STUDIES ON THE MIDGUT LECTIN GENE OF *GLOSSINA AUSTENI*

by

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A thesis submitted in fulfilment of the requirements for the award of the degree of Doctor of Philosophy of Kenyatta University

DECEMBER 2005

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

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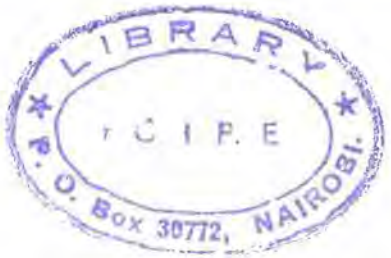
DEDICATION

To my beloved brother and sister, Caleb and Ruphina.



ACKNOWLEDGEMENT

I give thanks and praises to the Almighty God who made this work to be realized. The stipend to enable me carry out this work was provided by the German Academic Exchange Service (DAAD). To all the officials and staff of DAAD I express my sincere gratitude. I am also grateful to the authorities of the International Center of Insect Physiology and Ecology (I.C.I.P.E) for having given me the opportunity to carry out this research under the African Regional Postgraduate Programme in Insect Science (ARPPIS).

Special thanks and gratitude to all my supervisors, Dr. Ellie Osir (formerly of ICIPE) and Dr. Geoffrey Muluvi and Prof. Jesse Machuka (of Kenyatta University) for steering me through out the study. I particularly appreciate their continuous encouragement and guidance during the entire period of the study. I also would like to acknowledge Prof. Bruce Hammock and Dr. George Kamita, both of the University of California- Davis, for introducing me to the arena of protein expression in baculoviruses. I will always remain indebted to both of them. Sincere gratitude to Ms Jeanette Martin and her colleagues of Hammock Lab. UC Davis. Special thanks to Prof. Linda King of Oxford Brooks University for the kind donation of AcMNPV (chi-) vector.

I would like to express sincere thanks to Prof. Onesmo Ole-MoiYoi and Dr. Dan Masiga for their support during the work. At some stage in my studies, I received assistance from staff at Labs 5 and 6 of the International Livestock Research Institute-Kenya. Special thanks to Dr. Evans Taracha, Dr. Roger Pelle, Dr. Honda, Mr. Ferdinand Mbwika, Mr. Francis Chuma, Susan Musembi, Julius Osaso and Beatrice Anyona.

I would like to thank my fellow students in the Molecular Biology and Biotechnology Department and the staff at Capacity Building Unit, I.C.I.P.E. Special gratitude goes to Mr. James Kabii, Mr. Mark Kimondo, Mrs Rosekellen Njiru, Mr. Benson Nyambegah, Dr. Laila Abubakar, Ms. Lisa Omondi and Mrs Lucy Mwihaki for their support. Sincere thanks to all the staff and students at I.C.I.P.E. I am particularly grateful for the support and love from my family, and the entire Cameroonian community in Kenya for their moral and spiritual support.

ABSTRACT

Trypanosomiasis continues to be a serious problem in sub-Saharan Africa. The inadequacies of existing control strategies necessitate the development of alternative control measures. In most cases, the causative agents of African trypanosomiasis, *Trypanosoma spp.*, require an obligatory passage through the tsetse, *Glossina* spp (Diptera; Glossinidae), vector. This developmental step of the parasite leads to vector-parasite interactions, especially within the midgut. Among the vector molecules highly implicated in the interplay are carbohydrate-binding proteins known as lectins. This thesis describes studies on the midgut proteolytic lectin of *Glossina austeni*. The purification of a proteolytic lectin from the midgut homogenate of *G. austeni* was achieved through a 2-step chromatographic procedure, involving anion exchange and affinity columns. The purified protein was shown to cause the agglutination of *Trypanosoma brucei brucei* (Kinetoplastida, Trypanosomatidae) and washed rabbit red cells *in vitro*. It also showed some trypsin activity when the chromogenic substrate, chromozym-TRY, was used. Polyclonal antibodies to this molecule were used to isolate a putative gene encoding the protein from a *G. austeni* midgut cDNA library. The sequence of the isolated gene (GenBank Accession Number DQ060150) showed very high similarity to another gene previously obtained from *G. fuscipes fuscipes* midgut cDNA library. Analysis of the gene sequence, using basic bioinformatics tools, showed that the translated protein with 274 amino acids contains a signal peptide region and signature motifs for the serine protease trypsin family. The recombinant protein was expressed in *E. coli* BL 21 (DE2) cells and the purified product used to raise polyclonal antibodies. The recombinant *Glossina* proteolytic lectin was further expressed in *Spodoptera frugiperda* (Sf) 21 cell lines by the baculovirus expression system. This is the first *Glossina* protein to be expressed with this system. The baculovirus-expressed recombinant lectin was found in the medium of baculovirus-infected Sf-21 cell cultures indicating that the tsetse fly-derived signal peptide was recognized and cleaved by the Sf-21 cells. The recombinant protein was purified by immuno-affinity chromatography and shown by Periodic Acid Schiff stain to be post-translationally modified by glycosylation. Moreover, the glycosylation pattern was not via the classical O-linked and N-linked sugar attachment motifs as these were absent from the nucleotide sequence. Both the baculovirus- and bacteria- expressed lectin proteins showed agglutination and enzymatic properties. The two recombinant forms of the expressed lectins showed no significant differences in terms of biological activity indicating that the sugar moiety may not be crucial for these functions. It is plausible that the physiological relevance of the sugar moiety is to act in synergy with the signal peptide for proper targeting of the secreted protein and also enhancing its solubility within the midgut region of the fly. The findings provide an important contribution in the characterisation of *Glossina* proteolytic lectin. The constructed recombinant baculovirus, designated AcMNP.gpl, constitutes a very useful molecular tool for further investigation of both the structure of the protein as well as the mechanisms of action of *Glossina* proteolytic lectin (Gpl) upon African trypanosomes. Preliminary data from the present study has led to the suggestion that the initial part of interaction between Glossina proteolytic lectin and D-glucosamine, which is binding, may rely more on structural complementarities between the two molecules, in a pattern similar to a lock and key mechanism.

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ABBREVIATIONS

AcMNPV	Autographa californica multiple nucleopolyhedrosis virus
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BIS	Buffered insect saline
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Chromozym-TRY	Carbobenzoxy-val-gly-arg-4-nitrianilide acetate
DNA	Deoxyribonucleic acid
DEAE-	Diethylaminoethyl
Gpl	Glossina proteolytic lectin
h	Hour
Kbp	Kilobase pairs
M	Molar
mg	Milligramme
$MgCl_2$	Magnesium chloride
min	Minute (s)
mM	Millimolar
µ	Micro
µg	Microgramme
µl	Microliter
µM	Micromolar
NaCl	Sodium chloride
ng	Nanogramme
nm	Nanometer (s)

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phospahate buffered saline
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RBC	Red blood cells
RLOs	Rickettsia-like organisms
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
Sec	Second (s)
TBS	Tris buffered saline
Tris	Tris-(hydroxymethyl) amino methane
VSG	Variant surface antige

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Sub-Saharan Africa continues to be plagued by numerous tropical parasitic diseases including malaria, leishmaniasis, filariasis, schistosomiasis and trypanosomiasis. Trypanosomiasis is a debilitating vector-borne disease, of which the causative agent and vector have been known for more than a century, but no effective preventive treatment has been made available. There are no prospects for development of a preventive and/or protective vaccine in the foreseeable future. Trypanosomiasis is caused by protozoan parasites of the genus, *Trypanosoma* (Kinetoplastida; Trypanosomatidae). Most of these parasites live partly in the vertebrate host and partly in the tsetse fly, *Glossina* species (Diptera; Glossinidae) because trypanosome transmission from one vertebrate host to the next, for the cyclically transmitted forms, occurs through the vector (Bruce *et al.*, 1909). The disease is restricted to between latitudes 14°N and 29°S on the African continent because this is the tsetse belt, which, unfortunately, is the most agriculturally fertile part of the continent. The resulting economic losses in the agricultural sector of the continent are estimated at billions of U.S. dollars annually as trypanosomiasis deprives rural farmers of meat, milk, draught power and manure. Trypanosomiasis is a disease that involves the interplay of three

organisms, the parasite, vector and host. Disease management measures have targeted one, two or all of the three.

Drug treatment of infected cases has been an important component of such measures. Close to half a million people in the 36 affected African countries are infected each year, with less than a third of these diagnosed and treated. Of the about 60 million exposed, less than 4 million are under surveillance (WHO, 1995). The drug treatment approach has been hampered by numerous problems such as the high cost and serious side effects of the few available drugs, difficulties in accessing the target population who live mostly in rural areas, as well as the development of resistance to the drugs by the parasite (McNeil, 2000). A wide arsenal of vector control tools has also been used in an attempt to ease the problem of trypanosomiasis. Unfortunately, these control measures have not been fully effective with 60 million people and hundreds of millions of wild and domesticated livestock still exposed to these parasites.

These setbacks have stimulated increased interest in understanding vector-parasite interactions, and how these enhance or impede parasite transmission. Such studies have resulted in the identification of host bloodmeal (Moloo, 1981; Mihok *et al.*, 1995; Nguu *et al.*, 1996) as well as tsetse midgut factors that play a role in the transmission cycle of this disease. Among such midgut factors are lectins, proteases, trypanolysins (Ibrahim *et al.*, 1984; Stiles *et al.*, 1990; Imbuga *et al.*, 1992: Welburn and Maudlin, 1999; Osir *et al.*, 1999; Otieno *et al.*, 1983) and other yet uncharacterized molecules. So far, lectins, trypanolysins and trypsin have

been shown to be involved in the lysis of bloodstream forms of trypanosomes once the latter get into the tsetse midgut (Maudlin and Welburn, 1987; Welburn *et al.*, 1989; Stiles *et al.*, 1990; Imbuga *et al.*, 1992; Osir *et al.*, 1999). The midgut lectin, which is induced by a bloodmeal (Welburn *et al.*, 1989; Abubakar *et al.*, 1995) is now associated with two roles; promoting establishment and transformation of bloodstream forms to procyclics (midgut) forms as well as the lysis of trypanosomes (Maudlin and Welburn, 1987; Welburn *et al.*, 1989). The level of lectin in tsetse midgut determines whether it favours lysis (high levels) or establishment and transformation (low levels) (Maudlin and Welburn, 1988a; Maudlin, 1991; Imbuga *et al.*, 1992; Osir *et al.*, 1993).

The fact that lectins and trypsin play a crucial role in the establishment and transformation of trypanosomes in tsetse midgut has been demonstrated by *in vivo* and *in vitro* studies (Maudlin and Welburn, 1987; 1988a,b; Imbuga *et al.*, 1992; Osir *et al.*, 1993). This has led to isolation, purification and characterization of a midgut proteolytic lectin (formally referred to as lectin-trypsin complex) from *Glossina longipennis* (Osir *et al.*, 1995) and *G. fuscipes fuscipes* (Abubakar *et al.*, 2003). It may therefore be necessary to characterise the molecule further while confirming its presence in other species of this vector.

1.2 Justification and Significance of the study

So far vector control, through the use of insecticides, and drug treatment of infected cases has been the major way to control

trypanosomiasis (Allsop, 2001). However, wide use of these chemicals has led to insecticide resistance by the vector. Another set back is that there is the emergence of drug-resistant parasites. It is therefore necessary to improve on the existing strategies or design alternative measures to combat the disease.

There is general awareness among tsetse researchers that an integrated control strategy offers more hope, compared to dependence on use of insecticides and traps, if this vector will be contained. From the success achieved in Zanzibar, the sterile insect technique (SIT) is viewed as an important tool for any tsetse eradication programme. However, this will require that other existing tools like traps, screens, and tsetse repellents be employed. With the development of biotechnology, it is also becoming an acceptable strategy to reduce the ability of tsetse to transmit trypanosomes. One basic requirement for this approach is the identification of candidate molecules that could be used. Knowledge on how the tsetse is able to transmit trypanosomes and yet survive is an avenue for the isolation of candidate molecules. In this regard, the tsetse midgut proteolytic lectin is a potential molecule in view of its dual roles. However, for this to be realized the molecule needs to be further characterised biochemically.

Presently, manipulation of some disease vectors as means of effecting vector control is a common practice. For example, the potential of expressing antipathogen factors in transgenic mosquitoes has been attempted (Kokoza *et al.*, 2000). Working with *Aedes aegypti*, Kokoza *et al.* (2000) were able to create a stable transgenic organism with improved

systemic immunity. Application of this strategy in other insect disease vectors has led to the creation of transformed Rickettsia-like organisms that live symbiotically with *Glossina* species (Beard *et al.*, 1993). The goal in this approach was to enhance the anti-parasitic property of this disease vector using plasmids. This strategy is referred to as paratransgenesis as direct transformation of *Glossina* is not practicable as a result of its life cycle.

An important finding about the biochemical property of the purified *Glossina* midgut proteolytic protein is that it is glycosylated (Osir *et al.*, 1995). However, the functional significance, if any, of the glycosylation is not known. The nature of the glycosylation linkage is also not yet elucidated. If this molecule were to be used in a transgenic tsetse to reduce vector competence, issues such as glycosylation (pattern and role) need to be addressed. Answers to some of these points can be provided only when the gene encoding *Glossina* proteolytic lectin (Gpl) is cloned and expressed in a system that permits post-translational modification of proteins. The findings from such a study would provide baseline data in designing novel avenues of trypanosomiasis control, especially paratransgenesis.

1.3 Hypotheses

1) Protein glycosylation in *Glossina* may show some differences from the classically known O- or N-linkage.

2) The carbohydrate moiety is not crucial for the interaction of Gpl with trypanosome species

3) Shape is the main factor in the interactions between Gpl and D-glucosamine.

1.4 Objectives

1.4.1 Overall objective.

The overall objective of the present study is to undertake a functional analysis of midgut lectin in *G austeni*.

1.4.2 Specific objectives

i) To purify and biochemically characterize midgut proteolytic lectin from *Glossina austeni*

ii) To clone and express a functional midgut proteolytic lectin in a baculovirus expression vector system.

iii) To characterise the purified recombinant proteolytic lectin.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview on trypanosomiasis

Trypanosomiasis is a debilitating disease that affects man and many other mammals (both domesticated and wild). Technically, the form of the disease in animals is referred to as nagana while that in man is sleeping sickness (Human African Trypanosomiasis, HAT). Being a disease of the tropics, the variant in the new world is Chagas' disease. In man, trypanosomiasis has acute and chronic forms, and this depends on the species of the parasite. Parasites of the genus *Trypanosoma* are the causative agents of the disease. The human forms of the disease are caused by *Trypanosoma brucei rhodesiense* (acute form) and *T. brucei ganbiense* (chronic form). The diseases caused by the two species of the parasite are commonly known as Rhodesian sleeping sickness and Gambian sleeping sickness, respectively (Kreier, 1977).

The incidence of the disease is highest in rural areas of the affected countries, approaching a prevalence rate of about 60% in some villages in the Democratic Republic of Congo (DRC, Smith *et al.*, 1998; Seed, 2001). The scenario is also changing as HAT is fast becoming a huge public health problem in some big cities across Central (Kinshasa in DRC and Brazzaville in Congo Republic) and Western (Conakry in Guinea Republic) Africa (Miaka Mia Bilengue *et al.*, 2001). The most affected countries are mainly in sub Saharan Africa. In mammals other than man, other species of this parasite

cause a form of the disease known as nagana, which is derived from a Zulu term meaning "to be in low or depressed spirits"— a very apt description of the disease.

Though the symptoms of trypanosomiasis may vary, generally, it is characterized by an early fever stage (trypanosomal fever), when the parasites are mostly in the bloodstream, and at a later stage it affects the nervous system. In addition to being difficult to diagnose, the presence of trypanosome parasites in the nervous system often leads to personality changes in the infected individual (Kreier, 1977). An example is the change in the sleep-wake behaviour of the infected person. Gambian sleeping sickness is found in different areas of West and Central Africa, while Rhodesian sleeping sickness is found mainly in eastern and southern Africa and the Zambezi Basin. Transmission of trypanosomiasis from one host to another is by insect vectors of the genus *Glossina.* This vector is commonly referred to as tsetse (*Tsetse* is a word from Sechuana language which is spoken in Bechuana, Botswana). Several species of this vector exist. Examples include *G. fuscipes fuscipes, G. morsitans morsitans, G. austeni and G. pallidipes* (Kreier, 1977).

2.2 Therapy

For most tropical diseases, patients can readily be managed with a wide spectrum of drugs that meet certain safety standards. However, in trypanosomiasis, the story is different. The treatment of HAT patients depends on the species of the parasite and the how chronic it is. For example, first line treatment uses drugs like the suramin pentamidine,and

melarsoprol. Not only are these drugs toxic, they do not meet present day drug-safety standards (Atouguia and Costa, 1999). Eflornithine is a recently approved drug for trypanosomiasis. Another component of the treatment is the need for patients to be hospitalized, as treatment requires extensive and careful nursing, as well as follow-ups, to check for relapses (Molyneux and Ashford, 1983). The problem is complicated by the fact that there is little interest by pharmaceutical companies towards developing new anti-trypanosomiasis drugs as the disease affects only poor countries (McNeil, 2000) and within these countries, most of the sufferers are in rural areas (regions with extreme poverty). However, if there will be interest in novel drug discovery, the African rain forest may offer new anti-trypanosomal compounds as recently shown with extracts from *Kola acuminata* (Kubata *et al.*, 2005).

The epidemiology of the disease, the technicality in diagnosis as well as the absence of an adequate number of well-trained personnel in the affected areas are some of the other factors that contribute towards hampering the treatment of trypanosomiasis. Chemotherapeutic management of animal trypanosomiasis relies on the use of some relatively toxic chemicals such as suramin, quinapyramine, isometamidium chloride, and diminazene aceturate among others. In most cases, the administration of the drug causes local tissue necrosis in addition to the development of resistance by the parasite (Ross and Sutherland, 1997).

2.3 The Parasite

Trypanosomiasis is a disease caused by protozoan parasites of the genus *Trypanosoma*. The presence of a kinetoplast, long undulating flagellum and the existence of pleomorphic forms are some of the major characteristics of the cyclically transmitted trypanosomes (Lumsden and Evans, 1979) as indicated in Fig.1. Several species of this genus are known to date, and affect both man and other mammals, living extracellularly within the host (Smith and Hajduk, 1995). In addition to the species that affect man, other species are parasitic to other mammals and include *T. congolense, T. brucei brucei, T. vivax, T. simiae, T evansi* and *T. equiperdum* (Reduth *et al.*, 1991). *T.equiperdum* is a species of trypanosomes that is transmitted during coitus (Molyneux and Ashford, 1983), and so far, appears to have been isolated mainly from equines (horses, asses, and mules). In these animals the form of trypanosomiasis is referred to as dourine.

