFFKRMKAL VLEQSAYASL NEVEKQALFG IVVTKNTNHL SLVDVSVLSP VMHGNIWATV
SPRVQIMEWC NALLRFAKQN TEVCSTC

Peptide sequences for antibody production against $TbPLA_2$ in rabbit:

Peptide 1: LEENQNPVKGVSVKSVYTFD

Peptide 2: TVSPRVQIMEWCNALLRFAKQNTEV

The underlined peptides in the protein sequence were selected for antibody production as they revealed significant hydrophilic properties. These were analyzed using hydrophilic (Kyte-Doolittle) and surface (Emini) probability calculations.

3. Experimental organisms

3.1 *Escherichia coli*

Bacterial strains XL-1 (subcloning) and BL-21 (DE3) (expression) competent cells were bought from Stratagene, Germany and stored at -70°C. One shot TOP 10 and MAX Efficiency®DH10 Bac competent cells were bought from Invitrogen, Germany and stored at -70°C.

3.2 *Spodoptera frugiperda* (Sf9)

Insect cells *Sf9* (fall armyworm) stabilates were bought from Invitrogen, Germany, stored at -70°C and propagated at 27°C in an incubator.

3.3 *Trypanosoma brucei*

In this work two forms of trypanosomes, bloodstream and procyclic form were used. Bloodstream forms were from a monomorphic strain known as strain EATRO 427 clone MITat 1.2 (**M**olteno **I**nstitute **T**rypanozoon **a**ntigenic **t**ype, Cambridge) with the VSG (Variable Surface Glycoprotein) antigen variant 221 (Cross, 1975). Procyclic form was obtained from MITat 1.2 using a standard transformation protocol (Overath *et al.*, 1986).

3.4 Animals

Stabilates of bloodstream form trypanosomes were prepared with infection in Wistar rats bought from Charles River Laboratories, Germany in an animal model to propagate trypanosomes. The animals were maintained in the animal house facility of the Interfaculty Institute of Biochemistry, University of Tuebingen.

4. General chemicals

Acetone analytical grade	Fluka, Buchs (Switzerland)
Acrylamide/bisacrylamide (Rotiphorese Gel 30)	Roth, Karlsruhe
Adenosine	Sigma, Deisenhofen
Agar-agar	Roth, Karlsruhe
Agarose (Rotigarose)	Roth, Karlsruhe

Ampicillin	Sigma, Deisenhofen
Ammonium persulfate (APS)	Merck, Eurolab,GmBH, Mannheim
L- Arginine	Sigma, Deisenhofen
Bathocuproinedisulfonic acid disodium salt (BCS)	Sigma, Deisenhofen
Bovine serum albumin (BSA), Fat free	Sigma, Deisenhofen
Bradford protein assay reagent	Bio-Rad, Munich
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma, Deisenhofen
Calcium chloride	Merck, Eurolab,GmBH, Mannheim
Canusal® (Heparin-Na)	CP Pharmac. Ltd. Wrexham (UK)
Cellfectin® Reagent	Invitrogen, Karlsruhe
Chloroform	Merck, Eurolab,GmBH, Darmstadt
Coomassie brilliant blue R-250	Serva, Heidelberg
5´- [^{32}P] dATP	Amersham Biosciences, Freiburg
DEAE-Sephacel	Sigma,Deisenhofen
Dimethyl formamide	Sigma, Deisenhofen
Disodium hydrogen phosphate	Merck, Eurolab,GmBH, Darmstadt
DNA ladder 1kb	Fermentas, St. Leon-Rot
Dilinoleoyl phosphatidylcholine (DL-PC)	Sigma, Deisenhofen
dNTP mix	Fermentas, St. Leon-Rot
Dulbecco's PBS (1X)	PAA Labs., GmBH, Pasching, Austria
Ethanol	VWR International, Darmstadt
Ethidium bromide	Roth, Karlsruhe
Ethylenediamine tetraacetic acid (EDTA)	Roth, Karlsruhe
EX-CELL 420 serum free medium w/ L-Glutamine	Invitrogen, Karlsruhe
Fetal bovine serum (FBS)	Invitrogen, Karlsruhe
Formaldehyde (37 %)	Sigma, Deisenhofen
Hydrogen chloride	Merck, Eurolab,GmBH, Darmstadt
Gentamicin	Sigma, Deisenhofen
D-Glucose monohydrate	Sigma, Deisenhofen
L-Glutamine	Sigma, Deisenhofen
L-Glutamic acid	Sigma, Deisenhofen
Glycerine 87%	Sigma, Deisenhofen
L-Glycine	Sigma, Deisenhofen
Haemin	Sigma, Deisenhofen

HEPES	Sigma, Deisenhofen
L- Histidine	Sigma, Deisenhofen
Hypoxanthine	Sigma, Deisenhofen
Iscove's Dulbecco's medium	Sigma, Deisenhofen
L- Isoleucine	Sigma, Deisenhofen
Isopropanol	Merck, Eurolab,GmBH, Darmstadt
IPTG (Isopropyl-beta-D-thiogalactopyranoside)	Fermentas, St. Leon-Rot
Kanamycin	Merck, Eurolab,GmBH, Darmstadt
Leupeptin	Sigma, Deisenhofen
Lipoxygenase	Sigma, Deisenhofen
L- Lysine	Sigma, Deisenhofen
Magnesium chloride	Merck, Eurolab,GmBH, Darmstadt
Magnesium sulfate	Merck, Eurolab,GmBH, Darmstadt
MEM amino acids	Sigma, Deisenhofen
MEM vitamin solution	Sigma, Deisenhofen
Mercaptoethanol	Merck, Eurolab,GmBH, Darmstadt
Methanol	Merck, Eurolab,GmBH, Darmstadt
L- Methionine	Sigma, Deisenhofen
Myristic acid	Sigma, Deisenhofen
Nitro blue tetrazolium (NBT)	Sigma, Deisenhofen
L- Ornithine	Sigma, Deisenhofen
Paraformaldehyde	Fluka, Buchs
Penicillin/ Streptomycin	Sigma, Deisenhofen
Pepstatin	Sigma, Deisenhofen
Phenol red	Sigma, Deisenhofen
L- Phenylalanine	Sigma, Deisenhofen
Phospholipase A_2 from porcine pancreas	Sigma, Deisenhofen
Pfu DNA polymerase	Stratagene Europe Netherlands
Potassium chloride	Merck, Eurolab,GmBH, Darmstadt
Potassium dihydrogen phosphate	Merck, Eurolab,GmBH, Darmstadt
Prestained protein ladder	Fermentas, St. Leon-Rot
Protein G	Roche Diagnostics GmBH, Mannheim
Protein molecular marker	Sigma, Deisenhofen
L- Proline	Sigma, Deisenhofen

Pluronic® F-68	Invitrogen, Karlsruhe
Restriction enzymes and buffers	Fermentas, St. Leon-Rot
Roti-Load 1(4 X, non-reducing)	Roth, Karlsruhe
Roti-Load 1(4 X, reducing)	Roth, Karlsruhe
Sodium azide	Sigma, Deisenhofen
Sodium acetate	Sigma, Deisenhofen
Sodium chloride	Fluka, Buchs
Sodium dihydrogen phosphate	Merck, Eurolab,GmBH, Darmstadt
Sodium dodecyl sulfate	Sigma, Deisenhofen
Sodium hydroxide	Sigma, Deisenhofen
Taq DNA polymerase	Invitrogen, Karlsruhe
T4 DNA ligase	Fermentas, St. Leon-Rot
TEMED	Roth, Karlsruhe
Tetracycline	Sigma, Deisenhofen
L- Threonine	Sigma, Deisenhofen
Tris	Roth, Karlsruhe
Triton X- 100	Roth, Karlsruhe
Trypan blue	Sigma, Deisenhofen
L- Tryptophan	Sigma, Deisenhofen
Tween 20	Roth, Karlsruhe
L- Tyrosine	Sigma, Deisenhofen
L- Valine	Sigma, Deisenhofen
Yeast extract	Roth, Karlsruhe

5. Plasmids

pCR®2.1-TOPO (3931 bp)	Invitrogen, Karlsruhe
pMalC2e (6646 bp)	New England Biolabs, Frankfurt
pFastBac 1 (4775 bp)	Invitrogen, Karlsruhe
Bacmid (bMON14272)	Invitrogen, Karlsruhe

6. Kits

AccuPrime *Taq* DNA polymerase high fidelity	Invitrogen, Karlsruhe
Hot Star *Taq* master mix kit	Qiagen GmbH, Hilden

Pfu DNA polymerase	Stratagene Europe, Netherlands
QIAquick gel extraction kit	Qiagen GmbH, Hilden
QIAprep spin MiniPrep test kit	Qiagen GmbH, Hilden
NucleoBond AX (MaxiPrep)	Macherey-Nagel GmbH, Dueren
NucleoBond AX (MidiPrep)	Macherey-Nagel GmbH, Dueren

7. Antibodies

Anti-rabbit IgG alkaline phosphatase conjugate	Sigma, Deisenhofen
Alexa Flour 594 goat anti-rabbit IgG	Invitrogen, Karlsruhe
Anti-VSG (antigen variant 221, rabbit)	

8. Materials and Equipments

Amicon microcentrifuge MC 13	Heraeus, Osterode
Analytical plus fine weighing maschine	Ohaus
Beckman TL-100 ultracentrifuge	Beckman, Munich
Biofuge A	Heraeus, Osterode
Biometra standard power pack P25 power supply	Biometra analytik GmbH
Bluemarine 100 agarose electrophoresis	Serva electrophoresis
Centrifuge Sigma 3K12	Sigma, Deisenhofen
Cell culture flasks (T-12; T-25; T-75; T-175) sterile	Greiner, Frickenhausen
Chromatography paper 3MM Chr	Whatman GmbH, Dassel
Cuvettes semi-micro	Sarstedt, Nuembrecht
Cuvettes UV-micro	Brand GmBH, Wertheim
Electrophoresis minigel mighty small II SE 250	Hoefer, San Francisco
Eppendorf and PCR cups	Greiner, Frickenhausen
Falcon tubes (15 and 50 ml)	Greiner, Frickenhausen
Flouresence microscope Olympus BH2 RFCA	Olypmus, Hamburg
Gene power supply GPS 200/400	Pharmacia
GFL water bath shaker	Hampshire, UK
Glass slides with cover glass	Roth, Karlsruhe
Heidolph MR 2002 IKA RCT classic magnetic stirrer	IKA Labortechnik, Staufen
HLB 2448 GS weighing maschine	Mettler
Hybond –ECL nitrocellulose membrane	Amersham Biosciences, Freiburg
Inverted microscope ID 02	Zeiss, Oberkochen

Inverted microscope Axiostar plus	Zeiss, Oberkochen
Incubator Hera cell with CO_2 and 37°C	Hereaus, Osterode
Incubator with 27°C	Koettermann, Uetze-Haenigsen
Incubator	INFORS AG, Bottmingen
Lab shaker model Kuehner	B. Braun, Melsungen
Microtiter plates (6- well)	Greiner, Frickenhausen
Micropippetes SL-PetteXE	Nichiryo
Microwave oven	Panasonic
Neoblock 1	Neolab Miggie,Heidelberg
Neubauer Haemocytometer	Brand, Wertheim
Orbital shaker	
PCR minicycler PTC-150 MJ research	Biozyme, Oldendorf
PCR sprint	Hybaid, USA
pH meter (pH 539)	WTW, Weilheim
PP- sterile test tubes (15 ml, 50 ml)	Greiner, Frickenhausen
Pressure cooker	CS Solingen, Solingen
Round filter paper MN 615	Macherey-Nagel GmbH, Dueren
SFCA sterile filter membrane	Nalge, Hereford, UK
Shaker	Infors AG
Sonifier® Cell disruptor B-30	G. Heinemann, Schwaebisch Gmünd
Sterile pump	Microgon Inc., USA
Vaccum centrifuge	Bachofer, Reutlingen
Vortex genie	Bender & Hobein AG, Switzerland
Ultracentrifuge TL-100	Beckman, USA
Ultrospec 3000 spectrophotometer	Pharmacia Biotech, Cambridge, UK
Water bath	Schott, Mainz

9. Databank and softwares

THMM server version 2.0	http://www.cbs.dtu.dk/services/TMHMM/
Brenda Enzyme Information System	http://www.brenda-enzymes.info/
DNAMAN GATC Biotech DNA sequencing/ viewer	http://www.gatc-biotech.com/de/
GeneDB Hosted by Sanger Institute	http://www.genedb.org/
Invitrogen Primer OligoPerfect Designer	http://www.invitrogen.com/
PubMed (NCBI Homepage)	http://www.ncbi.nlm.nih.gov/

2. Media, Buffers and solutions

2.1 Media for different organisms

2.1.1 *Escherichia coli*

Luria-Bertani medium (LB-medium) pH 7.0

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

All components were mixed in distilled water and adjusted to pH 7.0 using NaOH. Afterwards, the final volume was made upto 1 liter and then autoclaved.

Transformation medium for E. coli (TSS)

Supplemented in LB-medium	
DMSO	5 %
$MgCl_2$ X 6 H_2O (50 mM)	10.2 g/l
PEG 6000	10 %

SOB medium

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g

Distilled water was added to above chemicals and mixed. The final volume of the solution was made to 1 liter and then autoclaved. Afterwards, 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$ was added to this solution prior to use.

SOC medium

To the autoclaved SOB medium, 1 ml of filter-sterilized 2 M glucose was added to a final volume of 100 ml. Subsequently, aliquots of 5 ml of the medium were made and stored at – 20°C till further use.

Ampicillin stock solution (1000 X)

Ampicillin (50 mg/ ml) was dissolved in 70 % ethanol and stored at -20°C.

X-Gal stock solution (500 X)

X-Gal (20 mg/ ml) was dissolved in dimethyl formamide and stored at -20°C.

Culture plates

Agar-Agar in LB-medium	1 %

IPTG dioxane free, freshly prepared

IPTG (0.1 M) was dissolved in sterile distilled water and filtered.

Column buffer pH 7.4

Tris	20 mM
NaCl	200 mM
EDTA	1 mM

The solution was adjusted to pH 7.4 using HCl.

2.1.2 Baculovirus Expression Vector System

Ex-Cell 420 Serum-Free with L-Glutamine liquid medium

The medium was bought from SAFC Biosciences. Before propagating and infection in *Sf9* insect cells, the medium was supplemented with following additives under sterile conditions

Fetal Bovine Serum (FBS)	10 %
Pluronic® F-68	0.01 %

Penicillin/ Streptomycin solution	5 ml/l

RIPA (Radio-Immunoprecipitation Assay) Lysis buffer pH 7.4

Tris	50 mM
NaCl	150 mM
NP-40	1%
Sodium deoxycholate	0.5 %
EDTA	1 mM
SDS	0.1 %
Sodium azide	0.01 %

The solution was adjusted to pH 7.4 using NaOH

2.1.3 *Trypanosoma brucei*

2.1.3.1 Citrate-Glucose-Anticoagulant (CGA) pH 7.7

Sodium citrate X $2H_2O$	100 mM
Glucose	40 mM

2.1.3.2 Separation buffer pH 8.0 (Lanham and Godfrey, 1970)

$NaHPO_4$ X H_2O	57 mM
KH_2PO_4	3 mM
NaCl	44 mM
Glucose	55 mM

2.1.3.3 Cell culture medium

2.1.3.3.1 Basic medium or Minimum Essential Medium (MEM) pH 7.4

The medium was prepared according to Eagle (1959) which was modified by Duszenko *et al.*, (1985 and 1992).

	[mg/ ml]
$CaCl_2$ X H_2O	265
KCl	400
$MgSO_4$ X 7 H_2O	200
NaH_2PO_4 X H_2O	140
NaCl	6800
HEPES	7140

Above chemicals were dissolved in distilled water and adjusted to pH 7.4 using NaOH.

	[mg/ ml]
L-Arginine/ HCl	126
L-Cysteine	24
L-Histidine/ HCl X H_2O	42
L-Isoleucine	52
L-Leucine	52
L-Lysine	73
L-Methionine	15
L-Phenylalanine	100
L-Threonine	48
L-Tryptophan	10
L-Tyrosine	100
L-Valine	46
L-Ornithine/ HCl	10
Adenosine	12

The amino acids and adenosine were dissolved in distilled water and then added the following commercial stock solutions:

	[mg/ l]
MEM non-essential amino acids solution	10
MEM vitamin solution	10
Phenol red	10

The medium was sterile filtered using pump and filter (SFCA membrane) and stored at 4°C. It can be used for up to 3 months.

2.1.3.3.2 Complete bloodstream culture medium (BF-medium) pH7.4

The following stock solutions were added to the basic medium for preparation of a complete culture medium:

		[ml/ l]
$NaHCO_3$	(750 mg/10ml distilled water)	30
L-Cysteine	(30.3 mg/10 ml distilled water)	10
BCS	(5.6 mg/10ml distilled water)	1
Hypoxanthine	(13.6 mg/10ml with 0.1N NaOH)	10
2'-Desoxythymidine	(3.9 mg/10 ml distilled water)	10
L-Glutamine	(292 mg/10 ml distilled water)	10
BSA/ Myristic acid	(50 X)	20
FBS	(Heat inactivated: 30 min. 56°C)	150
Penicillin/ Streptomycin solution		5
Glucose		5.4 g

Preparation of myristic acid linked to defatted BSA (Ferguson and Cross, 1984): 24 mg myristic acid was dissolved in 100 µl ethanol (95%) and gradually added to a BSA solution (1 g defatted BSA/ 20 ml distilled water). The aliquots of 20 ml were made and stored at – 20°C.

The BF-medium was adjusted to pH 7.4 using NaOH. Afterwards, it was sterile filtered and stored at 4°C which can be used for up to 4 weeks.

2.1.3.3.3 Procyclic culture medium pH 7.4

The following substances were added to the basic medium for the cultivation of procyclic parasites:

		[mg/ ml]
L-Glutamine	(29.2 mg/ ml distilled water)	10
Haemin	(2.5 mg/ ml in 0.1 M NaOH)	3
Pyruvate	(22 mg/ ml distilled water)	10
L-Proline	(60 mg/ ml distilled water)	10
FBS	(Heat inactivated: 30 min. at 56°C)	100
Penicillin/Streptomycin solution		5

The medium was adjusted to pH 7.4 using NaOH. Afterwards, it was sterile filtered and stored at 4°C which can be used for up to 4 weeks.

Trypanosoma Dilution Buffer (TDB) pH 7.7

Na_2HPO_4 X 12 H_2O	18 mM
NaH_2PO_4 X H_2O	2 mM
KCl	5 mM
NaCl	80 mM
$MgSO_4$ X 7 H_2O	1 mM
Glucose X H_2O	20 mM

Freezing medium for stabilates (TDB-Glycerin)

TDB	77 ml
87 % Glycerin (20 % end concentration)	23 ml

Phosphate Buffered Saline (PBS) pH 7.4

Na_2HPO_4 X 12 H_2O	10 mM
KH_2PO_4	10 mM
NaCl	120 mM
KCl	8.7 mM

Protease inhibitor mix (1000 X)

Pepstatin	1 mM
Chymostatin	1 mM
Leupeptin	1 mM

All three inhibitors were dissolved in DMSO and stored as aliquots at -20°C.

Cell lysis buffer

Na_2HPO_4 X 12 H_2O	10 mM
KH_2PO_4	10 mM
Protease inhibitor mix	1 %
Triton X-100	0.15 %

Both the phosphate components were dissolved in deionized water and titrated to pH 7.4. The protease inhibitor mix was added to the solution. Triton X-100 was only added for membrane fraction isolation.

$ZnCl_2$ stock solution (50 X)

$ZnCl_2$	6.85 mg / ml	(end concentration 0.2 mM)	10 mM

2.2 Buffers and solutions

2.2.1 Buffers and solutions for molecular biology

TE-buffer pH 8.0

Tris	40 mM
EDTA	2 mM

DNA gel electrophoresis

<u>DNA sample buffer pH 8.0</u>

Glycerol	5 %
Bromophenol blue	0.025 %
EDTA	0.1 M

<u>TAE buffer pH 8.0 (50 X)</u>

Tris	0.04 M
EDTA	0.002 M
Acetic acid	57.1 ml

The solution was adjusted to pH 8.0 with acetic acid.

2.2.2 SDS-PAGE (SDS-PolyAcrlyamide Gel Electrophoresis)

2.2.2.1 Resolving gel and separating gel solutions (according to Laemmli, 1970)

Stock solutions

APS (freshly prepared)	0.1 g/ ml in deionized water
1 M Tris-HCl pH 8.8	
Tris	24.2 g/ 200 ml in deionized water
1 M Tris-HCl pH 6.8	
Tris	12.1 g/ 100 ml in deionized water
SDS 10 %	10 g/ 100 ml in deionized water

Resolving gel

	(12 % T for 1 gel)
Acrylamide/ Bis (Rotiphorese gel 30)	2.15 ml
Deionized water	1.27 ml
1 M Tris-HCl pH 8.8	2.28 ml
10 % SDS	0.057 ml
10 % APS	0.019 ml
TEMED	0.006 ml

Stacking gel

	(5.1 % T for 1 gel)
Acrylamide/ Bis (Rotiphorese gel 30)	0.5 ml
Deionized water	2.1 ml
1 M Tris-HCl pH 6.8	0.375 ml
10 % SDS	0.03 ml
10 % APS	0.015 ml
TEMED	0.003 ml

Running buffer pH 8.3

Tris	3.03 g/l	(25 mM)
Glycine	14.4 g/l	(192 mM)
SDS	1 g/l	(0.1 %)

2.2.2.2 Coomassie staining

Staining solution

Coomassie Brilliant Blue R-250	1 g/ l
Ethanol	400 ml/ l
Acetic acid	100 ml/ l
Distilled water	500 ml/ l

Coomassie Brilliant Blue R-250 was dissolved in ethanol. Subsequently, other solutions were added after Coomassie was completely solublized and the volume was made up to 1 liter.

Destaining solution

Ethanol	400 ml/ l
Acetic acid	100 ml/ l
Distilled water	500 ml/ l

2.2.2.3 Silver staining

Fixation solution

Acetic acid	12 %
Ethanol	40 %
Formaldehyde (37 %)	0.05 %

Wash solution

Ethanol	50 %
Distilled water	50 %

Pretreatment

$Na_2S_2O_3$ X $5H_2O$ (10% in distilled water)	0.2 %

Impregnation

$AgNO_3$	0.2 %
Formaldehyde (37 %)	0.075 %

Development

Na_2CO_3	6 %
$Na_2S_2O_3$ X $5H_2O$ (10% in distilled water)	0.004 %
Formaldehyde (37 %)	0.005 %

Stop solution

Ethanol	40 %
Acetic acid	12 %

All solutions were made in distilled water.

2.2.2.4 Gel drying solution

Glycerol 87 %	2.3 %
Ethanol	25 %

The solution was made in distilled water and volume made up to 1 liter.

2.2.3 Western blotting

Transfer buffer pH 9.2

Tris	5.82 g/ l	(48 mM)
Glycine	2.93 g/ l	(39 mM)
SDS	3.75 ml	(10 %)
Methanol	200 ml/ l	(20 %)

Wash buffer pH 7.4

Tris	1.21 g/ l	(10 mM)
NaCl	8.766 g/ l	(150 mM)
Tween 20	1 ml/ l	(0.1%)

Tris Buffered Saline (TBS) pH 7.4

Tris	5.82 g/ l	(25 mM)
KCl	0.2 g/ l	
NaCl	8 g/ l	

Blocking solution

Milk powder	10 % in 2 X PBS

2 X Phosphate Buffered Saline (PBS) pH 7.4

Na_2HPO_4 X 12 H_2O	7.26 g/ l
KH_2PO_4	0.48 g/ l
NaCl	16 g/ l
KCl	0.40 g/ l

Alkaline phosphatase (AP buffer) pH 8.9

Tris	2.42 g/ l	(20 mM)
NaCl	8.77 g/ l	(150 mM)
$MgCl_2$	2.03 g/ l	(10 mM)

Color development solutions

BCIP, Toluidine salt (fresh solution in DMF)	7 mg/ 250 µl	(0.38 mM)
NBT (fresh solution in 70% DMF)	16.25 mg/ 500 µl	(0.40 mM)

The BCIP and NBT solutions were dissolved in 50 ml AP buffer for each blot.

Primary antibody (Anti-$TbPLA_2$) in TBS 1:2000

Secondary antibody (Anti-Rabbit IgG, alkaline phosphatase conjugated) in TBS 1:20 000

2.2.4 Northern blotting

10 X FA gel buffer pH 7.0

MOPS	41.86 g/ l	(200 mM)
Sodium acetate	4.10 g/ l	(50 mM)
EDTA	3.72 g/ l	(10 mM)

The solution was adjusted to pH 7.0 using NaOH

1 X FA Running buffer

10 X FA gel buffer	100 ml/ l
37% Formaldehyde	20 ml/ l

5 X Loading buffer

Bromophenol blue solution	16 µl
EDTA (500 mM, pH 8.0)	80 µl
37 % Formaldehyde	720 µl
100 % Glycerol	2000 µl
Formamide	3084 µl
10 X FA gel buffer	4000 µl
Ampuwa	volume made up to 10 ml

The loading buffer was stored at 4°C which can be used for 3 months.

20 X SSC pH 7.0

NaCl	175.3 g/ l	(3 M)
Sodium citrate X 3 H_2O	88.2 g/ l	(0.3 M)

500 mM sodium phosphate pH 7.2

Na_2HPO_4 X 12 H_2O	(500 mM)

The solution was adjusted to pH 7.2 using H_3PO_4.

Hybridmix sodium phosphate

SDS	70 g/ l	(7 %)

EDTA (pH 7.5, 93.05 mg/ml = 0.5 M)	4 ml/ l
Sodium phosphate (500 mM)	volume made up to 1000 ml

Phosphate wash

SDS	10 g/ l	(1%)
Sodium phosphate (500 mM)	80 ml/ l	
Ampuwa	volume made up to 1000 ml	

Stripping buffer

SDS	10 g	(1%)
Ampuwa	volume made up to 1000 ml	

2.2.5 Immunoflouresence

Fixation solution (Freshly prepared)

Paraformaldehyde	0.4 g	(4 %)
PBS	volume made up to 10 ml	

The solution was warmed (not boiled) in microwave oven till paraformaldehyde was solublized and put on ice.

