INDUCED PROTEINS PROFILE IN THE HAEMOLYMPH OF DESERT LOCUST (*Schistocerca gregaria*) FOLLOWING A TRYPANOSOMATID FLAGELLATE (*Trypanosoma brucei brucei*) CHALLENGE

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A thesis submitted to the Graduate School in partial fulfillment for the requirements of the degree of Master of Science in Biochemistry of Egerton University

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DECLARATION

I declare that this thesis is my original work and the contents described herein have not been presented to any institution for any award.

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DEDICATION

This work is dedicated to all members of my family for their undivided attention and patience during the entire period of my study.

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ABSTRACT

Innate immunity has a key role in the control of microbial infections in both vertebrates and invertebrates. In insects, including vectors that transmit parasites that cause major diseases such as trypanosomosis, leishmaniasis and filariasis, antimicrobial peptides and agglutinins form an important component of innate immunity and participate in regulating parasite development. In this study, induced haemolymph peptides from a non-vector, non-heamatophagous insect, *Schistocerca gregaria* were assessed using one and two dimensional gel electrophoresis following *Trypanosoma brucei* inoculation. The pattern of protein induction was assessed by inoculating the locusts with parasites followed by haemolymph collection at 0, 6, 18, 24, 30, 42, and 48 hour. The amount of protein in each sample was quantified and found to increase with time, with the 18 hour sample having the highest protein concentration. On analysis using SDS-PAGE, five peptides were found to differ in terms of their presence and relative abundance in all the samples following *T. brucei* challenge. Following 2D-PAGE, some peptides were found to be induced, enhanced or suppressed while others were unaffected. *In vitro* assays were performed to ascertain the extent of trypanosomes lysis by incubating the parasites with various haemolymph samples obtained after challenge with the parasites. This further indicated that lysis increased with increasing protein concentration, with complete lysis (100%) being attained in the 18 hour sample after 75 minutes. The effects of sugars on the induction of the proteins were determined by inoculating the insects with the parasites and introducing the sugars (500mM of D-glucosamine, D-galactose, D-glucose and N-acetylglucosamine) after 30 minutes. Samples collected 18 hours later were subjected to protein quantification followed by an *in vitro* lysis assay. The sugar, D-glucosamine was found to have the highest inhibition with D-galactose having the least effect on the induction. An approximated 80% lysis was observed with 0.5mg/ml of the 18 hour sample treated with D-galactose and only 10% lysis with D-glucosamine. Further analysis was carried out by subjecting the samples to immunodetection with antibodies raised against *Glossina* proteolytic lectin (*Gpl*), an induced midgut lectin found among the *Glossina spp.* and none of the samples collected post *T. brucei* challenge showed cross-reactivity. These induced proteins have the potential of being used to modulate tsetse fly vectorial competence.

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LIST OF ABBREVIATIONS

AMPs	Antimicrobial Peptides
BSA	Bovine serum albumin
Dif	Dorsal related immune factor
1D-PAGE	One-dimensional polyacrylamide gel electrophoresis
DTT	Dithiothreitol
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
Gpl	Glossina proteolytic lectin
HAT	Human African trypanosomosis
IEF	Isoelectric Focusing
Ig	Immunoglobulin
IMD	Immunodeficiency
IPG	Immobilized pH gradient
KDa	Kilo Daltons
LPS	Lipopolysaccharide
NAG	N-acetylglucosamine
NP40	Nonidet P40
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
pI	Isoelectric Point
PBSG	Phosphate Buffered Saline Glucose
PPO	Prophenoloxidase
RLOs	Rickettial-like organisms
SDS	Sodium Dodecyl Sulphate
TEMED	N, N, N', N'-tetramethylethylenediamine
VSG	Variable surface glycoprotein
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

More than 66 million people in 36 countries of sub-Saharan Africa suffer from human African trypanosomosis (HAT) (Maudlin, 1991). There are two forms of African sleeping sickness, caused by two different subspecies of *Trypanosoma* namely; *Trypanosoma brucei* ***gambiense*** causes a chronic infection lasting years and affects countries of Western and Central Africa. *Trypanosoma brucei rhodesiense***,** on the other hand causes acute illness lasting several weeks in countries of eastern and southern Africa. When untreated, trypanosomosis gives no respite from suffering and ultimately ends in death. HAT transmitted by tsetse of the order *Diptera* and genus *Glossina* remains a major cause of human mortality throughout the sub-Saharan Africa (SSA) and is a major constraint in livestock production (Allsopp, 2001). Tsetse fly (Figure 1) infests greater than 40% of the total land area of 37 countries in SSA, an equivalence of 8.5 million km^2 (Hao *et al*., 2001)

Figure 1: The tsetse fly of the genus, *Glossina* (Maudlin, 1991).

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Human trypanosomosis is therefore a vector-borne parasitic disease. The vector is found only in Africa, between the fifteenth parallels north and south. Its favored habitat is the vegetation along watercourses, lakes, forest edges and gallery forests, extending to vast areas of shrub savanna (Allsop, 2001).

Tsetse control is vital in attempts to reduce the impact of African trypanosomosis. Effective control methods include the sequential aerosol techniques (SAT), the sterile insect technique (SIT) and the most widely adopted trapping techniques (Allsop, 2001). Despite the success of the above methods, sustaining control achievements remain a problem. Re-invasion is inevitable unless the entire tsetse populations are eliminated. Active surveillance and treatment efforts are hampered by the high costs of sustaining such an effort, lack of new drugs and the adverse side effects of the ones currently in use as well as the emergence of drug resistance in patients (McNeil, 2000). The presence of wild and domestic animal reservoirs for *T. b. rhodesiense* further complicates the disease control efforts (Hide, 1998) and treatment of infected patients alone might not be sufficient to interrupt transmission. Prospects for control of African trypanosomosis by tsetse vector manipulation have been reported (Askoy *et al*., 2001). Recombinant DNA technique such as transgenesis aims at modulating vector competence of insects hence lowering their ability to transmit pathogens. This may be done through the introduction and expression of foreign genes with anti-pathogenic properties which then interferes with the parasites viability, development or transmission. The eventual goal of any transgenic approach is to replace the naturally susceptible population with their engineered refractory counterparts in the field.

Parasite control targets the parasite mainly in the vertebrate host. Chemotherapy of HAT is essentially limited to suramin, pentamidine, melarsoprol and eflornithine. Suramin and pantamidine are active in the haematological stages of the disease (Wery, 1994; Wang, 1995), while both melarsoprol and eflornithine have their efficacy against the terminal

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(meningoencephalitic) phase (Wery, 1994). Suramin and pantamidine however, are ineffective against trypanosomes once they traverse the blood-brain barrier. Melarsprol causes lethal encephalopathy while eflornithine necessitates an expensive protocol in the developing countries experiencing HAT (Zweygarth and Rotcher, 1989).

The tsetse fly feeds on the blood of animals and humans. Once inoculated by an infected fly, the trypanosomes (Figure 2) proliferate and gradually invade all the organs of the host. In the midgut, the mammalian bloodstream parasites rapidly differentiate to procyclic forms and begin to replicate. Once established, the parasites migrate forward to the proventriculus and the mouthparts where they colonize the proboscis or salivary glands, depending on the parasite species (Vickerman, 1985). Here they differentiate into the infective metacyclics, which can then be transmitted to the next host during haematophagy.