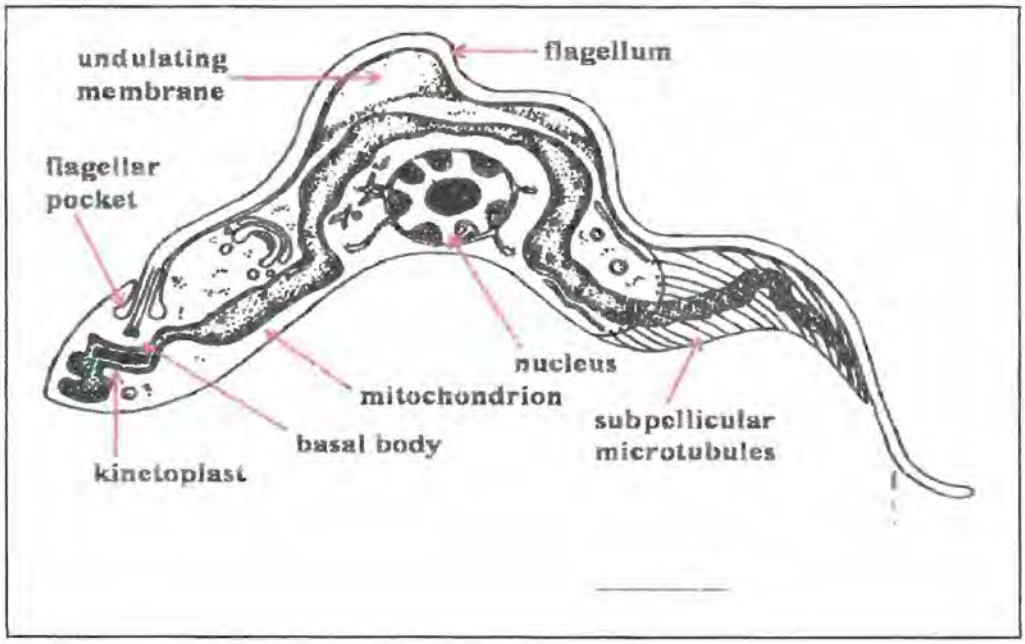


Figure 1. Diagram of *Trypanosoma* species. The distinguishing features of the organism such as long undulating flagellum, kinetoplast and a basal body are clearly indicated (*In* Parasitic diseases, chapter 6 edited by Despommier, Gwadz, Hotez and Knirsch).

The life cycle of cyclically transmitted trypanosomes involves stages or forms in the vertebrate host as well as forms in the insect vector. For these trypanosomes, the developmental cycle in the arthropod vector is an obligatory step, as transmission from one vertebrate host to the next via a vector is the major means of transmission. Additionally, mechanical transmission takes place on a small scale (Kreier, 1977), for example, transmission of *T. vivax*. A simplified diagram of the life cycle is shown in Fig. 2.

Essentially, *Trypanosoma* spp. can be classified as salivarian or stercorarian depending on the means of transmission. Salivarian are those that mature in the proboscis and salivary glands of the vector, and as such are injected into a new vertebrate host when the insect vector takes a blood meal. An example in this case is *T. brucei* complex. On the other hand, Trypanosoma species such as T. cruzi, are described as stercorarian since they undergo posterior development, which takes place in organs other than salivary glands and proboscis of the insect. Here contamination by the insect's feaces ensures transmission of this eukaryotic microbial parasite. Though salivarian and stercorarian mechanisms are the two major means by which trypanosomes are transmitted, mechanical transmission from one host to another, as is the situation with *T. evansi* and *T.vivax* (in areas out of the tsetse belt), also occurs. This usually involves the mouthparts of flies like the tabanids (*Tabanus* species) and *Stomoxys*, which therefore act as flying syringes. (Molyneux and Ashford, 1983).

Vertebrate bloodstream forms (long and slender as well as stumpy forms), when ingested during a blood meal, first establish and transform to procyclic forms within the midgut of the vector (Logan-Henfrey *et al.*, 1992). It is important to note that it is the short stumpy form, which is predetermined to continue development in the invertebrate host tsetse fly (Robertson, 1913). Thereafter, maturation to metacyclic infective forms takes place either in the mouthparts or salivary glands (Maudlin, 1985; Maudlin and Welburn, 1987; Abbelle *et a*l., 1999). The current accepted concept of bloodstream forms is that, at some time in their development within the mammalian host, some long slender forms are converted to short stumpy forms, in a population density-dependent (quorum-sensing) mechanism (Seed and Black, 1997; 1999). The process is believed to be promoted by a low molecular weight product, stumpy-inducing factor (SIF), which is of trypanosome origin (Reuner *et al.*, 1997). This is believed to reduce the number of parasites feeding on the host's circulating energy source, glucose (Seed and Wenck, 2003) as well as a means of preparing for invertebrate host (Robertson, 1913). This conversion from the long slender to the short stumpy form is an irreversible process. Similar phenomena of quorum sensing followed by an irreversible change are observed in the tsetse. For example, epimastigotes transform to metacyclics within the salivary glands of tsetse once a certain number is epimastigotes is attained (Seed and Wenck, 2003). While this process prepares the parasite for transmission through the vector, it also helps reduce the stress imposed on host's energy by the parasite, a means of

ensuring parasitism. Additionally, trypanosomes contribute to the pathogenesis by producing prostaglandins (Kubata *et al.*, 2000). One of these, PGD2, induces programmed cell death (apoptosis) in the parasite itself (Kubata *et al.*, 2005). The production of stumpy-inducing factor and PGD2 by trypanosomes, two molecules that maintain a check on the population, could be considered as strategies used by trypanosome parasites to ensure parasitism.

2.4. The vector

It is already an established fact, for more than forty decades, that tsetse of the genus *Glossina* are the vectors of cyclically transmitted trypanosomiasis. In a vast majority of the cases, transmission of trypanosomes from one vertebrate host to the other requires an obligatory developmental step that leads to the formation of infective metacyclic forms. This developmental differentiation and maturation from bloodstream (long slender and stumpy) forms to procyclics takes place within the tsetse vector (Welburn and Maudlin, 1989). The developmental process, of trypanosomes, within the tsetse vector is summarized on Fig. 3

There are twenty-two species of tsetse to date and these have been classified into 3 major groups: Morsitans, Palpalis and Fusca groups. Some of the species are again divided into subspecies. In total, there are thirty-three species and subspecies of tsetse.

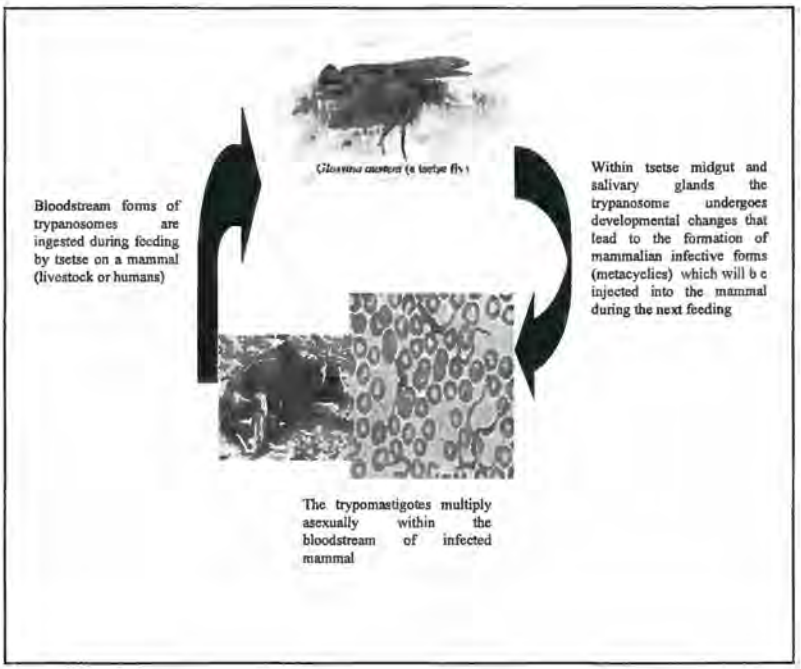


Figure 2. The life cycle of *Trypanosoma brucei* group involves stages in the mammalian bloodstream as well as stages within the vector, tsetse flies

The geographic distribution of the different groups is shown on Fig. 4. The Morsitans group is made up of flies that are mostly found in the savannah areas of Africa. This group is comprised of at least 7 species of *Glossina,* for example, *G. austeni, G. morsitans morsitans, G. swynnertoni* and *G. pallidipes.* The flies in this group are good vectors of all trypanosome species, most especially in eastern and southern Africa. The Palpalis group is found in the very humid areas of Africa, mangrove swamps, rain forests, lakeshores, and gallery forests along rivers. It comprises of 10 species, with examples such as *G. fuscipes fuscipes*, *G. palpalis* and *G. tachinoides.* This group is made up of vectors for trypanosomiasises in certain West Africa. The Fusca group, being the largest, is made up of at least 15 species, such as *G. fusca fusca and G. longipennis* (Kreier, 1977*).* Species of the fusca group inhabit areas ranging from dry land, for example Sudan, to forest regions (Pollock, 1982). The fusca group appears to comprise good vectors of *T. congolense* and *T. vivax.* In summary, *Glossina* of the morsitans and palpalis groups are the major vectors of trypanosomiasis (Pollock, 1982; Maudlin, 1991).

All adult tsetse flies (male and female) are strictly heamatophagous. i.e they feed solely on blood, principally vertebrate blood. Though all species of tsetse are capable of transmitting trypanosomiasis to man and other animals, it is important to note that different *Glossina* species have been reported to differ in their susceptibility to trypanosomes (Moloo *et al.*, 1992; 1998).

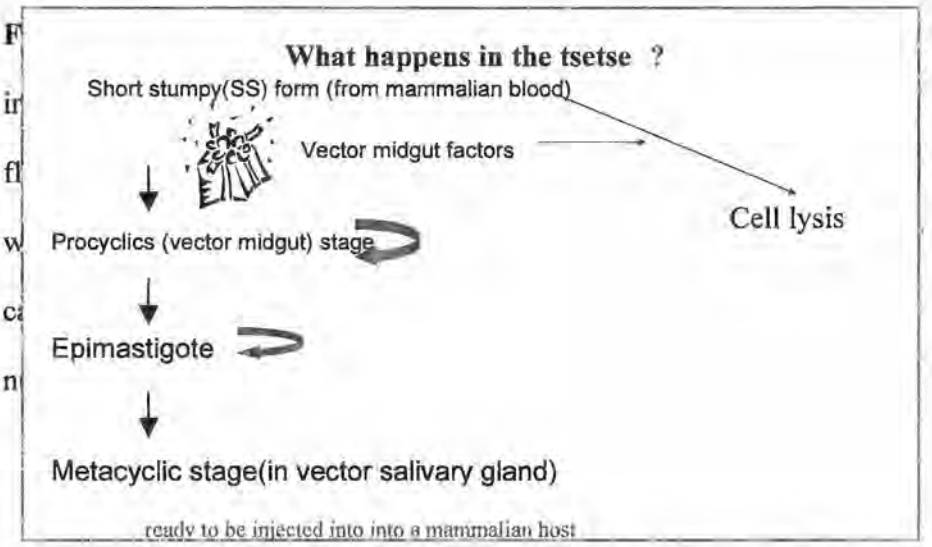


Figure 3. A summarized diagram showing the developmental process that ingested salivarian trypanosomes undergo once within the vector, tsetse flies. Factors of tsetse origin stimulate the different developmental stages while others, including midgut lectin and trypanolysin are capable of causing cell lysis. Also, amplification of trypanosome numbers at the level of procyclic and Epimastigote stages takes place.

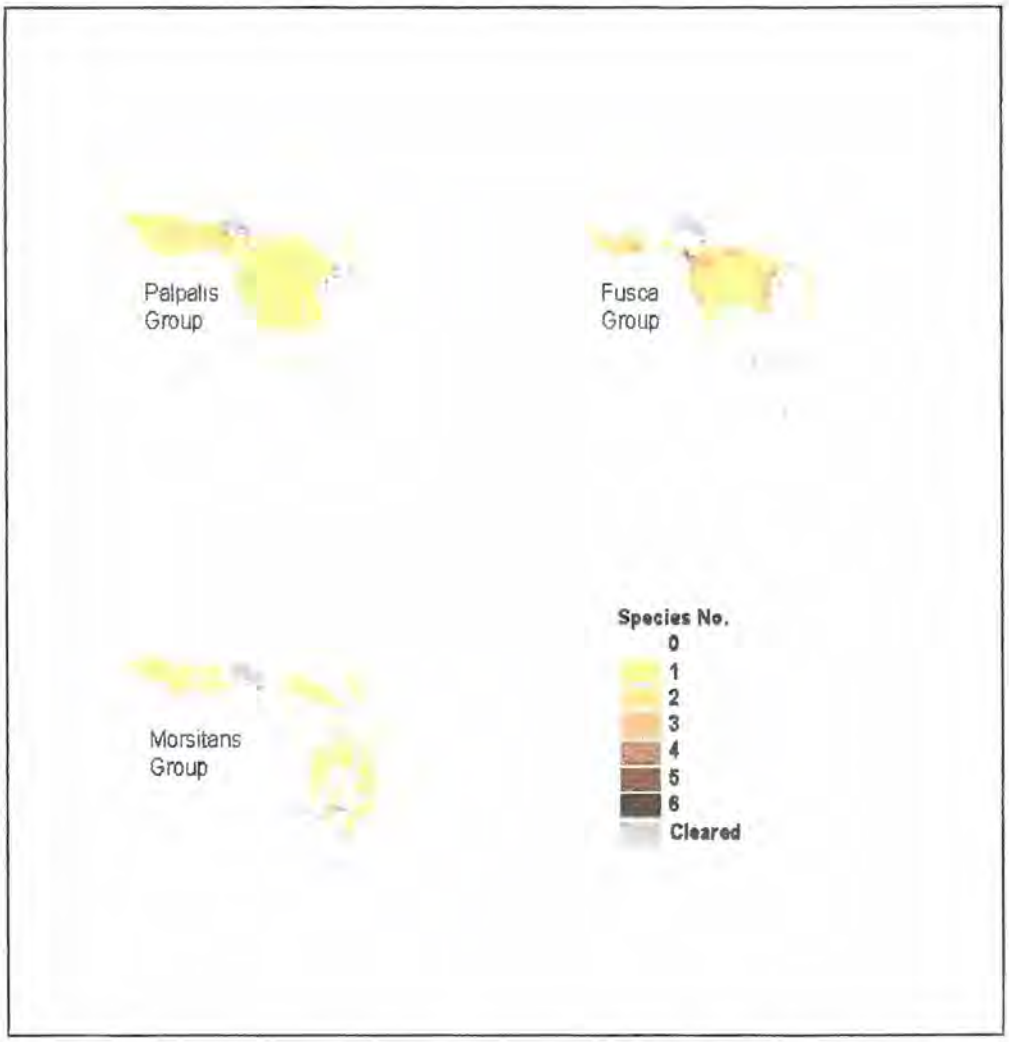


Figure 4 Distribution of the three groups of tsetse in Africa. The different species of tsetse are often classified into three groups, with each group mostly located within a specified geographical area (source: *Parasitic diseases*, chapter 6, 5th edidtion, published by Apple Tree Production).

For example, tsetse of the palpalis group is less susceptible compared to those of the morsitans group (Harley and Wilson, 1968; Roberts and Gray, 1972; Moloo and Kutuza, 1988; Moloo *et al.*, 1992; 1998; Reifenberg *et al.*, 1997). The major vectors of trypanosomes, therefore, are *G. morsitans morsitans*, *G. m. centralis*, *G. pallidipes*, *G. palpalis palpalis*, *G. fuscipes fuscipes*, and *G. tachinoides*. Despite this variation in susceptibility, tsetse is, in general, refractory to parasite (trypanosomes) transmission. For instance, only 1-20% infection rates have been observed in laboratory strains of tsetse, while in the field, infection rates of 1-5% only have been reported (reviewed by Hao et al., 2001). Lectin levels in the gut, fly species, sex, age and symbiotic associations within the fly are some of the factors accounting for this. *Sodalis glossinidius*, *Wolbachia* spp, and *Wigglesworthia* sp. are the endosymbionts of tsetse (Aksoy et al., 2001).

So far, the underlying mechanism of this refractoriness of tsetse is not yet fully understood, though it is highly believed to be lectins (Maudlin, 1991) that kill trypanosomes. In addition, some immune responsive genes have been characterized and fall into the class of antimicrobial peptides. The three groups of antimicrobial peptide genes characterized so far are attacins, defensins, and diptericins and were obtained from tsetse fat body tissue (Hao *et al.*, 2001). It has been reported that up-regulation of these genes prior to feeding on infective blood, can act to block trypanosome transmission (Hao *et al.*, 2001)

2.5 Vector control

The control of tsetse populations has been the pre-occupation of several international and national institutions/organizations for many decades now. Control strategies have employed both conventional and non-conventional approaches as outlined below.

2.5.1 Conventional approaches

For over forty years, trypanosomiasis control in Africa was based on aerial and ground spraying with DDT and other persistent insecticides as a result of the failure to control the flies by biological means. Such an approach led to considerable amounts of success in South Africa, Nigeria, Cameroon, Zambia (Allsopp, 2001) and a host of other African countries. In addition to aerial and ground spraying, with persistent insecticides, bush clearing was sought as a control strategy. However, due to the fact that these two methods were environmentally unacceptable, other strategies were developed. One of such strategies is the use of trapping techniques. Based on knowledge of tsetse behaviour and ecology, different traps have been developed that target specific species of tsetse. Some examples of the available tsetse traps are provided in Table 1. Generally, traps work by attracting tsetse flies, trapping them and killing them by the heat of the sun or by the use of an insecticide in the trap. To date, there are different variations of the tsetse traps. A common feature with these traps is the incorporation of blue and black colours. The blue colour serves to attract tsetse while the black is a 'deceptive resting 'place as these flies often rest

in shades in the wild. The different traps available are used for trapping different tsetse species. For example, the Ngu trap is used for trapping *G. pallidipes* (Table 1).

The efficiency of the trap can be greatly improved with the use of a tsetse attractant. An example in this case is a blend of cow urine and acetone. ICIPE has developed a synthetic repellent for tsetse, which is currently being tested in a push-pull strategy. This strategy relies on the use of two different chemical compounds, one of which repels the tsetse from hosts (push) while the other compound attracts the flies towards a trap where they can be killed (pull). The traps used originally have been simplified to cloth screens impregnated with insecticides. A further modification of this has led to the inclusion of live baits (for example, cattle) to which insecticide has been applied. Dipping, spraying and use of pour-on can apply insecticides on the live bait (Bauer *et al.*, 1992; Shereni, 1997; Baylis and Stevenson, 1998). A major advantage of this live bait technique is that a single farmer can comfortably use it on his livestock. In addition, the insecticide now widely used is a synthetic pyrethroid (for example, deltamethrin, and cypermethrin), which has a lower toxicity to mammals.

The sterile insect technique (SIT) is yet another tsetse control tool that has been experimented. It was used to successfully eliminate *G. austeni* from Zanzibar. The strategy here involves a mass release of sterile males into the environment with the aim that mating between these and females will lead to a systematic reduction in tsetse population. SIT does not

require the use of insecticides, and this makes it environmentally friendly (Feldmann and Hendrichs, 1998). Another technique recently developed has been the bait technique. It involves attracting tsetse flies with visual or olfactory bait to a point where the insect can be captured or killed (Vale, 1988).

Table 1. The different traps available for control of tsetse flies.