1% BSA solution (Freshly prepared)

BSA	10 mg/ml in PBS

Sodium phosphate pH 7.2 containing Glycine

Na_2HPO_4 X 12 H_2O	35.81 g/ l	
Glycine	7.51 g/ l	(0.1 M)

0.2% Triton solution (Freshly prepared)

Triton X-100	2 mg/ml in PBS

Bisbenzimide solution

Bisbenzimide	1 mg/ml in distilled water

2.2.6 PLA_2 enzyme assay

All chemicals were solublized in deionized water

Solubilization buffer for DL-PC

Tris	6.057 g/ l	(50 mM)
Sodium deoxycholate	4.15 g/ l	(10 mM)

Dilinoleoyl phosphatidylcholine (DL-PC) stock solution

DL-PC	12.5 mg/ml in solubilization buffer

Lipoxygenase stock solution

Lipoxygenase	3.6 µg/ µl

Stock solution was diluted to 1:100 with Ampuwa.

Phospholipase A_2 from porcine pancreas (stock solution)

Phospholipase A_2 from porcine pancreas	3 mg/ml

Stock solution was diluted to 1:100 with Ampuwa to 30 µg/ ml.

Reaction buffer 1: Tris-HCl pH 8.5 buffer

Tris	6.057 g/ l	(50 mM)

Reaction buffer 2: Tris-HCl pH 8.5 containing sodium deoxycholate buffer

Tris	6.057 g/ l	(50 mM)
Sodium deoxycholate	2.49 g/ l	(6 mM)

II. Methods

1. General methods

1.1 Protein determination with Bradford method

Protein concentrations were determined for all experiments using the Bradford method (Bradford, 1976) with BSA in 2 µg, 4 µg, 6 µg, 8 µg and 10 µg/ ml concentrations as standard. The concentrated Bradford reagent (Bio-Rad) was diluted to 1:5 with deionized water. The diluted reagent was pipetted in a volume of 900 µl in each cuvette. As reference, took 100 µl deionized water for standards or buffer for samples. All standards were performed in duplicate. The reaction mixtures were incubated for 5 min. at room temperature and then read against reference at 595 nm in an Ultrospec 3000 spectrophotometer.

1.2 SDS-PAGE

The glass plates, assembly, comb and spacers were cleaned with ethanol. The glass plates with spacers were fixed inside the assembly. As mentioned above in 2.2.2.1, resolving gel mixture was poured carefully in between glass plates taking care not to let air bubbles in the solution and the gel was polymerized (polymerization time was from 15 to 30 min.) with overlay of deionized water. After the gel had polymerized, stacking gel mixture was poured above the gel carefully and immediately added comb on top of it till completely immersed between plates. Polymerization time was 45 min. and during this time, protein samples were prepared after protein estimation from Bradford method (1.1) from 10 to 20 µg protein concentration per sample. Reducing sample diluting buffer was added to samples and adjusted the volume accordingly. The samples were boiled at 100°C for 5 min. and loaded into respective wells with protein molecular marker. The water cooling system was attached with the assembly and running buffer was added in upper and lower reservoir. The electrodes were attached accordingly into power supply and SDS-PAGE was run at constant voltage (160 V) till bromophenol boundary reached bottom of the gel. Gel was removed carefully from plates and placed in Coomassie staining solution for 1 h and then destained or in fixation solution for silver staining (2.2.2.3) or followed immediately for Western blotting.

1.3 Western blotting

The protein samples were resolved in SDS-PAGE (1.2) and immediately blotted. The gel was placed on an anode plate in Semi-Dry apparatus. It was overlaid onto blotting paper (Hybond–ECL nitrocellulose membrane) sandwiched between three layers of Whatman filter papers soaked with transfer buffer (2.2.3) on each side respectively. It was carefully assembled to remove any air bubbles in between the membrane, gel and filter papers. The electro transfer was carried out at about 10 V (400 mA) for 1 h with cathode side above the assembly. Thereafter, the membrane was blocked with 10 % milk powder in 2 X PBS (2.2.3) for overnight at 4°C. After overnight blocking, the membrane was washed for 5 X 5 min. in wash buffer with gentle shaking. It was incubated with the first antibody diluted in TBS buffer for 1 h at room temperature in fresh disposable bags with gentle shaking. Subsequently, the membrane was washed as above and incubated with alkaline phosphatase conjugated secondary antibody diluted in TBS for 1 h with gentle shaking. The blot was washed and finally, it was developed (2.2.3) and color development was observed carefully. When the bands appeared, the reaction was stopped with PBS and blot was scanned after drying on filter paper.

1.4 Northern blotting

1.4.1 Sample preparation

Samples of total RNA from bloodstream form out of the logarithmical and stationary phase were centrifuged at 3000 rpm for 5 min. Cells were washed twice in PBS (pH 7.4). The cell pellets were used for RNA isolation as per manufacturer's instruction using the RNeasy Mini Kit (Qiagen, Gemany). The RNA was eluted with 40 µl Ampuwa (Fresenius, Germany) and stored in liquid nitrogen.

1.4.2 RNA electrophoresis

Gel was prepared by weighing 0.6 g agarose, which was dissolved in 50 ml 1 X FA running buffer (2.2.4) by heating in microwave. To the warm agarose solution, 900 µl 37 % Formaldehyde and 1 µl Ethidium bromide was added and gently mixed. The agarose solution was polymerized in the gel apparatus and cooled down for additional 30 min at 4°C.

The RNA sample concentration was measured in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). A test agarose gel was loaded with the calculated amount of 1 µg RNA

in order to verify the measured concentrations. For experiments, samples of 10 -20 µg RNA were denatured at 65°C for 5 min. which were then cooled on ice. Samples were loaded in the gel for electrophoresis and run for 1-2 h at 80 V. Afterwards, the gel was photographed under UV light. The running pattern of three bands of rRNA (2250 bp, 1850 bp and 1350 bp) were used as control to monitor RNA loading and as an internal standard for the molecular weight.

1.4.3 Sandwich diffusions blot

For blotting, the gel apparatus was filled with 20 X SSC (2.2.4). The RNA capillary transfer was performed on Hybond N+ Nylon membrane (Amersham) as per standard protocol. After 16- 24 h, the blot was taken out and washed twice in 20 X SSC and left under dark to dry. The cross linking for fixation of RNA on the membrane was done with UV Stratalinker 1800 (program: Auto Cross Link).

1.4.4 Hybridization and marking with [^{32}P] dATP

Following the reverse transcription of total RNA with the Omniscript reverse transcription kit (Qiagen), unlabelled probes were amplified by PCR using the HotStar *Taq* PCR kit (Qiagen) after the manufacturer's instructions. The PCR product was run on a gel electrophoresis. The respective bands were cut out of the gel, purified with the QIAquick gel extraction kit and eluted with Ampuwa (deionized water). The probes were stored at -20°C and directly before hybridization, radioactively labeled with [^{32}P] dATP (Amersham) using the HexaLabel DNA labeling kit (Fermentas).

1.4.5 Development

Hybridization was performed in 1 % BSA in Hybrid mix sodium phosphate (2.2.4) with 50 µl hybridized probe (1.4.4) at 68°C (water bath) overnight. In order to prevent unspecific binding, 100 µg /ml Salmon sperm was added, which was previously denatured at 95°C. After hybridization, the membrane was washed three times for 20 min., once with 0.5 % BSA in phosphate buffer and then twice with washing buffer. For the development, the blot was packed in a fresh disposable bag, covered with an X-ray film and left at -70°C in a development cassette for 1-48 h. The development was done with X-ray film developer (SRX-101A, Konica).

1.4.6 Stripping

The stripping of the hybridized membrane was done for 20 min at 68°C in a water bath with 1% SDS (2.2.4). Afterwards, the membrane could be used for further hybridizations.

1.4.7 Analysis

The developed film was scanned and bands intensity was semi-quantitatively estimated with GelScan V5.1.

2. Standard protocols for molecular biology

The sense and anti-sense primers were solublized in TE buffer (2.2.1).

2.1 Reverse transcriptase PCR (RT-PCR)

Samples of total RNA from bloodstream form out of rat blood was isolated and following reaction mixture was prepared using Omniscript reverse transcription kit (Qiagen) according to manufacturer's instructions

Reaction mixture

10 X RT Buffer	2 µl	
dNTP mix	2 µl	
Oligo dT	2 µl	
RNase inhibitor (10 U/µl)	1 µl	
Template RNA (1.9 µg/µl)	1 µl	
Reverse transcriptase	1 µl	
Deionized water	11 µl	volume made till 20 µl

The reaction mixture was incubated at 37°C for 1 h and afterwards heat inactivated at 85°C for 5 min.

2.2 Polymerase chain reaction (PCR)

The PCR reactions were performed using AccuPrime *Taq* DNA polymerase high fidelity kit (Invitrogen) according to manufacturer's instructions. The reaction mixture was as follows

Reaction mixture

10 X AccuPrime PCR buffer 1	10 µl
Sense primer $TbPLA_2$ (1:10 diluted)	2 µl
Anti-sense primer $TbPLA_2$ (1:10 diluted)	2 µl
cDNA (1:100 diluted)	5 µl
Distilled water (Fermentas)	80.6 µl
AccuPrime *Taq* DNA polymerase	0.4 µl

The following program for PCR reaction was used

1. Initial denaturation	94°C	2 min.
2. Denaturation	94°C	30 sec.
3. Annealing	56°C	30 sec.
4. Extension	68°C	2 min.
30 cycles to step 2		
5. Final extension	68°C	10 min.
6. End	4°C	72 h

2.3 DNA digestion with restriction enzymes

The restriction enzymes and respective buffers (Fermentas or New England Biolabs) 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 the volume till 20 µl. The reaction mixture was incubated at 37°C and for different time span depending on enzyme and DNA concentration.

2.4 Agarose gel electrophoresis for DNA

The gel was polymerized by weighing 0.5 g agarose (1 % agarose gel) dissolved in 50 ml 1X TAE buffer (2.2.1) by heating in microwave oven until agarose was completely solublized and solution was clear. The solution was left to cool and immediately added 0.5 µg/ml Ethidium bromide. The solution was poured in gel casting tray with sample comb and allowed to solidify at room temperature. The sample was prepared by adding sample buffer (2.2.1) to the calculated amount of DNA. The sample was loaded into gel with 1 kb DNA ladder (Fermentas). The electrophoresis was run at 80 V constant for 1 h until the blue boundary reached the bottom of the gel. The gel was photographed under the UV light transilluminator (312 nm). The DNA bands were visualized and migration of DNA was estimated against DNA ladder.

2.5 DNA extraction

After DNA was resolved in agarose gel, the gel was placed on a UV transilluminator and immediately the required DNA band was cut out from the gel with the help of clean scalpel and placed in pre-weighed sterile eppendorf. The gel mass was measured and QIAquick gel extraction kit (Qiagen) used as per manufacturer's instruction. Finally, DNA was eluted with specific volume of elution buffer (Qiagen).

2.6 DNA estimation

After gel extraction, DNA purification and for DNA sequencing, DNA was quantitatively measured at λ = 260 nm, 280 nm and 320 nm with different volumes using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

2.7 Ligation

The vectors (pCR®2.1–TOPO, pMal-c2E, pFastBac-1) in concentration of 50 ng with DNA of interest ($TbPLA_2$) in different ratios of 1 to 3 or 1 to 6 were used for ligation reaction. The Rapid DNA ligation and transformation kit (Fermentas) was used according to manufacturer's instruction.

Ligation was performed using following reaction mixture

Vector DNA (50 ng)	volume depending on DNA concentration
Insert DNA	volume depending on DNA concentration

10 X ligation buffer	2 µl
Deionized water	up to 20 µl
T4 DNA ligase (5 U)	1 µl

The reaction mixture was incubated for 1 h at 22°C or for 17 h at 16°C.

2.8 Transformation

The freshly ligated DNA construct in a volume of 2 µl was transformed into One shot TOP 10 *E. coli* (Invitrogen) or expression grade BL-21 (DE3) competent cells using protocol from Stratagene. The competent cells were thawed on ice for 5 min. Then, the cells were gently flicked and 50 µl of competent cells were transferred into pre-chilled tubes.

The calculated amount of 50 ng experimental DNA construct was added into pre-chilled tubes with competent cells. As a control, pUC19 was added to other tube with competent cells and immediately swirled the tubes gently. The tubes were incubated on ice for 20 min. The cells were heat-shocked at 42°C in water bath for 45 seconds (One shot TOP 10 cells were treated at 42°C for 30 seconds). The duration for the heat shock was critical for maximum efficiency. Afterwards, the tubes were left on ice for 2 minutes. Prewarmed SOC medium in a volume of 250 µl was added to each tube and incubated at 37°C for 1 h with shaking at 225-250 rpm. The transformation mixture in volumes of 100 µl and 200 µl was streaked on two prewarmed LB-agar selective plates containing appropriate antibiotic (and containing X-gal and IPTG if blue-white screening was desired). The plates were incubated at 37°C for overnight (at least 17 h for blue-white color screening). The blue color of colonies was enhanced by further incubating the plates for 2 h at 4°C following the overnight incubation at 37°C.

2.9 Bacterial culture stabilates

The fresh starter culture of tested clones and sterile glycerol (50 %) were added in volumes of 700 µl and 300 µl respectively in cryo vials. The tubes were immediately frozen in liquid nitrogen and stored at -70°C.

2.10 DNA purification

2.10.1 MiniPreparation (MiniPrep)

After transformation, the colonies were selected and were inoculated into respective 3 ml liquid LB medium with ampicillin (50 mg/ ml). The cultures were propagated at 37^{o}C with

shaking at 225-250 rpm for overnight (17 h). All the buffers used were from QIAprep spin Miniprep test kit (Fermentas).The starter culture was harvested by centrifugation at 13,000 rpm (Amicon microcentrifuge MC 13) for 2 min. The supernatant was discarded and the pellet was re-suspended in 150 µl P1 buffer, incubated for 5 min. at room temperature. The re-suspended cells were lysed by adding 150 µl alkaline P2 buffer, gently inverting the solution 4 -6 times and stand for 5 min. The solution was neutralized with 210 µl N3 buffer, inverted and left on ice for 5 min. It was centrifuged for 10 min. at 13,000 rpm and the supernatant was pipetted out in new eppendorf. To this solution, 900 µl ice cold analytical grade (100 %) ethanol was added and centrifuged for 10 min. at 13,000 rpm. The supernatant was removed and the pellet was washed with 1 ml ice cold 70 % ethanol by centrifuging for 10 min. at 13,000 rpm. The supernatant was discarded and the pellet was left to dry for 30 to 45 min. by inverting the eppendorf with open lid. Finally, the DNA pellet was gently solublized in 50 µl elution buffer. The DNA was stored at -20°C which can be further used for gel extraction, restriction enzyme digestion or verification of DNA band on agarose gel electrophoresis.

2.10.2 MidiPreparation (MidiPrep)

The fresh starter culture of either selected colony or from stabilates were inoculated and cultivated in specific volume of liquid LB medium with ampicillin (50 mg/ml) in flask under shaking 225-250 rpm for overnight (17 h) at 37^{o}C. NucleoBond AX (MidiPrep) plasmid DNA purification kit from Macherey-Nagel was used. The main culture was harvested by centrifugation at 5000 rpm for 15 min. at 4^{o}C.

The medium was discarded and the cell pellet in falcon was carefully re-suspended in 8 ml buffer S1 with RNase A. Cells were lysed by adding 8 ml buffer S2 to the suspension. The suspension was mixed gently by inverting the tube for 6- 8 times. The mixture was incubated at room temperature for 5 min. without vortex, as this releases contaminating chromosomal DNA from the cellular debris into the suspension. The pre-cooled (4^{o}C) 8 ml buffer S3 was added to the suspension and immediately mixed the lysate by inverting the falcon 6 -8 times until a homogenous suspension containing an off-white flocculate was formed and incubated on ice for additional 5 min.

The lysate was clarified by centrifuging at 5000 rpm for 25 min. at 4^{o}C and simultaneously, the column AX 100 (Midi) included in the kit was equilibrated with buffer N2. The column was allowed to empty by gravity flow and flow through was discarded. The cleared lysate after centrifugation was filtered using folded filter paper pre-wet with buffer N2 in a small

funnel. The lysate was loaded onto the wet filter paper and flow through was collected. The clear lysate was loaded onto the column. The flow though from this step was saved for analysis. The column was washed with 12 ml buffer N3 and flow through was discarded. Finally, the plasmid DNA was eluted with 5 ml buffer N5. The DNA was immediately precipitated with 3.5 ml isopropanol and centrifuged at 10,000 rpm for 30 min. at 4^{o}C. The supernatant was discarded and the pellet was washed with 2 ml (70 %) ethanol, vortexed briefly and centrifuged at 10,000 rpm for 10 min. at room temperature. The supernatant was carefully removed and allowed the pellet to dry at room temperature for 30 min. The DNA pellet was reconstituted in appropriate volume of elution buffer with constant flicking till pellet was completely dissolved. The solublized DNA was used for further analyses like agarose gel electrophoresis, DNA estimation, restriction digestion with enzymes, transformation or DNA sequencing.

2.10.3 Bacterial colony PCR

The PCR was performed using HotStar *Taq* master mix kit from Qiagen. The following master mix was prepared

Master mix

	For 8 reactions (12.5 µl master mix/ reaction)
HotStar *Taq* master mix	100 µl
Sense primer	8 µl
Anti-sense primer	8 µl
RNase free water	84 µl

The master mix in volume of 25 µl was added to each PCR tube including positive and negative controls. The colonies were picked from LB-agar plates and inoculated into respective PCR tube. Simultaneously, the selected colony after inoculation was streaked on LB agar plates with ampicillin. The agar plates were incubated at 37^{o}C for overnight (17 h). The PCR reaction was performed as follows:

Program

1. Initial activation	95 ^{o}C	15 min.
2. Denaturation	94 ^{o}C	30 sec.
3. Annealing	56 ^{o}C	30 sec.

4. Extension	72 °C	1 min.
35 cycles to step 2		
5. Final extension	72 °C	10 min.
6. End	4 °C	9 h

After PCR reaction, 10 µl from each sample was loaded and run in agarose gel with DNA ladder in order to identify correct clones.

2.11 DNA sequencing

The isolated plasmid constructs after verification on agarose gel electrophoresis (2.4) and DNA quantification (2.6) were send for sequencing to GATC Biotech using single Run 24 supreme reaction in concentration of 30 -100 ng/ µl in a total volume of 30 µl. The results of sequenced DNA were analyzed with DNAMAN software by alignment with plasmid construct.

3. Cultivation and handling of organisms

3.1 *Escherichia coli*

3.1.1Cultivation on agar plates

The bacterial cells were cultivated on agar-agar with LB-medium and ampicillin (2.1.1). LB-medium was prepared as 2 X stock solution and mixed with pre-warmed 2 X agar-agar under sterile conditions. After cooling to room temperature, ampicillin (1 ml/ l) and X-gal (2 ml/ l) were immediately added and poured into culture plates. The plates were left to solidify. When the agar was solidified, the plates were packed under sterile conditions and stored at 4°C till further use.

3.1.2 Preparation of competent cells (Chung et al., 1989)

A single bacterial colony of XL-1 competent cells were picked from LB-agar culture plate containing tetracycline (5 mg/ ml in 70 % ethanol) that had been incubated at 37°C for overnight (17 h). This clone was inoculated into 3 ml liquid LB-medium with tetracycline and incubated at 37°C with shaking at 225 rpm for overnight (17 h). After the overnight incubation, 1 ml of the starter culture was inoculated into 100 ml LB-medium with tetracycline and propagated at 37°C with shaking at 225 rpm till the absorbance reached 0.8 at 600 nm. Then the cells were harvested at 5000 rpm for 5 min. at 4°C and the cell pellet was

re-suspended in 10 ml TSS medium (2.1.1). Subsequently, 100 µl aliquots were made in cryogenic vials which were immediately frozen in liquid nitrogen and stored at -70°C.

3.2 *Spodoptera frugiperda* (Sf9)

3.2.1 Culturing of Insect cells

Insect cells (Sf9) were bought from Invitrogen as frozen master stocks which were used as the host for the baculovirus transfer vector. The culture stabilates were washed with pre-warmed serum-free medium by centrifuging at 1700 rpm for 5 min. at 4°C. The cells were re-suspended in fresh pre-warmed serum-free medium in volume of 20 ml at a cell density of 8 X 10^5 cells/ ml. Insect cells were propagated under serum-free conditions at 27°C grown in passages of increasing concentration of FBS which provides protection from cellular shear forces in suspension culture. The growing passage of insect cells was grown till cell density of 4 X 10^6 cells/ ml reached. After which, cells were diluted with serum-free medium including Pluronic® F-68 and penicillin/ streptomycin. These cells were used for transfection or further propagated till 30 passages.

The stabilates of the insect cells were made by using culture passages. The cells were monitored to ensure that they were healthy and growing exponentially in the medium. The medium was replaced when the monolayer of cells reached 80 % confluence. Pre-warmed Ex-Cell 420 serum-free medium (2.1.2) including Pluronic® F-68 and penicillin/ streptomycin was added to the cells after harvesting with centrifugation. The cells were dispersed with careful pipetting. The viable cells were counted and adjusted the cell density to 4 X 10^6 cells/ ml with serum-free medium and were cooled to 4°C. Serum-free medium containing 20 % DMSO (v/v) was prepared. The cells were carefully suspended in serum-free medium containing DMSO. The suspension was cooled at 4°C and aliquots of 1 ml were made in cryogenic vials. The aliquots were placed immediately in liquid nitrogen. The vials were frozen at - 20°C for 12 h and then at -70°C for overnight. Afterwards, the vials were transferred to liquid nitrogen immediately and after a week, the viability of the stabilates were tested with cultivation in fresh Ex-Cell 420 serum-free medium (2.1.2) with increasing concentration of serum.

3.2.2 Vitality assay with trypan blue

The trypan blue (0.4 %) solublized in PBS was mixed with 100 µl cell suspension. The cells were then counted in Hemocytometer. Only the dead cells absorbed the trypan blue and therefore, cell vitality should be more than 80 % for transfection.

3.2.3 Transfection

The recombinant bacmid with the gene of interest was isolated and purified from DH10 Bac competent cells. The recombinant bacmid was used for transfection in insect cells. The main culture of insect cells passage was diluted with serum-free medium with defined cell density and seeded at 1 X 10^6 cells/ ml per well of 6 well tissue culture plate in 2 ml of growth medium containing antibiotics as stated above. The cells were allowed to attach at 27°C for 1 h and then bacmid DNA: Cellfectin® Reagent complex was prepared for each sample including negative control and Cellfectin® only. The recombinant bacmid and Cellfectin® in a volume of 6 µl were mixed separately in 100 µl serum-free medium. Both the solutions were combined to 200 µl total volume, gently mixed and incubated for 1 h at room temperature. During this incubation, the media from the attached cells were carefully washed twice with 2 ml of serum-free medium without antibiotics. Serum-free medium without antibiotics in a volume of 800 µl was added to each tube containing DNA: lipid complexes and gently mixed. This mixture was then added carefully to each well containing cells and the plate was incubated for 4 h at room temperature. Afterwards, the DNA: lipid mixture was removed and 2 ml of medium containing FBS and antibiotics were added to each well. The plate was incubated at 27°C for 72 h until there were microscopically signs of viral infection.

3.3 *Trypanosoma brucei*

3.3.1 Isolation from rat blood and stabilates production of bloodstream form

Trypanosome strain BF-221 kept as frozen rat stabilates in liquid nitrogen were used for the propagation of the parasites in Sprague-Dawley rats. The rat stabilates with cell density of 5 X 10^7 cells were intraperitoneally injected in anesthetized rat. After 3 days of infection, the parasitemia in rat at a cell density of 1 X 10^9 cells/ ml was achieved. The animal was prepared for sacrifice in CO_2 environment. The thorax of the animal was cut opened and washed with 2 ml CGA (2.1.3). The *vena cava inferior* was cut and the blood was sterile collected with

Pasteur pipette into fresh falcon tube. The rest blood was collected by puncturing 5 ml CGA in left ventricle and again collecting it in fresh falcon tube. Afterwards, the blood was centrifuged (Sigma 3K12) at 3000 rpm at 4°C for 10 - 15 min.

The blood plasma and erythrocytes were removed from top of the visible phase of buffy coat which consisted of parasites. The buffy coat was loaded onto DEAE-Sephacel in a column prepared with filter paper on lower and upper layer of material pre-equilibrated with separation buffer (2.1.3.2). The flow through was collected which was turbid due to the presence of parasites and this flow through was centrifuged (Sigma 3K12) at 3000 rpm at 4°C for 5 min. The supernatant was discarded and the cells were re-suspended in 5 ml TDB (2.1.3). The cells were washed twice by centrifuging (Sigma 3K12) at 3000 rpm at 4°C for 5 min. and then the cells were used for further experiments.

For stabilates production, the buffy coat was carefully transferred with the help of sterile Pasteur pipette and re-suspended in 4 ml CGA. Furthermore, 6 ml BF-medium (2.1.3.3.1) and 2 ml Canusal® (200 U, Heparin-Na) were added to the suspension and cell density was calculated. The cell density was adjusted at 2 X 10^8 cells/ ml and mixed in 1: 1 with freezing medium for stabilates (2.1.3). The aliquots were pipetted in volume of 1 ml each in cryo vials on ice. Finally, the stabilates were frozen at -20°C for 1 h and then at -70°C for overnight. Next day, the stabilates were transferred to liquid nitrogen until further use.

3.3.2 In vitro cultivation of bloodstream form

Trypanosome bloodstream form frozen stabilates were used for axenic cultivation. The stabilates were thawed under warm water and cell suspension was transferred into falcon tube with 9 ml pre-warmed bloodstream form medium (2.1.3.3.1) and washed by centrifugation (Sigma 3K12) at 3000 rpm at 4°C for 5 min. The supernatant was removed carefully till approximately 1 ml was left. The cells were re-suspended and counted. For counting the cells, 10 µl of the cell suspension were diluted to 1: 100 and counted in Hemocytometer. The starter culture in culture flask with bloodstream form medium was adjusted to cell density of 2.5 X 10^5 cells /ml and cultivated in an incubator at 37°C with 5 % CO_2. After 20 h incubation, the cell density in starter culture reached till 1 X 10^6 cells/ml. The starter culture was diluted with medium to main culture and this was propagated accordingly to experimental requirements to cell density of 2.5 X 10^5 cells/ ml. After 40 h incubation, the main culture reached a cell density of 2 X 10^6 cells/ ml (stationary phase) which was used for further experiments.