The host's natural defenses effectively destroy most of the parasites taken in with a blood meal, but some trypanosomes manage to evade the immune system by modifying their surface membrane, a process known as antigenic variation (McCulloch, 2004). The trypanosome can express thousands of variants, multiplying with each new surface change. In the midgut of the invertebrate host, a bloodmeal stimulates the release of different insect-derived molecules including proteolytic enzymes, trypsin-like enzymes (Imbuga *et al*., 1992), lectin-like molecules (Maudlin, 1991; Osir *et al*, 1995) and trypanolysins (Stiles *et al*., 1990) that constitute a biochemically and physiologically hostile environment for the establishment of trypanosomes. Innate immunity plays a key role in the control of microbial infections in both vertebrates and invertebrates. Antimicrobial peptides (AMPs) are important components of innate immunity in insects, including vectors that transmit parasites that cause major human and animal diseases. AMPs are induced upon parasitic infections and can participate in regulating parasite development in the digestive tract and in the haemolymph (Boulanger *et al.*, 2002). Injury in insects and other arthropods induces two main proteolytic cascades, which

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result in haemolymph coagulation and melanisation. The cellular reactions of insects in response to invading microorganisms constitute a diversity of processes including phagocytosis, cell aggregation, nodule formation and large-scale encapsulation (Nappi and Vass, 1993).

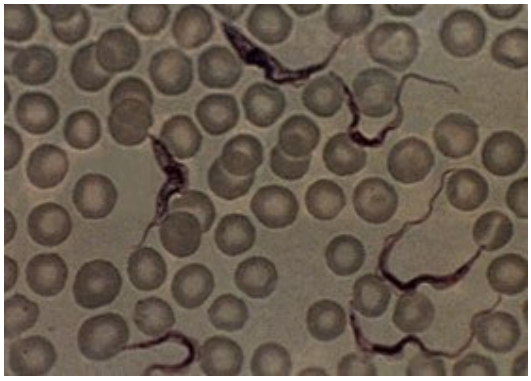

Figure 2: Trypanosomes proliferation in a mammalian blood (Pays *et al*., 2004).

In mammals, the parasite survives free in the bloodstream, being able to evade antibody responses through antigenic variation. The main initial clinical signs of human trypanosomosis include high fever, weakness and headache, joint pains and pruritus. Gradually, the immune defense mechanisms and the patient's resistance are exhausted. As the parasite develops in the lymph and blood of the patient, the initial symptoms become more pronounced and other manifestations such as anaemia, cardiovascular and endocrine disorders, abortion, oedema and kidney disorders appear (McNeil, 2000).

As the parasite invades the central nervous system in advanced stages of the disease, the patient's behavior changes and they can no longer concentrate and they become indifferent to their environment. Sudden and unpredictable mood changes become increasingly frequent, giving rise to lethargy with bouts of aggressiveness. Patients are then overcome by such extreme torpor such that eating, speaking, walking or even opening the eyes calls for an insurmountable effort. At night they suffer insomnia and during the day are exhausted by periods of sleep-like unconsciousness and finally the patients fall into a deep coma and death ensues (Roger and Packer, 1998)

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1.2 Statement of the Problem

African trypanosomosis is a disease of immense medical, agricultural and economic importance in sub-Saharan Africa. The existing control methods have not effectively contained the disease. Most recent studies have therefore focused on understanding the underlying vector-host and parasite-host interactions with the aim of identifying novel weak points that can be used to disrupt the transmission cycle. Many questions remain to be resolved in insect immunity and an efficient system involving hemocytes, well-controlled enzymes and inducible genes activity in relation to trypanosome differentiation as well as their elimination from the natural vector awaits elucidation. Immune molecules within vectors have been known to control parasites. There is however limited information about the presence, the characteristics and the role of these induced proteins in non-vector insects.

Previous *in vitro* studies have revealed the presence of parasite agglutinins against the trypanosomatid flagellates, *T. brucei* and *Leishmania hertigi* in the midgut extracts of *Schistocerca gregaria* and *Periplaneta americana* (Ingram *et al*., 1984). This study was therefore geared towards the elucidation of the patterns and the biochemical characteristics of the induced peptides in the haemolymph of *Schistocerca gregaria*, a non-vector, non-haematophagous insect. This analysis will lay a foundation for further research leading to further characterization and the actual identification of these immune molecules and hence their use in the management of trypanosomosis. This can be achieved by modifying the natural vectors of trypanosomatid flagellates the *Glossina spp* or it's endosymbionts by transgenesis and this involves the introduction and the expression of foreign genes whose product(s) have antiparasitic effects.

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1.3 Objectives

1.3.1 General objective

The aim of this study was to analyse induced haemolymph proteins in *Schistocerca gregaria* following *Trypanosoma brucei brucei* inoculation.

1.3.2 Specific objectives

1. To inoculate and quantify the anti-trypanosomal activity in the haemolymph at various time points.
2. To compare the electrophoretic profile of locust haemolymph proteins collected at specified time points following inoculation with *T. brucei brucei.*
3. To identify induced proteins in *Schistocerca gregaria* haemolymph following *Trypanosoma brucei brucei* challenge.
4. To analyse the effects of various sugars on the induction of haemolymph proteins.

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1.4 Hypotheses

This study was based on the following hypotheses:

a). The levels of haemolymph inducible proteins following *T. brucei brucei* challenge in *S. gregaria* does not vary with the time infection.

b). Electrophoretic analysis of induced haemolymph proteins show no variation in the pattern of induction.

c). *Trypanosoma brucei brucei* challenge does not produce any changes in protein levels in the haemolymph of *S. gregaria*.

d). Challenging *S. gregaria* with various sugars does not affect the levels as well as the activity of induced haemolymph proteins

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1.5 Justification

Human disease management relies on chemotherapy to treat infected individuals, whereas control of animal disease aims at reducing tsetse population and parasite clearance. The control strategies are carried out and financed by livestock owners with limited economic resources and in some cases, with no obvious economic incentives. Sustaining insecticide based control at the local level and relying on drugs for the treatment of a disease for which there is no evidence of acquired immunity can prove to be extremely expensive in the long run. At the same time, heavy reliance on insecticides and therapeutic drugs has resulted in the spread of drug resistance in both parasites and the vector, threatening the available tools for combating the disease. Lack of new drugs and the high cost, adverse side effects and emergence of resistance to the available drugs, hamper active surveillance and treatment efforts.