Trap name	**Target species**
Ngu	*G. pallidipes, G. longipennis, G. morsitans, G. fusciplueris*
Biconical	*G. pallidipes, G. longipennis, G. morsitans, G. fusciplueris,*
Nzi	*G. fuscipes, G. brevipalpis*
Pyramidal	*G. pallidipes, G. longipennis, G. morsitans*
Sticky trap	*G. fuscipes, G. austeni*
Vavoua	*G. palpalis*

2.5.2 Future prospects

The massive use of chemotherapy, insecticide and bush clearing brings with it numerous risks such as insecticide-resistant vectors and loss of biodiversity. This has led to the development of new vector control strategies that by-pass the old conventional methods. These novel strategies have as hallmark, the reduction of vector competence.

Transgenesis is one of such strategies (Aksoy *et al.*, 2001). This involves the elimination of the ability of vector insects to transmit pathogens. The principle behind this technique is the introduction and expression of foreign genes with anti-pathogenic properties that interfere with pathogen viability and transmission. Direct genetic manipulation of tsetse through conventional methods is not applicable with the tsetse vector. Conventional genetic modification of an organism relies on the introduction of the gene encoding for the trait into the embryonic stage of the target species. But in tsetse, the development from egg to late larval stage takes place within the female tsetse fly, and as such manipulation is not an easy task.

Fortunately, the tsetse vector is known to habour, in a symbiotic association, certain microorganisms. The three microbial organisms known to date are *Wolbachia* species, *Sodalis* species, and *Wigglesworthia* species. (Aksoy *et al.*, 2001). Though it is generally agreed that these microbes help supplement the highly proteinaceous diet of tsetse, it is now held that antitrypanosomal molecules could be expressed, via these microbes, in the tsetse (Cheng and Aksoy, 1999). This approach is termed paratransgenesis. However, this will require effector molecules to be identified. The fact that tsetse midgut proteolytic lectin has the capacity to lyse trypanosomes makes this molecule a potential target effector molecule for paratransgenesis.

2.6 Tsetse -Trypanosome interactions

2.6.1 General overview

The transmission cycle of African trypanosomiasis inevitably requires tsetse flies. The involvement of tsetse in this process was demonstrated through ingenious experiments nearly a century ago (Bruce *et al.*, 1909). When a tsetse fly, during a blood meal, feeds on an infected mammalian host, it ingests bloodstream forms of trypanosomes. The life cycle of this parasite, in the tsetse vector, goes through establishment (a necessary adaptation step) and maturation phases. First, trypanosomes in the tsetse blood meal get established in the midgut and later mature in mouthparts (*T. congolense*) or salivary glands (*T. brucei* group). Differentiation from bloodstream forms to procyclic (midgut) forms is the major change that takes place, and is now attributed to factors in the blood meal (Mihok *et al.*, 1995) and tsetse midgut (Otieno *et al.*, 1983; Imbuga *et al.*, 1992). Prior to these findings, several investigators had sought to account for the relationship between tsetse and trypanosomes, with the aim of explaining why only few tsetse infection rates could be obtained even in the wild (Maudlin, 1991). Among some of the tsetse midgut factors are digestive enzymes (Cheeseman and Gooding 1985), lectins (Welburn *et al.*, 1989), agglutinins (Ingram and Molyneux, 1990; Stiles *et al.*, 1990) and trypanolysins (Stiles *et al.*, 1990; Osir *et al.*, 1999). In a nutshell, these molecules interact with trypanosomes within the tsetse midgut. Lectins, which are carbohydrate-binding proteins, are capable of causing the agglutination (clumping) of trypanosomes and mammalian red blood cells

just like agglutinins. However, the latter do not bind to carbohydrates. Trypnaolysins are proteins of tsetse origin with the ability to lyse trypanosomes.

Once established in midgut, trypanosomes migrate towards the proventriculus and the mouthparts. Here, differentiation to epimastigotes occurs. This is followed by maturation, in the proboscis or salivary glands, to metacyclic forms (Maudlin, 1985; Maudlin and Welburn, 1987; Abbelle *et al.*, 1999). It stands out clear that establishment within the midgut is the first and most crucial step in the transmission cycle within the vector. This has led to numerous investigations on tsetse-trypanosome interactions, with the aim of identifying the factors involved.

Following earlier studies by Ibrahim *et al.* (1984) that reported on the presence of trypanoagglutinin in tsetse midgut homogenates, efforts, both *in vivo* and *in vitro*, have led to the association of trypsin (Imbuga *et al.*, 1992) and lectins (Welburn *et al.*, 1989; Osir *et al.*, 1995) as factors promoting establishment. However, certain levels of these factors, together with trypanolysins (Stiles *et al.*, 1990; Osir *et al.*, 1999) and other yet uncharacterized factors may also serve to lyse bloodstream forms of trypanosomes.

Imbuga *et al.* (1992), as well as Yabu and Takayanagi (1988), associated trypsin with the transformation of trypanosomes. Later a lectin with trypsin activity (Osir *et al.*, 1995: Abubakar *et al.*, 2003) was also shown to be a midgut factor involved in transformation of trypanosomes. Given the central role played by lectins in trypanosome establishment and

transformation, and lysis (Welburn and Maudlin, 1999) as well as the association of a trypsin moiety on lectin (Osir *et al.*, 1995), efforts have intensified towards clarifying this picture. Recently, a midgut proteolytic lectin, from *G. fuscipes fuscipes*, has been cloned and expressed in bacteria (Abubakar *et al.*, 2006). The general idea from the various findings points to the fact that tsetse midgut lectins play an important role in the transmission cycle of trypanosomes.

2.6.2 Blood meal induced changes in the tsetse

Numerous structural, biochemical as well as developmental changes take place in the tsetse midgut following ingestion of a blood meal. Structurally, a bilayered peritrophic membrane is secreted by the proventriculus and serves to protect the region that spans from the proventiculus to the midgut (Pollock, 1982; Lehane and Msangi, 1991).

The other changes that occur include secretion of proteases (Cheeseman and Gooding 1985; Imbuga *et al.*, 1992), lectins (Ibrahim *et al.*, 1984; Stiles *et al.*, 1990; Welburn *et al.*, 1989; Maudlin 1991) and trypanolysins (Stiles *et al.*, 1990; Osir *et al.* 1999). Secretion of these factors creates a hostile environment for trypanosomes, especially when these reach peak levels at 48-72 hours post blood meal (Onyango, 1993; Stiles *et al.*, 1990; Abubakar *et al.*, 1995; Osir *et al*, 1999). Most of the ingested trypanosomes are therefore eliminated.

Any surviving trypanosome will get established in the midgut region after getting to the ectoperitrophic space. This was initially thought to

occur either by circumnavigation or penetration of the bilayered peritrophic membrane. Recently, accumulated evidence, initially by electron microscopy (Evans and Ellis, 1983) and later by fluorescence microscopy (Gibson and Bailey, 2003), seem to point to the fact that direct penetration of the peritrophic membrane may be the major means by which trypanosomes get into the ectoperitrophic space Within the ectoperitrophic space, the successful parasite utilizes lectins to be transformed (Maudlin, 1985; Maudlin and Welburn, 1987).

2.7 Baculoviruses and the Baculovirus expression vector system

The *in vivo* expression of recombinant proteins is vital in many areas of research, especially in molecular biology. Expressed recombinant proteins have been, and continue to be, useful in the study of protein structure and function, protein-protein and protein-DNA interactions, antibody production as well as mutagenesis. Until recently, over-expression of such recombinant proteins relied on prokaryotic system. A major drawback of this system is that it does not offer for post-translational modification of proteins. In addition, the expressed proteins may be in insoluble form (inclusion bodies), making protein purification very difficult. These and other problems related to prokaryotic expression systems have been overcome through the development of eukaryotic expression systems. Several eukaryotic expression systems are commercially available now, and may either use yeast, insect or mammalian cells. Proteins expressed in eukaryotic systems are more likely

to be folded properly. In most cases, the correct post-translational modification as well as the proper targeting (for example, secretory) can be achieved. Of the different eukaryotic expression systems currently available, such as yeast and mammalian, the baculovirus expression vector system (BEVS) appears to be the most commonly used. Probably the reliance on this system is because the recombinant baculoviruses express high levels of eukaryotic gene products that are usually folded and processed in manner similar to the native proteins (Kamita *et al.*, 2001). BEVS relies on the use of some established insect cell lines together with a recombinant virus that carries the target gene to be expressed, to produce mass quantities of foreign proteins in insect cells.

2.7.1 Overview of baculoviruses

Baculoviruses all belong to the Baculoviridae family. Basically, this family is composed of virus pathogens infectious to arthropods. Though baculoviruses have been isolated from many insect orders, such as Diptera, Hymenoptera, Trichoptera and Lepidoptera, those from Lepidoptera are well characterised and are in greater use in the BEVS applications. Major characteristics of baculoviruses include an occluded structure containing a large rod-shaped virion in which is found a super coiled double stranded DNA. Usually, the genome size ranges from about 88 to over 160kbp.Viral particle occlusion, in a crystalline protein matrix, is a prominent feature among members of the Baculoviridae. Based on this, baculoviruses are classified into two genera; the nucleopolyhedrovirus (NPV) and

granulovirus (GV). A major difference between the two genera is the fact that, while the occlusion bodies of NPVs are polyhedron-shaped (polyhedrin is the major structural protein), the occlusion bodies in the latter are ellipsoidal in shape (the structural protein is granulin in this case) It is worth mentioning that occlusion bodies are particles containing viral nucleocapsids(s) embedded in a crystalline protein matrix.

NPVs can be found in a number of insect orders while GVs appear to be restricted to lepidoptera. Historically, baculoviruses have been used in the control of some insect pests, and with advances in molecular biology techniques, attempts have been made to improve upon their speed of kill. Experts believe that BEVS will be useful in the study of surface display of vaccine antigens (Boublik *et al.*, 1995; Grabherr and Ernst, 2001; Grabherr *et al.*, 2001), gene therapy (Condreay *et al.*, 1999; Kost and Condreay, 1999), as well as the generation of monoclonal antibodies (Lindley *et al.*, 2000)

2.7.2 Expression of foreign proteins in baculoviruses

The occlusion of a baculovirus requires polyhedra. The polyhedron gene found within the genome of a baculovirus encodes polyhedra. This gene is normally expressed at the very late stage of the baculovirus life cycle, under the influence of a very strong promoter. It is worth mentioning that just like any other viral life cycle, baculovirus life cycle can be divided into two phases: early and late (within this, there is the late and very late stages). In nature, polyhedra normally constitutes about 50% of total

protein. In as much as this protein is useful in the formation of occluded viruses, it is dispensable in cell culture. The BEVS therefore relies on the replacement of the polyhedron gene by a target gene, under the control of the polyhedrin promoter for mass expression. However, due to the large size of the baculovirus genome, its direct manipulation is problematic. This has been overcome by a strategy that exploits recombination of genetic materials. A target gene to be inserted into viral gnome is first cloned, in the proper orientation, into a baculovirus transfer vector, adjacent to either the p10 or the polyhedrin promoter. Numerous variants of baculovirus transfer vector are available commercially. In the next step, the recombinant transfer vector together with viral DNA will be used to co-infect susceptible cells. This process, co-transfection, leads to the production of a recombinant virus carrying the foreign target gene. The constructed recombinant virus is a powerful molecular tool, which can be used to express the recombinant protein either in an *in vivo* (in a larvae, e.g. *Bombyx mori* or *Trichoplusia. ni*) or *in vitro* (in cell lines, e.g. Sf-21, Sf-9, Hi 5 cells) system. The Sf21 and Sf9 cell lines are derived from ovarian tissues of the butterfly, *Spodoptera frugiperda.*

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental insects, animals and parasites

Male tsetse flies, *Glossina austeni*, were obtained from the Animal Rearing and Containment Unit (ARCU) of ICIPE. The *G. austeni* colony was established from pupae obtained from the Tanga Tsetse Laboratory based in Tanga, Tanzania. These flies were maintained at a relative humidity of 65%, and twelve hours of darkness followed by 12 h of light. The flies were fed, following a schedule, on pig blood through an artificial membrane. Male teneral flies were fed on pig blood, starved for 72 hours, fed again and then starved for 72 h before being dissected to obtain the midgut. Male wistar rats were also supplied by ARCU. The species of trypanosome parasite used in the studies was *T. brucei brucei*. The *T. brucei brucei* derived from ILTat 1.4 (Miller and Turner, 1981) were obtained from the International Livestock Research Laboratory (ILRI) based in Nairobi, Kenya. The *T. b. brucei* were maintained by both *in vivo* (in the male wistar rats) and in vitro passaging. The protocol for *in vitro* passaging was as described by Hirumi and Hirumi (1989) using both 24-well tissue culture plates and 25 ml T-flasks.

3.2 Preparation of midgut homogenates

Male teneral *G. austeni* that had been fed twice on pig blood, and then starved for 72 hours, were dissected to obtain midgut following the method

of Osir *et al.* (1993). Briefly, after brief chilling to immobilize the flies at +4 °C, midguts were dissected, washed in phosphate buffered-saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 8.0) to remove haemolymph and lipids, and resuspended in ice-cold PBS. Midguts were then homogenised using a homogeniser (Vertis, Gardiner, U.S.A) and centrifuged (10000rpm, 4°C, 30min) in a minifuge (Heraeus, Osterode, Germany). The supernatant of the homogenate was then filtered through a 0.22μm pore filter (Nalge, Rochester, N.Y., USA).

3.3 Preparation of parasite and red blood cells

Wistar rats (male) were infected with bloodstream forms of *T. brucei brucei* ILTat 1.4 intramuscularly (i.m.). Parasitised blood was collected from the rats by cardiac puncture, just before peak parasitaemia, into tube containing heparin (SIGMA, St. Louis, MO). Harvesting of parasites was achieved by the method of Lanham and Godfrey (1970). Essentially, the collected parasitised blood was spun (4000rpm, 10 min, RT) in a bench top medifuge (Heraeus Sepatech, Germany) and the buffy coat layer applied onto a packed diethyl amino ethyl (DEAE) Sephacel (SIGMA) column previously equilibrated with phosphate saline glucose (PSG, 10 mM Sodium phosphate, 150 mM sodium chloride, pH 7.4). The loaded column was washed with PSG and fractions collected into test tubes. Aliquots were applied onto a glass slide and covered with a cover slip. This was then observed under an inverted light microscope (Leitz Dialux, Germany). Washed parasite numbers were estimated using a haemocymeter fitted with

improved Neubauer ruling. Rabbit (New Zealand white strain) blood was collected and spun (2000rpm, 10 min, RT) in the Heraeus minifuge to isolate the red blood cells. Following the removal of plasma and buffy coat, the red blood cells pellet was washed five times in PBS (pH 7.4) with centrifugation (2000rpm, 10 min., room temp.).

3.4 Purification of the Glossina proteolytic lectin (Gpl)

Glossina proteolytic lectin (Gpl) was purified from the crude midgut homogenate of *G. austeni* by a 2-step chromatographic procedure involving anion exchange and glucosamine affinity chromatography.

3.4.1 Anion exchange chromatography

Diethylaminoethyl Sephacel (DEAE Sephacel; SIGMA, St. Louis, MO, USA) was used as an anion exchanger. The resin was soaked in 20 mM Tris-HCl, pH 8.0 (filtered through a 0.44μm pore filter and degassed) and later packed in a Pharmacia XK column (1.6x20 cm). The flow rate was adjusted to 10ml/h using a peristaltic pump (Micro tube pump MP-3, EYELA, Tokyo, Japan). This was connected to a fraction collector (Bio-Rad model 2128, Bio-Rad Richmond, CA, USA). The resin was equilibrated with the same buffer and crude midgut homogenate (approx. 90 mg) loaded. After washing unbound material with 20 mM Tris-Cl (pH 8.0), bound protein was eluted using a 0.0-0.5 M NaCl gradient in the same buffer. The gradient was created using a gradient maker G-1 (Pharmacia, Uppsala, Sweden). Fractions (2.0 ml) were collected and absorbance

values measured at 280 nm. The-collected fractions were assayed for trypsin and agglutinating activities. Those showing both activities were pooled and concentrated using polyethylene glycol (PEG). The excess salt, NaCl, was removed by extensive dialysis in 20 mM Tris-Cl, pH 8.0 (6 liters, 12 h)

3.4.2 Affinity chromatography

A glucosamine affinity column chromatography was used as a second step to further purify the proteolytic lectin. D (+) glucosamine (D-GlcN, SIGMA) was covalently coupled to epoxy-activated Sepharose 6B (SIGMA) following a protocol modified by Pharmacia from the methods of Sundberg and Porath (1974). Basically, the affinity column was prepared as follows: One gramme of epoxy-activated Sepharose 6B was swollen for 15 min in distilled water, and washed with 100 ml of distilled water using a sintered glass filter (Corning, Straffordshire, UK). The gel suspension (3 ml) was mixed with D (+) glucosamine (35 mg per ml of swollen gel) pre-dissolved in coupling solution [0.1 M carbonate/bicarbonate buffer (pH 9.0): dimethylformamide (50:50)] and incubated in a water bath with shaker (37°C, 16 h). Excess ligand was washed away with coupling solution, followed by distilled water, carbonate/bicarbonate (0.1 M, pH 8.0) and acetate buffer (0.1M, pH 4.0). Soaking the gel in 1 M-ethanolamine overnight at 45°C in a water bath with shaker blocked the remaining reactive groups. The coupled gel was washed finally with coupling solution followed by alternate washes with

0.1 M acetate buffer pH 4.0, containing 0.5 M NaCl and 0.1 M borate buffer (pH 8.0) containing 0.5 M NaCl.

The coupled resin was packed in a 10 ml Bio-Rad Econo-Pac column (Bio-Rad) and the column equilibrated with buffered insect saline (BIS; 10 mM Tris-Cl, pH 7.9, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) at a flow rate of 10 ml/h. Ion exchange sample that agglutinated trypanosomes (approx. 1 mg protein) was loaded onto the affinity chromatography column and unbound proteins were washed with BIS. Fractions (2 ml) were collected using a fraction collector (Bio-Rad model 2128) and absorbance values measured at 280 nm. Bound proteins were eluted from the column in BIS containing 0.2 M D-glucosamine. The collected fractions were analysed for trypsin and agglutinating activities. Only fractions that showed both activities were pooled. Following concentration with polyethylene glycol (PEG 40000, Serva, Uppsala, Sweden) samples were extensively dialysed in BIS at 4°C (8 liters, 18 h). The purified active sample was named *Glossina* proteolytic lectin (Gpl). Polyacrylamide gel electrophoresis was used to assess the purity of the sample, after protein concentration had been determined by the bicinchonic acid method using bovine serum albumin as standard (Pierce, Rockford, Ill, USA).

3.5 Determination of protein concentration

The Pierce BCA Protein assay kit determined the amount of protein in the crude midgut homogenate and the purified sample. Briefly, working reagent was prepared by mixing 50 parts of solution A (a solution of

sodium carbonate, sodium bicarbonate and tartrate in 0.02 N sodium hydroxide) with 1 part of solution B (an aqueous solution of 0.04 % copper sulphate). Two milliliters of the working solution was applied into test tube containing 30 µl of sample and 70 µl of 1 x PBS. The same volume of working solution was added into another tube containing 100 µl of 1 x PBS. The test tubes were incubated at 37°C for 30 min. At the end of the incubation the tubes were cooled to room temperature and the absorbance at 652 nm of each tube versus the blank (reference) read using the DU 640B Spectrophotometer (Beckman, CA, USA). The protein concentrations were then determined by extrapolating from the standard curve that had earlier been drawn with bovine serum albumin.