3.3.3 In vitro cultivation of procyclic form

The procyclic form frozen stabilates were used for axenic cultivation. The stabilates were thawed under warm water and running culture of procyclic form was prepared in procyclic culture medium (2.1.3.3) using the same procedure as described for bloodstream form (3.3.1). The culture cell density was adjusted at 5 X 10^5 cells/ ml and cultivated in an incubator at 27°C without CO_2. The running culture was diluted to a cell density of 2.5 X 10^5 cells/ ml during the week either for running or main culture depending on the experiment.

4. Cloning of phospholipase A_2 gene from *Trypanosoma brucei*

The complete gene of TbPLA$_2$ was cloned using total cDNA from bloodstream form with sense and anti-sense primers (see Materials 1.2) which were synthesized using DNA sequence (see Materials 1.1) from GeneDB , hosted by Sanger Institute (http://www.genedb.org/). This gene sequence included signal peptide and transmembrane sequence of protein.

4.1 Sub-cloning of TbPLA$_2$ gene in competent cells

After PCR with AccuPrime *Taq* DNA polymerase, the reaction product was verified on agarose gel electrophoresis and then it was used to clone into sub-cloning plasmid pCR®2.1-TOPO (Invitrogen). The following ligation reaction mixture was prepared according to the kit (Invitrogen)

PCR product	4 µl
Salt solution	1 µl
TOPO vector	1 µl

The reaction was gently mixed in PCR tube and incubated for 30 min. at 22°C in pre-programmed PCR machine with program ending at 4°C. The PCR tube was placed on ice. One Shot TOP 10 (Invitrogen) competent cells were thawed on ice and transformation protocol proceeded (2.8). The plates were incubated at 37°C for 17 h and thereafter, white colonies appeared. These colonies were selected, inoculated into starter culture and incubated at 37°C for overnight. MiniPrep (2.10.1) was performed and finally, the DNA plasmid was solublized in specific volume of elution buffer. The DNA was analyzed with restriction

analysis with *Nde1* to confirm for correct orientation of insert DNA and verified on agarose gel electrophoresis. The correct clone DNA was further double digested with *BamH1* and *Pst1* enzymes to cleave out the insert DNA with these specific sites. The reaction mixture was run on agarose gel electrophoresis and insert DNA band was cut out and proceeded with gel extraction protocol using QIAquick gel extraction kit (Qiagen) (see 2.5). The insert DNA was solublized in specific volume of elution buffer and DNA was quantified (see 2.6) and send for DNA sequencing (2.11) and verification. The stabilates of correct clone were made (see 2.9) and stored in liquid nitrogen till further use.

4.2 Cloning of $TbPLA_2$ gene in competent cells

The insert DNA was ligated with expression vector pMal-c2E (NEB) using ligation kit (Fermentas) (see 2.7) performed at 16°C for 17 h. Afterwards, the ligase was inactivated for 10 min. at 65°C. The ligation product was further used in transformation into One Shot TOP 10 (Invitrogen) sub-cloning competent cells using the transformation protocol (2.8). The colonies were picked from selective agar plate and verified with bacterial colony PCR (2.10.3). The reaction mixture was run on agarose gel electrophoresis to identify the correct clone. The correct clone with plasmid construct with gene of $TbPLA_2$ was picked and starter culture was cultivated for overnight. The starter culture 1.5 ml was used simultaneously for stabilate production and the rest of culture was used for MiniPrep. The construct DNA in a volume of 3 µl was used to transform (2.8) into BL-21(DE3) or Rossetta 2 expression competent cells. The colonies were picked and bacterial colony PCR (2.10.3) was performed to verify for correct insert DNA including positive and negative controls. These products were run on agarose gel electrophoresis and verified the correct clone. The starter culture of the clone was cultivated for stabilates and stored in liquid nitrogen until further use.

The gene of $TbPLA_2$ was also cloned into baculovirus expression system. The construct of $TbPLA_2$ with pMal-c2E and baculovirus vector pFastBac-1 were digested with *BamH1* and *Pst1* enzymes simultaneously to cleave out the insert DNA with these specific sites. Afterwards, pFastBac-1 and insert DNA was purified from agarose gel using QIAquick gel extraction kit (Qiagen) (see 2.5). The purified insert DNA and pFastBac-1 were used to perform ligation reaction (2.7) at 16°C for 17 h and then left at 4°C. The ligation reaction product of pFastBac-1 with $TbPLA_2$ was used to transform (2.8) into XL-1 competent cells. The colonies were verified by bacterial colony PCR (2.10.3) and starter culture cultivated for MiniPrep (2.10.1). The colonies were also verified for correct clone using restriction digestion

with *Nde1, HindIII* and *Eam1105 1*. The correct DNA construct was used to transform into MAX Efficiency®DH10Bac competent cells by using transformation protocol (2.8) on selective LB-agar plates containing kanamycin, gentamicin and tetracycline. The white colonies were picked and analyzed to verify successful transposition to the bacmid. The recombinant bacmid DNA with insert was 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 clone was picked and starter culture cultivated for MiniPrep (2.10.1). The DNA from MiniPrep was solublized in a specific volume of elution buffer and another starter culture from the same clone was used for stabilates.

5. Heterologous expression of phospholipase A_2 gene from *Trypanosoma brucei* in *Escherichia coli* and *Spodoptera frugiperda* (Sf9)

5.1 Expression in bacteria

The starter culture of construct of $TbPLA_2$ with pMal-c2E in either BL-21(DE3) or Rosetta 2 expression competent cells was cultivated for overnight (17 h) at 37°C with shaking at 225 rpm. The logarithmic phase growing bacterial cells from starter culture were used to inoculate into LB liquid medium (with ampicillin for BL-21(DE3) or chloramphenicol and ampicillin for Rosetta 2) in the ratio of 1:100. The cells were cultivated at 37°C with shaking at 225 rpm till the absorbance of culture reached 0.8 at 600 nm. Subsequently, the culture was divided into portions, one portion of the culture was designated as control culture (without induction) and other remaining cultures were induced with 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 time span of 9 h.

The cells were harvested subsequently by centrifugation at 5000 rpm for 15 min. at 4°C. The medium was discarded from control and induced cells. The cell pellets were frozen at -20°C. These were re-suspended in 5 ml of column buffer (2.1.1) and sonicated the suspensions for short pulses of 10 sec. for 5 times on ice in a Sonifier® Cell disruptor B-30 with following specifications:

Timer	Hold
Output control	5
Duty cycle	50 %
Continuous	

The lysates were centrifuged at 5000 rpm for 20 min. at 4°C. The supernatant was separated and protease inhibitors mix (2.1.3) was added immediately after centrifugation. The pellet was re-suspended in 5 ml column buffer with protease inhibitors mix (2.1.3). All supernatants and pellets were estimated with Bradford method (1.1) and analyzed on 12 % SDS-PAGE (1.2) in reducing conditions.

5.2 Expression in Sf9 insect cells

5.2.1 Isolation of P1 viral stock

The transfected cells from well of plate with signs of late stage infection were collected with the medium containing virus in a volume of approximately 2 ml and sterile transferred to falcon tube. The suspension was centrifuged at 1700 rpm for 5 min. at 4°C to remove cells and large debris. The clarified supernatant containing viruses was sterile transferred to another falcon tube and this was labeled the P1 (low viral titer) stock which was protected from light and stored at 4°C.

5.2.2 Amplification of Baculovirus stock

The culture passage of insect cells was diluted with serum-free medium with defined cell density and seeded at 2 X 10^6 cells/ ml. The multiplicity of infection (MOI) was calculated, whereas MOI ranges from 0.05 to 0.1:

$$\text{Inoculum required (ml)} = \frac{\text{MOI (pfu /ml) X number of cells}}{\text{Titer of viral stock (pfu / ml)}}$$

$$= \frac{0.1 \text{ pfu /ml X } (2 \text{ X } 10^7) \text{ cells}}{5 \text{ X } 10^6 \text{ pfu /ml}}$$

Inoculum required (ml) = 0.4 ml

The diluted 20 ml insect cells were infected with 0.4 ml P1 viral stock and incubated with shaking at 27°C for 72 h. After 72 h infection, the medium was collected by centrifugation at 1700 rpm for 5 min. at 4°C and the supernatant was transferred to fresh falcon tube and this was labeled the P2 (high viral titer) stock. The P2 viral stock was used for further infections for large scale volumes from 30 ml, P3 (highest viral titer) cultures. After every infection, the supernatant and cell pellet was preserved at 4°C.

Cell lysis

The cell pellet was washed twice with PBS pH 7.4 by re-suspending the cells. The cell suspension was centrifuged at 1700 rpm for 5 min. and the wash solution was carefully removed. After removal of the final wash solution from the cells, a volume of 1 ml RIPA lysis buffer was added. The cells were re-suspended and vortexed briefly. The cell suspension was incubated on ice for 5 min.

The lysate can be either used immediately or frozen at -20°C for further use. The lysate was centrifuged at 10,000 rpm for 10 min. at 4°C to pellet the cell debris and membranes. The supernatant was carefully transferred to fresh tube. Both the supernatant and the cells pellet were stored at -20°C till further use.

6. Immunoprecipitation

The insect cells lysate was centrifuged at 10,000 rpm for 10 min. at 4°C and immediately transferred the supernatant to an another eppendorf. The pellet was re-solublized in RIPA lysis buffer. Protein G agarose was prepared by washing the beads twice with PBS and then restored the suspension to 50 % slurry with PBS. The soluble pellet fraction was pre-cleared by adding 100 µl of washed Protein G agarose bead slurry (50 %) per 1 ml of cell lysate and incubating on an orbital shaker for 10 min. at 4°C. The Protein G beads were removed by centrifugation at 10,000 rpm for 10 min. at 4°C and transferred the supernatant to another eppendorf. The protein concentration of the supernatant was determined by Bradford method

by diluting it with PBS because of the interference of detergents from RIPA buffer. The protein concentration of supernatant was set at 500 µg. The anti-$TbPLA_2$ antibody in concentration of 10 µg was added to the supernatant. The mixture was gently rocked on an orbital shaker for overnight at 4°C.

Afterwards, the immunocomplex was captured by adding washed 100 µl Protein G agarose bead slurry (50 µl packed beads) and gently rocked on orbital shaker for overnight at 4°C. The agarose beads were collected by pulse centrifugation (i.e. at 10,000 rpm for 5 secs.). The supernatant was discarded and washed the beads 3 times with 800 µl PBS. The agarose beads were re-suspended in 50 µl PBS. The immunocomplex with beads were analyzed by adding 4 X reducing sample diluting buffer and gently mixed. The suspension was boiled for 5 min. to dissociate the immunocomplex from the beads. The beads were collected by centrifugation and either SDS-PAGE was performed with the supernatant or was frozen at -20°C for later use. The frozen supernatant was re-boiled prior to be loaded on SDS-PAGE.

7. Isolation of cytosolic and membrane fraction for analysis from bloodstream and procyclic form

The starter culture of *Trypanosoma brucei* BF-221 bloodstream form was cultivated (3.3.2). After 20 h, the starter culture reached a cell density of 1 X 10^6/ ml in 50 ml culture. The cells were harvested by centrifugation at 3000 rpm for 5 min. at 4^oC. The cell pellet was washed twice with 5 ml TDB (2.1.3) containing 100 µl 0.2 mM $ZnCl_2$ (10 mM stock solution) per 1 ml. The cells were lysed in 200 µl of first lysis buffer which consisted of 10 mM phosphate buffer containing $ZnCl_2$ and protease inhibitor for 15 sec. in sonicator and verified microscopically for lysis of cells. The lysate was incubated on ice for 1 h and centrifuged at 10,000 rpm for 15 min. at 4^oC. The supernatant was separated and the soluble cytosolic protein fraction was stored at 4^oC.

The cell pellet was washed twice in 10 mM phosphate buffer containing $ZnCl_2$ and protease inhibitor mix in a volume of 200 µl. The buffer was carefully added onto cell pellet and was not re-suspended. The pellet was washed by centrifugation at 10,000 rpm for 15 min. at 4^oC and the supernatant was discarded. The second lysis buffer consisted of 10 mM phosphate buffer containing protease inhibitor mix in a volume of 200 µl was added to cell pellet and vortexed for 1 min. at room temperature. Subsequently, the suspension was incubated for 20 min. at room temperature. The solution was centrifuged (Ultracentrifuge TL-100) at 60,000 rpm for 1 h at 4^oC. The supernatant was separated and stored at 4°C. The pellet was again

washed in 200 µl of 10 mM phosphate buffer containing protease inhibitor mix and was not re-suspended. The pellet was centrifuged at 60,000 rpm at 4°C for 1 h. The supernatant was discarded and the pellet was re-suspended in 200 µl third lysis buffer consisted of 10 mM phosphate buffer containing protease inhibitor mix and 0.1 % Triton X-100. The solution was vortexed and left at 4°C for overnight. All the fractions were precipitated with ice cold acetone in 1:3 ratio and left at -20°C for 30 min. The fractions were centrifuged at 10,000 rpm for 15 min. The pellet was solublized in 10 mM phosphate buffer containing protease inhibitor mix in a volume of 20 µl. The protein estimation was done with Bradford method. Subsequently, all fractions were stored at 4°C till further use.

8. Immunoflouresence microscopy

The trypanosomes from starter culture at a cell density of 1-5 X 10^6 cells per reaction were harvested by centrifugation and the medium was carefully removed. The cells were washed twice and carefully re-suspended in 200 µl PBS pH 7.4. Subsequently, the fixation solution (2.2.5) in volume of 300 µl was added to the cell pellet and carefully inverted. The cells were fixed for overnight at 4°C.

The fixed cells were centrifuged (Biofuge A) at 13,000 rpm for 20 sec. and the supernatant was carefully removed. The cell pellet was carefully washed in 1 ml PBS with centrifugation. Afterwards, the supernatant was discarded and the cells were re-suspended in 500 µl glycine-sodium phosphate buffer and the suspension was incubated for 15 min. at room temperature. The permeabilization was achieved by adding 500 µl 0.2 % Triton X-100 solution. The solution was inverted, incubated for 5 min and then centrifuged. The supernatant was discarded and the cell pellet was re-suspended in 100 µl primary antibody diluted in different concentrations in BSA/ PBS, thereafter incubated for 1 h at 4°C. After incubation, the cells were washed twice in 250 µl BSA/ PBS in bench top centrifuge. The cell pellet was re-suspended in 100 µl secondary antibody Alexa Flour 594 anti-rabbit in a working dilution of 1: 500 and incubated for 1 h in dark at 4°C. For visualization of nucleus and kinetoplast, freshly prepared 1 µl Bisbenzimide stock solution was added to each sample tube and incubated for additional 5 min. Finally, the pellet was carefully washed once with BSA/ PBS and then twice or thrice with 200 µl distilled water. The pellet was re-suspended in approximately 100 µl distilled water and pipetted out 10 µl from each sample on object slide and let it dry in dark under aluminium foil. After samples were dried on Immunomount object

slide, the samples were observed under the microscope (Olympus BH2 RFCA) and images were made with Olympus camera U-PMTVC and saved in computer.

9. PLA_2 enzyme activity assay

The PLA_2 enzyme activity assay was a modified assay from Jimenez *et al.*, 2003. The assay was based on a coupled enzymes using dilinoleoyl phosphatidylcholine (DL-PC) as phospholipase substrate and lipoxygenase as coupling enzyme. The substrate was prepared by solubilizing 5 mg dilinoleoyl phosphatidylcholine (Sigma) in 400 µl chloroform (analytical grade) at 4^oC taking care to pipette it in as quickly as possible and immediately vortexed. The aliquots were made with a final concentration of 1.28 mM and 80 µl each. The aliquots were then dried with speedvac to complete dryness for 15 min. and immediately re-suspended in 50 mM Tris-HCl pH 8.5 containing 10 mM sodium deoxycholate (2.2.6) in a volume of 983.6 µl each. The resulting substrate solution was equilibrated for 10 min. at 25^oC and stock substrate solutions were stored at -20°C.

The total reaction medium 1 ml consisted of 19.23 µl DL-PC dissolved first in calculated volume of reaction buffer 1(2.2.6). Subsequently, equal volume of the reaction buffer 2 was added to the mixture and mixed. The coupling enzyme 6.4 µl lipoxygenase was added to reaction tube. Finally, the reaction mixture was transferred in cuvette. Before measurement, the volume was made up to either with buffer for control or samples and mixed by inverting. Controls without either phospholipase or lipoxygenase were carried out. The reaction was followed spectrophotometrically in an Ultrospec 3000 spectrophotometer for 0 to 60 min. by measuring the increase in absorbance at 234 nm due to the formation of the hydroperoxides. Absorbance values from sample were subtracted from control for calculation. Specific activity was calculated by using the formula:

$\varepsilon = A/ c.l$

$c = A/ \varepsilon.l$

Where $\varepsilon = 25000\ M^{-1}\ cm^{-1}$, A = difference of absorbance, l = 1 cm.

3. Results

1. Characteristics of TbPLA$_2$ protein

The genome database of *T. brucei* contains the gene of a putative phospholipase A_2-like protein (TbPLA$_2$) and is located on chromosome 9. It has a gene length of 1344 base pairs (bp). Characteristically, this protein contains 447 amino acids in the open reading frame and has a molecular weight of 49.9 kDa including a signal and transmembrane sequence. This phospholipase A_2 does not contain a GPI anchor but has a predicted signal sequence. Sequence homology analysis shows 28.35 % sequence similarity with platelet-activating factor acetyl hydrolase (PAF-AH) from *Canis familiaris.*

The protein sequence of TbPLA$_2$ contains a conserved lipase motif of GHSFG as shown in Figure 3.1. It has a sequence identity with group VII PLA$_2$ PAF-acetyl hydrolase isoform II from residue number 75 to 443 (underlined in Figure 3.1) and belongs to the phospholipase A_2 family due to this similarity.

MVTWALKYFVRVVRWSTEAFLIWPTRPLFDYATSLHCVPISGTFITSVLL
CFYGFPLFWSTAMVAVGFIISGVLFLVSPLPLLKPIGGRYSVGLVHMNGC
RSQSIPPVAVFYPTNMVPEKKGLPYVPFGDDRFLRGVAAYANVPFFFIRD
FSFVRISASRNAVPAALLNQYERVPPIVVFSHGLAGYHLFYSCFALDLAA
RGAIVICLGHCDNSASFMRDSSGKESEVPLKDYGWEVPAREAQVAQRV
SEVRGTLQRLTEKDFWTTLGYINSDIDKFLSKPLQVHLA***GHSFG***GATVLA
AALEENQNPVKGVSVKSVYTFDPWMVPIQNEHFCNPLSDGRKSYTVPTV
TVHSDDWVKDSESWEFFKRMKALVLEQSAYASLNEVEKQALFGIVVTKN
TNHLSLVDVSVLSPVMHGNIWATVSPRVQIMEWCNALLRFAKQNTEVCS
TC

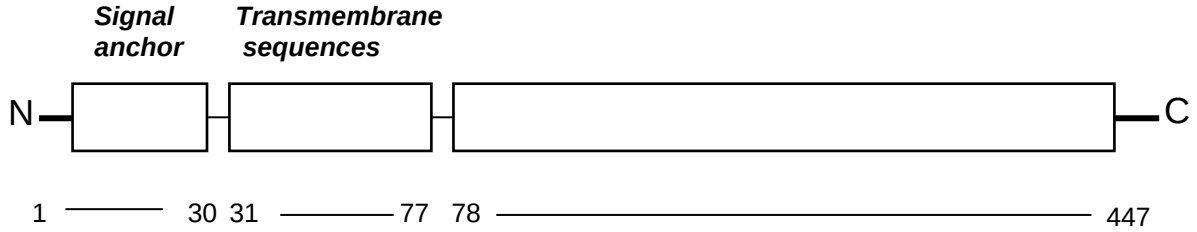


Figure 3.1 Predicted protein sequence of *T.brucei* phospholipase A_2-like protein (TbPLA$_2$) from GeneDB

An alignment of predicted amino acid sequence (Figure 3.2) of phospholipase A_2 from *T. brucei* with those of other putative phospholipase A_2-like proteins from *T. cruzi*, *Leishmania major* and characterized human platelet-activating factor acetyl hydrolase from suggests that this protein is a phospholipase A_2.

The protein contains a conserved GHSFG lipase motif (indicated by bar, Figure 3.2) in comparison with above mentioned proteins and apart from lipase motif, a FSHGL motif which is also conserved among these proteins and significantly elaborates the sequence similarity and relates them with phospholipase A_2 family. As calculated by TMHMM server 2.0 (transmembrane helix topology prediction method), this protein possesses a transmembrane helix (46 amino acids) from amino acid 31 till 77 on its N-terminus as shown in Figure 3.3. The transmembrane sequence predicts that the protein is associated itself with either the cell or an organelle membrane. In addition, $TbPLA_2$ contains 24 amino acids on its N-terminus which comprises a signal sequence used for targeted secretion after protein synthesis. The most likely cleavage site for signal sequence in this protein is between position 19 and 20 as predicted by SignalP 3.0 server (signal peptide prediction). The analysis with OGPET v 1.0 (O-glycosylation prediction electronic tool) in this protein predicted that it is o-glycosylated on T (Threonine), at position 266 in the sequence which suggest that it is a glycosylated protein. Analysis of the phylogenetic tree (Figure 3.4) indicates that $TbPLA_2$ (highlighted with light grey box) is closely related with other phospholipase A_2 like proteins from different organisms.

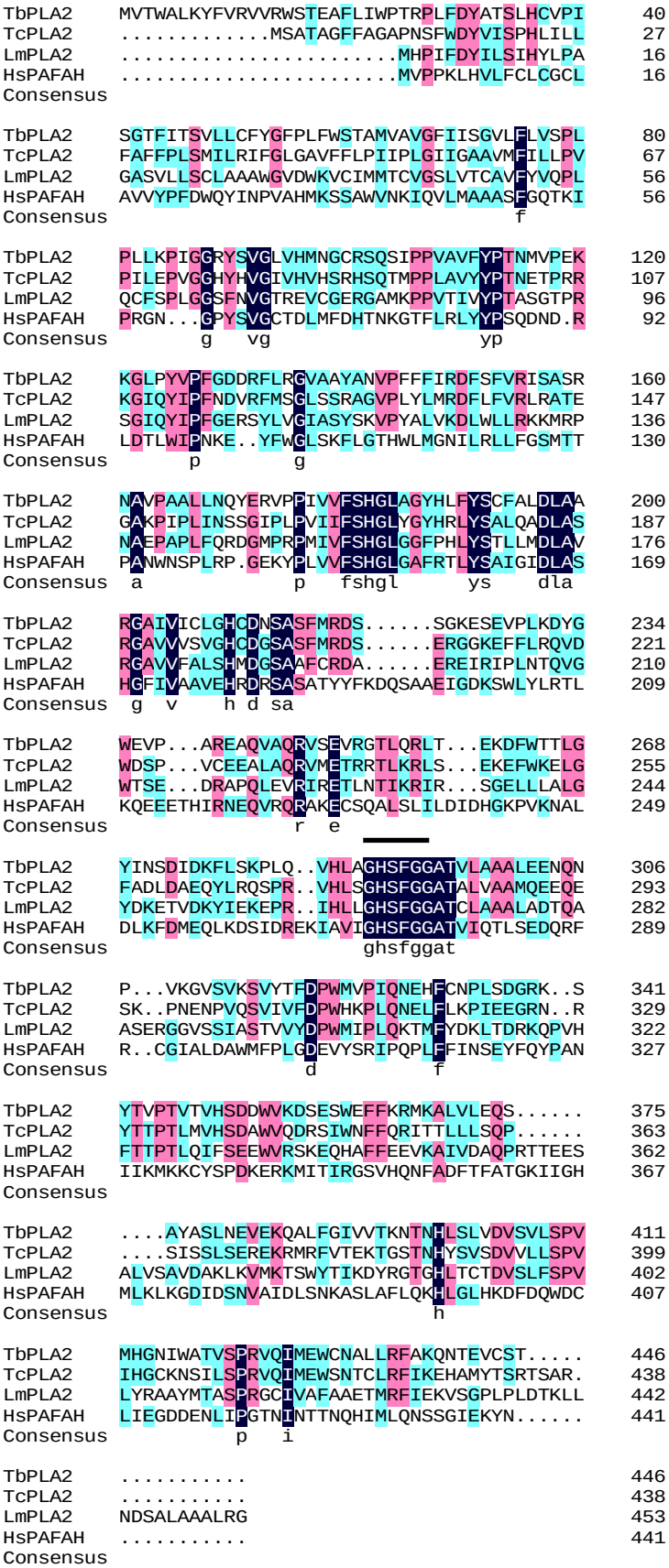
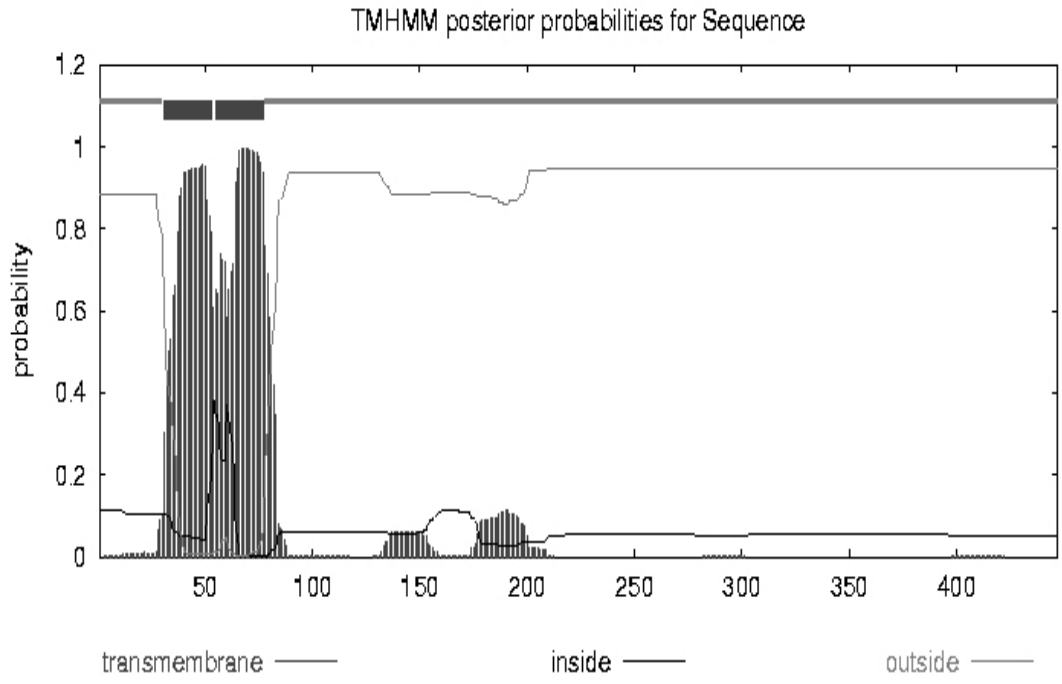


Figure 3.2 Amino acid sequence alignment of TbPLA$_2$ with phospholipases A$_2$ from TcPLA$_2$ (*T. cruzi*), LmPLA$_2$ (*Leishmania major*) and HsPAF-AH (human platelet activating factor-acetyl hydrolase). Conserved motifs are marked with black boxes and lipase consensus motif labeled with a bar above it.