In view of the above mentioned, most studies have now focussed on parasite-vector interaction in an attempt to control the problem. For example, it is not clear why most tsetse flies are refractory to trypanosome infection. The complete exclusion of trypanosomes in non-vector insects coupled with the finding that midgut homogenates of some non-vector insects contains trypanosome lysing factors suggests that these factors may be associated with this exclusion (Ogoyi *et al.*, 2003). A better understanding of the complex biochemical and molecular interactions between insects and parasites will help to develop new strategies like novel antiparasitic drugs and the use of transgenic or paratransgenic insects. Some antimicrobial peptides have been found to have a direct antiparasitic activity on flagellate parasites and a lethal effect has been shown in several heterologous systems. *Hyalophora* cecropin has been shown to have a lethal effect on *T. cruzi* (Durvasula *et al*., 1997). In the sandfly, *Phlebotomus duboscqi*, a defensin was found to be active specifically on the insect forms of *Leishmania major* (Boulanger *et al.,* 2006). A recombinant *Glossina* attacin has also been shown to have trypanolytic activity against the blood stages and the insect forms of *T. brucei in vitro* and *in vivo* (Hu and Askoy, 2005). Collectively, these approaches will help control this important often-fatal disease.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Trypanosomes

Trypanosomes are haemoflagellate parasites i.e. actively motile flagellated parasites that live in the blood and lymph. Trypanosomatidae is one of the groups of organisms that spend their time in the gut of the vector host, interacting with potential immune molecules more than at any other site. There is sometime the involvement of the haemocoel and the salivary glands, but this is infrequent (Molyneux *et al*., 1986). Trypanosomes can be classified according to their morphology, biochemistry and the disease they cause. The salivarian group of parasites is normally found in Africa and comprises of *T. brucei rhodesiense* and *T. brucei gambiense* causing sleeping sickness in humans and *T. brucei congolense, T. brucei brucei* and *T. brucei vivax* causing nagana in animals, which are entirely dependent on tsetse (*Glossina spp*) for their transmission between hosts (Roger and Packer, 1998).

2.2 Life Cycle of African Trypanosomes

The African trypanosome has a life cycle alternating between the tsetse vector (*Glossina spp.)* and mammalian host (Touray *et al*., I992). In the tsetse fly host, the development is characterized by several rounds of differentiation and proliferation. At each stage, the survival and successful replication of the parasite improves the chance of continuing with the cycle. The life cycle (Figure 3) in the vector begins when tsetse fly feeds on an infected mammalian host. The vector picks up a pleomorphic population of trypomastigotes consisting of long slender, intermediate and short stumpy forms of the parasite. The short stumpy bloodstream forms of the parasite are pre-adapted for life in the invertebrate vector. They rapidly differentiate into procyclic forms in gut lumen; lose their variable surface

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glycoprotein (VSG) coat and express a new coat composed of procyclin proteins (Ingram and Molyneux, 199I).

The procyclic form is an important stage in the establishment of midgut infection. *In-vitro* studies have linked transformation from bloodstream forms to procyclic forms to temperature shift from 37 ºC to 27ºC. The procyclic trypomastigotes then migrate anteriorly to the paired salivary glands where they differentiate into attached epimastigote forms and finally into the infective free living metacyclic forms which are secreted in saliva and transmitted to the next host when the vector takes a blood meal (Vickerman *et al*., 1988). *Trypanosoma brucei* takes a minimum of two weeks to complete its life cycle in the tsetse fly but the fly continues to produce infective metacyclics for the rest of its life (Vickerman, I985). This developmental cycle requires 20-30 days before parasites can be transmitted to another host via tsetse bite. The fly therefore must be at least 20-30 days old in order to transmit the disease. The average life of a female fly in the field is about 40 days but usually less for males (Askoy *et al*., 2001).

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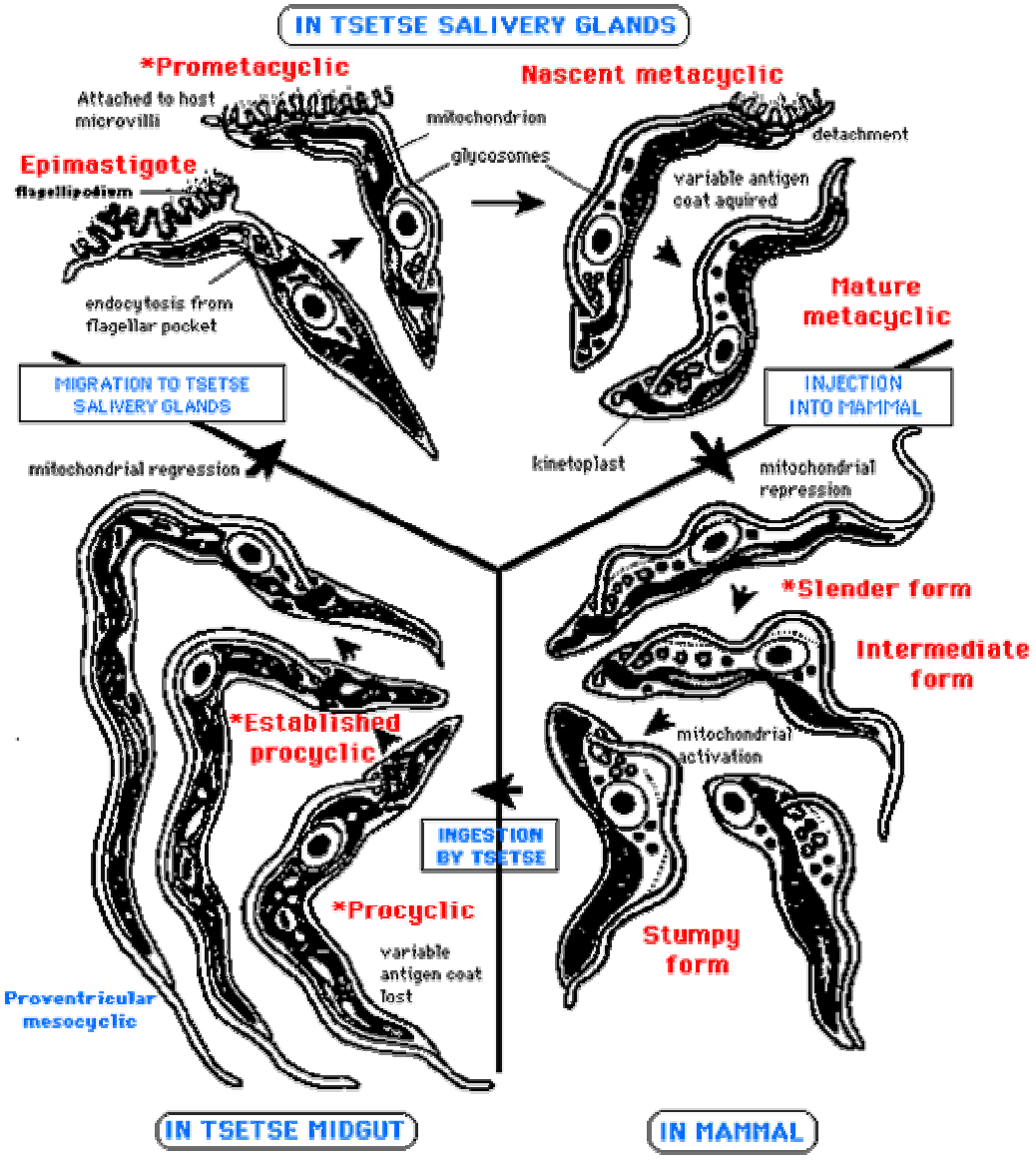

Figure 3: The life cycle of *T. brucei brucei*

Schematic representation of developmental cycle of *Trypanosoma brucei brucei* in a mammal and in the tsetse fly vector (Vickerman, 1985)

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2.3 Factors Influencing Transmission of Trypanosomes by Tsetse Fly

Successful transmission of *T. brucei* complex involves two developmental stages in the insect host. The first stage involves differentiation of ingested mammalian form parasite to insect-stage procyclic stage in the midgut and proventriculus tissues while the second involves invasion and maturation to metacyclic forms in the salivary glands from where they are transmitted to the mammalian host during a blood meal (Moloo, 1981).