3.6 Agglutination assay

The agglutinating property of the protein on trypanosome parasites and rabbit red blood cells was carried out in flat-bottom and round-bottom plates as described by Abubakar *et al.* (1995). Briefly, two fold serial dilutions of the sample were made with PBS mixed with an equal volume of washed parasites (approx. 5 million parasites/ml) or 2% washed rabbit red cells. Plates were incubated at 27°C for 2 h and agglutination scored using an inverted microscope (Leitz Dialux, Germany). The experiments were done in duplicates. The reciprocals of the highest dilutions that showed complete agglutination of parasites or red blood cells were taken as agglutination titer. Washed parasites or red cells in PBS only constituted the negative control.

3.7 Trypsin assay

The enzymatic (trypsin) activity was assessed following the procedure described by Imbuga *et al.* (1992). This used the chromogenic substrate, chromozym-TRY (Roche Molecular Biochemicals, Germany). The reaction mixture (950 µl of 100 mM Tris-Cl, pH 8.0; 10 µl eluted sample) was equilibrated at 37°C for 5 min (or 30°C for 10 min.). The reaction was initiated by addition of 40 µmol (4 µl) chromozym-TRY. The total assay volume was maintained at 1.0 ml. The change in molar absorbance at 410 nm was monitored using a DU 640B spectrophotometer. The change in molar extinction coefficient at 410 nm (ε_{410}=8800; Erlanger *et al.*, 1961) was used to determine the amount of substrate hydrolysed; 1 unit trypsin was considered as the amount of enzyme required to hydrolyse 1 µmol chromozym-TRY/min at 30°C.

Enzyme activity =[(change in A_{410}/min)/ ε_{410}] x (V/v) x dilution factor

Where v= volume of chromozym-TRY, and V=total assay volume

3.8 Polyacrylamide gel electrophoresis

Protein samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Four to twenty percent gradient polyacrylamide gels were casted using a gradient maker (BRL, Gaithersburg, MA, USA), while the stacking gel consisted of 3.13% polyacrylamide. Samples were dissolved in equal volume of 1 x loading buffer (0.13 M Tris-Cl, pH 6.8, 20% glycerol,

0.002% bromophenol blue, 4% SDS, 1% beta-mercaptoethanol) and boiled for 5 min at 100 °C. After centrifugation (10,000 rpm, 5 min.), the denatured samples in the supernatant were loaded on the wells of the gel. Electrophoresis was carried out as follows; stacking at 25 mA, and the current stepped-up to 35 mA. The running buffer consisted of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. The molecular weights of electrophoretically separated samples were determined by co-electrophoresing marker proteins from molecular weight calibration kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A). At the end of the run, the Coomasie Brilliant Blue R250 or Silver staining method was used to stain the gels.

3.9 Coomasie Brilliant Blue R250 staining

At the end of the electrophoretic separation of proteins, the gels were carefully removed from the glass plates and subjected to Coomasie Brilliant Blue staining according to standard procedures (Ausubel *et al.*, 1989). Briefly, gels were stained in 0.6% Coomassie Brilliant Blue R 250 (w/v) in 50% methanol (v/v) and 9.2% acetic acid (w/v) for 2 h. Next, they were destained with several changes of destain solution 1 [50%methanol: 9.2% acetic and: 48.2% distilled water (v/v)] followed by destain solution 2 [5% methanol: 7.2% acetic acid: 87.5% distilled water (v/v)].

3.10 Silver staining

The procedure used was modified from that of Wray *et al.* (1981). The polyacrylamide gel was removed from the casting unit and fixed for 2 minutes in a fixative [50% methanol, 10% acetic acid (v/v)]. The gel was then washed twice in 50% methanol, 10 min each, and rinsed in distilled water for 5 min. After a second round of washing twice in 50% methanol (10 min per wash), the fixed gel was incubated in 5% of 50% glutaraldehyde solution for 30 min. This was followed by five washes in distilled water (5 min per wash). Next, the gel was washed twice in 50% methanol for 10 min each, and incubated in silver stain for 15 min. The silver stain was prepared as follows: solution A (0.8 g of $AgNO_3$ in 2.5 ml of water) was added in drops onto solution B (1.0 ml NaOH, 1.6 ml NH_4OH in 20 ml of distilled water), while solution B was continuously stirred. The volume of the solution mix was adjusted to 100 ml with distilled water. At the end of 15 minutes, the stained gel was rinsed three times in distilled water (5 min per rinse). Protein bands were revealed by finally soaking the gel in colour developer (2.5 ml of 1% citric acid, 125 µl of formaldehyde, diluted to 250 ml). Once the right protein band intensity was achieved, soaking the gel in 5% acetic acid halted colour development.

3.11 Effect of sugars on the agglutinating property of the proteolytic lectin

The effect of four different sugars (D-glucosamine, N-acetyl-glucosamine, D-glucose and D–galactose) on the agglutinating property of the purified molecule was investigated. In each case, three different

concentrations were used (100 mM, 200 mM, 500 mM). One microgram of purified protein was applied into wells of culture plates containing approx. 5 million parasites/ml in PBS. After addition of the sugar, the agglutination procedure was done as described above.

3.12 Periodic Acid Schiff (PAS) staining

Periodic acid Schiff stain is used for the detection of carbohydrate groups. The procedure employed here was as described by (Kapitany and Zebrowski, 1973) with slight modifications. Essentially, the protein sample was run on a SDS-PAGE (12.5%). At the end of the electrophoretic separation, the gel was rinsed in 7.5% acetic acid (v/v) (10 min, 25°C). Next, it was incubated in 1% periodic acid (w/v) for 15 min at four degrees centigrade. Then, it was washed with distilled water (6 washes at 5 min per wash) at 25°C. The washed gel was then incubated in Schiff's reagent for 15 min in the dark (4°C), after which it was washed 3 times (10 min per wash), at 25°C, with 0.5% (w/v) sodium metabisulfite. The gel was finally rinsed extensively in distilled water (6 washes of 5 min per wash) at 25°C.

3.13 Western blotting

Protein samples were separated on a gradient polyacrylamide gel (4-15%) by denaturing gel electrophoresis according to the method of Laemmli (1970). Gradients were cast using a gradient maker (BRL, Gaithersburg, Md., USA). Proteins separated by SDS PAGE were

transferred to nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Dassel, Germany) using a 2117-250 NOVABLOT Electrophoretic Transfer Kit. Blotting was performed for 2 h at 0.8 mA/cm^2 at RT. After rinsing in distilled water, the blot was stained for proteins with 1%(w/v) Ponceau red, incubated for 4 h in blocking buffer (25 mM Tris-Cl, pH 7.4, 3 mM KCl, 137 mM NaCl, 1% albumin). Incubation with primary antibody (diluted 1:300) was carried out at RT for 2 h in the same buffer. This was followed by three washes, 5 min each, with Tris/NaCl/KCl/Tween-20, and then 2 h of incubation in secondary antibody (anti-rabbit IgG, ALP-linked, 1:1000 dilution) The enzyme reaction was developed in the dark, at 25°C for 4 min, using 4-nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoly-phosphate 4-toluidine salt (BCIP, SIGMA, USA).

3.14 Isolation of RNA from *G. austeni* midgut

Fifty male teneral *G. austeni* were fed 24 hours after emergence on pig blood through an artificial membrane and starved for 72 h. These were fed again following the same procedure and starved for another 72 h. Next, their midguts were dissected and homogenized in a 1 ml pre-chilled denaturing solution (26 mM sodium citrate, pH 4.0, 0.5% N-lauryl sarcosine, 0.215 M β-mercaptoethanol, 4 M guanidine thiocyanate) using a homogeniser (Vertis, Gardiner, NY, U.S.A). Total RNA was then extracted using an RNA extraction kit (Promega, Madison, WI, U.S.A). Briefly, 100 μl of 2 M sodium acetate (pH 4.0) was added to the homogenate and mixed thoroughly by inversion, followed by 1 ml phenol: chloroform:

isoamylalcohol (25:24:1), and the content chilled on ice for 15 minutes. The suspension was then transferred to a sterile micro centrifuge tube and spun (14000 rpm, 4°C, 10 min) in a Heraeus 2 minifuge. The top aqueous layer was carefully transferred to a clean sterile tube and an equal volume of isopropanol added. The suspension was mixed by inversion and RNA precipitated by incubating at -70°C for 30 min. At the end of the 30 min period, the tube was spun (14000 rpm, 4°C, 10 min) to pellet the RNA. After decanting the supernatant, the pellet was resuspended in 200 µl of denaturing solution. Following the addition of an equal volume of isopropanol, the solution was mixed and incubated again at -70°C for 30 mi. After centrifugation as described above, the resulting pellet (total RNA) was washed twice with 2.5 volumes of pre-chilled 70% ethanol, air-dried in an RNase-free environment and aliquots were immediately at -70°C. An aliquot was used to check the integrity and purity of the RNA by agarose gel electrophoresis.

3.15 cDNA synthesis from total RNA

A SMART^{TM}cDNA library construction kit and protocol (Clontech, Palo Alto, CA) was used to prepare the cDNA according to the manufacturer's instructions.

3.15.1 Synthesis of first strand complementary DNA (cDNA)

The enzyme employed in this process was a Powerscript™ reverse transcriptase (RT), a point mutant of Moloney murine leukemia virus (MMLV) reverse transcriptase, which lacks RNase H activity but has polymerase and terminal transferase activities. Also, a modified oligo (dT) primer (CDSIII/ 3' PCR primer) and an oligo (G) primer (SMART IV oligonucleotide) provided in the kit were used. Three microliters of total RNA (~1.0 μg) was mixed with 1.0 μM of each primer and incubated (72°C, 2 min) in a thermal cycler (PTC 100, MJ Research, NJ, USA). The contents were then cooled on ice and spun briefly before addition of 1 x first strand buffer (250 mM Tris, pH 8.3, 30 mM $MgCl_2$, 375 mM KCl), 2.0 M dithiothreitol (DTT), 0.25 mM dNTPs and 1.0 μl Powerscrpot RT. The 10 μl content was mixed gently and spun briefly before incubation at 42°C for 2 h. The reaction was terminated at the end of the incubation period by cooling in ice. The resulting single-stranded cDNA was used in a PCR reaction to generate full-length ds cDNA.

3.15.2 Generation of double stranded cDNA by polymerase chain reaction

Long distance polymerase chain reaction (PCR) was used to prepare the full-length ds cDNA from the first strand. The 100 μl reaction mixture contained 1x Advantage 2 PCR buffer, 0.25 mM dNTPs, 200 pmol of each primer, 1x Advantage 2 polymerase mix and 2 μl of the first strand single

stranded (ss) cDNA as template. PCR was performed on a preheated (95°C) PTC-100™ (MJ research Inc.) under the following cycling programme: initial denaturation at 95°C for 20 sec., followed by 24 cycles of denaturation at 95°C for 5 sec. and annealing at 68°C for 6 min. The product was analysed on a 1.2% agarose/EtBr gel by electrophoresis.

The DNA polymerase used for ds cDNA synthesis was inactivated by Proteinase K treatment of the PCR product. Three micrograms of amplified ds cDNA was mixed with 0.8 μg of Proteinase K and incubated (45°C, 20 min). The digest was diluted twice with deionised water and cDNA extracted with an equal volume of phenol: chloroform: isoamyl alcohol. The phases were separated by centrifugation (14000 rpm, 5 min) and the top aqueous layer carefully transferred into a clean tube. To precipitate the cDNA, 3 M sodium acetate, 20 μg/μl glycogen and 95 % ethanol (at RT) were added and the mixture immediately centrifuged (14000 rpm, 20 min, RT). The cDNA pellet was washed with 80% ethanol, air-dried (10 min) and resuspended in nuclease-free deionised water

3.16 Construction of midgut cDNA expression library

The cDNA expression library was constructed from the synthesized ds cDNA following the SMART™ cDNA Library construction kit protocol (Clontech, CA, USA).

3.16.1 Sfi 1 restriction enzyme digestion

Following the verification of the cDNA quality by agarose/EtBr gel electrophoresis, the product was digested by Sfi 1 restriction endonuclease in order to incorporate asymmetric Sfi 1 restriction sites at the 5' and 3' cDNA ends. Two units of Sfi 1 restriction enzyme, 1x Sfi buffer and 1xBSA were added to the cDNA and the mixture incubated (50°C, 4 h). At the end of the incubation period, 1% xylene cyanol was added to the total reaction mixture (100 µl) and the digested cDNA size-fractionated as described in the next section.

3.16.2 cDNA size fractionation

Sfi-1-digested cDNA was size fractionated using a CHROMA SPIN-400 column (Clontech, CA, USA). The columns were equilibrated with 700 µl of column buffer at a flow rate of 1 drop/50 sec. The 100 µl reaction mixture from the Sfi 1 digestion reaction, containing xylene cyanol, was applied to the top of the column and allowed to absorb. The cDNA was eluted using 600 µl of column buffer and 35 µl fractions collected. The collected fractions were monitored on a 1.1% agarose/Ethidium Bromide gel by electrophoresis. Peak fractions were determined by visualizing the intensity of the cDNA bands under Ultra Violet (long wavelength). These were pooled (fractions 1-4), and precipitated in 1/10 volumes of sodium acetate (3 M, pH 4.0), 0.25 µg/µl of glycogen and 2.5 volumes of 95 % ethanol at -20°C overnight.

Recovery of digested and fractionated cDNA was achieved by centrifugation (14000 rpm, 20 min, RT), air-dried (10 min) and resuspended in 7 µl of nuclease-free deionised water.

3.16.3 Ligation of cDNA to pTriplEx2 vector

The size fractionated and precipitated cDNA was ligated into an Sfi 1-digested and dephosphorylated λTriplEx2 vector. Two ligation reactions were set up to determine the optimal ratio of cDNA to vector required. The prepared cDNA (1.0 µl, 1.5 µl) was each mixed in a 100 ng/µl vector, 1xligation buffer, 1.0 mM ATP, 40 units T4 DNA ligase and the volume adjusted to 5.0 µl with nuclease-free deionised water. The ligation mixtures were incubated at 16°C overnight. A control reaction (10 ng control insert with Sfi1 A & B) was also set up.

3.17 Screening of cDNA library with antibody against proteolytic lectin

The pTriplEx2 cDNA library was screened with polyclonal antibodies against *Glossina* proteolytic lectin that had been prepared. The screening procedure was according to Sambrook *et al.* (1989). Briefly, the serum was preadsorbed with *E. coli* lysate at 1:10 dilution to reduce nonspecific binding to bacterial proteins. The preadsorbed serum was further diluted to a working dilution at 1:500.The library (in *E. coli* DH5α) was plated on Luria-Bertani agar/ampicillin plates and about 5000 colonies were transferred to nitrocellulose (Hybond C, Amersham, Cleveland, OH). After blocking with 5% non-fat dry milk (25°C, 2 h), the filters were incubated

with polyclonal antibody to *Glossina* proteolytic lectin (4°C,overnight). At the end of the incubation, the filters were washed twice (5 min. per wash) in Tris buffered saline (TBS) and incubated with goat anti-rabbit conjugated alkaline phosphatase secondary antibody (Bio-Rad, CA, USA). Positive clones were identified by incubating with 5 bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) substrate (Sigma, MO, USA).

3.18 Plasmid purification (mini-prep) by alkaline lysis

Following the identification of positive clones by antibody screening, plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, Germany) according to the manufacturer's protocol. Essentially, 5 ml overnight cultures of *E.coli* carrying the plasmid were prepared by inoculating a single positive colony in Luria-Bertani broth (LB medium, 10g/l bactotryptone, 5g/l bacto-yeast extract, 10 g/l NaCl, pH 7.0) with 125 μg/ml ampicillin. Bacterial cells were then pelleted by centrifugation (10,000 rpm, 5 min) and resuspended in 250 μl of buffer P1 containing RNase A. Two hundred and fifty microliters of buffer P2 was added to the cell suspension and mixed gently by inversion. The lysate was neutralized by addition of 350 μl of buffer N3 and mixed thoroughly by inversion. Plasmid DNA was separated from chromosomal DNA and other cell debris by centrifugation (14000 rpm, 10 min). The supernatant was applied on a QIAprep column membrane and spun briefly (1 min) to bind plasmid DNA. The column was washed with buffer PE (750 μl) and spun again

(14000 rpm, 1min) and column centrifuged another 1 min (14000 rpm). The bound plasmid DNA was finally eluted in 50 µl of sterile distilled deionised water.

3.19 Gene sequencing and bioinformatics analyses

The recombinant plasmids were submitted for sequencing at ILRI Nairobi automated sequencing. The National Center for Biotechnology Information (NCBI, Bethesda, MD) BLAST computer search program was used to perform sequence homology searches against public databases (Altschul *et al.* 1997). Sequence alignment and statistical analysis of alignments were performed using the MultiAlign program (Corpet 1988).

3.20 Recombinant Protein expression in *E. coli*

Recombinant Gpl was expressed in *E.coli* strain BL21 from a pTriplEx2 vector containing the full-length cDNA of gpl. The transformed bacteria were cultured in LB medium containing 125 µg/ml ampicillin and incubated at 37°C until they reached a log phase of growth. At this point 5 ml of cells were transferred aseptically to a flask containing 300 ml of TYP broth (16 g Bactotryptone, 16 g yeast extract, 5 g NaCl, 2.5 g K_2HPO_4). Protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG; 1 mM final concentration) when the OD_{600} reached 0.6 (approximately 2 h). The induced cells were allowed to grow overnight (12 h) in a vertical incubator shaker (Environ-shaker 3597-1, Lab-line instruments, Inc.). The cells were pelleted by centrifugation (3000 xg, 4°C, 10 min), and resuspended in PBS (pH 8.0) and sonicated on ice as described above.

Next, the lysate was centrifuged (8000 xg, 20min, 4°C) in the same centrifuge and the resultant supernatant extensively dialysed against 6 liters of BIS (10 mM Tris-Cl, pH 7.9, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) for 8 h. The recombinant Gpl was purified by anion exchange and affinity chromatographic techniques as described above.

3.21 Preparation of polyclonal antibodies

Polyclonal antibodies to purified recombinant Gpl were raised in a male New Zealand white rabbit following the procedure described by Osir *et al.* (1989). The rabbit was obtained from ARCU. Briefly, pre-immune serum was obtained from the rabbit by bleeding the marginal ear vein. Three hundred microgramme of the purified recombinant Gpl was emulsified in an equal volume of Freund's Complete Adjuvant and injected subcutaneously at the rabbit hind thigh. A booster dose (100 μg of sample), mixed with an equal volume of Freund's Incomplete Adjuvant, was administered 3 weeks later in the other hind thigh. The animal was bled a week later through the main ear artery, and the collected blood allowed to clot at 4 °C overnight. The serum was separated from the clot and crude immunoglobulin mixture isolated from the serum. Basically, the serum was diluted in an equal volume of PBS (pH 7.2), precipitated in 50% saturated ammonium sulphates, and the precipitate collected after centrifugation (9000 rpm, 4°C, 15 min). It was then dissolved in PBS and dialysed extensively in PBS (8L, 12h) at 4°C.