Sequence length: 447

Sequence number of predicted transmembrane helices (TMHs): 2

Sequence expected number of amino acids (AAs) in TMHs: 46.75847

Possible N-terminal signal sequence

Sequence	TMHMM2.0	outside	1	30
Sequence	TMHMM2.0	TMhelix	31	53
Sequence	TMHMM2.0	inside	54	54
Sequence	TMHMM2.0	TMhelix	55	77
Sequence	TMHMM2.0	outside	78	447

Figure 3.3 Predicted transmembrane and helix analysis in $TbPLA_2$

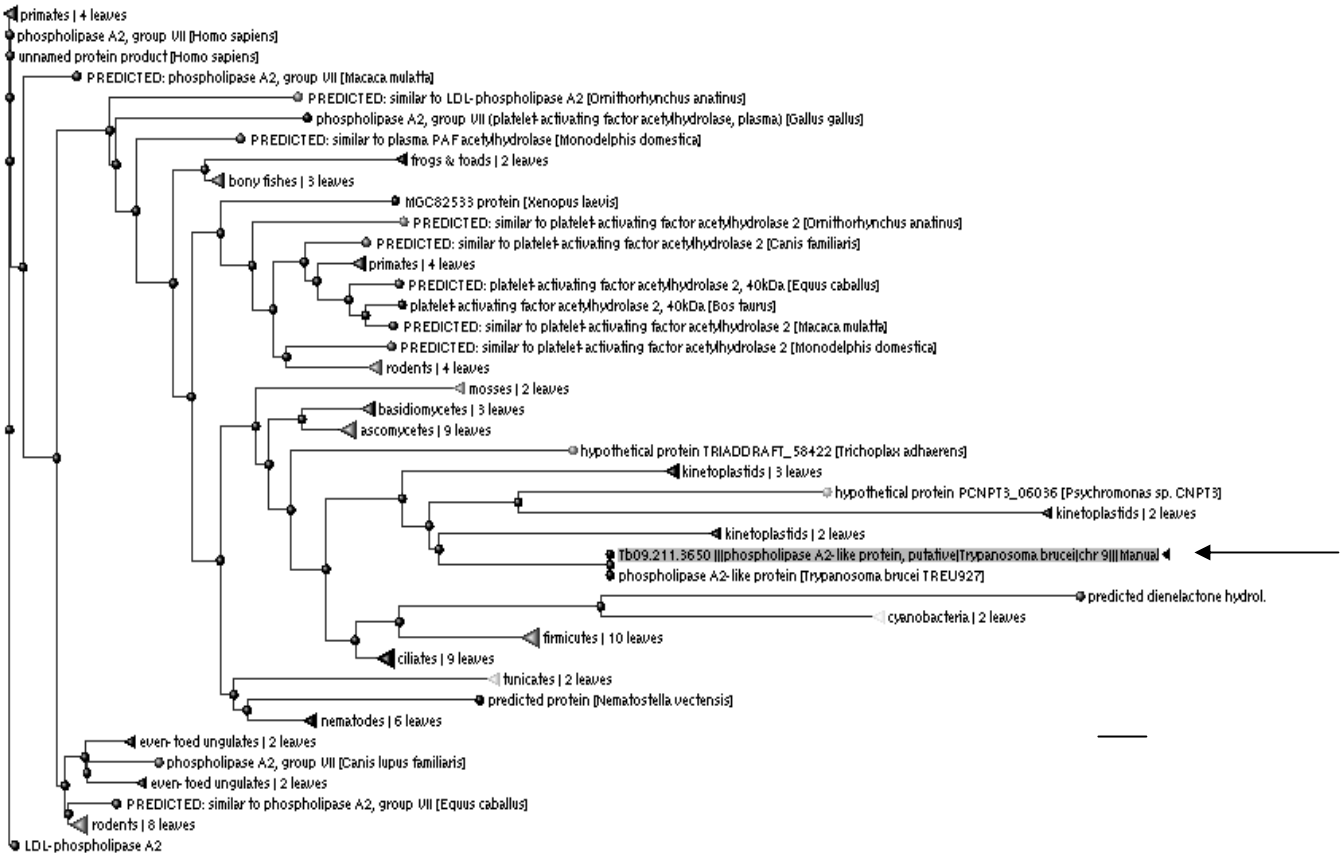


Figure 3.4 Phylogenetic tree analysis of $TbPLA_2$. The tree was calculated using the NCBI BLAST tree view widget. The arrow indicates the location and homology of TbPLA2with other phospholipases A_2. Maximum sequence difference and distance is 0.85. Bar represent distance of 0.85.

2. Cloning of $TbPLA_2$ in E.coli

The PCR reaction with AccuPrime *Taq* polymerase using sequence-specific primers yielded a product between 1000 and 1500 bp which corresponded to the open reading frame (ORF) of $TbPLA_2$ (Figure 3.5). After gel extraction, this PCR product was ligated into subcloning vector pCR 2.1 using the TOPO-TA cloning kit (Invitrogen). The DNA construct was transformed into TOP 10 competent cells and this yielded colonies. Positive clones were propagated and the DNA was isolated from the cells using the method of MiniPreparation (MiniPrep) (see Material and methods). The DNA construct obtained was verified using restriction enzyme *Nde1*.Analysis on agarose gel revealed bands at 4453 bp and 841 bp as expected (Figure 3.6). The clone containing this DNA construct was further propagated and the DNA was obtained by the method of MiniPreparation. Afterwards, this DNA was analyzed and digested with *BamH1* and *Pst1* to cleave out the $TbPLA_2$ gene fragment. Additionally, $TbPLA_2$ was extracted from the agarose gel and the DNA concentration was estimated spectrophotometrically at λ = 260 nm.

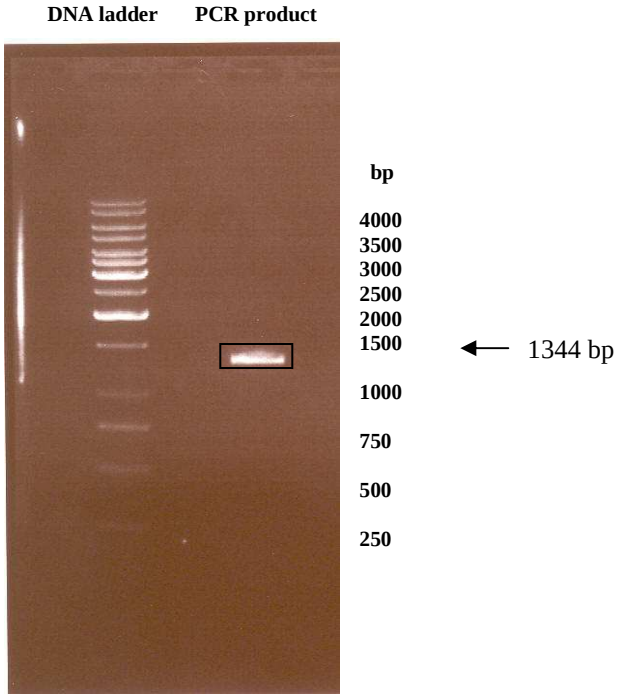


Figure 3.5 PCR analysis on 1 % agarose gel of $TbPLA_2$ with AccuPrime *Taq* polymerase

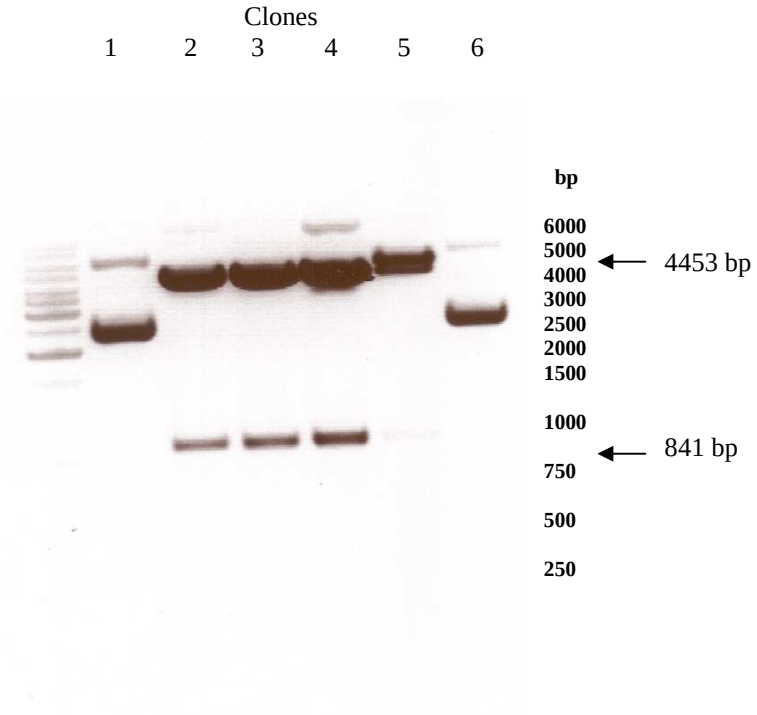


Figure 3.6 Analysis on agarose gel of plasmid constructs of pCR 2.1 containing $TbPLA_2$ from clones in TOP 10 competent cells after *NdeI* digestion

The $TbPLA_2$ was ligated into the expression vector pMal-c2e which was previously digested with *BamH1* and *Pst1*. The ligated construct was successfully transformed into XL-I competent cells and propagated. Positive clones from this transformation were picked and further cultivated. The DNA construct from the clones were obtained using the method of MiniPrep and analyzed using *BamH1* and *Pst1* (Figure 3.7). The confirmed clone was further selected and cultivated to gain the respective DNA construct which was further transformed into BL-21 (DE3) expression competent cells. The clones obtained were analyzed and verified using the method of bacterial colony PCR (see Material and method). As shown in Figure 3.8, this was performed including a negative control (without any template DNA), a positive control (template DNA for primers) and the respective clones to be analyzed. The obtained DNA construct was sequenced by GATC Biotech. and aligned with known gene database sequence to confirm sequence homology. Using DNAMAN®, this DNA sequence was converted into the respective protein sequence and aligned with known database sequence in order to evaluate and verify the sequence.

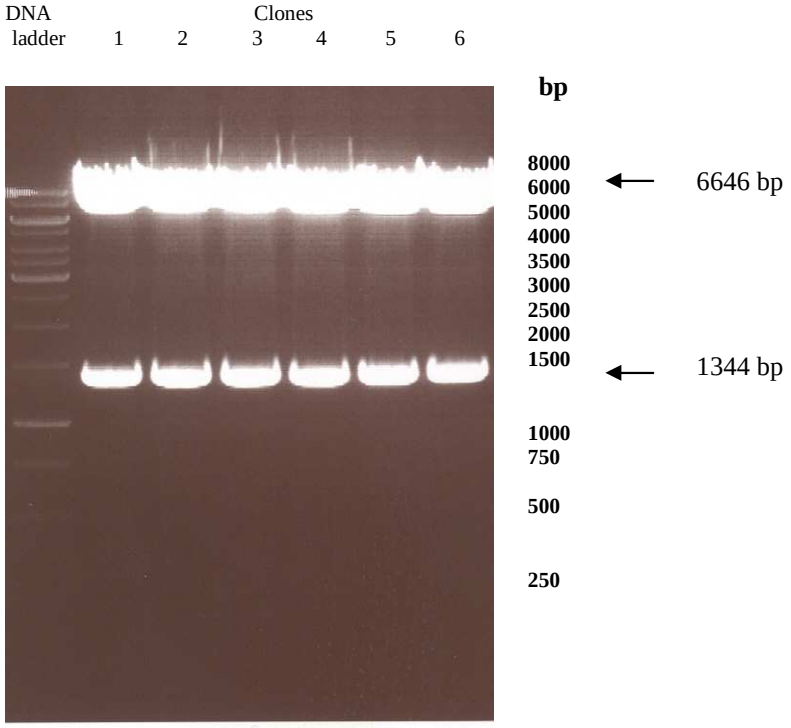


Figure 3.7 Plasmid constructs of $TbPLA_2$ in pMal-c2E from clones in XL-1 competent cells after *BamH1* and *Pst1* digestion analysis on 1 % agarose gel

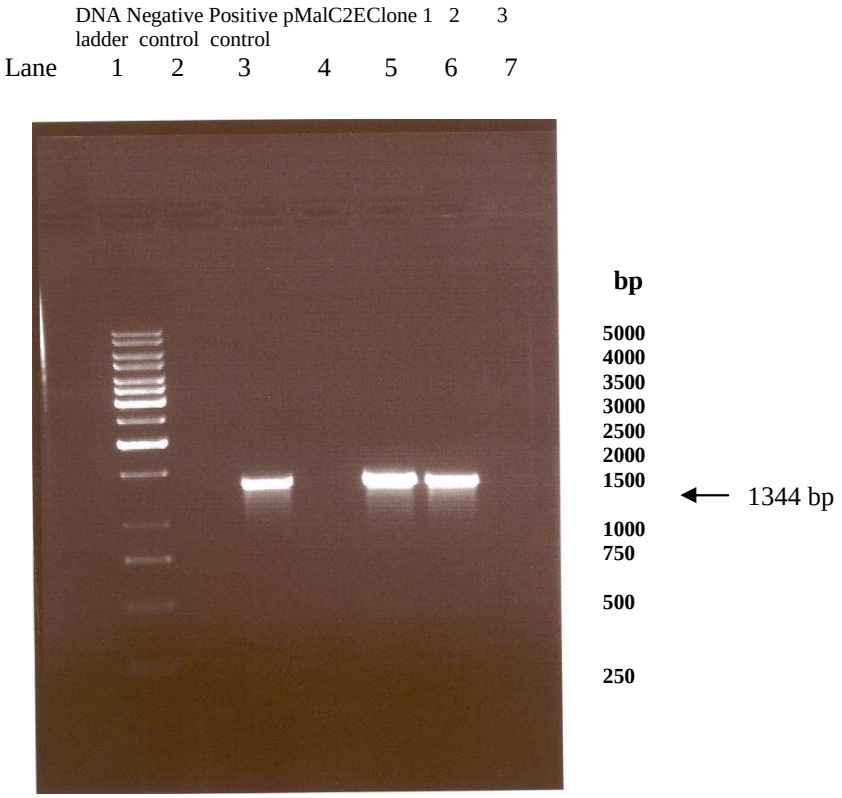


Figure 3.8 Bacterial colony PCR of clones from BL-21 (DE3) competent cells with construct of pMal-c2E - $TbPLA_2$ analysis on 1 % agarose gel

As a result of unsuccessful heterologous over-expression attempt, the verified pMal-c2E-$TbPLA_2$ construct was transformed into Rosetta 2 expression competent cells and positive clones obtained were verified with bacterial colony PCR (see Material and method) as shown in Figure 3.9.

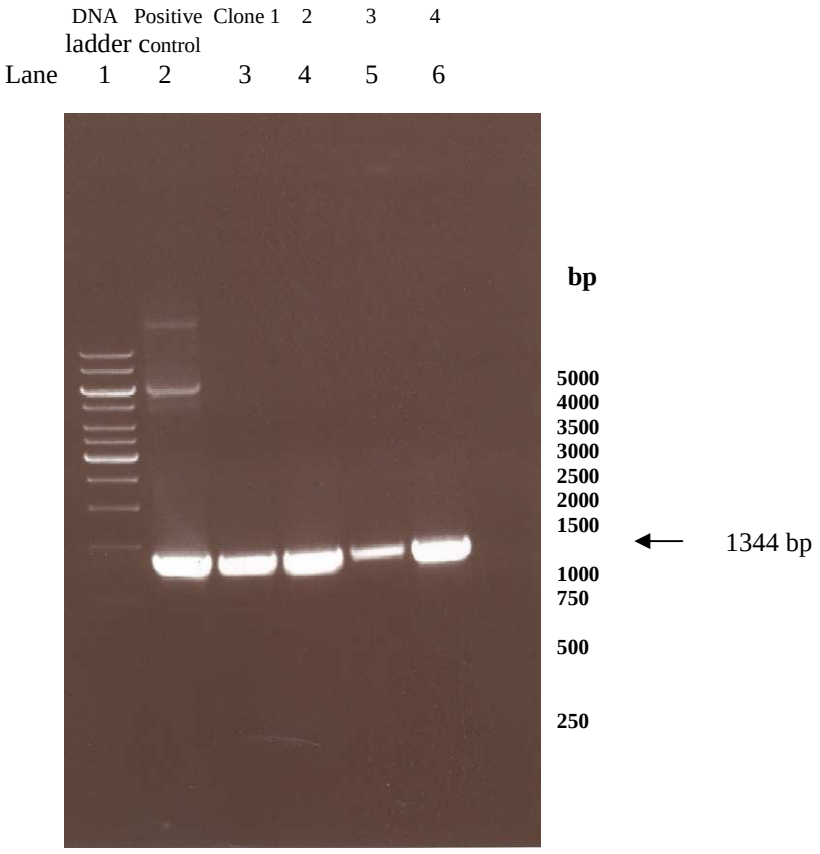


Figure 3.9 Bacterial colony PCR of clones from Rosetta 2 competent cells with construct of pMal-c2E - $TbPLA_2$ analysis on 1 % agarose gel

3. Gene and protein sequence alignment of clones

The multiple gene sequence alignment of clones from different cloning experiments indicated constant alterations (point mutations) at three positions as compared to the known gene sequence from GeneDB of *T. brucei* (Figure 3.10). These alterations had been verified by translating the several sequenced $TbPLA_2$ gene (Figure 3.10) to amino acid sequence using the software DNAMAN®. The amino acid multiple sequence alignment showed that there was not only a single but rather three constant amino acids alterations as compared with known database which included, instead of phenylalanine (F) to leucine (L), isolecine (I) to serine (S) and valine (V) to leucine (L) as indicated with asterisks in Figure 3.11. The conserved sequence was highlighted in black in Figure 3.11. On the other hand, characterized *T. brucei*

phospholipase A_1 ($TbPLA_1$) (Richmond and Smith, 2007), had also contained alteration at two position as compared to database sequence before its characterization. The $TbPLA_1$ amino acid sequence reported had alterations from known database instead of proline (P) to serine (S) and alanine (A) to glycine (G). This suggests that the amino acid sequence of $TbPLA_2$ as indicated in database can have alterations. Similar to $TbPLA_1$ in the case of $TbPLA_2$, the clones having alterations at three positions instead of F, I and V to L, S and L respectively are constant as verified from several separate clone sequences.

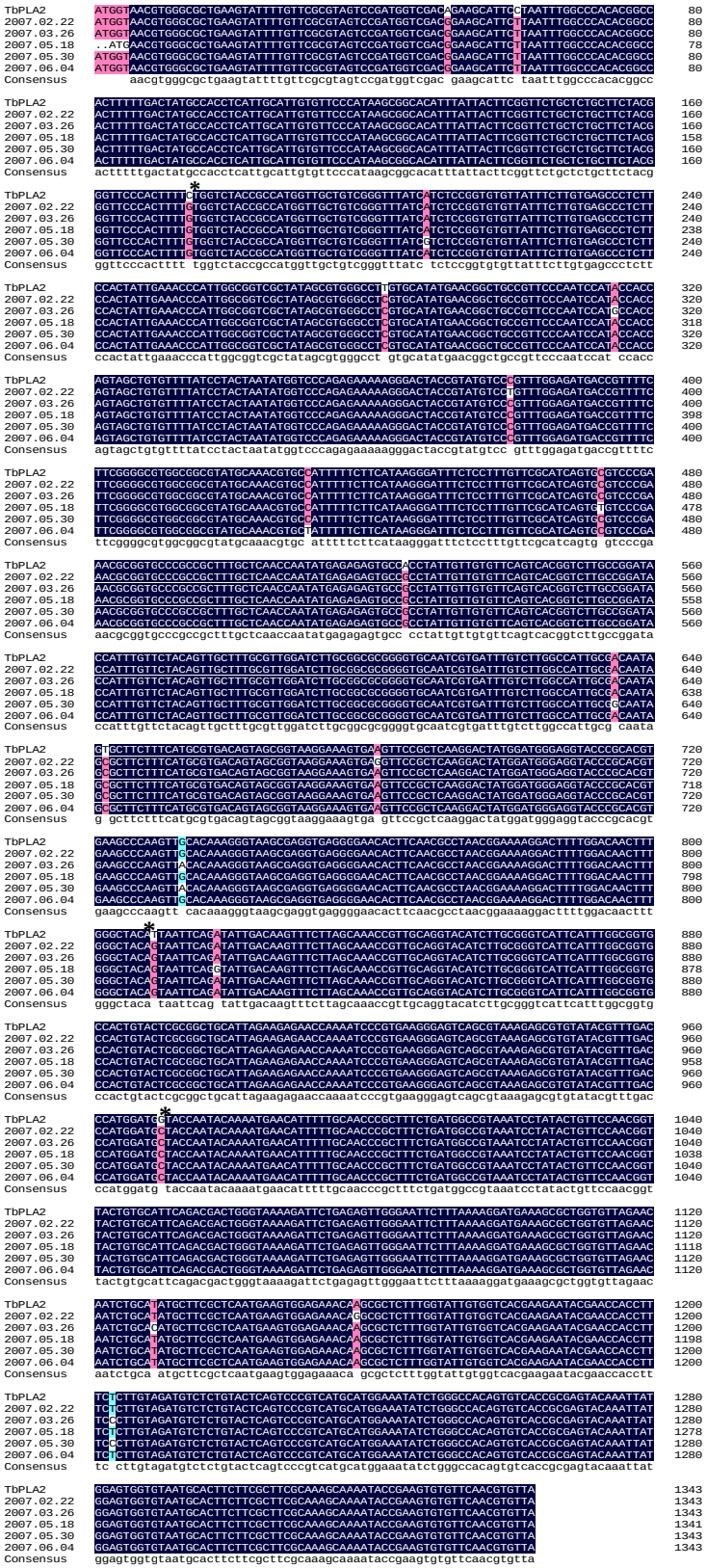


Figure 3.10 Gene sequence alignment of different clones in comparison to $TbPLA_2$ database sequence and constant alterations are marked with asterisks in sequence

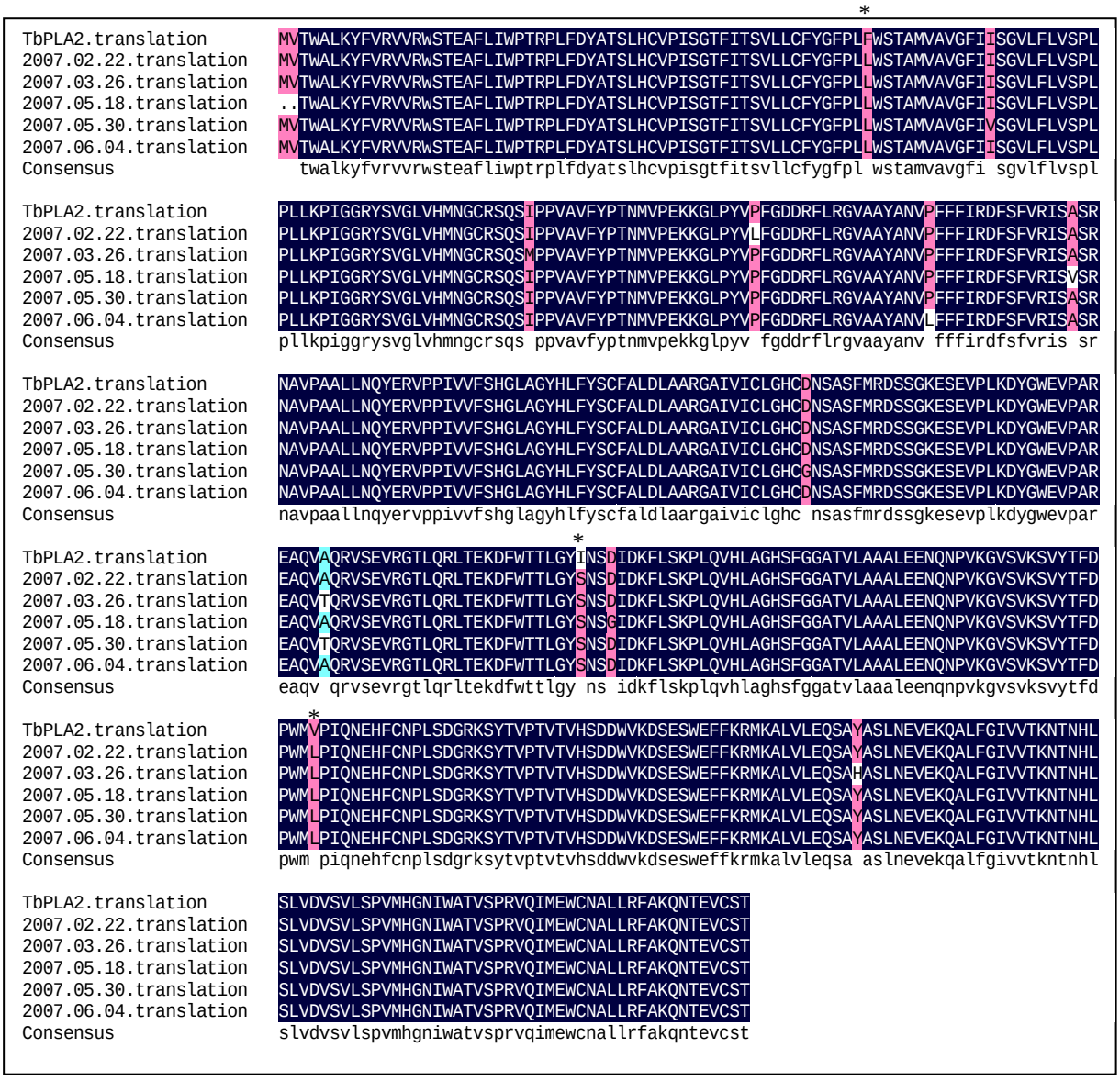


Figure 3.11 Protein sequence alignment from several sequenced clones in comparison with TbPLA$_2$ known database sequence. The constant alterations at three positions instead of F, I and V to L, S and L respectively are marked with asterisks

4. SDS-PAGE analysis of heterologous bacterial expression

Heterologous expression of $TbPLA_2$ in pMal-c2E plasmid within the correct clone was attempted in BL-21 (DE3) and Rosetta 2 expression competent cells at different conditions by induction of IPTG at final concentrations of 0.1 mM, 0.3 mM, 0.5 mM and 1mM as well as varying temperatures of 16°C, 27°C and 37°C for time period of 9 h and 12 h. The induced bacterial cells were harvested by centrifugation, lysed in buffer (see Material and method) and fractionated into soluble and membrane fractions. The fusion protein MBP (Maltose binding protein) with $TbPLA_2$ expression was expected to be at the molecular size of 100 kDa in the fractions of the induced cells as compared to the control cells. The corresponding fractions obtained from different parameters were resolved on SDS-PAGE as shown in Figures 3.12, 3.13, and 3.14. The SDS-PAGE gels did not showed the expected band at a molecular size 100 kDa in induced cells. Although at low temperature condition, the bacterial growth is not optimum as compared to 37°C but there was no expression of target fusion protein. On the whole, the expression of $TbPLA_2$ in BL-21 (DE3) competent cells was not successful as seen in SDS-PAGE gels.