Susceptibility to infection in *Glossina* is linked to a number of midgut factors and recent investigations with tsetse fly have yielded interesting information concerning an interaction between a host symbiont and successful trypanosome development (Kaaya and Darji, 1988). The presence of endosymbionts in the midgut cells appear to be linked with enhanced susceptibility (Maudlin and Ellis, 1985; Welburn and Maudlin, 1989; Welburn and Maudlin, 1999). It is suggested that these organisms produce chitinases that result in increased carbohydrate concentrations in the midgut lumen. These glucosamine-like sugars bind and effectively mop up midgut lectin and so prevent the lectin binding to the trypanosome surface. This in turn blocks parasite agglutination, allowing establishment and subsequent success of the trypanosome infection. Despite this inhibition of lectin activity, it is also proposed conversely, that different carbohydrate specific lectins are positively involved in the successful establishment of infection in the midgut (Maudlin and Welburn, 1988).

2.3.1 Immune Mechanism in Insects

Vector insects are known to posses both cellular and humoral defense capabilities (Rowley *et al.,* 1986; Dunn, 1990). The successful maturation of a parasite within the vector thus requires the circumvention of its immune system, which theoretically might be due to the parasite being insensitive to, evading or suppressing the immune response (Loker, 1994). The specific mechanisms operating in vectors is generally not well known, although in some cases

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of refractoriness it is clear that immune defenses are rapidly and specifically activated upon parasite invasion. This immune defense localized to the microenvironment of parasite surface may be targeted to specific stages of parasite development (Götz, 1986; Scalzolichtfouse *et al.,* I990).

Of the various stages of development, the midgut stage is thought to be very crucial for successful parasite development (Jacobson and Doyle, 1996). Bacteria and *T. brucei* provoke both cellular and humoral responses when injected in the tsetse fly haemocoel (Kaaya *et al.,* 1986; Kaaya *et al*., 1987). Insects present various general and specific barriers to infection. General barriers can block opportunistic infections and include anatomical features such as the cuticle and cibarial armature (Ham, 1992). While physiological processes include peritrophic membrane formation (Miller and Lehane, 1993) and synthesis of digestive proteases in the midgut (Feldmann *et al*., 1990).

2.3.2 Inducible Antibacterial and Antifungal Peptides

More than 50 antimicrobial molecules, which are all cationic peptides, have been isolated from the blood of immune-challenged insects (Cociancich *et al*., 1994). Two main factors normally present in the haemolymph are prophenoloxidase and lectins. Inducibility has been reported for antibacterial peptides, proteases as well as prophenoloxidase and lectins.
Upon infection, a rapid response is mounted in the insects and this involves the synthesis of a battery of potent antimicrobial molecules. These are known to be an array of small peptides synthesized and/or released into the haemolymph, following an infection (Dunn, 1990) or from trauma (Boman *et al*., 1981). These peptides include; cecropins, defensins and attacins.

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2.3.2.1 Cecropins

Cecropins act on Gram-positive and Gram-negative bacteria and are present in Diptera and Lepidoptera. They constitute a family of basic proteins with a molecular weight of about 4-8 kDa (Hultmark *et al*., 1980). Induction occurs when lipopolysaccharide present on the Gram-negative bacteria is recognised by the receptors in the immunodeficiency pathway (IMD) leading to the translocation of the transcriptional factor Relish and induction of AMPs such as cecropins, attacins, defensins and diptericin. Cecropins are membrane active antibiotics and experiments conducted with artificial membranes have indicated that they have channel-forming properties and can perforate the lipid bilayer (Chouristensen *et al*., 1988).

2.3.2.2 Attacins

Attacins are larger than cecropins, and have a molecular weight of 20-23 KDa. They are secreted by the hemocytes and the fat body. Like the cecropins they constitute a protein family of six molecules and in addition they are glycine rich and active on Gram-negative bacteria (Engstrom *et al.*, 1984). Through a double stranded RNA-based interference approach, it has been established that the pathogen-induced expression profile of attacins is under the regulation of the immunodeficiency pathway (Changyun and Askoy, 2006).

2.3.2.3 Defensins

Insect defensins are widely distributed among insects and are about 4KDa peptides consisting of thouree domains namely; a flexible amino-terminal loop, central amphipathic α-helix and a carboxyl-terminal antiparallel β-sheet. The α-helix is stabilized via two disulfide bridges linked to one of the strands of the β-sheet, and the amino-terminal loop is linked via one disulfide bridge to the other β-strand (Bonmatin *et al.*, 1992). These molecules form voltage-dependent channels, leading to the rapid leakage of K^+ and other ions (Cociancich *et al.*, 1993).

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2.4 Anti-parasitic Responses in Insects

Tsetse flies have been shown to possess midgut lectins that are capable of killing trypanosomes *in vivo* by a process resembling programmed cell death or apoptosis (Welburn and Maudlin, 1999). Lectins are defined as carbohydrate binding proteins other than antibodies and enzymes (Lehane and Msangi, 1991). The sources of lectins in nature are not only plants, but also viruses, bacteria, fungi, parasites, invertebrates and vertebrates (Lis and Sharon, 1998). Generally, the physiological functions of lectins and their interactions with the transmitted pathogens include; regulation of differentiation processes and morphogenesis, self and non-self recognition in immune and defense reactions, regulatory role of vector infections by transmitted pathogens and as differentiation factors of a vector-specific developmental stage of the pathogen (Wallbanks *et al.*, 1986; Maudlin, 1991). The predominant molecules in the haemolymph of insects include lectins or lectin-like molecules and prophenoloxidases. Injury in insects and other arthropods induces two major proteolytic cascades, which result in haemolymph coagulation and melanisation (Nappi and Vass, 1993). Specific recognition of parasites by the insect vectors through lectin-like molecules has been observed in mosquitoes, blackflies, sandflies and tsetse flies (Ham, 1992). Among the tsetse species, variation in susceptibility has been attributed partly to inherent differences in titer and specificity of the lectins (Wellburn and Maudlin, 1989; Maudlin, 1991). Differentiation of bloodstream form trypanosomes into procyclic forms is accompanied by complex morphological and physiological changes that enable the parasites to adapt to the harsh environment within the fly (Ghiotto *et al*., 1979; Roditi and Pearson 1990).

In vivo feeding experiments have demonstrated that D-glucosamine is an effective inhibitor of tsetse midgut lectins thus potentiating trypanosome establishment in the midgut (Wellburn and Maudlin, 1989). *In vitro* lysis assay indicated that D-glucosamine has the

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highest inhibition on the trypanolysin activity with N-acetyl glucosamine, D-glucose and D-galactose having limited inhibition (Ogoyi *et al*., 2003). This is an indication that a 100% infection rate could be achieved with the right inhibitory sugar (Maudlin and Wellburn, 1988).