3.22 Baculovirus expression of protein

3.22.1 Cloning of Gpl into transfer vector, pAcUW21

3.22.1.1 Amplification of Gpl with specific primers containing Bgl II sites

The complete open reading frame of *Glossina* proteolytic lectin from *G. austeni* (Gpl) was amplified from pTriplEx2gpl with gpl specific primers, designed with *Bgl* II restriction sites, by PCR. The forward primer sequence was 5'GCA GAT CTA TGA AGT TCT TTG CAG TGT TC 3' while the reverse primer had the sequence 5'GCA GAT CTT TAC AAA AGT TGC GCA TAG TT 3'. The underlined bases represent the recognition sequence for the enzyme, *Bgl* II. Both primers had a GC clamp. The 100 µl PCR reaction mix consisted of 1 ng of template (template), 1 x *Pfu* buffer, 8 µl of 2.5 mM dNTP mix, 100 pg of each primer, 2 units of *Pfu* polymerase and 76 µl of sterile triple distilled deionised water. The amplification conditions were; an initial denaturation at 94°C for 2 min., 39 cycles of 94°C for 1 min, 50°C for 1min, and 72°C for 1 min. The final extension step was performed at 72°C for 3 min. At the end of the run, the sample was analysed on a 1.2% agarose/EtBr gel by electrophoresis. The amplified band was gel purified and cloned into a Sma1-digested and dephosphorylated pUC 19 vector (Clontech).

3.22.1.2 Gel purification of amplified fragment and cloning into pUC 19

The amplified PCR product was analysed on a 1.2% agarose/EtBr gel by electrophoresis (80V, 1h). The gel casting and running buffer was 1 x TAE (0.4 M Tris acetate, 0.002 M EDTA)). After 1 h of run, the gel was

exposed briefly over long wave UV light and the amplified band excised with a sterile scalpel. Purification of the target band from the gel slice was achieved by using a Qiagen gel extraction kit (Qiagen, Valencia, U.S.A) following the manufacturer's protocol. Essentially, 1ml of buffer QG was added onto the 0.250 mg gel slice. Heating at 50°C, for about 10 min made the gel slice to dissolve. Next, 100 µl of isopropanol was added and the mixture applied into the spin column. This was spun at 13000 rpm for 1 min and the column washed with 500 µl of buffer QG. After a final wash with 750 µl of buffer EB (13000 rpm, 1 min), the flow through was decanted and the column spun again at 13000 rpm for 1 min. Elution of the band from the column was done with 50 µl of buffer EB (10 mM Tris-Cl, pH 8.0).

To clone this product into pUC 19 vector, approx. 4 µg of the eluted product was ligated to 5 ng of a previously Sma I digested and alkaline phosphatase dephosphorylated pUC 19. The ligation mix contained 20 µl of insert, 2 µl of vector, 3 µl of 10x ligase buffer, 5 µl of water and 3 µl of T4 DNA ligase. This mixture was incubated at RT for 3 h. At the end of the incubation, 6 µl of the ligation mix was added to 60 µl of chemically competent cells (E.coli XL-1 blue; Strategene?). Following incubation in ice for 30 min, cells were heat-shocked at 42 °C for 30 sec and 1 ml of SOC medium (2.0g Bactotryptone, 0.5g yeast extract, 1 ml 1 M NaCl, 0.25 ml 1 M KCl, 1 ml 2 M $MgSO_4$, 1 ml 2 M glucose) was added. The mixture was incubated for 1 hour at 37°C. After pelleting the cells (13000 rpm, 4

min), the cell pellet was plated on an LB/ampicillin plate and incubated at 37°C overnight.

3.22.1.3 *Bgl* II digestion and cloning into pAcUW21 vector

Ten clones from the overnight culture above were selected and screened by PCR, using Gpl-specific primers. Replicas of the selected clones were made on a fresh LB/ampicillin plates. Eight of the ten clones contained the recombinant pUC carrying gpl. A clone from among the positive was picked from the replica plate and used to inoculate a 5 ml LB broth. After overnight incubation at 37°C in an incubator shaker (Environ-shaker 3597-1, Lab-line Instruments Inc., IL, U.S.A), plasmid was extracted by the miniprep procedure using a Qiagen plasmid miniprep kit. Approximately 5 μg of the extracted plasmid was subjected to restriction digestion with 3 units of Bgl II. The 20 μl reaction mix also included 2 μl of 10 x reaction buffer 3 and 5 μl of water. The reaction mix was incubated at 37°C for 2 h. At the end of the incubation, the sample was analysed on a 1.2% agarose gel and the target band excised. The band was purified from the gel slice using a Qiagen gel extraction kit. In the next step, the eluted band was ligated to Bgl II-digested and dephosphorylated pAcUW21. The ligation mix contained 4 μg of insert, 2 ng of vector, 2 μl of 10xligation buffer, 3 μl of water and 3 units of T4 DNA ligase. After mixing, the tube was incubated at RT for 3 h, at the end of which 5 μl was used to transform competent cells as earlier described. After overnight culture, clones were

picked and insert verified by PCR. Further verification, by restriction digestion with *Bgl* II and *Eco* RI, was performed on the plasmids, which were extracted using Qiagen plasmid extraction kit as earlier described. The plasmid was also submitted for sequencing to verify the integrity of the sequence as well as the correct orientation.

3.22.2.0 Generation of recombinant baculovirus containing Gpl

3.22.2.1 Co-transfection of SF 21 cell lines

Co-transfection mediated by Cellfectin, was used to introduce DNA into Sf-21 cells. The co-transfection was conducted as described by Choudary *et al.* (1995), with slight modifications. Briefly, about half a million Sf-21 cells (approx. 50% monolayer) were seeded in a 35 mm-diameter culture dish and allowed to attach within 1 h. During this period, solutions A and B were prepared: For solution A, 5 µg of recombinant transfer vector plus 100 ng of linearised viral DNA (chitinase negative, AcMNPV [chi-]) were added into a 1.5 ml microfuge tube containing 100 µl of serum free medium. Solution B consisted of 6 µl of Cellfectin (Invitrogen, Calabasas, CA) basically as described by O'Reilly *et al.* (1992) and 100 µl of serum free medium. Solutions A and B were then combined in a polystyrene tube, mixed gently and incubated at RT for 30 min to allow complex formation. Near the end of the 30 min. RT incubation, the seeded cells were washed once with serum free medium. At the end of the incubation, 800 µl of serum free medium was added to the mixture of solutions A and B, gently mixed, and the solution added drop-

wise onto the washed cells. This was then incubated at 27°C for 5 h. At the end of this incubation period, the inoculum was exchanged with 2.0 ml of complete medium containing 2x kanamycin (4 µl of 1000 x per 2.0 ml), and further incubated at 27°C for 3 days. The medium was then harvested at the end of the 3 day period and used for plaque assay.

3.22.2.2 Purification of recombinant virus by Plaque Assay

Recombinant virus carrying Gpl was purified from the co-transfected cells by standard plaque assay procedures as described by King and Possee (1992) with slight modifications. Briefly, a 6 well plate (Costar, U.K) was seeded with $1.5x10^6$ Sf21 cells per well in a 2.0 ml TC-100 medium (complete with 5% Foetal calf serum). The seeded cells were incubated at 28°C for 2 h to allow the cells to settle. Serial dilutions of the virus – containing medium from co-transfection were made; the dilutions ranged from 10^{-5}-10^{-9} and were made with TC-100 medium without serum. At the end of the 2 h incubation, the medium was carefully removed and the monolayer of attached cells washed once with TC-100 without serum. The cells in each well were then overlaid with 100 µl of the appropriate dilutions by drop wise addition of the medium with virus to the center of the well. Wells were labeled A to E and the dilutions 10^{-5},10^{-6},10^{-7},10^{-8},10^{-9} used in the respective wells. The plate was incubated at room temperature for 1 h to allow the virus to adsorb to the cells. At 15 min interval, during the incubation period, the plate was rocked gently to ensure even coverage of the virus. During the 1 h incubation an agarose overlay was prepared.

3.22.2.3 Isolation of viral DNA for PCR

Recombinant virus from the picked plaque was isolated and used for PCR as described below. One thousand microliter of viral supernatant was spun at 25000 rpm (30 min, 5°C) without a sucrose cushion. The pellet was then resuspended in 100 µl of Tris/EDTA (10:1). Next $1/20^{th}$ volume (5 µl) of 20 mg/ml Proteinase K and $1/10^{th}$ volume (10 µl) of 10% SDS was added to release the viral DNA and the mixture incubated at 57°C (water bath) for 1 h. At the end of the incubation, the DNA was extracted twice with phenol, once with phenol: chloroform, and twice with chloroform. One microliter was then used for PCR.

3.22.2.4 *In vitro* expression of Gpl in susceptible insect cell lines

The plaque purified recombinant virus AcMNP.gpl was used to express Gpl in relatively large amounts in a suspension culture. First, a monolayer of Sf-21 cells in a T75 flask was infected with the recombinant virus AcMNP.gpl at a titer of $2.5x10^8$ following the procedure described by King and Possee (1992). At 72 hpi (hours post infection), the medium was harvested and spun at 300 rpm for 5 min, and the supernatant transferred into a 500 ml flask containing a suspension culture of cells at 10^6cells/ml. The multiplicity of infection was 10pfu/cell. The virus-infected suspension culture was incubated at 28°C for 72 h, at the end of which the medium was harvested. Cell debris was removed by spinning at 3000 rpm for 5 min, and the supernatant used for biological assay and protein purification.

Western blot was carried out as earlier described (section 3.13). The negative control experiment was protein harvested from AcMNPV.LacZ-infected Sf-21 cell lines.

3.24. Immuno-affinity purification of baculovirus-expressed Gpl

In order to purify the baculovirus-expressed Gpl, an affinity matrix was generated by coupling polyclonal antibodies that were generated against the recombinant bacteria-expressed Gpl to cyanogen bromide–activated sepharose 4B (CNBr-activated Sepharose 4B; Pharmacia, Piscataway, NJ) following the manufacturer's protocol. The affinity column (3.5 ml) was equilibrated with wash buffer (10 mM Tris-Cl, pH 8.0, 0.14 M NaCl, 0.025% NaN_3). There after, 1.5 ml of the concentrated supernatant of virus-infected cells (see above, containing approximately 3 mg of crude protein) was loaded onto the column. The gel was first washed with 50 ml of wash buffer and secondly with 40 ml of buffer A (50 mM Tris-Cl, pH 8.0, 0.1% triton X-100, 0.5 M NaCl). The bound proteins were eluted with 40 ml of buffer B (50 mM Tris-Cl, pH 9.0, 0.1% Triton X-100, 0.5 M NaCl). The flow rate was maintained at 250 µl/min using a peristaltic pump for all of the washes and elution. The absorbance (A_{280}) of the eluate (1 ml per fraction) was measured using a DU 640B spectrophotometer (Beckman, Fullerton, CA). The eluted sample was first dialysed against 6 L of PBS (pH 8.0) for 12 h and then concentrated with PEG 40,000 (Serva), then further dialysed against 6 L of PBS (pH 8.0) for 8 h.

3.25 Purification of recombinant Gpl by a glucosamine coupled to CNBr-activated Sepharose 4B

An affinity column in which glucosamine was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) was also used to purify the baculovirus expressed protein. The glucosamine was covalently linked to the activated Sepharose 4B through the amino group of the sugar following the procedure provided by the manufacturer. Essentially, 2 g of CNBr-activated Sepharose 4B were weighed and swollen in 1M HCl. The swollen gel was washed for 15 min on a sintered glass and mixed with D-glucosamine in coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl). The mixture was rotated end-over-end for 2 h at RT. At the end of the rotation, excess ligand was washed away with coupling buffer and any remaining active group blocked with 0.1 M Tris-Cl buffer (pH 8) by incubation at room temp for 2 h. Finally, the coupled gel was washed with three alternating cycles of acetate buffer (0.1 M, pH 4) containing NaCl (0.5 M) followed by 0.1 M Tris buffer (pH 8.0) containing 0.5 M NaCl.

3.26 Functional characterisation of recombinant Gpl

Purified bacterially expressed and baculovirus expressed proteins were assayed for their ability to cause agglutination of washed rabbit red cells and trypanosome bloodstream forms. The recombinant proteins were also assayed for trypsin activities. Both experiments were carried out as described above.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 CHARACTERISATION OF NATIVE PROTEOLYTIC LECTIN

4.1.1 Purification of authentic *Glossina* proteolytic lectin

A two–step purification procedure involving anion-exchange and affinity chromatography was employed to isolate Gpl from *G. austeni* midgut homogenate. The homogenate, with 90 mg of total protein, was prepared from 200 twice-fed and starved (72h) male teneral *G. austeni*. The first step, anion exchange chromatography, produced the profile shown in Fig.5. Fractions from peaks III and IV, which showed agglutination activity, were pooled and applied on the D-glucosamine affinity column. The agglutinating and trypsin activities were co-eluted in the bound fraction (peak II, Fig. 6). The successful binding of the purified protein to both columns is a clear indication that the authentic protein has an overall net negative charge and it also interacts with sugars. Ion exchange chromatography facilitates the separation of a mixture of samples on the basis of selective interaction of the components in the sample with the stationary phase (which is charged). In the present situation, only molecules with a net negative charge, at the pH used (pH 8.0) interacted with the DEAE Sephacel column.

The second step in the purification was an affinity chromatographic procedure in which separation was based on the selective interaction between a component in the sample and the D-glucosamine in the column.

Some proteins can interact with sugars, and this interaction is very selective. In most of the reported cases, one protein sample will interact strongly with just one type of sugar. The purified protein sample from the midgut homogenate of *G. austeni* is therefore a lectin, which binds selectively to D-glucosamine. The use of this sugar in preparing the affinity column stems from the initial report by Ibrahim *et al* (1984), which described the very strong inhibitory activity of the crude midgut homogenate of *G. austeni* to agglutinate *Trypanosoma brucei brucei* AnTaT serodeme and mammalian red blood cells.

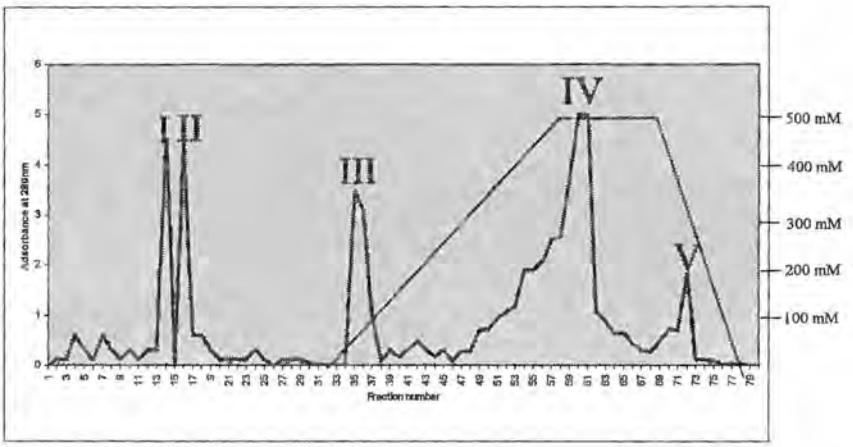


Figure 5. Anion exchange chromatographic profile for the purification of authentic Gpl. Crude midgut homogenate (90 mg protein) was applied onto a DEAE Sephacel column. Unbound proteins (fractions 1-33) were removed from the column with 20 mM Tris-HCl, pH 8.0, while bound proteins (fractions 34-75) were eluted using a salt gradient (0-0.5M NaCl) in the same buffer.

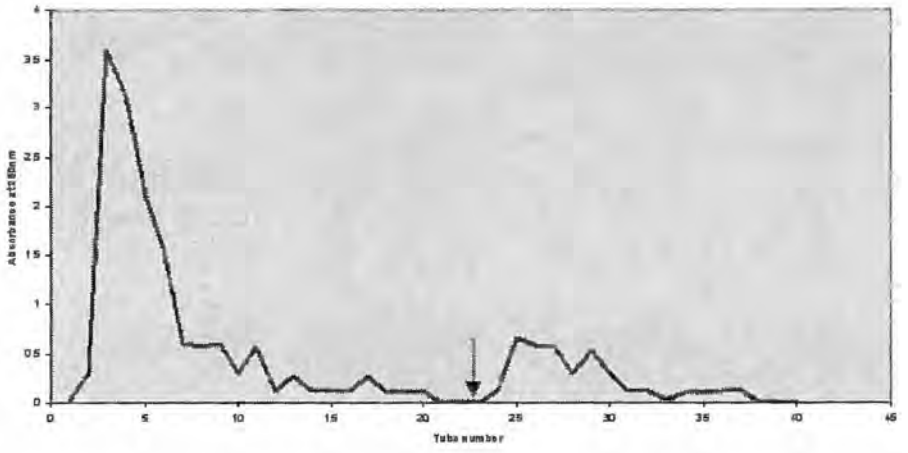


Figure 6. Glucosamine affinity column. Eluted bound material from the first column was applied onto a glucosamine affinity column, which had earlier been equilibrated with BIS (pH 7.9). Non-specifically bound material was washed with same buffer (tubes 0-20). Bound protein (tubes 24-38) was eluted with BIS containing 0.2 M glucosamine

4.1.2 Biological and biochemical characterisation of purified native Gpl

About 7.2 μg of purified protein was obtained following the two-step procedure of anion exchange and affinity chromatographic techniques. A sample of the eluted material from the affinity column, when incubated with *T. brucei brucei* and washed rabbit red cells (in separate microtiter plates) showed an agglutination titer of 2048 and 1024 respectively (Table 2). The fact that the active purified sample from the midgut homogenate was able to cause the agglutination of the parasites and rabbit red cells is further indication of lectin activity, and ties very well with findings obtained from other species of tsetse (Abubakar *et al.*, 1995; Osir *et al.*, 1995; Abubakar *et al.*, 2003) and the initial study on *G. austeni* midgut homogenate by Ibrahim *et al.* (1984). However, this is the first report in which a lectin has been purified from the midgut of *G. austeni.* Ibrahim *et al.* (1984) used crude midgut homogenate in their study. To investigate whether this purified protein had enzymatic activity, it was used in a trypsin assay, with chromozym –TRY as substrate. An average trypsin activity of $4.8x10^{-2}$μmol/min/ml was recorded. When analysed by SDS PAGE, the eluted bound protein from the affinity column gave two bands with molecular weights of 27,000±277da (n=3) and 35,500±425da (n=3) (Fig. 7, lane 3).

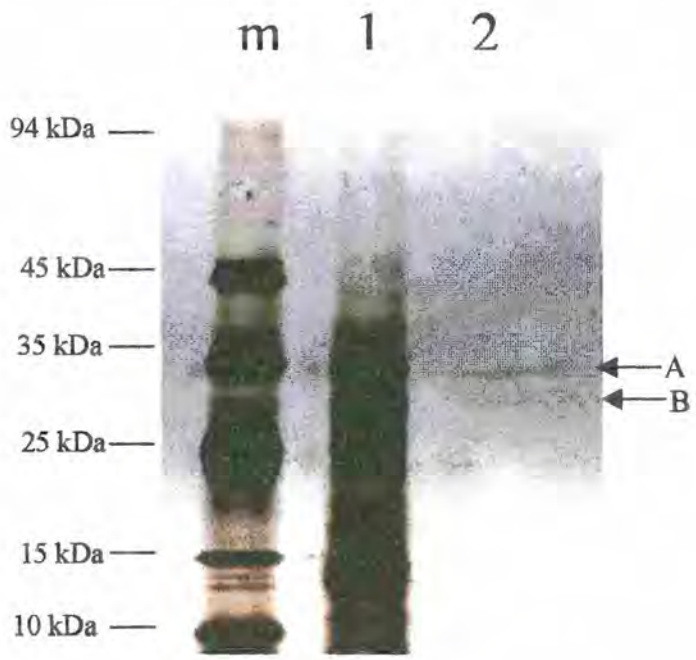


Figure 7. Denaturing gel electrophoresis Gpl purified from midgut. Protein samples were separated by gradient SDS PAGE (4-15%). Lane 1-low molecular weight standards, lane 2-crude midgut homogenate (10μg), lane 3-purified proteolytic lectin (3μg; arrows A and B showing the two chains).