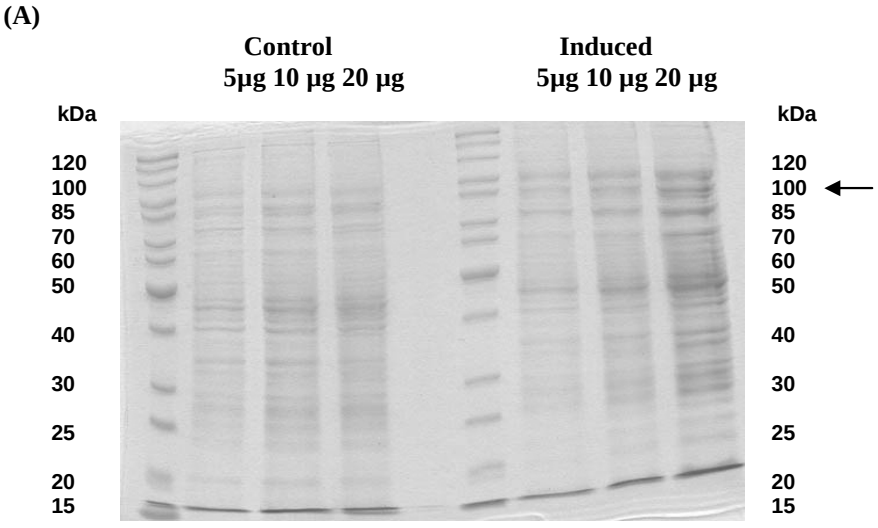


Figure 3.12 Coomassie stained 10 % SDS-PAGE to analyze heterologous expression of $TbPLA_2$ in BL-21 (DE3) competent cells induced with 0.3 mM IPTG at room temperature for 12 h. (A) control and induced cells lysate soluble fraction in different protein concentrations.

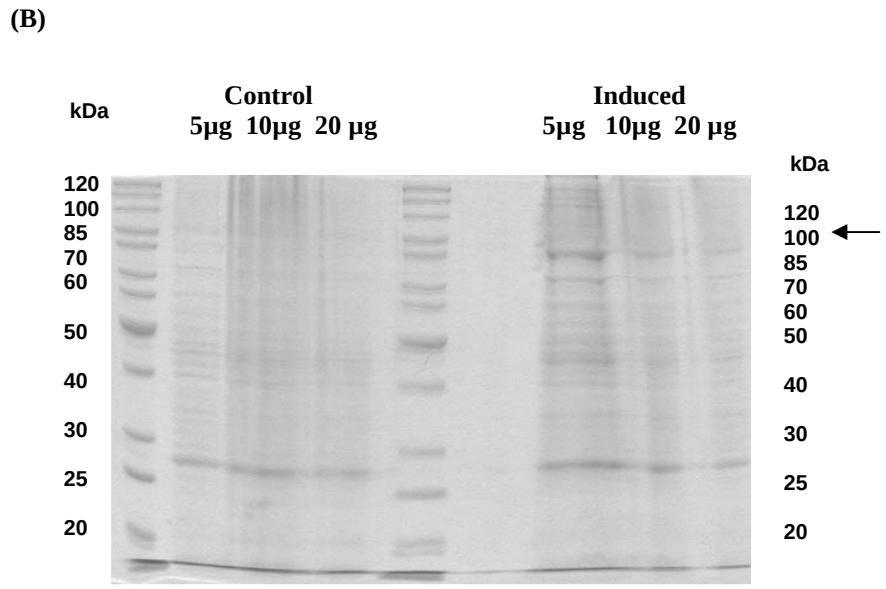


Figure 3.12 (B) Control and induced cell lysates membrane fraction in different protein concentrations.

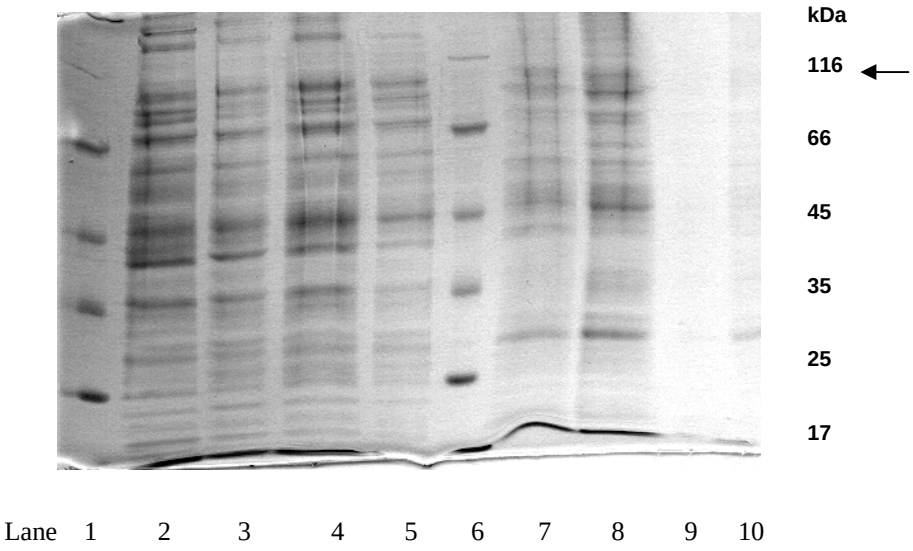


Figure 3.13 SDS-PAGE 10 % gel coomassie stained of heterologous expression in BL-21 (DE3) competent cells. Cells were induced with IPTG and incubated at room temperature for 12 h. Lanes (1) protein molecular marker (2) control cell lysates soluble fraction, (3) induced cell lysates soluble fraction with 0.1 mM, (4) induced cell lysates soluble fraction with 0.2 mM, (5) induced cell lysates soluble fraction with 0.5 mM, (6) protein molecular marker, (7) control cell lysates membrane fraction, (8) induced cell lysates soluble fraction with 0.1 mM, (9) empty, (10) induced cell lysates soluble fraction with 0.5 mM

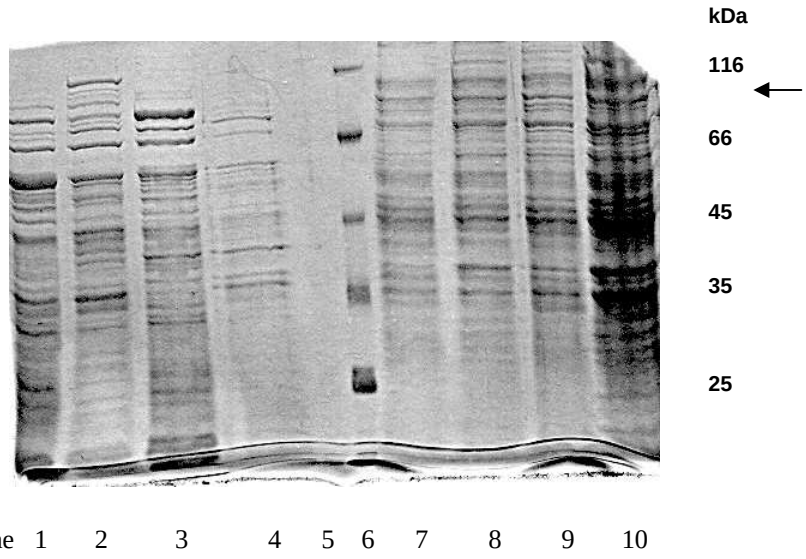


Figure 3.14 Heterologous over-expression in BL-21 (DE3) competent cells and resolved on 10 % SDS-PAGE Coomassie stained. Cells were induced with 1 mM IPTG for 12 h. Where lanes (1 and 2) were control and induced cell lysates soluble fraction at 37°C, lanes (3 and 4) control and induced cell lysates soluble fraction at 25°C, lane (5) empty, lane (6) protein molecular marker, lanes (7 and 8) control and induced cell lysates membrane fraction at 37°C and lanes (9 and 10) control and induced cell lysates membrane fraction at 25°C.

Due to toxic nature of the protein and possibly membrane lytic activity of the $TbPLA_2$, the BL-21 (DE3) competent cells were not able to over-express this protein. This led to an attempt to express the protein in Rosetta 2 expression competent cells. As Rosetta 2 cells contained specific tRNAs for the codons rarely found in *E. coli* for enhanced eukaryotic protein expression. Therefore, the construct of $TbPLA_2$ in pMal-c2E was transformed into Rosetta 2 competent cells. The cells were induced at varying IPTG concentrations, temperatures and for the time period of 9 h. Afterwards, both the control and induced cells were harvested by centrifugation and lysed with hypotonic buffer (see Material and method) .The cell lysates were split into soluble and membrane fractions. These fractions each from control and induced cells were resolved on 12 % SDS-PAGE under reducing conditions as shown in Figure 3.15, 3.16 and 3.17.The fusion protein band of MBP and $TbPLA_2$ in all gels was expected at a molecular size of 100 kDa. The gels did not show this band of 100 kDa in induced cell lysates as compared to control cell lysates. These competent cells were also unable to over-express the $TbPLA_2$ protein due to toxic nature of this protein for the bacteria.
On the whole, the heterologous expression of $TbPLA_2$ in bacterial cells was not successful although the cloning of the gene was confirmed. So, the problem remained to express the protein in a suitable host or use another expression system for this eukaryotic membrane protein.

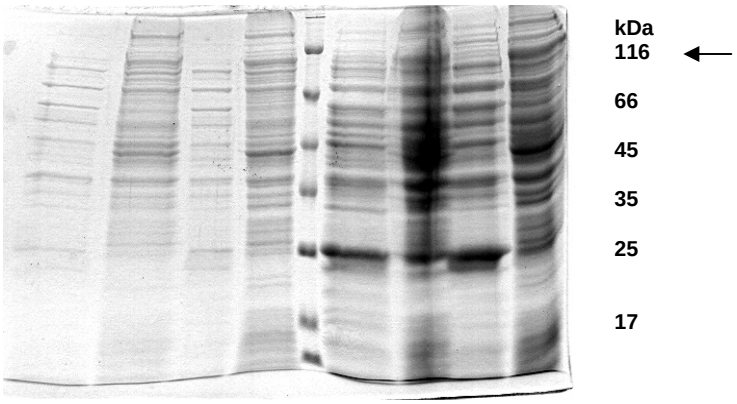


Figure 3.15 Analysis of heterologous expression in Rosetta 2 competent cells on coomassie stained 12 % SDS-PAGE. The cells were induced with IPTG in the concentration of 1 mM for 9 h. where lanes (1 and 2) were control cell lysates soluble and membrane fractions at 16°C, lanes (3 and 4) induced cell lysates soluble and membrane fractions at 16°C, lane (5) empty, lanes (6 and 7) control cell lysates soluble and membrane fractions at 37°C and lanes (8 and 9) induced cell lysates soluble and membrane fractions at 37°C.

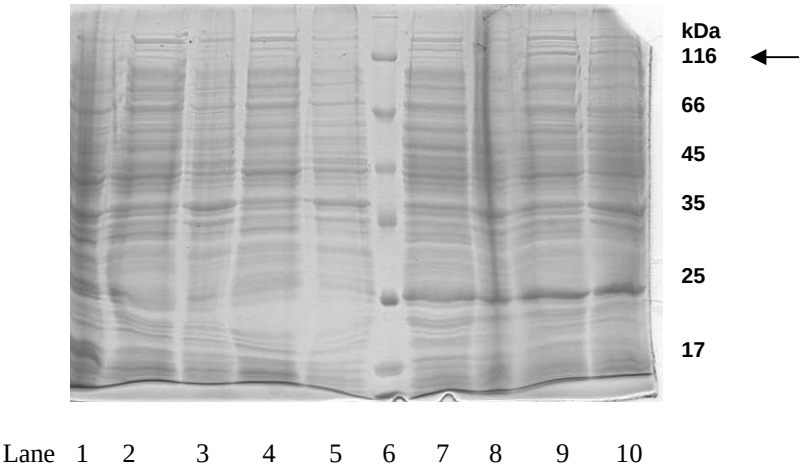


Figure 3.16 Heterologous expression in Rosetta 2 competent cells and fractions resolved on coomassie stained 12 % SDS-PAGE. The cells were induced with 0.1 mM IPTG for 9 h. Lane (1) control cell lysate after 3 h, lanes (2-3) control cell lysates soluble and membrane fraction at 16°C, lanes (4-5) induced cell lysates soluble and membrane fraction at 16°C, lane (6) protein molecular marker, lanes (7-8) control cell lysates soluble and membrane fraction at 37°C and lanes (9-10) induced cell lysates soluble and membrane fraction at 37°C.

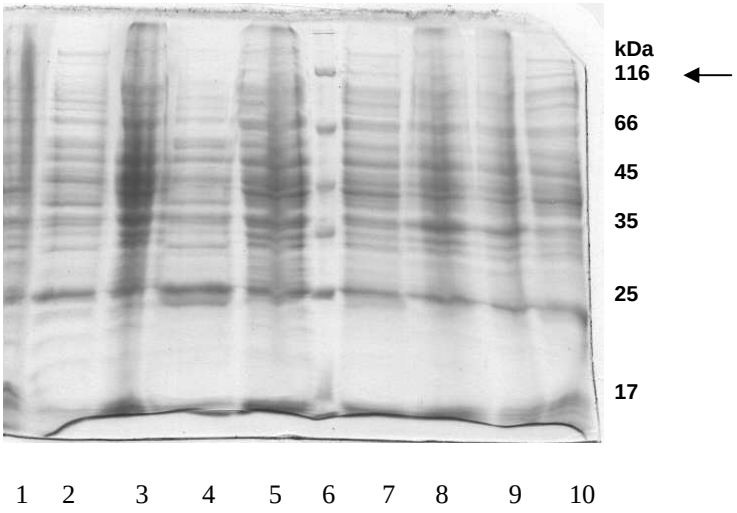


Figure 3.17 Analysis of heterologous expression in Rosetta 2 competent cells on 12 % SDS-PAGE stained with coomassie brilliant blue. The cells were induced at 27°C for 9 h. Lane (1) control cell lysate after 3 h, lanes (2 and 3) control cell lysates soluble and membrane fractions, lanes (4 and 5) 0.1 mM IPTG induced cell lysates soluble and membrane fractions, lanes (7 and 8) 0.5 mM IPTG induced cell lysates soluble and membrane fractions and lanes (9 and 10) 1 mM IPTG induced cell lysates soluble and membrane fractions.

5. Expression of $TbPLA_2$ in Sf9 insect cells

The Bac-to-Bac Baculovirus expression system provides the efficient method to generate recombinant baculoviruses and permits the high-level expression of protein in insect cells. This method utilizes the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of recombinant bacmid DNA that can be used to infect insect cells for large-scale expression of the protein. For this purpose, the sequenced construct of $TbPLA_2$ in pMal-c2E and the pFastBac-1 plasmid for Baculovirus expression were simultaneously restriction digested with *BamH1* and *Pst1* as shown in Figure 3.18 (A) and (B) respectively. The pFastBac-1 after digestion revealed a band of 4692 bp and 83 bp as expected. This $TbPLA_2$ gene after digestion from construct revealed a size of 1344 bp and then utilized for gel extraction. The purified DNA of pFastBac-1 and $TbPLA_2$ were spectrophotometrically estimated to determine the concentration. This DNA of $TbPLA_2$ was used for cloning in the Baculovirus expression system. Afterwards, this $TbPLA_2$ gene was ligated into pFastBac-1 plasmid. The ligated construct was verified by restriction digestion with *Nde1* with expected bands at 5214 bp and 841 bp. The expected bands were confirmed on agarose gel as seen in Figure 3.19. The ligation construct from lane 5 in Figure 3.19 was used for transformation into XL-1 competent cells. The colonies obtained were selected for confirmation of correct construct and orientation of gene.

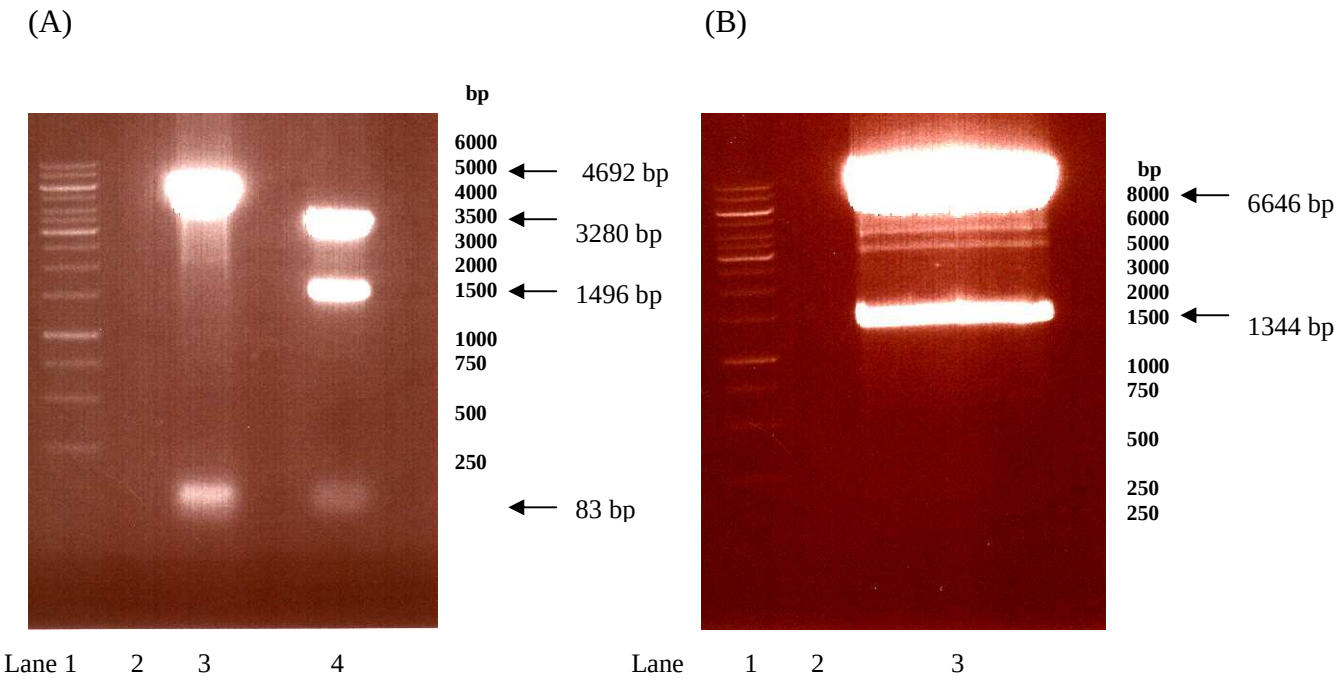


Figure 3.18 Analysis of restriction digestion on 1% agarose gel (A) lane (1) DNA ladder, lane (2) empty, lane (3) pFastBac-1 double digested with *BamH1* and *Pst1*, lane (4) pFastBac-1 digested with *Eam11051*. (B) lane (1) DNA ladder, lane (2) empty, lane (3) construct of $TbPLA_2$ in pMalC2E double digested with *BamH1* and *Pst1*. Note the band at 1344 bp.

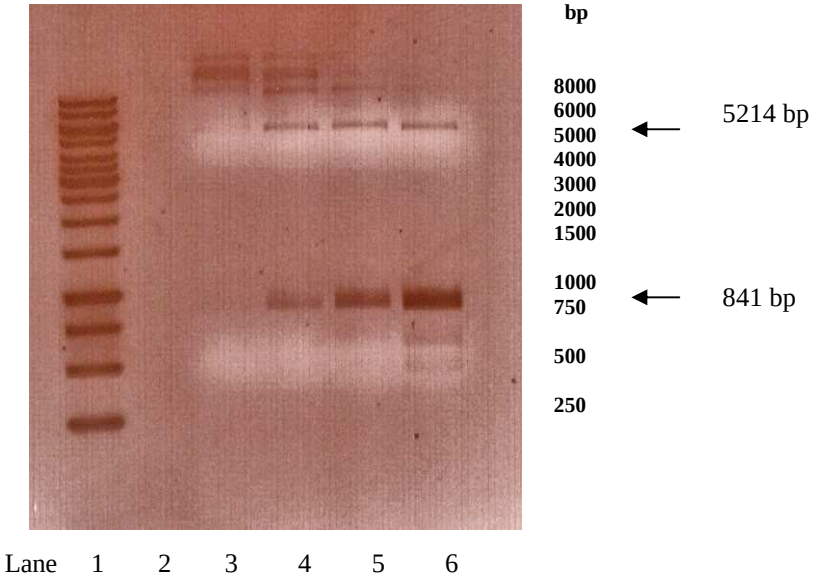


Figure 3.19 Ligation reaction of $TbPLA_2$ with pFastBac-1 analyzed l and digested with *Nde1* on 1 % agarose gel. Lane (1) DNA ladder, lane (3 and 4) contains ligation reactions which were performed at 16°C with ratio of insert to plasmid in 1:3 and 1:6 respectively, lane (5 and 6) contains ligation reactions which were performed at 22°C with ratio in 1:3 and 1:6 respectively.

The colonies were verified for correct constructs with the method of bacterial colony PCR. This reaction included negative control (without any template DNA) and positive control

(pMal-c2E-TbPLA$_2$). The correct clones were verified on agarose gel as shown in Figure 3.20.

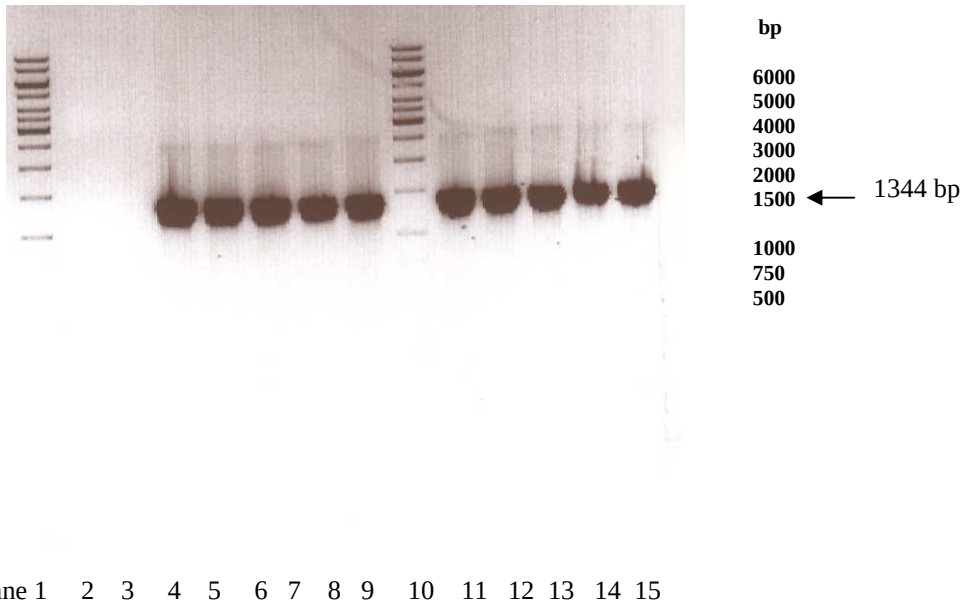


Figure 3.20 Bacterial colony PCR of construct of TbPLA$_2$ in pFastBac-1transformed into Xl-1 competent cells on 1 % agarose gel. Lanes: (1) DNA ladder, (2) negative control, (3) positive control, (4-9) clones, (10) DNA ladder, (11-15) clones.

The clone containing the correct construct from lane 5 was selected. This clone was propagated in LB-medium with ampicillin and the method of MiniPrep was performed to obtain DNA construct. The DNA orientation was verified using restriction enzymes like *Nde1* and *Eam11051*. After the digestion with *Nde1*, the bands were expected at 5214 bp, 841bp and for *Eam11051* at 4559 bp, 1496 bp (Figure 3.21). This implicated that the gene was ligated in correct orientation in the plasmid which can be further utilized.