Through co-evolution parasites have managed to exploit vector-derived lectins, which have been implicated in their differentiation. Insect lectins, which are carbohydrate-binding molecules, have also been involved in non-self recognition, phagocytosis, encapsulation, melanisation and agglutination (Ingram *et al.,* I984; Sharon and Lis, I989). *Glossina* proteolytic lectin (*Gpl*), is one of the induced peptides among the members of the *Glossina spp*. Some AMPs have direct effect on flagellate parasites. A lethal effect has been shown in several heterologous systems. *Hyalophora cecropin* has a fatal effect on *T. cruzi* (Durvasula *et al.*, 1997) while *Phormia diptericin* has a fatal effect on *Trypanosoma spp.* (Hao *et al.*, 2001). In the sand fly, *Phlebotomus duboscqi*, a defensin was found to be active specifically on the promastigote forms (insect forms) of *Leishmania major* (Boulanger *et al.,* 2006). A recombinant *Glossina* attacin was also shown to have trypanolytic activity against the blood stages and the insect forms of *T. brucei*, *in vitro* and *in vivo* (Askoy and Hu, 2005). This has suggested that antimicrobial peptides might contribute to the specificity of the parasite-vector interaction.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Animals and Parasites

Adult Winstar rats were supplied by the Department of Zoology, University of Nairobi and maintained on a rat pellet diet. These were used for maintaining serial passages of *Trypanosoma brucei brucei.* Adult desert locusts, *Schistocerca gregaria*, were obtained from the insectary at the Department of Zoology, University of Nairobi and were maintained on cereal grasses and kept at a temperature of 28-34ºC, 40-50% relative humidity and photoperiod of 12 h: 12 h (light: dark). The trypanosome used was *Trypanosoma brucei brucei* (ILTAT 1.4), a strain originally obtained from the International Livestock Research Institute, Nairobi.

3.2 Parasite Maintenance and Counting

The parasites were passaged severally in the rats within the Department of Biochemistry where the rats were subcutaneously inoculated with approximately 10^3 trypanosomes suspended in 2 ml phosphate buffered saline (PBS) (0.1 M phosphate, 0.15 M NaCl), pH 8.0 containing 1% glucose (PBSG).

The levels of the parasites in the rats were monitored every two days by tail snips to obtain blood that was then examined for parasites under the inverted microscope at a magnification of x40. To isolate the parasites, infected rat blood was drawn by cardiac puncture with a 21-gauge needle into a 10ml syringe using heparin as the anticoagulant in PBSG. The obtained blood was then centrifuged at 12000 *g* for 15 minutes and the buffy layer containing mainly the parasites re-suspended in PBSG. The trypanosomes were then separated from the contaminating cells by anion-exchange chromatography on DEAE-cellulose (Lanham and

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Godfrey, 1970). Parasite count was carried out using an Improved Neubauer ruled haemocytometer and the final concentration adjusted to 10^7 parasites/ ml in PBSG.

3.3 Locust Inoculation

Locusts were injected into their abdomen with 20 µl of $5.0x10^6$ trypanosomes/ml using an inoculation needle as described by Paskewitz and Riehle (1994). The effect of sugars (D-glucose, D-glucosamine, D-galactose and N-acetylglucosamine) on the induction of the protein was further assessed through inoculation of parasites followed by sugars at a concentration of 20 µl of 500 mM solutions.

3.4 Haemolymph Collection

At different time points post inoculation with trypanosomes, (6, 18, 24, 30, 42 and 48hours), haemolymph samples were collected by gently squeezing the locust thorax after piercing the exoskeleton at the base of the metathoracic legs. This was immediately mixed with PBS in eppendorf tubes prior to centrifugation at 5,000 *g* for 10 minutes at 4^oC to remove hemocytes and cellular debris. The cell free haemolymph was aliquoted and stored at -20^0C prior to protein estimation and analysis.

3.5 Protein Quantification

Protein estimation was carried out according to Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as the standard for the calibration curve. Two milliliters of the working reagents was added to each of the test tubes and thereafter the samples were added to appropriately labeled tubes. This was followed by a further addition of 450 µl of reagent A (70 mM Sodium–Potassium tartarate, 0.81 M Na_2CO_3, 0.5 N NaOH). The samples were mixed and incubated in a water bath at 50^oC for ten minutes then cooled to room temperature and 50 µl of reagent B (70 mM Sodium-Potassium tartarate) added. Upon incubation of the samples at

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room temperature for 15 minutes, 1.5 ml of reagent C (2N Folin- Ciocalteau phenol) was added and the samples incubated for a further 10 minutes in a water bath at 50^{o}C and absorbance measured at 650nm. Protein concentrations in the haemolymph samples were obtained from a standard curve constructed using BSA.

3.6 Trypanolysin Assays

About 20µl of haemolymph samples collected at 0, 6, 18, 24, 30, 42, and 48 hours following *T. brucei brucei* challenge was used to prepare doubling serial dilutions in PBSG and the total volume maintained at 0.02ml. The level of the activity was monitored after a further addition of 20µl of 10^7 parasites/ml in each well.

To analyze the effects of various sugars on the induction of haemolymph proteins, trypanolysin assay was carried out with a starting protein concentration of 1mg/ml for the test samples collected after 18 hours following *T. brucei brucei* and D-glucosamine, N-acetylglucosamine, D-glucose and D-galactose challenge. This time point was selected because induction of the proteins in the haemolymph post inoculation was found to peak after 18 hours. Haemolymph samples were serially diluted on a microtitre plate using 20µl of PBSG. An equal volume of the trypanosomes (bloodstream forms) containing approximately 10^7 parasites/ml was added to each dilution. After mixing, the plate was incubated at 27^{o}C for two hours and the level of lysis scored using an inverted microscope. Control experiments were set up using PBSG in place of haemolymph.

3.7 Electrophoretic Analysis of Haemolymph Proteins

3.7.1 Native and SDS-Page analysis

Native PAGE was used to asses the purity and the molecular weight of the target protein under non-denaturing conditions. Using this approach, the molecular weight of the

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native assembly rather than that of the component subunits of the proteins were estimated using high molecular weight markers ranging from 14.2 kDa- 272 kDa (Sigma, USA). The Tris buffers used for preparing stacking and the resolving gels and the electrophoresis running buffer were prepared as described for SDS-PAGE except that sodium dodecyl sulphate was left out for non-denaturing gels. Samples were not boiled before loading and SDS and 2-mercaptoethanol were not added.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemli (1970). Gradient denaturing gels ranging from 5 - 15% acrylamide (w/v) were cast using a gradient maker (BRL, USA) in a mini gel system and stacked using 4% acrylamide (w/v). Samples were then dissolved in an equal volume of sample loading buffer (0.0625 M Tris-HCl (pH 6.8), 10% SDS, 2% ß-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and boiled for about 5 minutes in a water bath prior to loading. The samples were then resolved at room temperature alongside SDS-low molecular weight standards ranging from 14.2 kDa-66 kDa (Sigma, USA). A current of 18 mA was applied to concentrate the samples in the stacking gel and electrophoresis carried out for 45 min at a constant current of 20 mA. SDS-PAGE was used to estimate the molecular weights of the differentially expressed proteins in various haemolymph samples collected at different time points (0, 6, 18, 24, 30, 42, 48hours).