These findings confirm earlier reports (Osir *et al.*, 1995 and Abubakar *et al.*, 2003), which indicated that the purified lectin from the midgut of *Glossina* sp. is a dimeric protein with enzymatic activity. Importantly, this enzymatic activity is similar to that of the serine protease, trypsin, because the purified protein sample was active on the chromogenic substrate, chromozym-TRY. Imbuga *et al.* (1992) had earlier established the substrate specificity.

Since the initial attempts to characterise the lectin in *Glossina* midgut employed crude protein sample, it was important to determine whether the purified sample sugar specificity was restricted to D- glucosamine or to related sugars. The effect of four different sugars on the agglutinating property of the purified proteolytic lectin was therefore investigated. Of the four sugars used, D-glucosamine was the only sugar that completely inhibited the agglutinating activity. On the other hand, glucose, galactose and N-acetyl-glucosamine showed very little or no effect on the agglutinating activity of the proteolytic lectin at the concentrations used (Table 2). This demonstrates that the *Glossina* midgut proteolytic lectin is indeed a D-Glucosamine-specific lectin.

Table 2. Inhibitory effect of sugars on agglutination activity

Sugar	Concentration tested (nM)	Agglutination titer	Inhibitory Effect on agglutination
D-Glucosamine	100	0	Very strong
	200	0	Very strong
	500	0	Very strong
N-acetyl-glucosamine	100	1024	Negligible
	200	1024	Negligible
	500	1024	Negligible
Glucose	100	1024	Negligible
	200	1024	Negligible
	500	1024	Negligible
Galactose	100	2048	No effect
	200	2048	No effect
	500	2048	No effect

4.2 MOLECULAR CLONING AND EXPRESSION OF PROTEOLYTIC LECTIN

4.2.1 Cloning and sequencing of proteolytic lectin

Seventy-eight micrograms of total RNA was isolated from the midguts of 50 *G. austeni*. The integrity of the RNA sample was confirmed by analyzing an aliquot on a denaturing agarose gel (Fig. 8). Ten microliter aliquots of this sample were preserved immediately at -70°C. One of the vials was used to synthesis ds cDNA by use of a Clontech SMART™ cDNA synthesis kit, which after confirmation on an agarose gel was size fractionated with Sfi I enzyme. The constructed ds cDNA and the size-fractionated digest were of the right size, i.e 300bp-3000bp (Fig. 9).

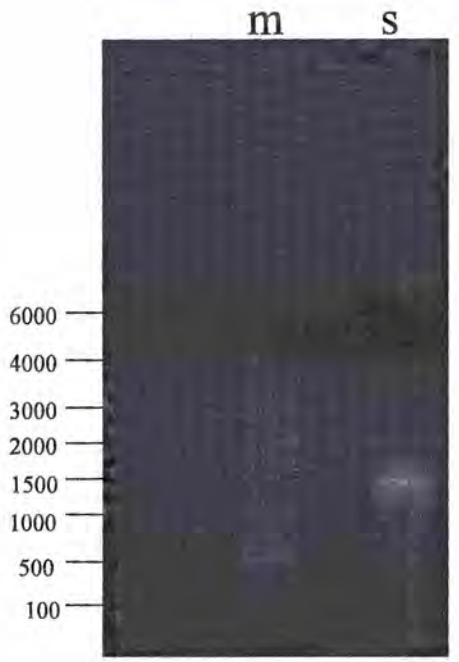


Figure 8. Analysis of RNA by denaturing agarose gel (1.2%) electrophoresis. Total RNA was extracted from midgut tissue of 50 male *G. austeni* as described in materials and methods. The integrity of an aliquot of the RNA was checked by electrophoresis. The agarose gel was stained after the run with ethidium bromide. Lane m RNA size standard, lane s aliquot of extracted RNA.

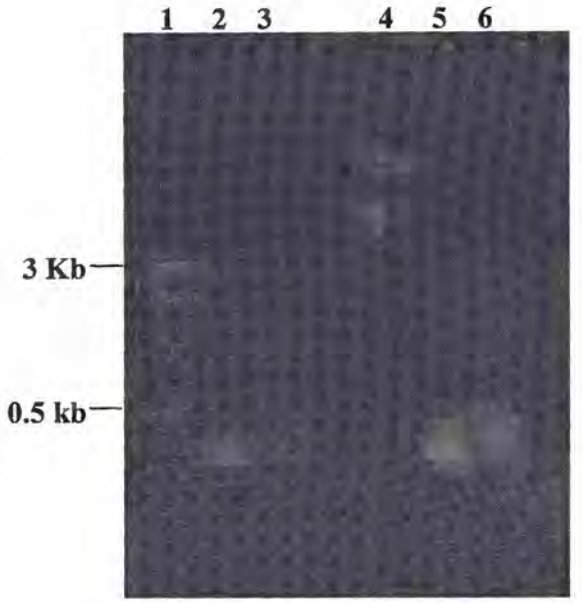


Figure 9. Agarose gel electrophoresis. An aliquot of the PCR-constructed ds cDNA was analysed for integrity before and after Sfi I-digestion followed by size fractionation on a 1.2% agarose gel, which was stained with ethidium bromide. Lane 1 DNA 100bp ladder, lane 2 constructed ds cDNA of positive control sample, lane 3 constructed ds cDNA of *G. austeni* before digestion, lane 4 DNA high molecular weight marker, lane 5 digested and size-fractionated positive control, lane 6 Sfi I-digested and size-fractionated ds cDNA of *G.austeni*

Following the confirmation of the right size of the fractionated Sfi I digest, the ds cDNA was ligated to an *Sfi* I-digested pTriplEx2 vector of the SMART cDNA library construction kit. This system allows for construction of high quality cDNA libraries with full-length clones, which are useful for mapping transcription start sites. Additionally, the use of pTriplEx2 vector and Sfi I enzyme leads to directional cloning, and the insert, which is easily screened by blue/white selection technique, can be readily expressed in all three reading frames. An aliquot (6μg) of the ligation mix was used to transform *E. coli* XL-1 blue cells, which were plated on an LB agar plate with IPTG and X-gal. The plates were observed to have thousands of white colonies with some blue colonies (in a ratio of about 50:3). Thus, the blue/white screening procedure indicated the successful construction of the midgut cDNA library of *G. austeni*. Next, an equal aliquot of the ligation mix was used to transform *E. coli* BL 21 (DE 3). Following plating and 18 h incubation on 5 LB plates without IPTG and X-gal, the plates were screened with polyclonal antibodies raised against native Gpl. Four Gpl-positive clones (ca. 0.002%) were detected from five filters containing about 4500 colonies. The plasmids containing putative Gpl cDNAs were isolated and screened with TriplEx2 and SP6 primers, and shown to contain inserts (Fig. 10). The sizes of the inserts were determined by PCR using Gpl-specific primers (Fig. 11), which were observed to have the same size. The clones were purified to homogeneity and the inserts sequenced. The sequences of all the inserts were identical. The cDNA sequence of the proteolytic lectin from *G. austeni* is shown in

Fig. 12 (GenBank accession number DQ060150). The cDNA contained an open reading frame (nucleotides 30 to 825 in Fig. 12) that encoded a predicted protein of 274 amino acid residues. The predicted molecular weight of the deduced protein was 29 kDa. A putative signal peptide (amino acid residues 1 to 16) was identified with signal peptide database of Swiss-Prot/EXPASY proteomics bioinformatics tools. Sequence motifs conserved in serine proteases, were also found between amino acid residues 68 to 73 and 213 to 224 (underlined in Fig. 12). In a comparative sequence analysis with other gene sequences in the GENBANK, BLASTP (Altschul *et al.*, 1990) with the deduced protein sequence for G. austeni proteolytic lectin showed 98% identity to a Gpl that was previously cloned from *G. fuscipes fuscipes* midguts (Abubakar *et al.*, 2006), and 91% identity to a chymotrypsin-like serine protease from *G. morsitans morsitans* (Yan *et al.*, 2001) (Fig. 13).

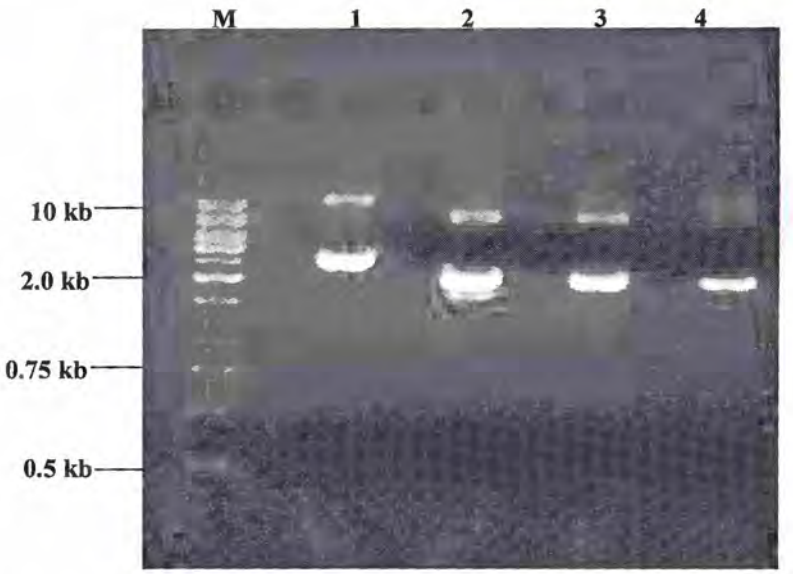


Figure 10. A 1.2% agarose gel electrophoresis of selected plasmids. Following the identification of putative clones with Gpl cDNA, plasmids were isolated the selected clones as described in materials and methods and analysed on agarose gel. Staining was with ethidium bromide. Lane m DNA 1kb ladder, lanes 1-4 selected clones

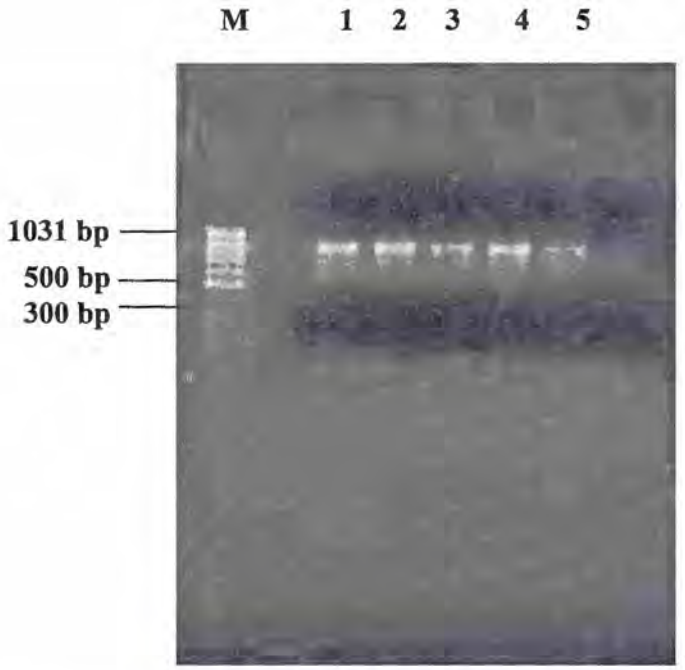


Figure 11. PCR screening of positive selected clones. Plasmids from the selected putative clones were isolated as described. These were used as template in a screening PCR with Gpl-specific primers and the PCR products analysed on a 1.2% agarose gel, which was stained with ethidium bromide. Lane m DNA 100bp ladder, lane 1 positive control sample, lanes 2-5 extracted plasmids, lane 6 negative control.

Figure 12. Nucleotide and deduced amino acid sequences of the proteolytic lectin cDNA of *G. austeni*. The arrowhead indicates the predicted signal peptide cleavage site. The putative serine protease active

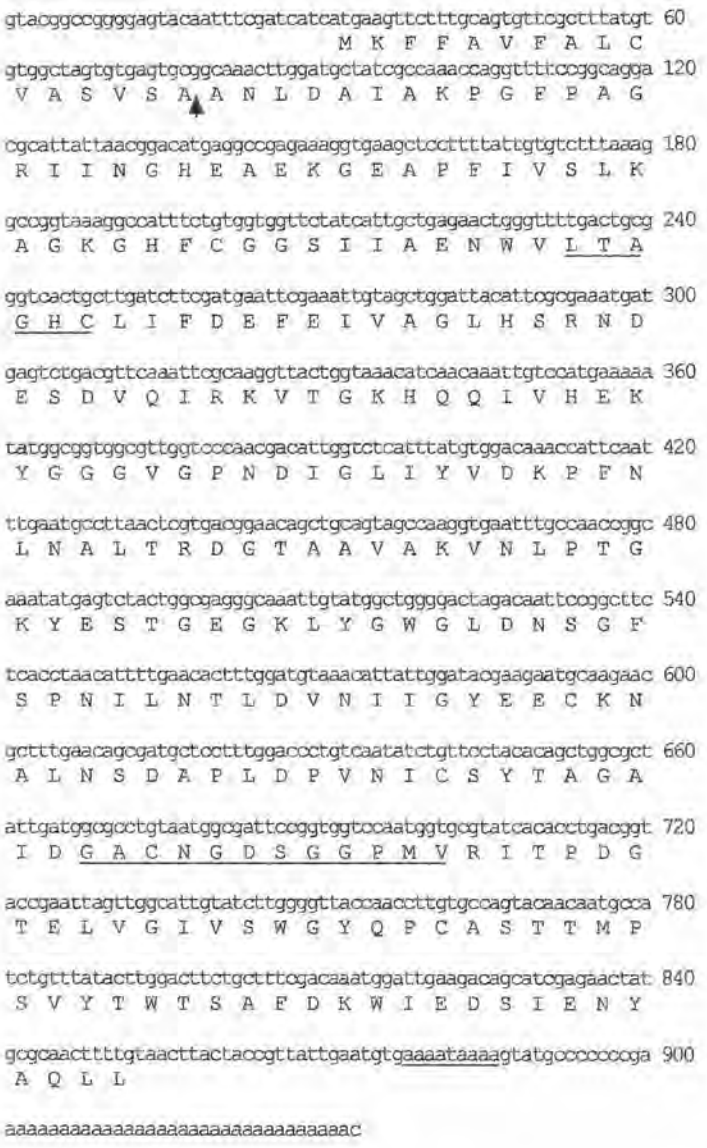


Figure 12. Nucleotide and deduced amino acid sequences of the proteolytic lectin cDNA of *G. austeni*. The arrowhead indicates the predicted signal peptide cleavage site. The putative serine protease active site residues are underlined. A potential polyadenylation signal is at the end of sequence

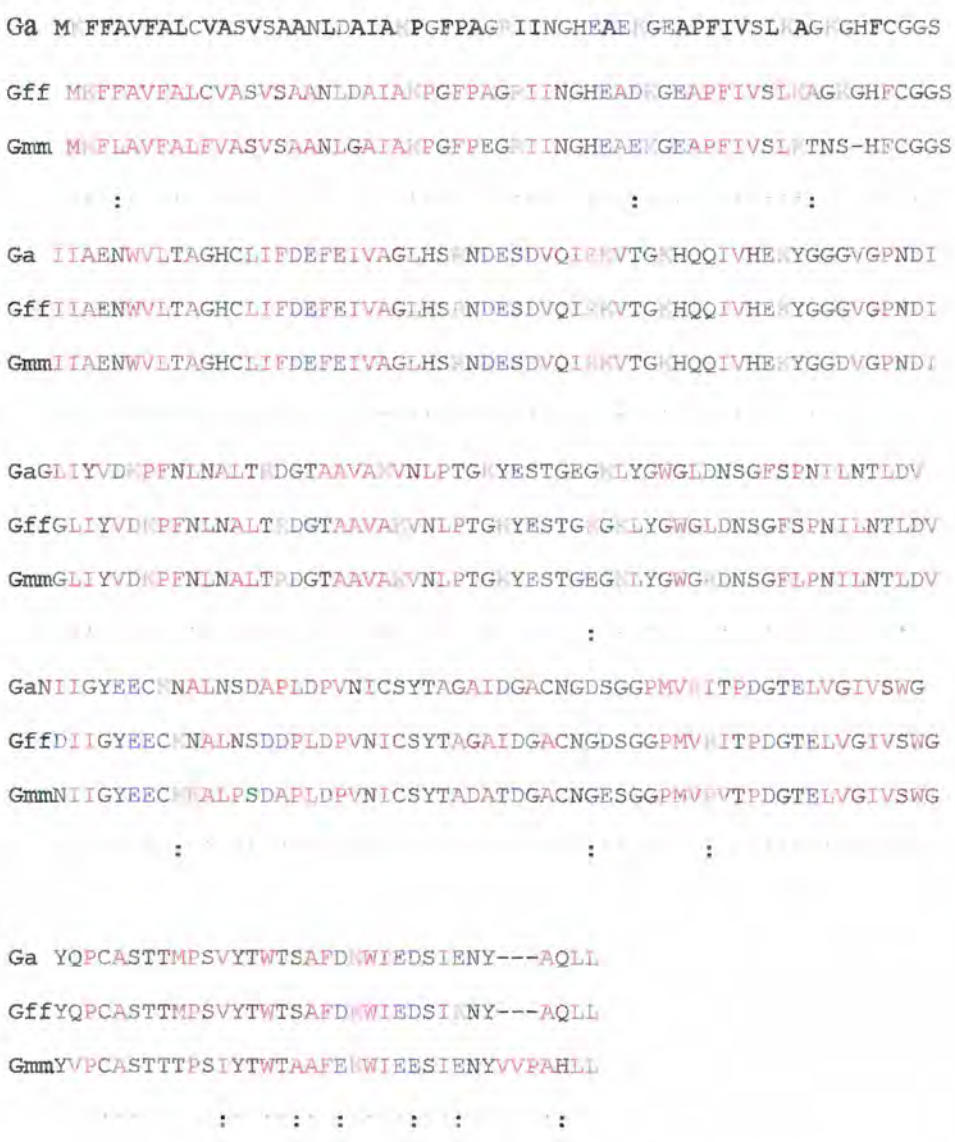


Figure 13. Alignment of the deduced protein sequences of proteolytic lectins from *G. austeni* (Ga) and *G. fuscipes fuscipes* (Gff), and a chymotrypsin-like serine protease from *G. morsitans morsitans* (Gmm). Identical (*) and similar (:) amino acids residues are indicated.