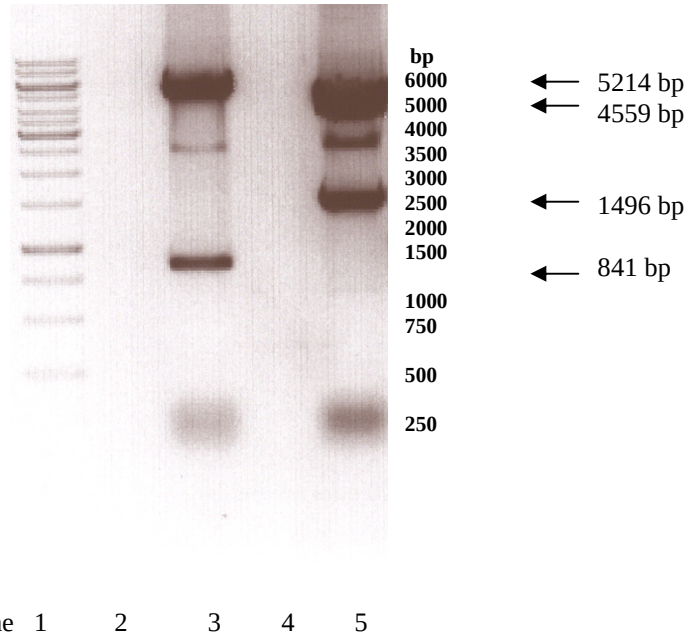


Figure 3.21 Analysis on 1 % agarose gel verification of construct of $TbPLA_2$ in pFastBac-1. The construct was digested with *Nde1* (lane 3) and *Eam11051* (lane 5).

After confirmation of the correct construct, this recombinant plasmid of $TbPLA_2$ in pFastBac-1 was transformed into DH10Bac competent cells. The clones containing recombinant bacmid transposed with $TbPLA_2$ were verified with the help of PCR method using M13 sense and anti-sense primers with HotStar *Taq* polymerase. This PCR reaction yielded a product with the size of 3644 bp. Furthermore, these recombinant bacmids were purified with Qiagen PCR purification kit and verified with PCR using M13 sense and anti-sense primers as depicted in Figure 3.22 (A). The recombinant bacmid was further verified using restriction enzyme *EcoR1* which depicted a band at 3644 bp as shown in Figure 3.22 (B). Certainly, this confirms the correct orientation of the gene of $TbPLA_2$ in the plasmid which was utilized for infection in insect cells.

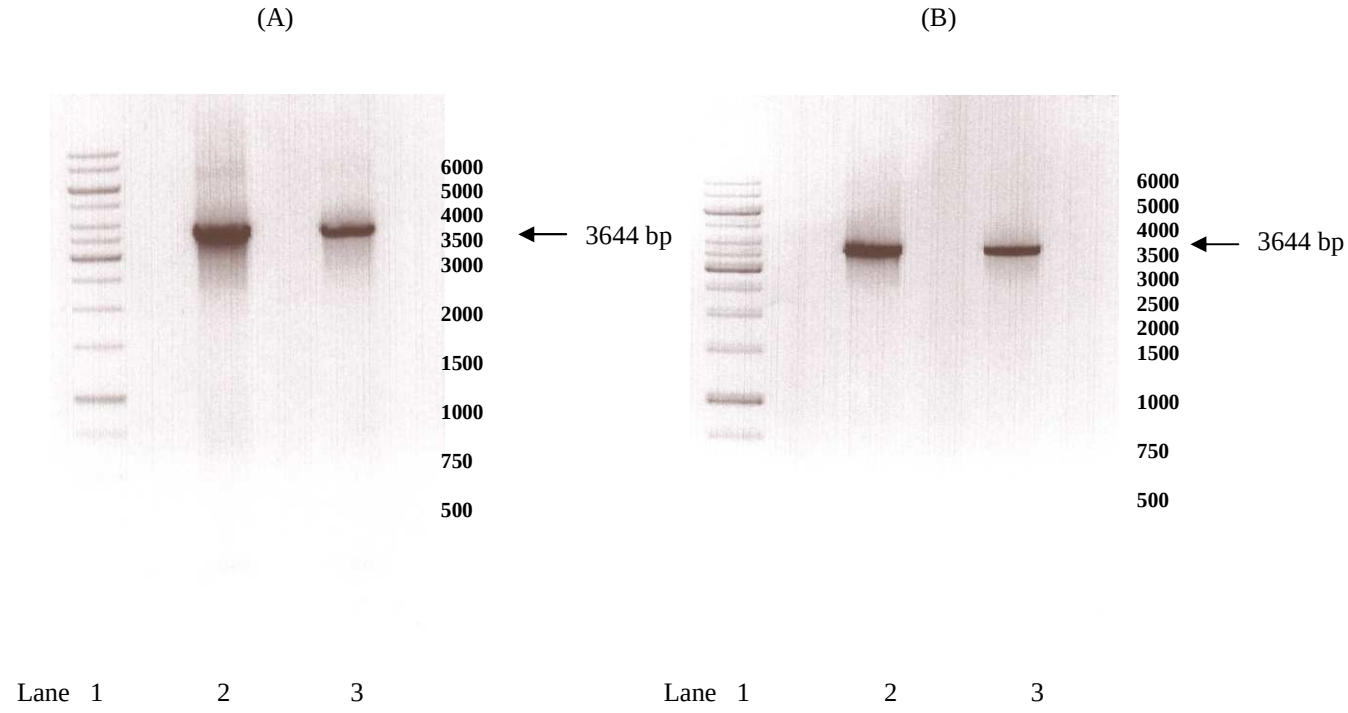


Figure 3.22 Analysis of transposed recombinant bacmid in DH10Bac competent cells on 1 % agarose gel. (A) PCR using M13 sense and anti-sense primers for recombinant bacmid in clones. Lane (1) DNA ladder, lanes (2 and 3) contains purified recombinant bacmids with Qiagen gel extraction kit. (B) lane (1) DNA ladder, lanes (2 and 3) contains recombinant bacmid digested, analyzed with *EcoR1* and purified with Qiagen gel extraction kit.

6. Insect cell expression analysis with SDS-PAGE

The purified recombinant bacmid transposed with plasmid containing $TbPLA_2$ was used for infection in *Sf9* insect cells. The insect cells were grown at a cell density of 2.8 X 10^6/ ml and infected with the recombinant bacmid. After 72 h of infection, the cells were centrifuged and the P1 (low viral titer) supernatant was used for further infections. Exponentially growing *Sf9* insect cells were infected with the P1 viral stock. Following an established infection, the suspension was centrifuged and the P2 (high viral titer) supernatant was separated and pellet was stored at 4°C. Furthermore, this P2 viral stock supernatant was utilized in infection in growing cultured *Sf9* insect cells for P3 (higher viral titer). After this, the culture was centrifuged to separate supernatant and cell pellet. The P3 viral stock supernatant and pellet were preserved at 4°C for further analysis. The infected cells from all infections were lysed with RIPA buffer (see Material and method). After the lysis, cell lysates were centrifuged at

13.000 rpm for 15 min. at 4ºC to separate the soluble and membrane fractions which were analyzed including the supernatant of infected cells by SDS-PAGE using a 12 % gel (Figure 3.23). The gel depicted a prominent band at molecular size of 58 kDa in only the membrane fraction of P2 viral stock cell lysates (lane 7 in Figure 3.23).

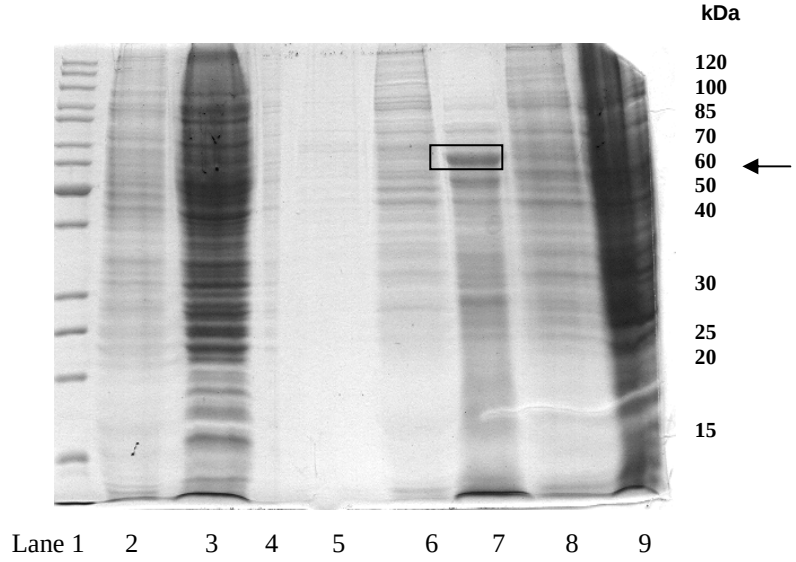


Figure 3.23 Heterologous expression of $TbPLA_2$ in insect cells after 72 h infection and resolved on 12 % SDS-PAGE gel stained with coomassie brilliant blue. Lane (1) protein molecular marker, lane (2) *Sf9* cells supernatant, lane (3) *Sf9* cell lysates soluble fraction, lane (5) P1 viral stock supernatant, lane (6) P2 viral stock supernatant, lane (7) P2 cell lysates membrane fraction, lane (8) P3 viral stock supernatant and lane (9) P3 viral stock cell lysates membrane fraction.

For the optimization, the exponentially growing insect cells were infected with the recombinant bacmid transposed with $TbPLA_2$ for the time period of 120 h. After 120 h of infection, the culture was centrifuged and supernatant and cell pellet were separated. Similar procedure was followed for 72 h infected insect cells. The P2 and P3 viral stocks supernatant and cell pellet were preserved at 4°C.On the whole, the cells were lysed with RIPA buffer as described in Material and method. The cell pellets from each infection were fractionated into soluble and membrane fractions. These fractions including the supernatant of infected cells were analyzed on 12% SDS-PAGE as stated in Figure 3.24. Additionally, this gel suggested a band of 58 kDa in P3 viral stocks cell lysates membrane fraction (lane 7, Figure 3.24). This certainly meant that the infection period may play a role in the expression of protein in insect

cells. This comparative infection period was intended to evaluate the expression level of this protein and to determine the optimum time for infection to yield the maximum expression of $TbPLA_2$ in insect cells.

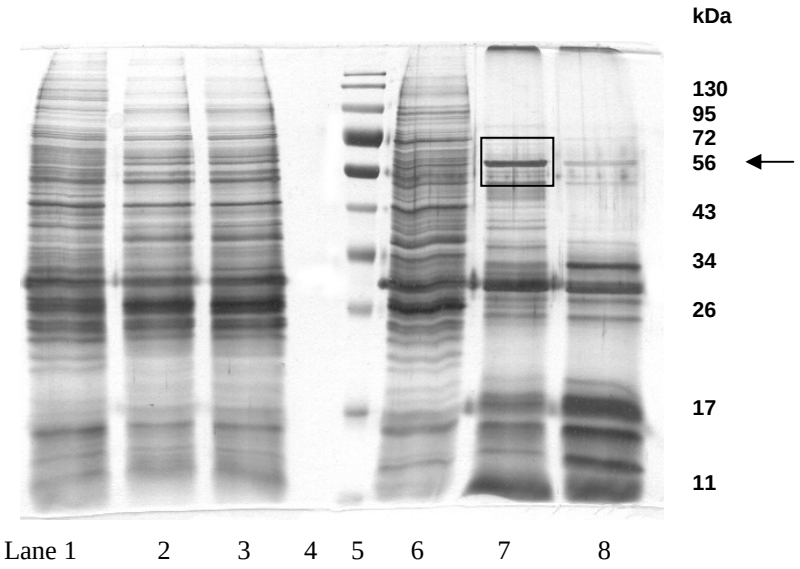


Figure 3.24 Analysis of heterologous expression in Sf9 insect cells after 120 h on silver stained 12 % SDS-PAGE. Lane (1) Sf9 control cells supernatant, lane (2) P2 viral stock supernatant, lane (3) P2 cell lysates membrane fraction, lane (4) empty, lane (5) protein molecular marker, lane (6) P3 viral stock supernatant, lane (7) P3 cell lysates membrane fraction, lane (8) P3 cell lysates pellet.

7. Western blot analysis of heterologous expression

In order to ascertain that this protein band in the gel (Figure 3.23 and 3.24) was definitely $TbPLA_2$, the Western blot analysis was performed. The soluble and membrane fractions from P1, P2 and P3 viral stocks after 72 h of infection in insect cells were analyzed by Western blotting using an anti-$TbPLA_2$ in a working dilution of 1: 2000. The SDS-PAGE Coomassie stained gel and blot of the gel certainly defines that the band observed at molecular size of 58 kDa (denoted with an arrow, Figure 3.25) was the band of $TbPLA_2$. The band was observed only in the membrane fraction of cell lysate from P2 viral stock.

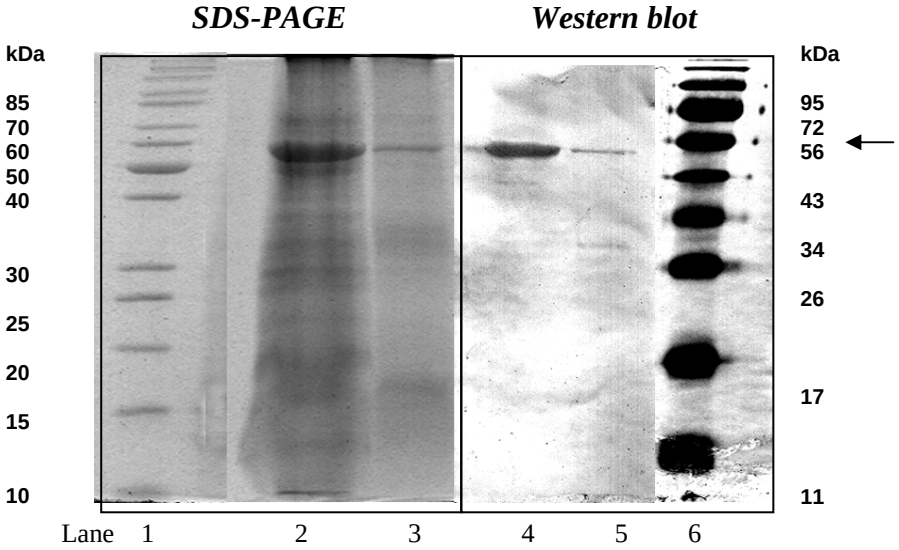


Figure 3.25 Heterologous expression of $TbPLA_2$ in insect cells after 72 h and analysis with 12 % SDS-PAGE coomassie stained and Western blot; lane (1) protein molecular marker, lane (2) P2 viral stock membrane fraction, lane (3) P2 viral stock cell supernatant, (4) P2 viral stock membrane fraction, lane (5) P2 viral stock cell supernatant, (6) protein molecular marker.

In comparison, insect cells after 120 h of infection were analyzed with Western blot. The cell pellets from P2 and P3 viral stocks were lysed and fractionated into soluble and membrane fractions. These fractions and the cell supernatant from each infection were resolved on 12 % SDS-PAGE. Then the gel was blotted and analyzed using anti-$TbPLA_2$. The soluble and membrane fractions from P2 and P3 viral stocks after 120 h from two independent infections were verified to determine the intensity of expression of this protein as shown in Figure 3.26.

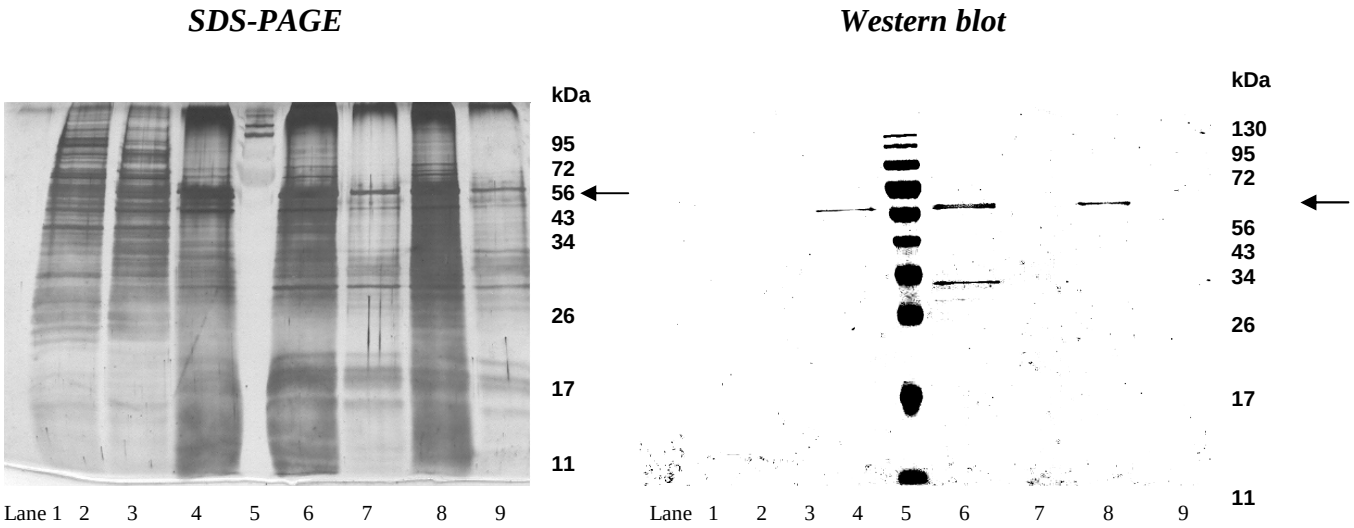


Figure 3.26 Detection of $TbPLA_2$ with 12 % SDS-PAGE silver stained and Western blot analysis of heterologous expression in insect cells after 120 h. lanes: (1) empty lane, (2) cell supernatant, (3) P2 infected cells supernatant, (4) P2 infected cell lysates membrane fraction, (5) protein molecular marker, (6) P3 (independent infection 1) infected cell lysates membrane fraction, (7) P3 (independent infection 1) infected cell lysates soluble fraction, (8) P3 (independent infection 2) infected cell lysates membrane fraction, (9) P3 (independent infection 2) infected cell soluble fraction.

8. Immunoprecipitation

The insect cell lysates membrane fraction was solublized in RIPA lysis buffer and subjected to immunoprecipitation. The anti-$TbPLA_2$ antibody was used to capture the expressed protein from insect cell lysate. For this purpose, Protein G coupled agarose bead was utilized. After immunoprecipitation of the lysate with antibody, the immunocomplex was obtained. The fractions obtained at every step of this method were resolved on 10 % SDS-PAGE under reducing conditions. Two different bands were observed in lane 6 which contained immunocomplex at the molecular size of 50 kDa and 25 kDa respectively (Figure 3.27).

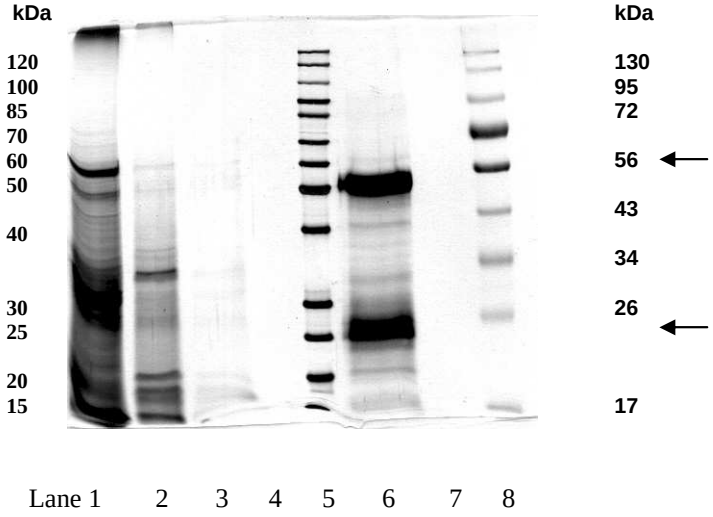


Figure 3.27 Immunoprecipitation of $TbPLA_2$ from infected insect cells. Resolved on 10 % SDS-PAGE and silver stained. Lanes (1) P3 supernatant, (2) P3 soluble membrane fraction, (3) P3 supernatant of immunocomplex, (4) P3 insoluble pellet, (6) immunocomplex of protein with anti- $TbPLA_2$ antibody.

9. Western blot analysis of bloodstream and procyclic form cells for $TbPLA_2$

The bloodstream and procyclic form cells were cultivated at a cell density of 1 X 10^7 cells/ ml. The cells were centrifuged and afterwards lysed with hypotonic buffer. The lysates obtained from both forms of cells were used for further analysis. The cell lysates were fractionated into soluble, membrane and VSG fractions for bloodstream form using the protocol described (Material and method). The procyclic form cell lysates were fractionated into soluble and membrane fractions. These fractions from both forms of parasites were resolved on 12 % SDS-PAGE and subsequently analyzed using Western blot with anti-$TbPLA_2$ to identify $TbPLA_2$ in both forms of the parasites. Both forms of parasite cell lysates were analyzed separately onto two blots. The blots revealed a single band at a molecular size of 50 kDa and visible only in membrane fraction of both parasite forms, indicated in Figure 3.28 (A) and (B). Bloodstream form membrane fraction from rat blood in the cell density at 3 X 10^9 was analyzed on the blot as shown in Figure 3.29 lane 2 depicting a band at 50 kDa.

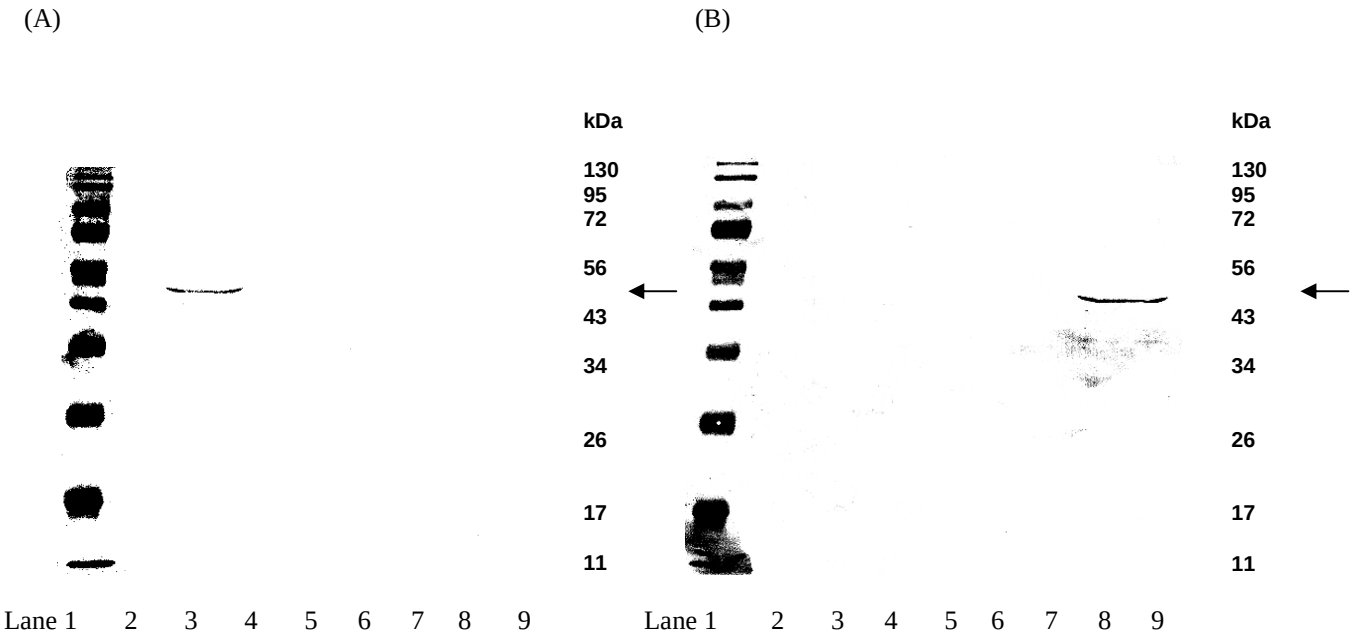


Figure 3.28 Western blot analysis of TbPLA$_2$ in *T.brucei* bloodstream and procyclic form. (A) blot showing bloodstream form cells lysate fractions; lanes: (1) protein molecular marker, (2) empty lane, (3) cell lysates membrane fraction, (4) empty, (5) VSG fraction, (7) cell lysates soluble fraction, (8) empty lane, (9) PLA$_2$ from porcine pancreas. (B) blot with procyclic form cell lysates fractions; lanes (3) cell lysates soluble fraction, (9) cell lysates membrane fraction.

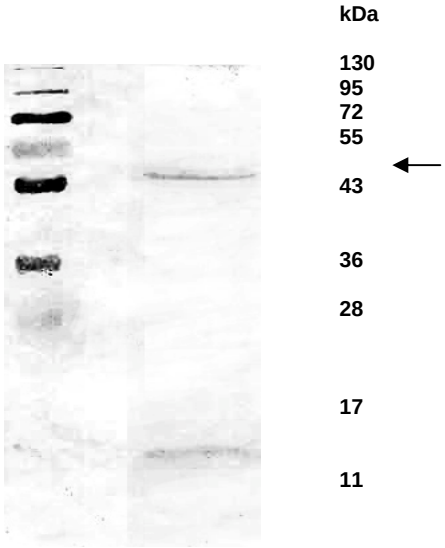


Figure 3.29 Western blot analysis of TbPLA$_2$ in *T.brucei* bloodstream form. Blot showing (1) protein molecular marker, (2) membrane fraction.

For a comparative study, logarithmic and stationary phases bloodstream as well as procyclic form cell lysates were analyzed. The lysates were fractionated into cytosolic and membrane

fractions simultaneously analyzed on the same blot. The blot revealed a prominent band at a molecular size of 50 kDa in only membrane fractions in all analyzed forms (Figure 3.30). A single band at the molecular size of 50 kDa was observed on the blot in the cell lysates membrane fraction (lane 3, Figure 3.31). This experiment was performed several times (results not shown) till the same reactivity at a molecular size of 50 kDa was observed. This revealed that this protein is expressed in every life stage of the parasite. In spite of antibody recognition of the protein in cell lysates, it was imperative to investigate the preimmune serum from rabbit and its reactivity with the parasite cell lysates. The preimmune serum was analyzed on blot with the cell lysates from bloodstream form. The bloodstream form cell lysates were fractionated as described previously. Figure 3.32 depicts the analysis of preimmune serum against the membrane fraction from bloodstream form from rat blood in the cell density at 3 X 10^9 showing a band at 36 kDa only in the cytosolic fraction.