3.7.2 Two-Dimensional PAGE

3.7.2.1 ***Sample preparation***

Haemolymph samples collected at 0, 6, 18, 24, 30, 42, and 48 hours were centrifuged at 10,000 *g* for 15 minutes and mixed well with the solubilization buffer (8M urea, 4% NP-40, 0.5% Triton x-100, 1.6% Ampholine pH 5.0 to 7.0, 0.4% Ampholine pH 3.0 to 10 (Uppsalla, Sweden) and 20mM Dithiothoureitol (DTT)) in the ratio of 2:1 respectively to enhance

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complete solublization and denaturation of the proteins. Some of the modifications used to optimize protein solubilisation included the use of DTT in place of β-mercaptoethanol for complete reduction of the disulphide bonds. NP-40 instead of SDS since the latter is not compatible with the first dimension where proteins are separated purely according to their pIs. Reduction of urea concentration from 9.5M to 8M aimed at reducing crystallization of the tube gel during the first dimension run.

3.7.2.2 ***First dimension electrophoresis***

Glass capillary tubes (70mm x 3mm) thoroughly cleaned in chromic acid and rinsed with de-ionized water then completely dried were used. The casting tube was sealed at the bottom using a thick layer of parafilm and the tubes placed in a vertical position.

In the first dimension, isoelectric focusing was carried out according to the procedures of O'Farrell, (1975). A 4% acrylamide monomer solution comprising of 9.2M urea, 2% NP-40, 1.6% ampholine (pH 5-7) and 0.4% ampholine (pH 3-10) was cast onto capillary tubes and allowed to polymerize. After polymerization, pH gradient was established by pre-electrophoresis of the rod gel in 10mM H_3PO_4 anolyte and 20mM NaOH catholyte at 500V for 10 minutes. The haemolymph samples (10µl containing 50µg of protein) were then loaded onto the gels and overlaid with the sample overlay buffer (7M urea, 0.8% ampholine (pH 5-7) and 0.2% ampholine (pH 3-10) and 0.05% bromophenol blue). Using fresh electrolytes electrophoresis was carried out at 500V for 10 minutes followed by 750V for 3.5 hours.

3.7.2.3 ***Second dimension electrophoresis***

After isoelectric focusing, the gels were extruded from the capillary tubes using a 1ml syringe tube filled with the equilibration buffer and attached to the ejector. After ensuring that

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the tube was gripped tightly, the gel was extruded onto a piece of parafilm by gently increasing the pressure on the syringe plunger until the gel started to emerge. After noting the orientation of the gel (basic and the acid end), the equilibration buffer (6M urea, 2% SDS, 375mM Tris pH 8.8, 20% glycerol and 2% DTT) was added onto the parafilm together with the SDS sample buffer in the ratio of 1:1 and allowed to settle for 15 minutes.

For the second dimension, a 12% SDS slab gel was prepared as described earlier for SDS-PAGE. This was stacked using a 4% acrylamide gel and after pouring off the equilibration buffer from the isoelectrofocusing gel, the tube gel was carefully laid on the second dimensional gel noting the cathode and the anode end and resolved alongside SDS-molecular weight standards. Separation was carried out according to the protocol described for SDS-PAGE at a constant current of 20 mA/gel until the bromophenol blue dye reached the bottom of the gel.

3.8 Protein Visualization and Analysis

3.8.1 Silver staining

After electrophoresis, the gels were silver-stained according to the procedures of Gottlieb and Chavko (1987) as detailed in the table (appendix). This involved protein fixation where the gels were soaked in 50% methanol and 10% acetic acid in distilled water for two minutes. The gels were then rinsed twice in 50% methanol for twenty minutes each and a further incubation for 30 minutes in 2.5% glutaldehyde. In the reaction step, solution A (0.8g $AgNO_3$ in 2.5ml distilled water) was used to titrate solution B (1ml of 2M NaOH and 1.6ml NH_4OH adjusted with distilled water to a volume of 20ml). The gels were placed in the solution for fifteen minutes and later rinsed thoroughly in distilled water. Stain development involved immersing the gels in a color developer (2.5ml of 1% citric acid (w/v) and 125µl of 38% formalin adjusted to 250ml with distilled water) until a clear coloration was achieved.

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This reaction was stopped using a 5% acetic acid and the gels stored in 7% acetic acid prior to photographing. This technique is up to 100 times more sensitive than Coomassie Blue R-250 and is able to detect as little as 0.38ng/mm^2 of bovine serum albumin.

3.8.2 Coomassie staining

Coomassie staining was considerably less complicated than silver staining and an additional advantage is that different proteins tend to stain to the same extent with this dye, thus making it possible to roughly quantify the relative amounts of different proteins by comparison of the intensities of the bands (Hames and Rickwood, 1990). Staining was done using 0.6% (w/v) Coomassie brilliant blue in 45% methanol, 10% acetic acid and allowed to go on for a couple of hours. Destaining was enhanced by including pieces of foam packings in the destain solution (45% methanol and 10% acetic acid).

3.8.3 Analysis of 2-D gels

Gel scoring was done visually using a light box. Protein spots outstanding in shape and intensity were marked first in different areas of the gels. The homology of the spots was assigned by the relative positions (pI and molecular weight) and the appearance of the protein spots (colour intensity). Replicate gels were used to confirm enhancement, reduction or the presence/absence of spots. Comparison of various gels scans and identification of unique spots or over expressed proteins was done using the Flicker program, computer software).

3.9 Western Blotting

In this study, antibodies against Glossina proteolytic lectin (Gpl) previously raised in a New Zealand white rabbit were used (Osir *et al*., 1995). Haemolymph samples collected

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from *Schistocerca gregaria* following *T. brucei brucei* challenge were used in immunoblotting experiments. The samples obtained were separated by SDS-PAGE as previously described. The separated proteins were then transferred onto nitrocellulose membrane using a semi dry blotter (Biorad) in blotting buffer (25mM Tris, 192mM glycine and 20% methanol, pH 8.3). The sandwich was then placed between two parallel electrodes and electroblotting carried out at a constant voltage (10V) for two hours.

Following blotting, the success of the transfer was confirmed by staining the membrane in 0.2% ponceau S in 10% acetic acid for five minutes where the proteins appeared as red bands on a white background. After destaining the membrane with distilled water, the nitrocellulose paper was placed in a blocking solution (20µg/ml BSA in coating buffer containing 1% gelatin) for 30 minutes to block the remaining sites on the membrane. The membrane was washed four times for five minutes each in Tween-PBS (2% Tween 80 in PBS, pH 7.4) and soaked for one hour in a solution of the primary antibody diluted in a ratio of 1:1000 (courtesy of Dr. Abubakar of the Department of Biochemistry, University of Nairobi). After washing the membrane for five times for five minutes each in Tween-PBS to remove any traces of unbound primary antibody, the nitrocellulose membrane was incubated for a further 45 minutes with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted in a ratio of 1:2500 in coating buffer. The sheet was then washed four times for five minutes each in Tween-PBS and the bands revealed by incubating with the substrate, 4-Chloro-1-Napthol (1 part diluted in 6 parts of 0.05M Tris/HCl, pH 7.4) from a stock solution containing 3 mg/ml of 4-chloro-1-Napthol in absolute methanol and 10µl of 30% hydrogen peroxide. Incase of a cross-reaction, the substrate produces a blue deposit. Further colour development was stopped by rinsing the membrane in distilled water.