4.2.2 *In vitro* expression and purification of recombinant protein

Following the isolation and sequencing of the gene encoding Gpl, the recombinant protein was expressed in both prokaryotic and eukaryotic expression systems. In the prokaryotic system recombinant protein expression was achieved with *E. coli* while for eukaryotic expression, the recombinant protein was expressed in Sf21, (a cell line from *Spodoptera frugiperda)* following transfection of the cell line with a constructed recombinant baculovirus containing Gpl, designated AcMNP.gpl.

4.2.2.1 Purification of bacterially expressed Gpl

A two-step purification scheme was used to purify the recombinant Gpl expressed in bacteria. In the first step, the crude protein sample was applied onto a packed DEAE Sepharose 6B column, and the bound proteins were eluted with a NaCl gradient (Fig. 14). In the second step, proteins eluted from the DEAE Sepharose column were applied onto a glucosamine affinity column and eluted with 0.2 M D-glucosamine in equilibration buffer (Fig. 15). The sample purity was ascertained by SDS-PAGE (containing 2% β-mercaptoethanol), which yielded a single protein band (Fig. 16). The estimated molecular weight of the bacterially expressed Gpl was about 32 kDa by SDS-PAGE.

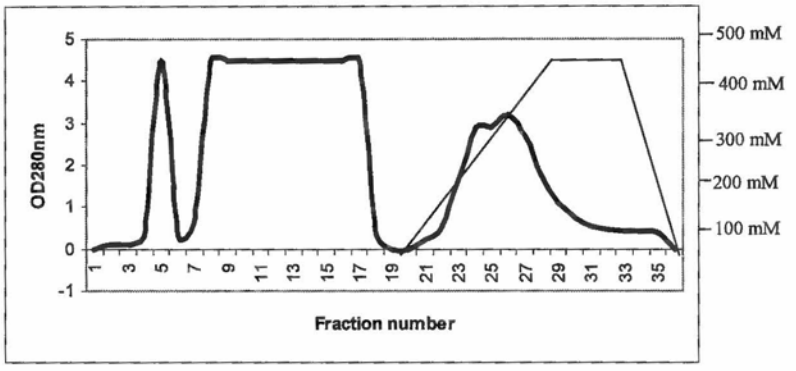


Figure 14. Purification of bacterial expressed Gpl. Lysate of *E. coli* cells transformed with pTriplEx.gpl was applied onto a DEAE Sephacel column. Unbound proteins (fractions 1-19) were removed from column with 20 mM Tris-Cl, pH 8.0, while bound proteins (fractions 21-36) were eluted using a salt gradient (0-0.5 M NaCl) in the same buffer.

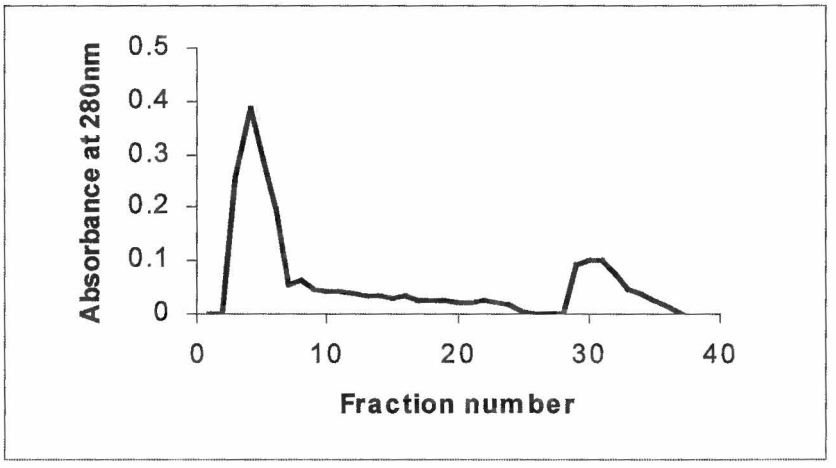


Figure 15. Glucosamine affinity purification of recombinant Gpl expressed in bacteria. Eluted bound material from first column was applied onto a Buffered Insect Saline (BIS)-equilibrated glucosamine affinity column. The column was washed with BIS to remove unbound material (fractions 1-26, peak I) and bound proteins (fractions 28-35, peak II) were eluted with 0.2 M glucosamine in BIS

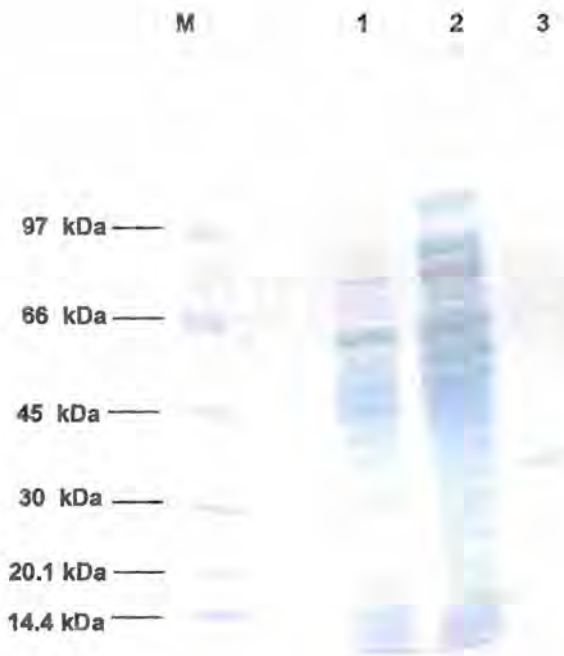


Figure 16. A gradient (4-15%) SDS-PAGE analysis of the non-induced bacterial cell lysate (lane 1), pTriplEx2.gpl-transformed bacterial cell lysate (lane 2), and purified rGpl from pTriplEx2.gpl-transfromed bacterial cell lysate (lane 3). Each lane was loaded with 30 μg of protein. The sizes of the molecular weight standards (lane M) are shown to the left. The proteins were visualized by Coomassie Brilliant Blue staining.

4.2.2.2 Immuno-affinity purification of baculovirus expressed Gpl

A recombinant baculovirus containing Gpl, AcMNP.gpl, was constructed. This was confirmed by PCR using Gpl-specific primers (Fig. 17). The culture medium of insect Sf-21 cells infected with AcMNPV-gpl (Fig. 18A) exhibited the ability to agglutinate washed rabbit red cells beginning about 48 h post infection (p.i.). This activity peaked around 72 hours post infection. Very little agglutinization activity was found in the supernatant of Sf-21 cells infected with AcMNPV-lacZ compared to that observed with supernatant from AcMNPV-gpl infected Sf-21 cells. Polyclonal antibodies that were generated against Gpl expressed in bacteria were used to prepare an immuno-affinity column that was used to purify the baculovirus-expressed Gpl (Fig. 19, peak III). This was carried out after the same antibodies had been used in a western blot to confirm expression of Gpl in AcMNPV-gpl infected Sf 21 cells (Fig. 20, lane 4). Analysis of the immuno-affinity purified protein by SDS-PAGE gave a single band, with an approximate molecular weight of 33 kDa (Fig 20).

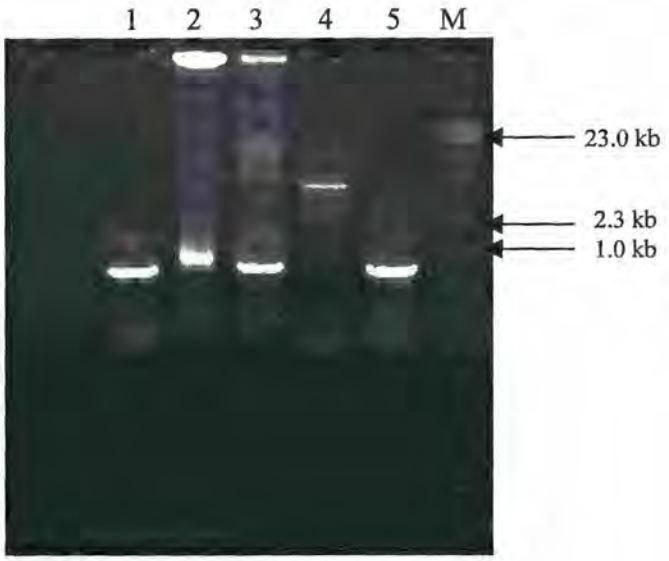
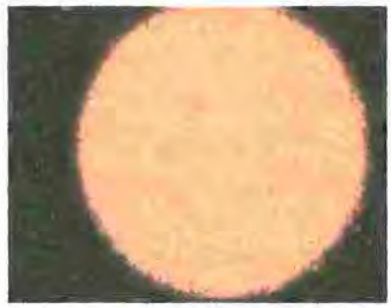


Figure 17. A 1.2% agarose/TAE gel electrophoresis to verify recombinant virus. The gel was stained with ethidium bromide. Lane 1, recombinant virus with *Glossina* proteolytic lectin gene (AcMNP.gpl); lanes 2 and 3, recombinant transfer vector with gpl; lane 4, negative control (AcMNP.LacZ); lane5, positive control 1 (pTriplEx2.gpl); Lane m, DNA high molecular weight marker.

A

B



C

Figure 18. Panel A shows SF21 cells growing on a monolayer culture, while panel B is a photograph of a monolayer culture of SF21 cells infected with recombinant virus (AcMN.Gapl), and panel C is AcMNP.LacZ-infected Sf-21 cells. Pictures were taken at 72hpi. Observe the cytological changes on the cells in B and C

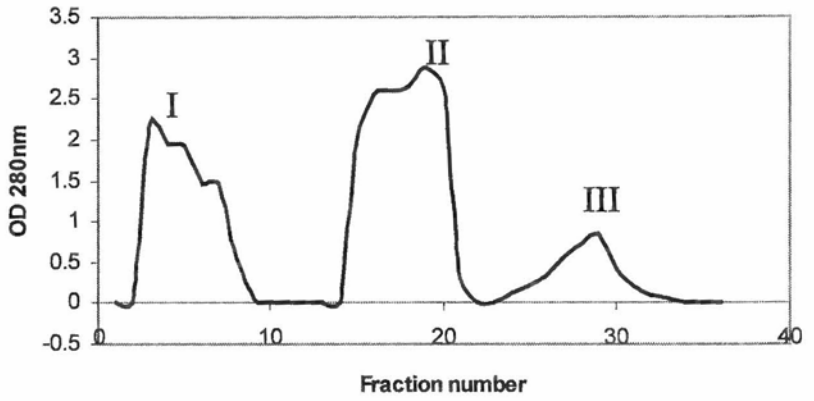


Figure 19. Immuno affinity chromatographic purification of recombinant Gpl expressed in Sf21 cell lines. The immuno-affinity column was loaded with the culture medium of AcMNPV.gpl-infected Sf-21 cells. Following elution of unbound proteins (peak I) with wash buffer (10 mM Tris-Cl, pH 8.0, 0.14 M NaCl, 0.025% NaN_3), non-specifically bound proteins were eluted with buffer A (50 mM Tris-Cl, pH 8.0, 0.1% triton X-100, 0.5 M NaCl) (peak II), and bound proteins were eluted with buffer B (50 mM Tris-Cl, pH 9.0, 0.1% Triton X-100, 0.5 M NaCl) (peak III).

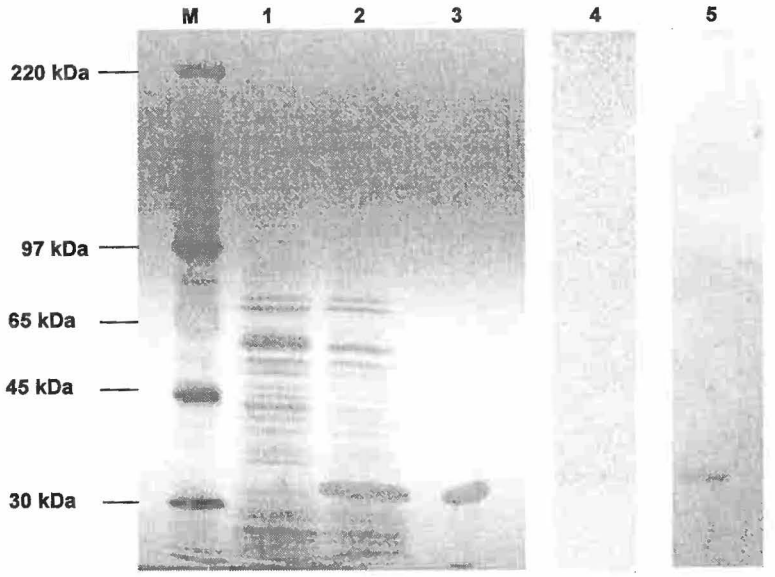


Figure 20. SDS-PAGE (4-15%) analysis of proteins in the cell culture medium of Sf-21 cells infected with AcMNPV-LacZ (lane 1) or AcMNPV-gpl (lane 2), and affinity purified, AcMNPV-gpl-expressed Gpl (lanes 3 and 4). Each lane was loaded with 15μg of protein. The sizes of the molecular weight standards (lane M) are shown to the left. The proteins were visualized by silver staining (lanes M, 1, 2, and 3) or carbohydrate staining with PAS {lane 4). AcMNPV.gpl expressed Gpl was separated by SDS-PAGE and then transferred onto nitrocellulose paper. The blot was then reacted with antiserum to the purified bacterial expressed Gpl (lane 5).

4.3 CHARACTERISATION OF RECOMBINANT PROTEIN

4.3.1 Comparison of the biological activities of the native and recombinant Gpl

The ability of the native (from the tsetse fly midguts) and recombinant (bacterially or baculoviral expressed) Gpls to agglutinate trypanosomes (purified from either *in vivo* or *in vitro* sources) and red blood cells are shown in Table 3. The agglutination assay was carried out at 27°C since a previous study indicated that this was the optimum temperature for this assay (Ibrahim *et al.*, 1984). The agglutination activities of the recombinant Gpls appeared to be identical, but only half as strong as that of the native Gpl. There was no difference in the agglutination of trypanosomes maintained by serial passage in rats (i.e., *in vivo*) or purified from blood (i.e., *in vitro*). The agglutination titer reported is the reciprocal of the highest protein dilution that showed clumping of cells. The trypsinization activities of the authentic and recombinant Gpls are also given in Table 3. The authentic Gpl showed higher (ca. 1.5-fold) trypsinization activity in comparison to that of the recombinant Gpls. The addition of 200 mM D-glucosamine to the assays strongly (60- to 250-fold) inhibited agglutinization activity. There was no significant difference between any of the three Gpl proteins in terms of the biological activities investigated.

4.3.2 Partial chemical characterization of the purified baculovirus-expressed protein

The presence of covalently bound carbohydrate moieties on the purified baculovirus-expressed Gpl was determined by PAS staining following SDS-PAGE (Fig. 20, lane 4). The baculovirus-expressed Gpl was shown to be glycosylated on the basis of PAS staining.

Table 3. Agglutination and trypsin activities of the native and recombinant Gpls

Source of purified Gpl	Agglutination Activity[1]			Trypsinization Activity[2] ± s.d. (nmol/ml/min)
	parasites		Red blood cells	
	In vivo cultured	*In vitro* cultured		
Native	2048	2048	1024	48.0 ± 3.0
Native+D-glucosamine	8	8	8	0
Bacterially expressed	1024	1024	512	32.1 ± 4.0
Bacterially expressed+D-glucosamine	8	8	8	0
Baculovirally expressed	1024	1024	512	33.4 ± 2.0
Baculovirally expressed+D-glucosamine	8	8	8	0

[1]Average value of two independent assays

[2]Average value of three independent assays

4.3.3 Interaction of Gpl with D-glucosamine

The effect of glucosamine on the biological and biochemical activities of the recombinant Gpl was assayed as reported above. The results are in agreement with previous reports on a midgut lectin from *Glossina* spp. However, there has not been any suggestion as to how this tsetse midgut protein interacts with this sugar. A preliminary investigation was therefore carried out here to determine whether this interaction is based on the shape or relies solely on the amino group of the sugar.

Several studies on tsetse midgut proteins have shown that complete inhibition of the effect of midgut lectin on trypanosomes could readily be achieved *in vivo* (Maudlin, 1991) or in vitro (Ibrahim *et al.*, 1984; Abubakar *et al*, 2003; Amin *et al.*, 2006) with D-glucosamine. For example, Maudlin *et al.* (1991) showed that high tsetse infection rates with trypanosomes could be attained if D-glucosamine was added onto the bloodmeal. The same results could not be obtained with other related sugars including glucose or N-acetylglucosamine. This had remained intriguing since charge was ruled out after lysine failed to produce similar effects (Maudlin, 1991). From these studies, the arising question has therefore been how the inhibitory property of glucosamine comes about? And is there any role of the amino group in the process of binding prior to the inhibition of the biological property of the protein? Cyanogen bromide activated Sepharose 4B binds to molecules that have free amino groups with lone pair electrons on the nitrogen atom. The purification of the recombinant Gpl with a glucosamine-affinity column, in which the amino

group was immobilised, covalently to the Sepharose is an indication that structural complementarities between Gpl and D-glucosamine is the major factor towards the binding of the two, and therefore constitute the initial step in the specific inhibition. This binding may precede the electronic interaction between the two molecules, probably involving the amino group of the sugar and other groups on the protein. This interaction is likely to be structural. The immobilised sugar was used to purify the expressed recombinant Gpl from the harvested supernatant from AcMNP.gpl-infected Sf-21 cells (Fig. 21, peak II and Fig. 22, lane 2). Though the present findings may not be conclusive, it could be hypothesised that Gpl interacts with glucosamine in a manner similar to the lock and key, in which the glucosamine (the key) fits perfectly into a crevice within Gpl (the lock). The 3-dimensional structure of D-glucosamine is different from that of the other sugars. It is, therefore, suggested that electronic interactions between the nitrogen of D-glucosamine and Gpl may not account for the initial phase of the binding that leads to the specific inhibition of this proteolytic lectin by D-glucosamine. Nevertheless, this remains to be confirmed with crystallographic analysis of the two molecules. It is worth mentioning that unlike what has been described here, the covalent attachment of D-glucosamine to epoxy activated Sepharose 6B is via the hydroxyl groups of the sugar and not the amino group (Pharmacia, 1986).

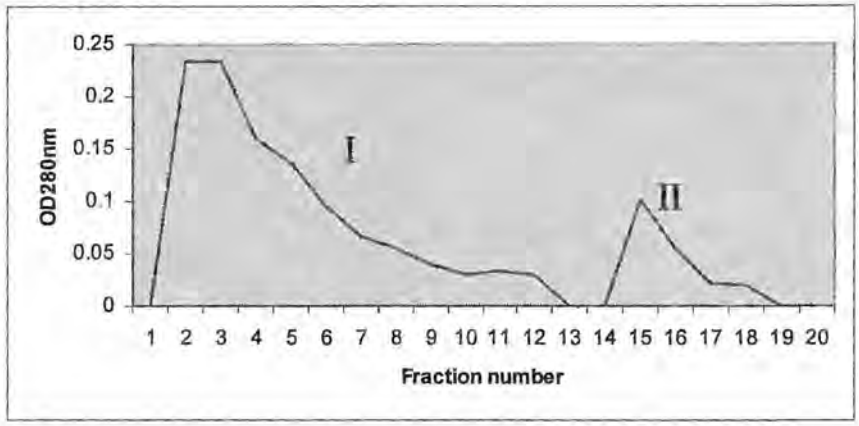


Figure 21. Affinity chromatographic purification of recombinant Gpl expressed in Sf21 cell lines. Glucosamine was covalently coupled to CNBr-activated Sepharose 4B through its amino group according to the protocol provided by manufacturer. The coupled gel was packed into a Pharmacia XK column and equilibrated with buffered insect saline (BIS). The protein sample was applied and after incubation for two hours, unbound proteins (peak I) were washed out with the same buffer. Bound protein (peak II) was eluted with BIS containing 0.2 M D-glucosamine.