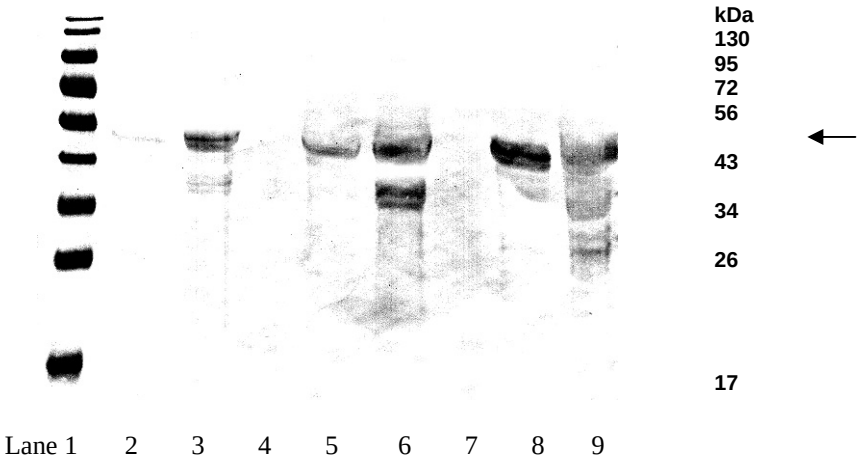


Figure 3.30 Comparative Western blot of bloodstream and procyclic form. 12 % SDS-PAGE gel ; lanes: (1) protein molecular marker, (2) cell lysates soluble fraction from logarithmic phase of bloodstream form, (3) cell lysates membrane fraction from logarithmic phase of bloodstream form, (4) empty lane (5) cell lysates soluble fraction from stationary phase bloodstream form, (6) cell lysates membrane fraction from stationary phase bloodstream form, (7) empty lane, (8) cell lysates soluble fraction from procyclic form, (9) cell lysates membrane fraction from procyclic form.

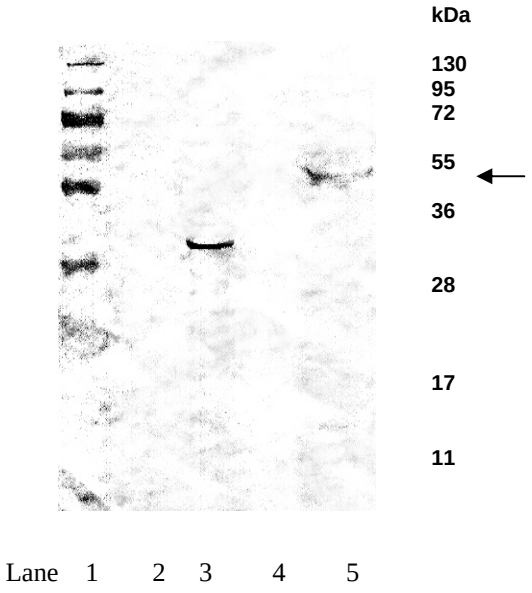


Figure 3.31 Analysis of bloodstream form cell lysates against anti-TbPLA$_2$. Lanes: (1) protein molecular marker, (2) empty lane, (3) cell lysates soluble fraction from bloodstream form, (4) empty lane, (5) membrane fraction from bloodstream form.

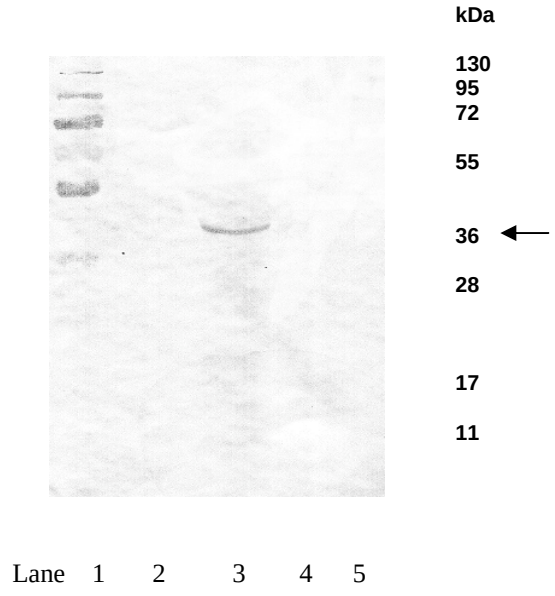


Figure 3.32 Analysis of bloodstream form cell lysates against preimmune serum. Lanes: (1) protein molecular marker, (2) empty lane, (3) cell lysates soluble fraction from bloodstream form, (4) empty lane, (5) membrane fraction from bloodstream form.

10. Transcription of $TbPLA_2$ gene from T. brucei

The expression level of $TbPLA_2$ gene in different life cycle stages of the parasite was also determined using Northern blot analysis. The monomorphic strains of *T. brucei* 221 cells were obtained from rat blood as well as from *in vitro* grown cultures in logarithmic and stationary phase were recovered and their total RNA was obtained (see Material and Methods). The total RNA from these stages was used for Northern blot analysis. The sequence specific-primers for the gene of $TbPLA_2$ were used for the probe as stated in Material and method. This definitely indicated the transcriptional expression and level of this protein throughout all growth phases of the parasites as depicted in Figure 3.33 (A). The comparison of band intensities in case of $TbPLA_2$ in three stages (Figure 3.33 B) showed that the expression of $TbPLA_2$ was significantly regulated in *in vitro* cultured logarithmic phase of the parasites as compared to other growth stages. Therefore, it can be proposed that $TbPLA_2$ gene is expressed throughout the life stages of parasite and specifically prominent in logarithmic phase. The Northern blot complemented the data of Western blot with *T.brucei* cell lysates indicating the transcriptional and translational expression of this protein throughout the life cycle of this parasite. On the whole, this speculates the significance of this protein in the parasitic life cycle.

A

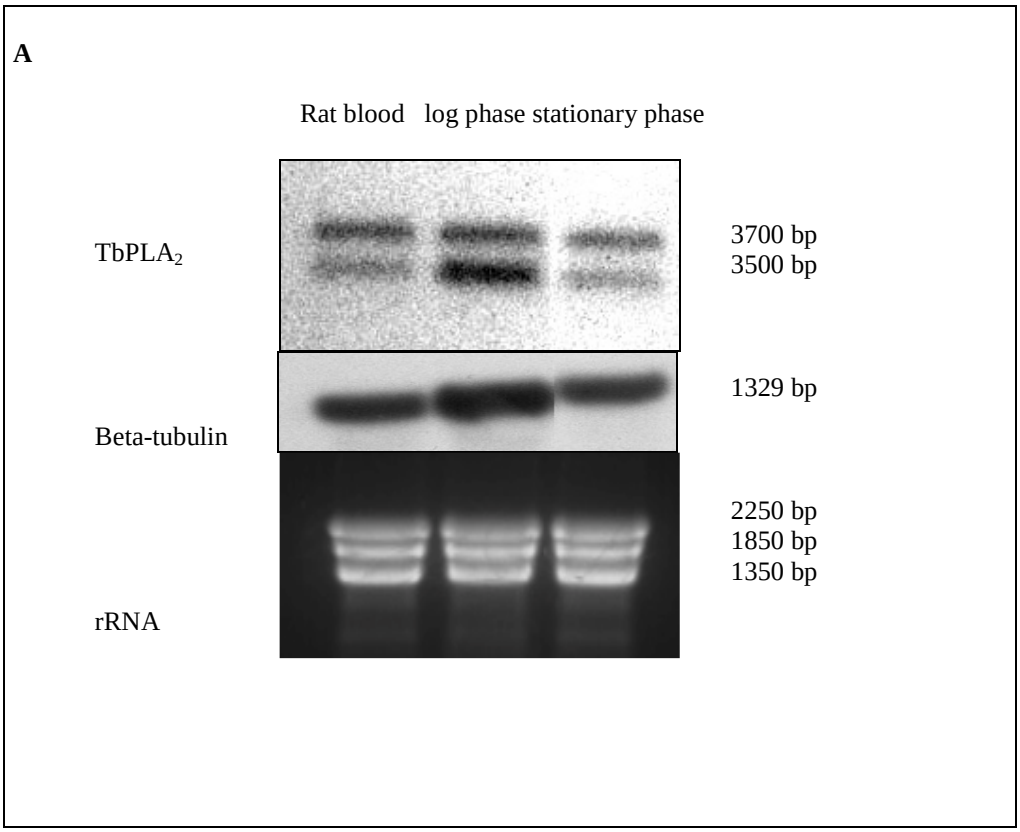


Figure 3.33 (A) Comparison of transcriptional expression of $TbPLA_2$ in rat blood, logarithmic phase and stationary phase of *T.brucei*. (A) Blot with $TbPLA_2$, loading control as beta tubulin and rRNA of *T.brucei* from rat blood, bloodstream log phase (16 h) and stationary phase (40 h) respectively.

B

	Rat blood logarithmic phase	Logarithmic phase	Stationary phase
$TbPLA_2$	0.5	0.7	0.5
	0.3	1.0	0.3
Beta tubulin	0.6	1.0	0.6
rRNA	1.0	0.81	0.97

Figure 3.33 (B) Semi-quantitative analysis of blots with GelScan V5.1 and value of the most intense band as standard was taken as 1.

11. PLA_2 enzyme activity assay with cell lysates of bloodstream and procyclic forms of T. brucei

The phospholipase A_2 activity was determined using a coupled assay which utilizes dilinoleoyl phosphatidylcholine (DL-PC) as a substrate and lipoxygenase as a coupling enzyme. The PLA_2 activity was constantly monitored spectrophotometrically at λ = 234 nm. The phospholipase A_2 activity released the product linoleic acid which was further oxidized by lipoxygenase giving rise to the corresponding hydroperoxide derivative. The standard reaction mixture contained 25 µM DL-PC, 0.23 mg/ ml and lipoxygenase in 50 mM Tris-HCl pH 8.5 containing 3 mM deoxycholate. The reaction was started by adding either known phospholipase A_2 from porcine pancreas or bloodstream as well as procyclic form cell lysates soluble and membrane fractions. The control was taken as the buffer in which the cell lysate was prepared. The reaction was proceeded for 5 min. with measurement at λ = 234 nm. Then, the value of specific activity was calculated (see Material and method) using the absorbance values. This activity assay was established with PLA_2 from porcine pancreas in increment of concentrations (Figure 3.34). This standard curve obtained was to demonstrate the optimum condition for the assay and further utilize this assay for activity estimation in parasite cell lysates.

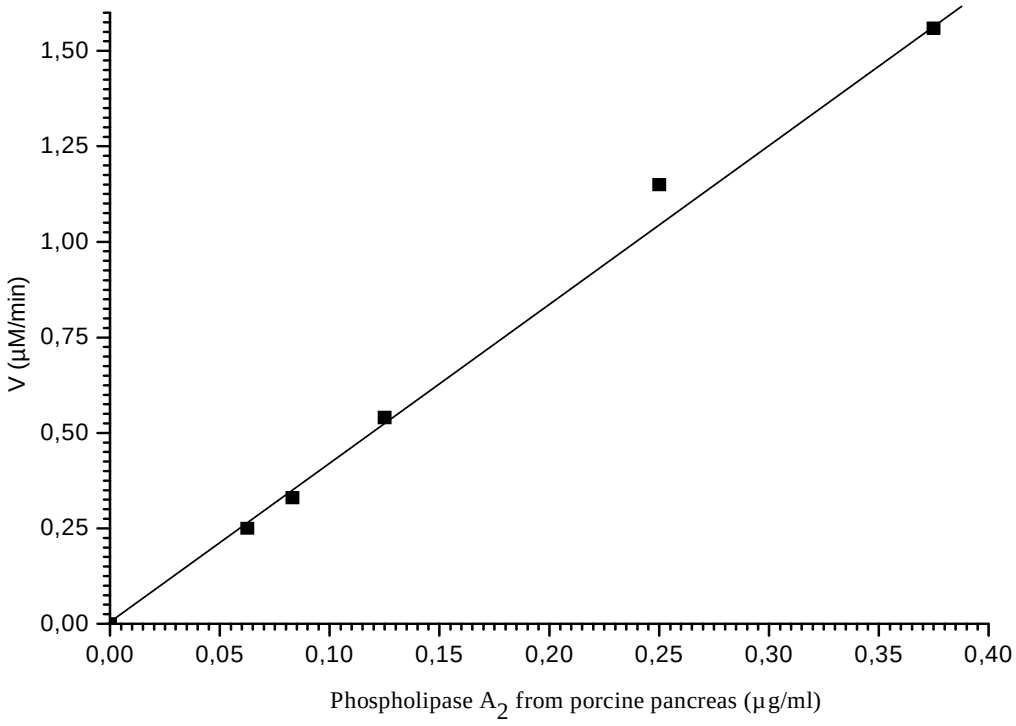


Figure 3.34 Activity assay standard curve for known and commercially available PLA_2 from porcine pancreas. Different concentrations of PLA_2 used for the activity assay. The PLA_2 from porcine pancreas in concentration of 0.0625 µg, 0.083 µg, 0.125 µg, 0.25 µg and 0.375 µg were used.

The soluble and membrane fractions from *T. brucei* bloodstream and procyclic forms were used for comparative activity assay to determine the expression and activity level of phospholipase A_2 in both forms. Both the forms of parasite were cultured at a cell density of 1 X 10^6 /ml. The bloodstream form cells from two phases namely logarithmic and stationary phase were taken and cell lysates were prepared. The cell lysates of both forms of parasites were fractionated into soluble and membrane fractions in order to evaluate the regulation of activity of this protein in different fractions of cells and throughout the life cycle of this parasite (Figure 3.35).

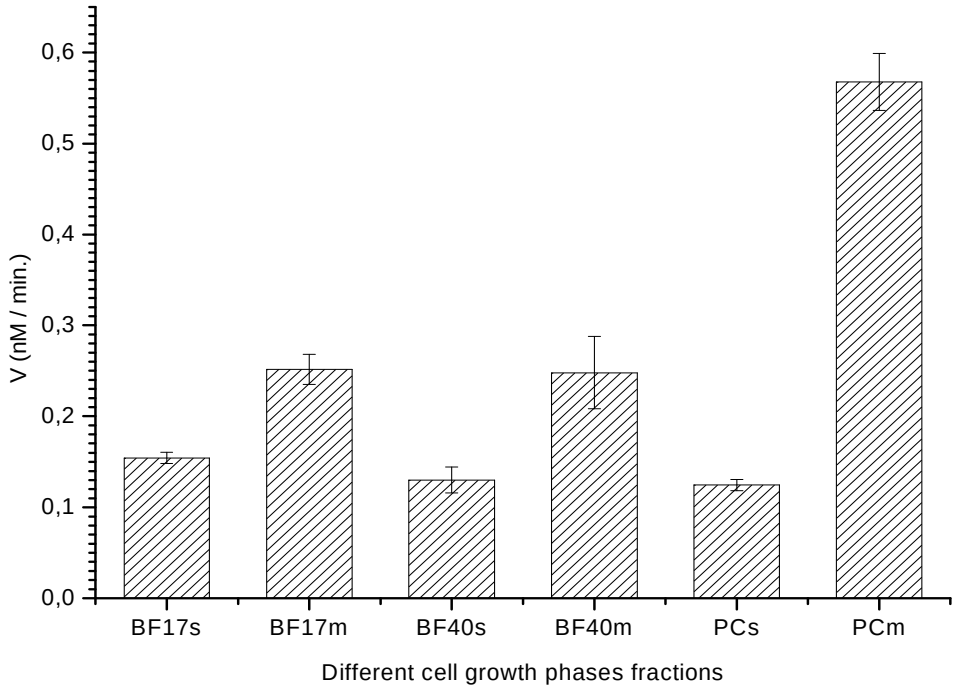


Figure 3.35 Comparative activity assay for PLA_2 with different cell phases of parasites. BF stands for bloodstream form and PC for procyclic form. Whereas BF 17s was cell lysates soluble fraction after 17 h cultivation, BF 17m was cell lysates membrane fraction after 17 h cultivation, BF 40s was cell lysates soluble fraction after 40 h cultivation, BF 40 m was cell lysates membrane fraction after 40 h cultivation, PCs and PCm were cell lysates soluble and membrane fractions respectively. Each fraction's activity was measured with 25 µg protein concentration.

4. Discussion

4.1 Characterization of TbPLA$_2$

The phospholipase A_2 superfamily constitutes enzymes which are ubiquitous in nature. They have several functions in living organisms, like remodeling membrane phospholipids and formation of lipid-derived second messengers. Considering the vast distribution and significance in nature, phospholipase A_2 is a valuable target and worth to be studied in detail in *T. brucei*. Especially, as it has not been identified or investigated till now in this organism.

PLA_2 is important for the liberation of arachidonic acid from phospholipids and synthesis of prostaglandins in *T. brucei*, which urged for a thorough investigation into the characterization of this enzyme. Trypanosomes produce prostaglandins from free arachidonic acid (Kubata *et al.*, 2000) and prostaglandin synthesis has also been discovered in *T. cruzi* (Kubata *et al.*, 2002) and Leishmania (Kabututu *et al.*, 2003), which gives an idea of distinct similarity among most of the kinetoplastids. Figarella *et al.*, (2005 and 2006) reported that the prostaglandin D_2 and its metabolites are involved in apoptosis and population density regulation in *T. brucei*. Involvement of prostaglandins in host-parasite interactions and virulence of the parasite could help to understand the various pathways. Prostaglandin $F_{2\alpha}$ synthases from *T. brucei* (Kubata *et al.*, 2000 and Okano *et al.*, 2002), *T. cruzi* (Kubata *et al.*, 2002) and Leishmania (Handman *et al.*, 1995 and Kabututu *et al.*, 2003) have been identified, which show analogy in the kinetoplastids. In *T. cruzi* and Leishmania, the putative phospholipase A_2-like protein has not been characterized until now but bioinformatic analysis has predicted that respective proteins are encoded within the genome of these organisms. This surely indicates that the kinetoplastids have evolved a metabolism for arachidonic acid and prostaglandin similar to higher eukaryotes. The existence of these enzymes in kinetoplastids elaborate further that these parasites do not completely depend on the host's metabolic machinery for prostaglandin synthesis and regulation (Kubata *et al.*, 2007).

T. brucei procyclic form synthesizes the largest fatty acid (FA) product which is stearate (C18), however in the bloodstream form is predominantly myristate (C14) (Morita *et al.*, 2000). It has been revealed that *T.brucei* make the bulk of their fatty acids by a mechanism involving endoplasmic reticulum (ER)-based elongases (ELOs) which is a type II FA-synthase system and has a specialized role in these parasites. Four ELOs have been found in these parasites encoded by *ELO 1-4* genes. It has been shown that the genes *ELO 1-3* together account for synthesis of saturated FAs up to a chain length of C18. *ELO1* converts C4 to C10, *ELO 2* extends the chain length from C10 to myristate (C14), and *ELO 3* extends myristate to

C18 (Lee *et al*., 2006). One remarkable feature of the trypanosome ELO system is that it utilizes short-chain acyl-CoA primers and this distinguishes the ELOs of trypanosomes from conventional ELOs and allows the former to synthesize FAs *de novo*.

ELOs synthesize lipids containing polyunsaturated FAs (PUFA). *T.brucei* expresses *ELO 4* which is specific for PUFAs and elongates arachidonate (C20:4) by two carbon atoms. Although arachidonate is available for salvage from the host serum, other PUFAs must be synthesized which are incorporated into phospholipids. The myristate requirement for VSG production by the *T.brucei* bloodstream form and the need for longer *de novo* synthesized FAs in membrane phospholipid biosynthesis, and the nutrients available in the environment, are all factors that have to be balanced by the parasite when regulating the ELO pathway (Englund *et al*., 2007). Regulation of the ELO pathway in these parasites could also influence the FA composition of membrane phospholipids. As the membrane lipid composition is altered when the parasite moves between the different temperatures that exist in the insect vector and the mammalian host consequently the lipid composition changes are needed to adjust for the effects of temperature on membrane fluidity. Triacylglycerols and steryl esters have been proposed to act as FA reservoirs in *T.brucei*. The nutrient-rich environment of the host, the parasite stockpiles lipids by uptake, synthesis or both. However, despite these FA reserves, it seems that the ELO pathway of *T.brucei* provides an essential supply of FAs for incorporation into phospholipids (Englund *et al*., 2007).

Apart from this, Richmond and Smith (2007) have identified and characterized a cytosolic phospholipase A_1 from *T. brucei*. It has a specific activity towards *sn*-1 esters of diacyl phospholipids, exhibits lysoPLA_1 activity and shows very little activity towards neutral lipid substrates. This study proposed that *T. brucei* PLA_1 has 21 % homology with PLA_1 from proteobacterium *Sodalis glossinidus*; a bacterial secondary endosymbiont of Glossina (tsetse) flies (Toh *et al.*, 2006). Furthermore, it has been postulated that *T. brucei* acquired this enzyme through horizontal gene transfer (HGT) after/during its adaptation to a parasitic lifestyle in its tsetse host (Richmond and Smith, 2007). Similar sequence homology to phospholipase A_1 is absent from the protein databases for *T. cruzi* and Leishmania. Due to this observation, it has been proposed that this HGT did occur only in *T. brucei* because the other kinetoplastids do not use Glossina as a host. HGT between prokaryote-eukaryote is still presumed to be rather rare and atypical but is most common in the protists (Boucher and Doolittle, 2000; Field *et al.*, 2000; de Koning *et al.*, 2000). PLA_1 in *T. brucei* has been extensively studied and biochemically characterized (Richmond and Smith, 2007) and its preferred substrate is Glycerophosphatidylcholine (GPCho). One important observation is that

the amino acid sequence of *T. brucei* PLA_1 is not homologous to *T. brucei* PLA_2. They have sequence identity only within the lipase conserved motif GXSXG.

It has been claimed by Richmond and Smith (2007) that a search of the *T. brucei* genome database did not reveal any cytosolic PLA_2 homologues. However, our study found that the *T. brucei* genome database does contain a putative PLA_2-like protein, which possesses a transmembrane domain in its sequence designating it most probably as a membrane protein rather than a cytosolic protein. Indeed, the enzyme was characterized by us using cloning and heterologous expression in insect cells and with the measurements of the respective enzyme activity. In addition, our study has identified a $TbPLA_2$ in the membrane fractions of *T. brucei* bloodstream and procyclic forms.

$TbPLA_2$ amino acid sequence from database shows ~40 % homology with phospholipases A_2 from *T. cruzi* and *Leishmania major* as well as with human PAF-acetyl hydrolase. Apart from these phospholipases A_2, $TbPLA_2$ shows considerable homology with PLA_2s from bacteria, ciliates, nematodes and plants as seen from BLAST tree view widget (see page 78 in Results). The amino acid sequence of $TbPLA_2$ contains the lipase consensus motif GHSFG, which indicates that it might be a member of the lipase family. The identification of phospholipase A_2 in *T. brucei* plays a significant role in understanding the metabolic pathway of arachidonic acid and formation of prostaglandins in these parasites.

4.2 Heterologous expression of $TbPLA_2$

Heterologous expression of a recombinant protein is an extremely powerful tool in the analysis of membrane proteins and several expression systems have been developed so far. Most proteins from a variety of organisms have been heterologously expressed either in bacterial cells (Laage and Langosch, 2001) or insect cells (Luckow *et al.*, 1993; Ciccarone *et al.*, 1997). The general strategy is to genetically fuse to the N- or C-terminus of the protein of interest with certain tags, e.g. His-tag, GFP (Green fluorescent protein) or MBP (Maltose binding protein). These fusions are designed to allow for efficient expression, detection and especially affinity purification of the expressed protein (Laage and Langosch, 2001). Both of the above stated heterologous expression systems were utilized for expression of $TbPLA_2$ in this work.

Efficient expression requires the use of an efficient promoter driving transcription of the plasmid-borne protein and the choice of an appropriate expression host cell system. In case of bacteria, combination of the bacteriophage T7 promoter with BL-21 (DE3) or Rosetta 2 cells

are the most frequently used expression systems. Cloning of the $TbPLA_2$ gene into bacterial subcloning and expression plasmids was successful, as judged by restriction enzyme digestion. Bacterial colony PCR with sequence-specific primers was used to confirm the correct plasmid construct and its correct orientation. Sequence identity of the $TbPLA_2$ gene in the construct was verified by DNA sequencing. The interesting finding from these cloning experiments was that the gene sequences of the constructs from several independently derived clones possessed constantly altered bases at 20 positions in comparison with the known database sequence. When these base alterations (point mutations) were translated in silico, then it was interpreted into only three constant amino acid residue alterations in the one construct analyzed. Translation of these alterations into the amino acid residues indicated that these three constant alterations were at positions 58 (F to L), 270 (I to S) and 324 (V to L). These constant alterations did not appear in the active center of the enzyme (i.e. GHSFG) and probably the activity of this enzyme. The clone carrying the confirmed construct was used for heterologous expression in bacteria, BL-21 (DE3) and Rosetta 2 competent cells. Protein expression was carried out using final concentrations of IPTG ranging from 0.1 to 1 mM and varying temperatures. The induced cell were lysed hypotonically and separated into cytosolic and membrane fractions by centrifugation. Unfortunately, however, neither fraction contained the fusion protein when resolved on SDS-PAGE, at 100 kDa in case of the MBP.

The unsuccessful attempts to express $TbPLA_2$ in bacterial cells were most likely due to the toxic nature of this protein for bacteria. It might be that the transmembrane sequence of this protein was toxic to the bacterial cells and the bacterial protease system may have degraded this protein immediately upon its synthesis to prevent the cells from its deleterious effects. A high-level expression of membrane proteins containing hydrophobic protein domains remains a difficult task in *E.coli* due to their toxic effects exerted on the host cells (Laage and Langosch, 2001). Therefore, the bacterial expression of membrane proteins had frequently been restricted to their soluble domains. On the other hand, over-expression of full-length membrane proteins is highly desirable since their transmembrane domains (TMD) often contain important structural information for folding, oligomerization or subcellular sorting (Laage and Langosch, 2001). Consequently, it was decided to heterologously express the protein in the Baculovirus expression system using *Sf9* insect cells. The verified $TbPLA_2$ construct from previous cloning experiments in the bacterial system was obtained and the $TbPLA_2$ gene fragment was extracted out of the plasmid. This gene fragment was introduced into the Baculovirus plasmid by subcloning in bacterial cells. After successful cloning, the recombinant construct was transposed into a bacmid, using transformation into DH10Bac

competent bacterial cells. The recombinant bacmid was extracted and confirmed by bacterial colony PCR using sequence-specific primers. The purified recombinant bacmid was then introduced into the growing culture of insect cells by viral infection.