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CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Trypanolytic assay following *T. brucei brucei* inoculation

In order to determine protein concentration in the haemolymph samples, a standard curve was constructed using bovine serum albumin as the standard (Figure 4). Following *Trypanosoma brucei brucei* challenge, haemolymph samples collected at 0, 6, 18, 24, 30, 42, and 48 hours were subjected to protein quantification, with the 0 hour sample acting as the control. The level of proteins in the various samples were found to increase with increasing time and peaking after 18 hours but thereafter decreasing in the 24, 30, 42, and 48hours sample (Figure 5). To determine the trypanolytic activity in various samples an *in vitro* lysis assay was carried out starting with an equal volume of each test sample. Lysis activity was detected in all the samples with the 18 hour sample having the highest activity at all times (Figure 6) with complete lysis (100%) being observed after 75 minutes of incubation. Trypanolysin activity was also detected in the other haemolymph samples and the level of activity starting with the highest to the lowest was as follows; 18 hour, 6 hour, control, 24 hour, 30 hour, 42 hour, and 48 hour respectively.

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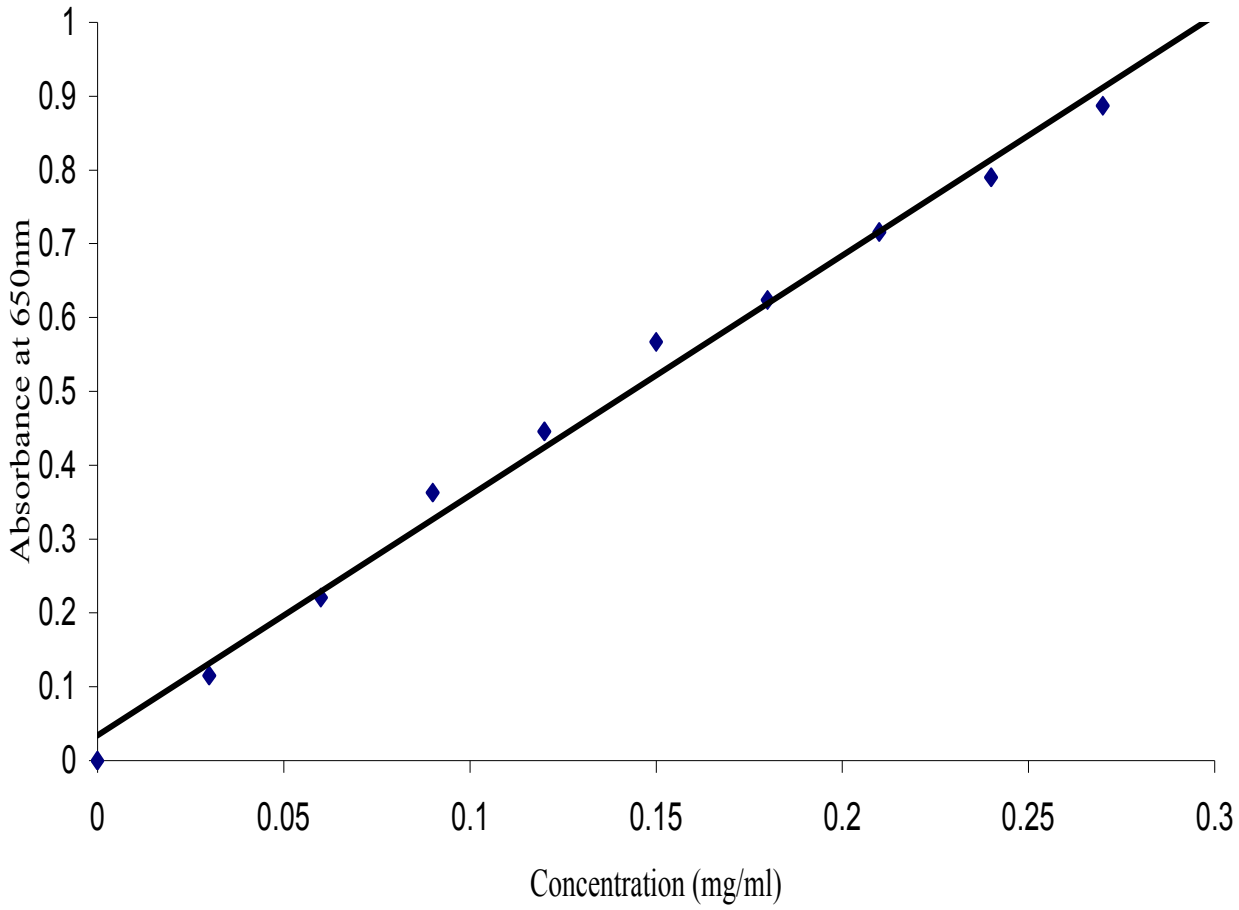

Figure 4: Protein quantification standard curve for estimating protein concentration of various haemolymph samples. Lowry-Hatree assay (Lowry *et al*, 1951) was used and BSA as the standard.

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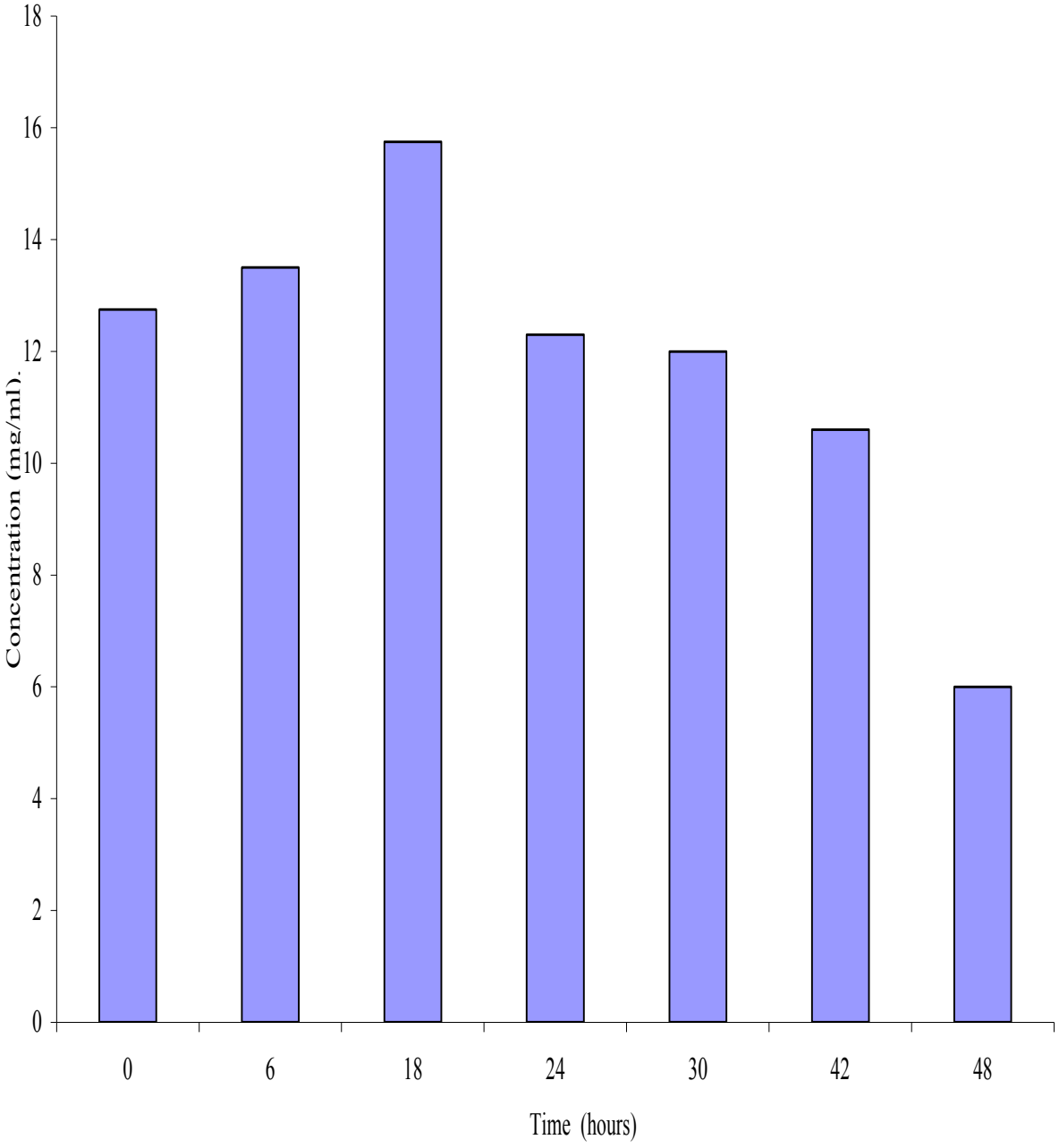