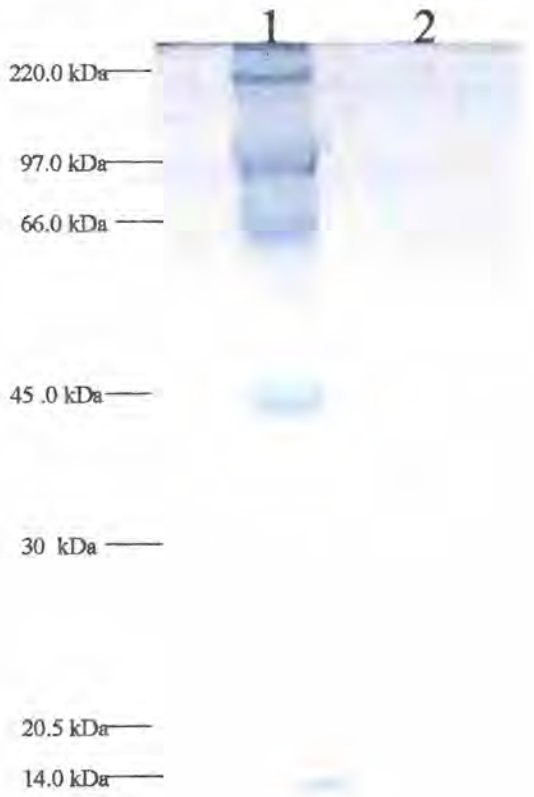


Figure 22. A gradient (4-15%) SDS-PAGE analysis of rGpl (expressed from AcMNP.gpl-infected Sf 21 cells) purified on the column in which the amino group of glucosamine was immobilised onto CNBr-activated Sepharose 4B. Each lane was loaded with 30 μg of protein. The sizes of the molecular weight standards (lane 1) are shown to the left. Lane 2 purified rGpl. The proteins were visualized by Coomassie brilliant blue staining.

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Biochemical and molecular biology techniques have been employed in this study to isolate both a native *Glossina* proteolytic lectin (from the midgut homogenate) and the encoding gene (from a midgut cDNA library) from the tsetse fly, *G. austeni,* which was expressed *in vitro.* Comparative sequence analysis, both at nucleotide and deduced amino acid levels, showed that the gene cloned from *G. austeni* is very similar to that previously reported for *G. fuscipes fuscipes (*Abubakar *et al.,* 2006) and a chymotrypsin-like protein (Yan *et al.,* 2001). As expected a proteolytic lectin has been purified from the midgut homogenate of both species. The physiological role of the molecule in both species is the same as reported in literature; it is associated with the differentiation of trypanosome bloodstream forms to procyclic (midgut) form within tsetse (Welburn and Maudlin, 1989; Logan-Henfrey *et al.,* 1992; Abubakar *et al.,* 2003). Also, this thesis is the first report on the purification of a proteolytic lectin from *G. austeni* that agglutinates trypanosomes *in vitro.* The level of this bloodmeal-induced proteolytic lectin is directly linked to the level of susceptibility or refractoriness of the tsetse species, either in the wild or in the laboratory (Maudlin, 1991). For example, the level of the protein is now known to be far higher in *G. fuscipes fuscipes,* a more refractory

species, compared to *G. austeni*, as was observed in the course of the experiments reported here. Even with reverse transcriptase polymerase chain reaction (RT-PCR), a more sensitive analytical tool, it has been shown that the gene transcript is several times higher in *G. fuscipes fuscipes* compared to *G. austeni* (Osir and Abubakar, unpublished data). This may explain why the amount of the midgut proteolytic lectin from *G. austeni* was found to be lower than that from *G. fuscipes fuscipes* in this study.

This report also deals with the expression of a functionally active *Glossina* proteolytic lectin (Gpl) in insect cells using a recombinant baculovirus expression vector. Recombinant protein expression in lepidopteran cell lines, especially *Sf*-21, is fast becoming a common practice because of the numerous advantages (Kamita *et al.*, 2001). The gene encoding Gpl was obtained from a midgut derived cDNA library from *G. austeni* and used to generate the recombinant baculovirus AcMNPV-gpl. To date, this is the first tsetse gene to have been cloned and expressed in a baculovirus genome. The recombinant protein expressed by this virus was secreted into the culture medium of Sf-21 cells, indicating that the *G. austeni*-derived signal peptide was properly recognized in the lepidopteran-derived cells.

The recombinant Gpls (rGpls) expressed in insect Sf-21 cells and in bacteria agglutinated washed trypanosomes and rabbit red blood cells. The rGpls produced an agglutination titer of 1024, which was comparable to that obtained with the native Gpl from midgut homogenates. The

recombinant Gpls also showed trypsin activities (32.1-33.4 nmol/ml/min) against the substrate chromozym-TRY. This trypsin activity was similar to that produced by the authentic Gpl (48.0 nmol/ml/min). Both agglutinization and trypsin activities were strongly inhibited by D-glucosamine, a strong indication that the protein is a lectin. Recently, a bacterially expressed Gpl was characterized and shown to promote the transformation of *T. brucei brucei in vitro* (Abubakar *et al.* 2006). This transformation was observed with the aid of immuno-fluorescence microscopy. The important role of D-glucosamine as the main physiological inhibitor of Gpl has been used to explain why teneral tsetse flies are more susceptible to infections with trypanosomes than adult tsetse (Maudlin, 1991). This affinity for D-glucosamine has been exploited as a biochemical tool to purify the protein, both from the midgut homogenate and the recombinant expressed protein as reported in this thesis and elsewhere (Abubakar *et al.*, 2003). Though how the interaction between Gpl and D-glucosamine occurs has been obscure, this thesis shows that the interaction might rely on structural complementarities between the two molecules. From this work, it can be postulated that the two interact in a lock and key pattern, with the amino group of the sugar not involved in any specific bond formation (neither weak nor strong) prior to binding. Rather, this group may help to confer a specific shape to the sugar that enables it to fit into some crevice in the native structure of the midgut protein. However, this remains to be shown by crystallographic analysis.

In view of the drawbacks of vector-control measures that are currently in use, it has been suggested that the creation of transgenic tsetse flies that are refractory to trypanosomes will significantly contribute towards the control of trypanosomiasis, if used together with a sterile insect release program. At present, transgenesis of tsetse is not possible since its eggs are nurtured within the female and only released at the final stage of larval development. However, in paratransgenesis endosymbionts of the tsetse fly can be used to express a foreign gene within the adult fly (Crampton 1994, Aksoy et al. 2001).

In this study, the recombinant Gpl expressed in Sf-21 cell lines with the recombinant baculovirus, AcMNP.gpl was investigated for glycosylation, a protein post-translational process present in most eukaryotes. PAS staining confirmed that the purified baculovirus-expressed Gpl was glycosylated. This is not surprising as the native Gpl from midgut homogenate of *G. longipennis* was shown to be dimeric, with the β-chain reported to be glycosylated (Osir *et al.*, 1995). Here, the baculovirus-expressed recombinant has a size comparable to this β-chain. The glycosylation pattern is different. There is a generally accepted view that most glycoproteins have O-linked or N-linked glycosylation pattern, which occurs through serine and asparagine residues, respectively. However, this appears not to be the case in Gpl. The deduced amino acid sequence of Gpl does not have the classical consensus signature sequences for these types of glycosylation. A number of studies (Haltiwanger *et al.* 1992, Previato *et al.* 1994, Hart *et al.* 1995, Mehta *et al.* 1997, Haynes

1998) have presented evidence for the existence of saccharide-protein linkages that do not follow the prevailing dogma. For example, in *Dictyostelium* spp. (Mehta *et al.* 1997), phosphoglycosylation occurs via the linkage of the sugar to a phosphate attached to a serine moiety on the protein backbone. However, there is no evidence as yet of a consensus recognition motif for this type of glycosylation (Haynes 1998).

The lack of a carbohydrate moiety on the bacterially expressed Gpl had essentially no effect on its agglutinization and trypsinization properties. Although not essential in these functions, glycosylation of Gpl may serve a structural role or ensure proper synthesis and positioning of Gpl within the organism. For example, appropriate glycosylation may be required for the proper targeting of the protein in the midgut region. The carbohydrate moiety of glycoproteins generally contributes a net negative charge and increased solubility to a protein (Nachon *et al.* 2002). Thus, it is also plausible that glycosylation plays a role in the proper solubility of Gpl within the midgut cells. Currently, Gpl is associated with two roles in the tsetse-trypanosome relationship (i.e., anti-trypanosome by causing an apoptosis-like cell death and, pro-trypanosome by promoting establishment). This protein may have evolved as an adaptation by tsetse to reduce the physiological stress on it brought about by trypanosomes, which compete for some of the components within tsetse bloodmeal, for example proline (Leak, 1999). This may explain why tsetse flies show low infectivity to trypanosome parasites in nature.

5.2 Conclusion

Characterisation of *Glossina* proteolytic lectin has been the pillar of this study. The proteolytic lectin was isolated from G. austeni midgut homogenate, and the encoding gene was identified midgut cDNA library and sequenced (GENBANK accession no. DQ060150). After a close study of the native and recombinant forms of Gpl, it was observed through in vitro studies that Gpl is a tsetse midgut protein that interacts with *Trypanosoma brucei brucei* ILTat 1.4, by bringing about agglutination. This agglutinating property was also observed when washed rabbit red blood cells were used. In addition, both forms of the protein showed trypsin-like enzymatic activity. These two important biological activities were strongly inhibited by the amino sugar, D-glucosamine. Though not very conclusive, results obtained also indicate that the interactions between Gpl and D-glucosamine may rely more on the structural complementarities between both molecules, with the sugar (key) fitting perfectly into a crevice on Gpl (lock), in a manner described as lock and key.

Following the expression of Gpl with the baculovirus expression vector system, the recombinant Gpl was found in the medium of AcMNP.gpl-infected cell, a strong indication that the tsetse signal peptide was properly recognized by the protein synthetic machinery of *Sf*-21 cell lines. This recombinant protein was shown by PAS stain to be glycosylated. This confirms that Gpl is indeed a glycoprotein. Interestingly, the present findings also indicate that the carbohydrate moiety on Gpl is not attached through the classical N- or O-linkage as

observed in most mammalian glycoprotein. It is therefore important to express eukaryotic proteins in systems that allow for post-translational modification in order to have such groups attached to the mature protein. And because the results from agglutination and enzymatic assays with both the unglycosylated and the glycosylated forms of the protein were not significantly different, it is concluded that the carbohydrate moiety may not play an important with respect to these biological activities of Gpl. However, it may be useful in conferring a net negative charge to the molecule for proper targeting.

Recombinant Gpl was expressed both in bacteria and in Sf-21 cells. Interestingly, far more recombinant Gpl was produced in Sf-21 compared to the use of bacteria cells in terms of volume of culture medium employed. Additionally, the expression of Gpl in Sf-21 cells made it possible for post-translational modification of the protein to be achieved.

5.3 Recommendations

This study has come up with very vital additional information on the nature and activity of Glossina proteolytic lectin. However, due to the budgetary constraints as well as time the length of the understanding could not be extended. Other key areas of research on this molecule, which have now been identified, will need further studies. It is therefore recommended that the following be looked into

i) The structure of the sugar moiety and the type of saccharide-protein linkage.

ii) The 3-D structure of the recombinant protein and the way Gpl interacts with D-glucosamine.

iii) The present study may be useful in terms of generating a transgenic endosymbiont that expresses Gpl in a paratransgenic insect. The expression of Gpl in a paratransgenic tsetse should produce the same effects as a transgenic Gpl-expressing insect.

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APPENDICES

APPENDIX 1 SOME FORMULATIONS

5 x SDS /electrophoresis buffer

15.1 g Tris base

72.0 g Glycine

5.0 g SDS

Add water to 1000 ml. Do not adjust pH of the stock; since this pH is 8.3 when the solution is diluted to 1x for use in the protocol.

NB For non-denaturing do not include SDS.

2 X SDS/sample buffer

To 40 ml of distilled water add

1.52 g Tris base,

20 ml Glycerol,

2.0 g SDS

2.0 ml 2-mercaptoethanol

1 mg Bromophenol blue

Adjust to pH 6.8 with 1N HCl and add water to 100 ml mark.

NB Exclude SDS when dealing with non-denaturing.

4 X Tris-Cl/SDS pH 6.8 (0.5M Tris-Cl containing 0.4% SDS)

To 40ml distilled water add,

6.05 g Tris base

0.4 g SDS

Adjust pH to 6.8 with 1N HCl, and add water to 100 ml. Filter solution through a 0.45 μm filter and store at 4 degrees centigrade.

NB For non-denaturing protocol prepare this solution without SDS.

4X Tris-Cl/SDS pH8.8 (0.5M Tris-Cl containing 0.4% SDS)

To 300 ml of distilled water add

91 g Tris base

2 g SDS

Adjust pH to 8.8 with 1N HCl. Add water to 500 ml.

Filter the solution through a 0.45 μm filter and store at 4 degrees centigrade.

NB Omit SDS for non-denaturing protocol.

30% Acrylamide/0.8% bisacrylamide

30 g acryl amide

0.8 g bisacrylamide

Add water to 100 ml

Filter through 0.45 μm filter and store at 4°C in the dark.

Tris buffered Saline(TBS) pH 7.4

8 g NaCl

0.2 g KCl

3 g Tris Dissolve in 500ml deionised distilled water, adjust pH to 7.4 with HCL and make vol. to 1000ml.

PBS (phosphate buffered saline), pH 7.4

8.0 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Dissolve salts in 600ml deionised distilled water, adjust pH to 7.2 and make vol. to 1L.

BCIP and NBT as revealing substrates

NBT stock solution: prepare NBT stock soln. By dissolving one tablet of NBT in 1ml of deionised water

BCIP stock solution: prepare BCIP stock soln by dissolving one BCIP tablet (25mg) in 0.5ml 100% DMF.

Substrate buffer: 0.1M Tris (12.11g/L), 100mM NaCl (5.84g/L), 5mM $MgCl_2$ (1.02g/L). Adjust pH to 9.5 with HCL

Substrate solution: prepare substrate solution by adding 330µl of NBT stock soln. To 10ml of substrate buffer, mix, and then add 33µl of BCIP stock soln., mix.

NB All solutions should be protected from light.

Immunoblotting (western blotting) formulations

Transfer buffer

48 mM Tris-Cl, pH 8.3

29 mM Glycine

20 % Methanol

0.037 % SDS

Ponceau stain

0.5 g Ponceau S

1 % glacial acetic acid

Add water to 100ml mark

Blocking buffer

25 mM Tris

137 mM NaCl

3 mM KCl

5% Non fat dry milk or 1% BSA (gelatin)

Washing buffer

TBS, pH7.4

0.3% Tween 20

SOC medium (1L)

2.0 g Bactotryptone

0.5 g Yeast extract

1 ml IM NaCl

0.25 ml 1M KCl

1 ml 2M Mg^{2+} stock (filter sterilized)

1 ml 2M glucose (filtered sterilized)

Procedure: Add tryptone, yeast extract, NaCl & KCl to 97 ml dH_2O. Stir to dissolve. Autoclave and cool to room temp. Add 2 M Mg^{2+} stock and 2 M glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile distilled water. Filter the complete medium through a 0.2 μm filter unit. The final pH should be 7.0

2M Mg^{2+} stock

20.33 g $MgCl_2.6H_2O$

24.65 g $MgSO_4.7H_2O$

Add distilled water to 100 ml, filter sterilize.

IPTG stock solution (100 mM)

1.2 g IPTG, add water to 50ml final vol. Filter to sterilize and store at 4°C

X-gal (2ml)

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Dissolve in 2 ml N, N-dimethyl formamide. Cover with aluminium foil and store at -20°C.

LB broth (1000ml)

10 g Bactotryptone

5 g Yeast extract

5 g NaCl

Adjust pH to 7.0 with NaOH. Autoclave

LB agar (1000ml)

10 g bactotryptone

5 g Yeast extract

5 g NaCl

Adjust pH to 7.0. Add 15 g agar and autoclave. Allow medium to cool to 50°C. Pour plates after adding antibiotics (if necessary).

Preparing ampicillin plates/broth

Prepare LB agar or broth; autoclave, allow to cool to 50°C. Add ampicillin to a final concentration of 100 μg/ml. Pour plates (30-35 ml/plate). Store at 4°C for up to one month.

Plates with IPTG and X-gal

Add 100 μl of 100 mM IPTG, 20μl of X-gal (50 mg/ml), spread on plate and allow to adsorb at 37°C prior to use.

TYP broth (1000ml)

16 g Bactotryptone

16 g Yeast extract

5 g NaCl

2.5 g K_2HPO_4

Transformation of competent cells

Add 50 µl of competent cells to ligation mix (1:5) and incubate on ice for 30 min.

Heat shock at 42°C for 30 seconds.

Add 500 µl of SOC medium and incubate at 37°C for 1 hour.

Centrifuge for 4 min., decant supernatant and resuspend cells in 200 µl of SOC medium.

Plating

Equilibrate LB/ampicillin plate to room temp.

Add 100 µl IPTG and spread, and then add 30 µl of X-gal. Spread and allow adsorbing.

Add 50 µl of sample (transformed cells), spread and incubate at 37°C overnight (at least 18 hours)

Insect buffered saline (BIS)

10 mM Tris-Cl pH 7.9	1.211 g
130 mM NaCl	7.597 g
5 mM KCl	0.373 g
1 mM $CaCl_2$	0.147 g

Dissolve salts in 600 ml distilled- deionised water, adjust pH to 7.9 with HCL, and make up vol. to 1000 ml.Filter and degas.

Acetate buffer (0.1M, pH 4.0)

0.6 M Sodium acetate 49.2 g/l

0.6 M Acetic acid 34.4 ml glacial acetic acid per Liter of distilled water

Mix 435 ml of 0.6 M acetic acid with 130 ml of 0.6 M sodium acetate. Adjust to 1000 ml with distilled deionised water.

Borate saline buffer (0.1M,pH 8.3-8.5)

Boric acid 6.18 g/l

Sodium tetra borate (borax) 9.54 g/l

Sodium chloride 4.38 g/l

Carbonate-bicarbonate buffer (0.1 M)

Stock solution A 0.2M Na_2CO_3 (21.1 g/l)

Stock solution B 0.2M $NaHCO_3$ (16.8 g/l)

X ml of solution A + y ml of solution B, diluted to a total of 200 ml will yield the approximate pH shown below (0.1 M)

Solution A	Solution B	pH
5.0 ml	45 ml	9.2
7.5	42.5	9.3
9.5	40.5	9.4

13.0	37.0	9.5
16.0	34.0	9.6
19.5	30.5	9.7
22.0	28.0	9.8
25	25.0	9.9
27.5	22.5	10.0
30	20.0	10.1
33	17.0	10.2
35.5	14.5	10.3
38.5	11.5	10.4
40.5	9.5	10.5
42.5	7.5	10.6
45	5.0	10.7