The infected insect cells were cultured in passages to increasing volumes in order to generate high viral titers. The supernatant from infected cells and the cell pellet were separated by centrifugation and stored at 4ºC for further analysis. According to the amplification rate of the viral titer, the supernatants from recombinant Baculovirus stocks were labeled P1 (low viral titer), P2 (higher viral titer) and P3 (highest viral titer). In order to establish the optimum conditions of infection, the time span for infection was established to be best at 120 h. After the infection period, the infected cells were lysed and fractionated into soluble and membrane fractions. These fractions along with control insect cell supernatants were analyzed on SDS-PAGE. The infected insect cell lysates membrane fraction depicted a prominent protein band at 58 kDa on SDS-PAGE which revealed the over-expression of a protein. This protein band had an increased molecular size in comparison to the expected size of 50 kDa. This difference in molecular weight could be due to post-translational modifications of the protein within insect cells. The expressed $TbPLA_2$ from membrane fraction of the infected insect cells was further verified by Western blotting using a polyclonal antibody. The $TbPLA_2$ antibody reacted with the prominent band seen at 58 kDa on Western blots. It can thus be concluded that the *T. brucei* PLA_2 protein was cloned, over-expressed and identified.

4.3 Biochemical and immunological characterization in T. brucei cells

In order to investigate whether phospholipase A_2 is expressed in *T.brucei* bloodstream and/or procyclic forms, biochemical characterization methods were used to determine its function. Cells from the logarithmic and stationary phase of bloodstream and procyclic forms were analyzed by Western blot to evaluate the presence of $TbPLA_2$ and by immunoflouresence to determine the possible localization in the cell. Western blots of membrane fractions from bloodstream and procyclic forms showed evidence of $TbPLA_2$ by reaction of the protein with the anti-$TbPLA_2$ antibody. Furthermore, this analysis revealed that $TbPLA_2$ was expressed in both the logarithmic and the stationary phases in bloodstream as well as in procyclic forms. As judged from Western blots, the protein is expressed in rather low concentrations in membrane fractions of different life cycle stages. Hypothetically, when the level and activation of this enzyme reaches a high concentration inside the cell, then it could potentially lyse the parasite from within by producing excess amounts of fatty acids and

lysophospholipids. The appropriate level of $TbPLA_2$ and consequently the products of this enzyme should be crucial for the normal functioning in *T. brucei*. The detection and recognition of $TbPLA_2$ with specific antibodies in the membrane fractions of bloodstream and procyclic cells at a molecular size of 50 kDa apparently directs towards the presence of this protein in these parasites. The membrane fractions of bloodstream form cells at a cell density of 3×10^9 depicted the similar molecular size and intensity band. The band on the blot appeared with less intensity due to low level of this protein in the cells and even though with prolonged development time period of the blot to enhance but could not intensify the band.

To further elaborate and ascertain that $TbPLA_2$ is a true phospholipase A_2, a specific activity assay (Jimenez *et al.*, 2003) was established using the known and commercially available phospholipase A_2 from porcine pancreas. This activity assay utilizes a coupled enzymatic assay in which phospholipase A_2 releases linoleic acid from the *sn*-2 position of DL-PC substrate. Linoleic acid is then a substrate for lipoxygenase, which further oxidizes it into hydroperoxides. This activity was followed spectrophotometrically by measuring the increase in absorbance at $\lambda = 234$ nm due to the appearance of hydroperoxides as products of the assay. Cell lysate fractions from bloodstream and procyclic forms corresponding to a cell density of 1×10^6/ ml were examined using this assay to determine the level of activation of the phospholipase A_2 in the parasites. Cell lysates from the logarithmic and stationary phases of bloodstream form as well as from the procyclic form were fractionated into soluble and membrane fractions. The specific activities of these fractions in nM/min were measured as shown below in the Table 3:

	Bloodstream form nM/ min		Procyclic form nM/ min	
	Membrane	Cytosolic	Membrane	Cytosolic
Logarithmic phase	0.25	0.15	0.57	0.12
Stationary phase	0.25	0.13	n.d.	n.d.

Table 3 Phospholipase A_2 specific activity values with cell lysate fractions from bloodstream and procyclic forms

The specific activity of the procyclic form membrane fraction was 0.57 nM/min as compared to the cytosolic fraction which was 0.12 nM/min. On the other hand, the specific activity of bloodstream form logarithmic phase and stationary phase membrane fractions determined were both 0.25 nM/min. In comparison, the specific activities of the cytosolic fractions from logarithmic and stationary phases were 0.15 nM/min and 0.13 nM/min respectively.

The specific activity in membrane fraction of procyclic to both phases of bloodstream form has activity factor of 2.28. The cytosolic fraction of procyclic to bloodstream form has activity factor of 1.25. In addition, procyclic form membrane to cytosolic fraction has specific activity factor of 4.75 and both phases of bloodstream form membrane to cytosolic fraction has specific activity factor of 1.92. This indicates that the specific activity is high in membrane fraction of procyclic as compared to bloodstream form. The cytosolic fractions in both procyclic and bloodstream form contains equal specific activities. On the whole, the results suggest that there is a phospholipase A_2 activity in *T. brucei* logarithmic and stationary phases of bloodstream and procyclic forms as determined from the specific activities.

In order to inhibit $TbPLA_2$ specifically it has been attempted to find an appropriate specific inhibitor (Lucas and Dennis, 2005). However, since each phospholipase A_2 has a group-specific inhibitor, the search for $TbPLA_2$ specific inhibitor/s was so far unsuccessful.

4.4 Regulation and expression of $TbPLA_2$ in T. brucei

Mature mRNA production in trypanosomes is a step-wise process that differs in several aspects from the biogenesis of mRNA in most eukaryotes (Ullu *et al.*, 1996). The trypanosome gene arrangement is highly compact with small intergenic regions separating one gene from the next. Trypanosome protein-coding genes are organized as polycistronic rather than monocistronic transcription units. Furthermore, the 5´ ends of all mature mRNAs are formed by trans-splicing contrary to transcription initiation. The partners in trans-splicing are the polycistronic pre-mRNA, which contains the mRNA coding regions preceded by a 3´ splice site, and the spliced leader (SL) RNA, which provides the capped SL sequence and the 5´ splice site (Mair *et al.*, 2000). Individual mature mRNAs are generated from polycistronic precursors by 5´ trans-splicing of a 39-nt capped leader RNA and 3´ polyadenylation. There is no consensus polyadenylation signal in the 3´-untranslated region (3´-UTR). Instead, experimental evidence suggests that polyadenylation occurs within a short region of 100-400 nt upstream of the polypyrimidine trans-splice signal (Benz *et al.*, 2005).

T. brucei phospholipase A_1 ($TbPLA_1$) production from mRNA level to the protein translation was examined in detail (Richmond and Smith, 2007) and it had been stated that it is controlled at a certain required level considering the potential damage a membrane lytic enzyme such as $TbPLA_1$ can invoke, if it was allowed to be expressed at uncontrollable levels in the cell. Similarly, the evidence for phospholipase A_2 in *T. brucei* at the transcriptional level and the regulation in different life stages of the parasite could give a clue to the synthesis of arachidonic acid and its metabolites. In order to compare and evaluate the transcriptional regulation of the $TbPLA_2$ gene in the bloodstream form, the transcriptional expression level was analyzed using the total RNA from the logarithmic and stationary phases as well as the logarithmic phase isolated from rat blood. The experiment was conducted with the beta-tubulin gene from bloodstream form cells as a loading control. The rRNA from each of the bloodstream form cells were obtained and it showed the three expected bands at positions of 2250 bp, 1850 bp and 1350 bp .

Using sequence-specific probes, Northern analysis of $TbPLA_2$ showed two bands at approximately 3,700 bp and 3,500 bp in each stage mentioned above. The transcripts from $TbPLA_2$ blot showed two mRNAs which had different sizes, positions and strengths of signals. These two transcripts may be alternate splicing forms of a single gene, which are expressed in the bloodstream forms. In addition, multiple alternatively processed products could be produced from intergenic regions with several polypyrimidine tracts (Benz *et al.*, 2005). In addition, it can be postulated that the different mRNA transcripts (Benz *et al.*, 2005) could be due to the transcription of complete length of intergenic regions starting from 5´ UTR including ORF of the gene and 3´ UTR region downstream till the next ORF of other gene as seen in the gene database (GeneDB).

The band intensity of the transcripts from each stage stated above was performed with semi-quantitative densitometric calculations of the respective gene. The results indicated that the most intense band among the three respective stages of parasite was from logarithmic stage of parasite which was depicted as the lower band designated with an intensity value of 1, and the size of 3,500 bp as compared to the upper band having an intensity value of 0.7 and a size of 3,700 bp. These values were calculated in comparison of bands from beta-tubulin as loading control which were similar in expression level in each life stage of the parasite.

The Northern blot analysis indicated a stage-specific regulation of $TbPLA_2$ gene transcripts. The gene was expressed at a low level in the stationary phase of parasites in culture and cells in the logarithmic phase isolated from rat blood. It was relatively more expressed and regulated in the logarithmic phase. This can be interpreted as the parasite's requirement of

building up resistance against the host immune system during the infection stage or cell density regulation of the parasites. The expression profile of the transcripts indicates a distinct significance of $TbPLA_2$ throughout the life cycle of the parasites. Although, these results brings us a step closer in understanding the expression level of $TbPLA_2$ mRNA, it is still not proven that $TbPLA_2$ functions in a pathway to release arachidonic acid from phospholipids within cell or organelle membranes, nor if this arachidonic acid liberation or $TbPLA_2$ itself is essential for the cellular viability and stability of the cell. Moreover, there is a need for further experiments to understand and answer some fundamental questions concerning the nature of $TbPLA_2$ and to resolve the structure-function relationship of this enzyme using site-directed mutagenesis, generation of deletion mutants for $TbPLA_2$, as well as substrate-specificity and inhibitor kinetic analysis.

Conclusion

Sleeping sickness vector *T.brucei* is an eukaryotic organism whose metabolism is the subject of extensive searches for the possible targets in the drug development against this disease. On the other hand, there are several proteins involved in different pathways still not investigated or identified. One of such a pathway is the phospholipid metabolism and the enzymes involved in it. This study was conducted to identify and characterize a *T. brucei* phospholipase A_2 which metabolizes phospholipid substrate from *sn*-2 position resulting in the production of arachidonic acid and eicosanoids. These products play a vital role in the intracellular metabolism and regulation of the parasite's cell membrane. This work concentrated on the cloning, heterologous over-expression of the phospholipase A_2 and simultaneously characterization of this enzyme in *T. brucei*.

The cloning of $TbPLA_2$ was successful in the bacterial plasmids. The heterologous expression of $TbPLA_2$ in bacterial cells did not yield the expected protein as seen on SDS-PAGE. Eventually, the expression was attempted in Sf9 insect cells which yielded the expression of this protein and specifically in the membrane fraction. This expression was verified with Western blot analysis depicting a molecular size of 58 kDa. The expression at the transcriptional level regulation of $TbPLA_2$ was analyzed with the Northern blot. The blot showed two transcript bands of different size, intensity and strengths in logarithmic, stationary phase from *in vitro* culture and *T. brucei* cells in logarithmic phase from rat blood. The transcript in the logarithmic stage from cell culture showed high expression level at a size of approximately 3500 bp. Phospholipase A_2 activity assay with DL-PC substrate indicated

the evidence of specific activity in nanomole concentrations in cell lysates soluble and membrane fractions of logarithmic, stationary phase of bloodstream and procyclic forms of the parasites. The specific activity was relatively high in cell lysates membrane fractions of both the forms of parasites. Among the fractions, procyclic form membrane fraction had twofold activity in comparison to bloodstream form. In general, this study concludes the evidence of PLA_2 in *T. brucei* through the cloning, over-expression and characterization in parasites.

5. Summary

Human African trypanosomiasis or sleeping sickness is caused by infection with the tsetse-fly-transmitted protozoan *Trypanosoma brucei*. It lives exclusively as an extra-cellular parasite unlike other Trypanosomatidae. The sub-species of this parasite include *T. b. rhodesiense* (in East and Southern Africa) and *T. b.gambiense* (in West and Central Africa). These parasites, along with the non-human infective *T. b. brucei* are zoonoses, and disease control is impeded by substantial wild and domestic animal reservoirs of infection. Historically, sleeping sickness had caused massive loss of life, and related animal diseases (Nagana) which have had a crucial impact on the development of sub-Saharan Africa (Sternberg, 2004). Currently, the incidence of sleeping sickness is rising, and the control is restricted due to inadequate development of drugs and these parasites are gaining resistance against current medicines.

The parasite lacks various core metabolic pathways present in the mammalian hosts, but also shows a set of parasite specific pathways. The enzymes and their metabolites involved in the lipid metabolism have been proven to be potential targets for the development of novel chemotherapeutics. In this context, elucidation of the parasite's specific metabolism is a prerequisite for drug development (Borza *et al.*, 2005; Van Hellemond *et al.*, 2006; Ginger, 2006).

One of the enzymes of lipid metabolism which plays a significant role in the cell is the phospholipase A_2. Phospholipases A_2 are the enzymes which catalyzes the hydrolysis of the fatty acid ester at the *sn*-2 position of the phospholipids to produce free fatty acids, such as arachidonic acid and lysophospholipids. Both the products represent precursors for signaling molecules that can exert a multitude of biological functions (Schaloske and Dennis, 2006). The phospholipase A_2 consists of 15 groups and includes five distinct types of classes, namely the secreted PLA_2 ($sPLA_2$), the cytosolic PLA_2 ($cPLA_2$), the Ca^{2+} independent PLA_2 ($iPLA_2$), the platelet-activating factor acetyl hydrolases (PAF-AH) and the lysosomal PLA_2s (Burke and Dennis, 2009). They perform various functions in living organisms including lipid modification in the cell membrane, lipid-derived second messengers. The PLA_2 releases arachidonic acid from phospholipid which is further metabolized to form prostaglandins, thromboxanes, or leukotrienes.

Although a PLA_1 was described in *T. brucei* earlier (Richmond and Smith, 2007), a PLA_2 has not been investigated so far. Figarella *et al.*, (2005 and 2006) have reported that the prostaglandin D_2 and its metabolites exerted a role in apoptosis and cell density regulation in

T. brucei. This encourages in the investigation and identification of a PLA_2 in these parasites. This work deals with the identification and characterization of PLA_2 in *T. brucei*. The $TbPLA_2$ gene sequence from GeneDB was utilized using sequence-specific primers to amplify the DNA which was sub-cloned in the bacterial cells. Furthermore, the verified recombinant plasmid was cleaved with restriction enzymes to extract the gene of $TbPLA_2$. This gene fragment was inserted into the expression plasmid and transformed into the BL-21(DE3) and Rosetta 2 expression competent cells for heterologous expression. The attempt for heterologous expression in the bacterial cells did not yield the target protein due to the toxic nature of this protein for the bacterial cells. Due to this, another heterologous expression strategy was followed namely Baculovirus expression system using Sf9 insect cells. The verified fragment of $TbPLA_2$ was introduced into the plasmid of Baculovirus system and amplified. The recombinant bacmid obtained containing $TbPLA_2$ was used to infect the growing insect cells. After the completion of the infection period, the cells were lysed and fractionated. The insect cell lysates soluble and membrane fractions were resolved on SDS-PAGE which showed a protein band only in membrane fraction at a molecular size of 58 kDa verified with Western blot using polyclonal antibody against $TbPLA_2$. In general, the $TbPLA_2$ was successfully cloned and heterologously expressed in the Sf9 insect cells.

In order to investigate the expression of phospholipase A_2 during the course of the life cycle of *T. brucei*, cells from the logarithmic and the stationary phase (equivalent to slender and stumpy forms) of bloodstream trypanosomes and the procyclic form were analyzed by Western blotting to evaluate the presence of $TbPLA_2$ within these cells. The respective blots showed that the enzyme is present in the membrane fractions from all forms at a molecular size of 50 kDa. As judged from these results, the protein is expressed in rather low concentrations, which seems to be a consequence of its high "toxicity", as its products (free fatty acids and lysophosholipids) are both membrane active and would inevitably lyse the cells from within by exceeding an acceptable threshold concentration. On the other hand, the appropriate level of $TbPLA_2$ activity seems to be crucial for the parasite's survival.

The Northern blots of different bloodstream forms revealed two bands at sizes of 3,500 bp and 3,700 bp, respectively. Interestingly, the latter band was rather similar in all forms, while the former band was strongly expressed only in cell culture forms from the logarithmic phase. These two transcripts are most probably alternate splicing forms of the single gene (Benz *et al.*, 2005). The expression profile of the transcripts indicates a distinct significance of $TbPLA_2$ throughout the life cycle of the parasites.

To ascertain that the analyzed protein is a true phospholipase A_2, a specific activity assay was performed using commercially available PLA_2 from porcine pancreas as control (Jimenez *et al.*, 2003). Bloodstream and procyclic forms were used to determine the enzyme activity in different cell fractions. The results show that $TbPLA_2$ is a membrane located enzyme expressed throughout the life cycle with a more than twofold higher expression in the procyclic insect form. From these observations together with the data from the Northern and Western blotting it can be postulated that $TbPLA_2$ exists in *T. brucei* as a membrane protein. Because of its importance for the parasite throughout its life cycle, it seems to be a promising target for drug development to be explored in more detail.

Zusammenfassung

Die Afrikanische Trypanosomiasis oder Schlafkrankheit wird ausgelöst durch die Infektion mit dem durch die Tsetsefliege übertragenen *Trypanosoma brucei*. Der Erreger lebt ausschließlich als ein extrazellulärer Parasit anders als andere Trypanosomatida. Die Unterarten dieses Parasiten bestehen aus *T. b. rhodesiense* (in Ost- und Südafrika) und *T. b. gambiense* (in West- und Zentralafrika). Diese Parasiten zusammen mit dem nicht humanpathogenen *T. b. brucei* sind Zoonosen und die Eindämmung der Krankheit wird erheblich erschwert durch substanzielle Reservoirs in Wild- und Nutztieren. In der Vergangenheit hat die Schlafkrankheit zu einem massiven Verlust an Leben geführt und die verwandte Tierkrankheit (Nagana) hatte einen entscheidenden Einfluss auf die Entwicklung des Bereichs Afrikas südlich der Sahara (Sternberg, 2004). Zurzeit steigt die Anzahl der Erkrankungen wieder und die Bekämpfung ist limitiert durch fehlende Medikamentenentwicklung und durch die Tatsache, dass die Parasiten Resistenzen gegen aktuelle Medikamente entwickeln.

Dem Parasiten fehlen verschiedene zentrale Stoffwechselwege verglichen mit dem Säugetierwirt, aber sie haben auch einige parasitenspezifische Wege. Die im Lipid-Stoffwechsel beteiligten Enzyme und ihre Metaboliten haben sich als potentielle Ziele für die Entwicklung neuer Chemotherapeutika herausgestellt. In diesem Zusammenhang ist die Aufklärung der parasitenspezifischen Stoffwechselvorgänge eine grundlegende Vorraussetzung für die Medikamentenentwicklung (Borza, 2005; Van Hellemond, 2006; Ginger, 2006).

Eines der Enzyme des Lipidstoffwechsels, das eine signifikante Rolle in der Zelle spielt, ist die Phospholipase A_2. Phospholipasen A_2 sind Enzyme, die die Hydrolyse von Fettsäureestern an der *sn*-2 Position des Phospholipids katalysieren um freie Fettsäuren, wie Arachidonsäure und Lysophospholipide herzustellen. Beide Produkte sind Vorläufer für Signalmoleküle die eine Reihe von biologischen Funktionen ausüben können (Schaloske and Dennis, 2006). Die Phospholipase A_2 gliedert sich in 15 Gruppen und beinhaltet fünf eindeutig zu unterscheidende Typen von Klassen, nämlich die sekretierte PLA_2 ($sPLA_2$), die cytosolische PLA_2 ($cPLA_2$), die Ca^{2+} unabhängigen PLA_2 ($iPLA_2$), die Plättchenaktivierender Faktor Acetyl-hydrolasen (PAF-AH) und die lysosomale PLA_2 (Burke and Dennis, 2009). Sie üben verschieden Aufgaben im lebenden Organismus aus, einschließlich der Modifikation von Lipiden in der Zellmembran und der Bildung von von Lipiden abstammenden Second

Messangern. Die PLA_2 spaltet Arachidonsäure von Phospholipiden ab, welche weiter zu Prostaglandinen, Thromboxanen oder Leukotriene verstoffwechselt wird.

Obwohl eine PLA_1 in *T. brucei* schon beschrieben wurde (Richmond and Smith, 2007) wurde bis heute noch keine PLA_2 untersucht. Figarella et al., (2005 and 2006) haben berichtet, dass Prostaglandin D_2 und seine Metaboliten eine Rolle bei Apoptose und der Zelldichteregulation in *T. brucei* spielen. Dies ermutigt die Suche und Identifizierung der Phospholipase A_2 in diesen Parasiten.

Diese Arbeit beinhaltet die Identifikation und Charakterisierung von PLA_2 in *T. brucei*. Die $TbPLA_2$ Gensequenz von GeneDB wurde verwendet um mittels sequenzspezifische Primer die DNA zu amplifizieren, welche dann in Bakterienzellen subkloniert wurde. Weiterhin wurde das verifizierte rekombinante Plasmid mittels Restriktionsenzymen geschnitten um das PLA_2-Gen herauszuschneiden. Diese Genfragment wurde in das Expressionsplasmid einligiert, in BL-21(DE3) und Rosetta 2 Zellen transformiert um für die heterologe Expression zur Verfügung zu stehen. Der Versuch der heterologen Expression in Bakterienzellen lieferte nicht das erhoffte Protein, sehr wahrscheinlich weil das Protein toxisch für Bakterien ist. Deshalb wurde eine andere Strategie für heterologe Expression verfolgt, nämlich das Baculovirus Expressionssystem unter Verwendung von Sf9 Insekten Zellen. Das überprüfte Fragment von $TbPLA_2$ wurde in das Baculovirus System einkloniert und weiter amplifiziert. Das rekombinierte Bacmid mit PLA_2 wurde mittels Infektion in die Insektenzellen gebracht. Nach Abschluss der Infektionsperiode wurden die Zellen lysiert und fraktioniert. Die lösliche und die Membranfraktion der Insektenzelllyse wurden auf einer SDS-Page aufgetrennt, welche nur in der Membranfraktion eine 58kDa Proteinbande zeigte. Die Membranfraktion der Insektenzellen zeigte auch im Western blot mit polyklonalen Antikörpern gegen $TbPLA_2$ eine Bande bei 58kDa. Zusammenfassend lässt sich sagen, dass $TbPLA_2$ erfolgreich kloniert und heterolog in Insektenzellen exprimiert wurde.

Um die Expression der Phospholipase A_2 während der verschiedenen Stadien des Lebenszyklus von *T. brucei* zu untersuchen wurden Zellen aus der logarithmischen und stationären Phase von Blutfom Trypanosomen (äquivalent zu slender und stumpy Formen) und aus prozyklischen Fliegenformen mittels Western blot untersucht, um die Level an $TbPLA_2$ in den Zellen beurteilen zu können.

Die entsprechenden Blots zeigen, dass das Enzym in der Membranfraktion aller Trypanosomenformen eine Bande bei 50 kDa vorliegt. Ausgehend von diesen Ergebnissen ist das Protein in eher geringer Konzentration exprimiert, was eine Folge seiner hohen ‚Toxizität' zu sein scheint. Seine Substrate (freie Fettsäuren und Lysophospholipide) sind

beide membranaktiv und bei zu hoher Konzentration (über einem vertretbaren Schwellenwert) würden sie unweigerlich zur Lyse der Zellen von innen heraus führen. Andererseits scheint ein gewisser Level an PLA_2 Aktivität absolut notwendig für das Überleben des Parasiten zu sein.

Der Northern blot der verschiedenen Blutform Trypanosomen zeigt je zwei Banden von 3.500bp und 3.700bp Größe. Interessanterweise war die letztere Bande in allen Formen sehr ähnlich, während die Bande bei 3.500bp nur in Blutform Trypanosomen aus Zellkultur stark exprimiert war. Diese zwei Transskripte sind höchstwahrscheinlich alternative Splicing Formen desselben Gens (Benz et al., 2005). Das Expressionsprofil der Transskripte zeigt die eindeutige Signifikanz der $TbPLA_2$ während des gesamten Lebenszyklus des Parasiten.

Um sicher zu stellen, dass das untersuchte Protein wirkliche eine Phospholipase A_2 ist, wurde ein spezifischer Enzymassay mit gekaufter PLA_2 aus der Bauchspeicheldrüse eines Schweins als Kontrolle (Jimenez et al., 2003) durchgeführt. Blut- und Fliegenformen der Trypanosomen wurden verwendet um die Enzymaktivität in verschiedenen Zellfraktionen zu bestimmen. Die Ergebnisse zeigen, dass $TbPLA_2$ ein in Membranen lokalisiertes Enzym ist das während des gesamten Lebenszyklus exprimiert wird, mit einer mehr als zweifach höheren Expression in Fliegenformen.

Ausgehend von diesen Beobachtungen zusammen mit den Daten vom Northern blot und Western blotting kann man postulieren, das $TbPLA_2$ *in T. brucei* als Transmembranprotein vorliegt. Wegen seiner Bedeutung für den Parasiten während des ganzen Lebenszyklus scheint $TbPLA_2$ ein viel versprechendes Ziel für die Arzneimittelentwicklung zu sein, das es sich lohnt noch näher zu untersuchen.

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1994-1995	Bachelor of Science (B.Sc.)	Govt. Islamia Science College, University of Karachi, Pakistan
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Scholarship

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