Figure 5: Protein concentration of various haemolymph samples collected at different time points following *T. brucei brucei* challenge.

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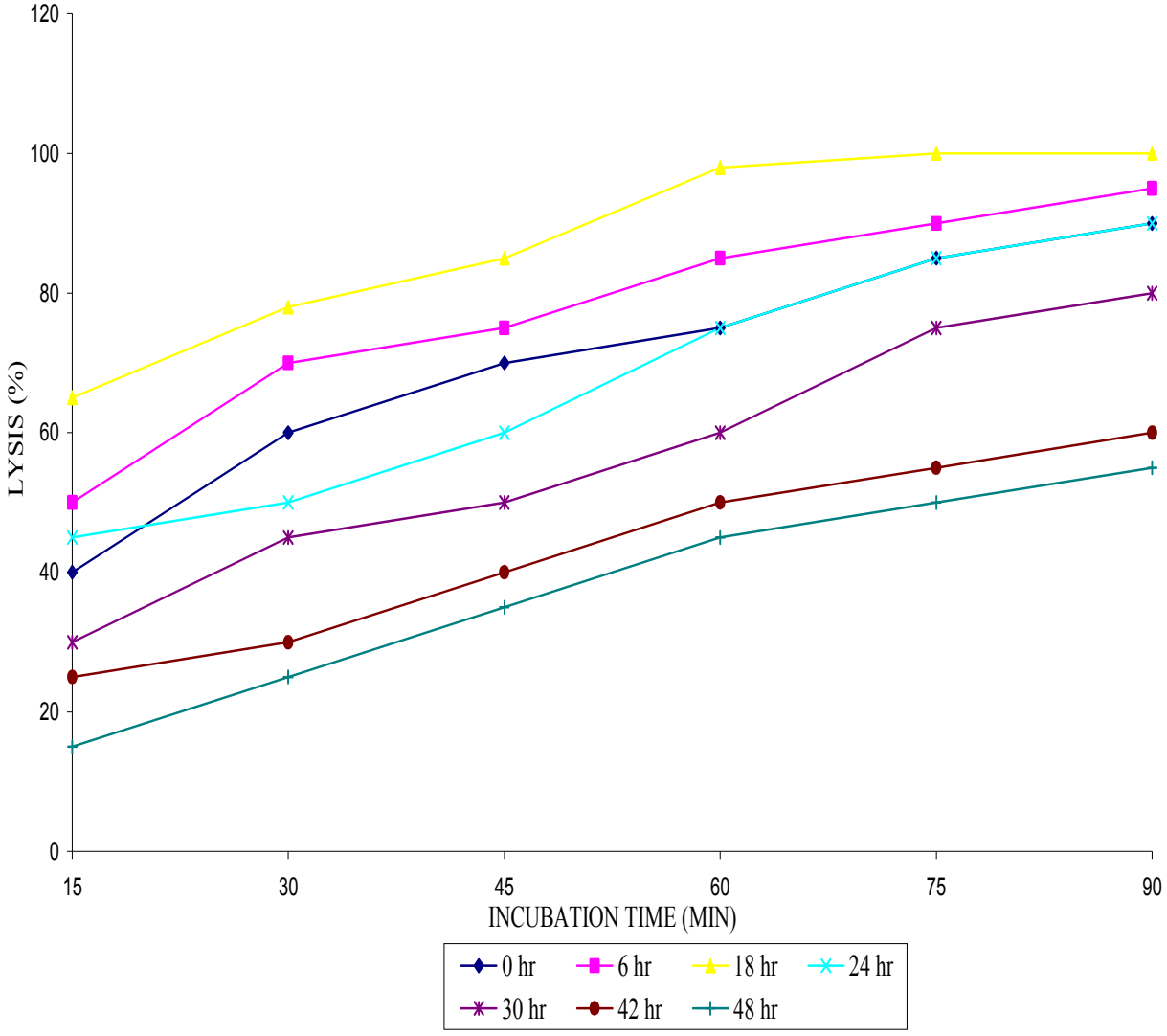

Figure 6: *In vitro* lysis assay of haemolymph samples collected at different time points following *T. brucei brucei* challenge. Equal volumes of the samples were incubated with parasites and the percentage lysis scored using an inverted microscope at a magnification of X40.

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4.1.2 Electrophoretic profile of induced haemolymph proteins

4.1.2.1 ***Native and SDS-PAGE***

Haemolymph proteins profiles were evaluated by separating them on native and SDS-PAGE followed by staining using coomassie and silver stains. This was followed by molecular weight estimation using a standard calibration curve (Figure 7). After separating the proteins on a non-denaturing gel (Figure 8), four proteins (*w, x, y, z)* were enhanced or induced with time. Protein *W* (331.8 kDa) was found to be up regulated with time, whereas protein *X* (269.2 kDa) intensified in 6 and 18 hour and then faded out in the 24 and 30 hours. There was also evidence of the same protein in the 42 and 48 hour almost comparable to that of 6 and 18 hours samples. Protein *Y* (52.5 kDa) on the other hand faded out with time upon peaking after 18 hours while protein *Z* (20.9 kDa) was enhanced in the 6 hour sample then faded out with time.

Haemolymph profiles of the samples collected at 0, 6, 18, 24, 30, 42, and 48 hours post *T. brucei brucei* challenge were compared using SDS-PAGE to examine the extent of polymorphism in proteins. Five polypeptides of approximately 53.7, 48, 21.9, 17.4, and 16.6 kDa were consistently different in the seven samples (Figure 9). Band *a* (53.7 kDa) was found to fade out with time being completely suppressed in the 48 hour sample. Band *b*, (48 kDa) was absent in 0, and 6 hour sample but induced thereafter intensifying with time and faded out in the 48 hour sample. Band *c*, (21.9 kDa) was absent in 0 hour but present in all other samples with induction being suppressed after 48 hours. Band *d*, (17.4 kDa) was constitutively present in all samples but suppressed in the 48 hour sample. Band *e*, (16.6 kDa) was present in the 18, 24, 30 and 42 hour samples but absent in the 48 hour sample .

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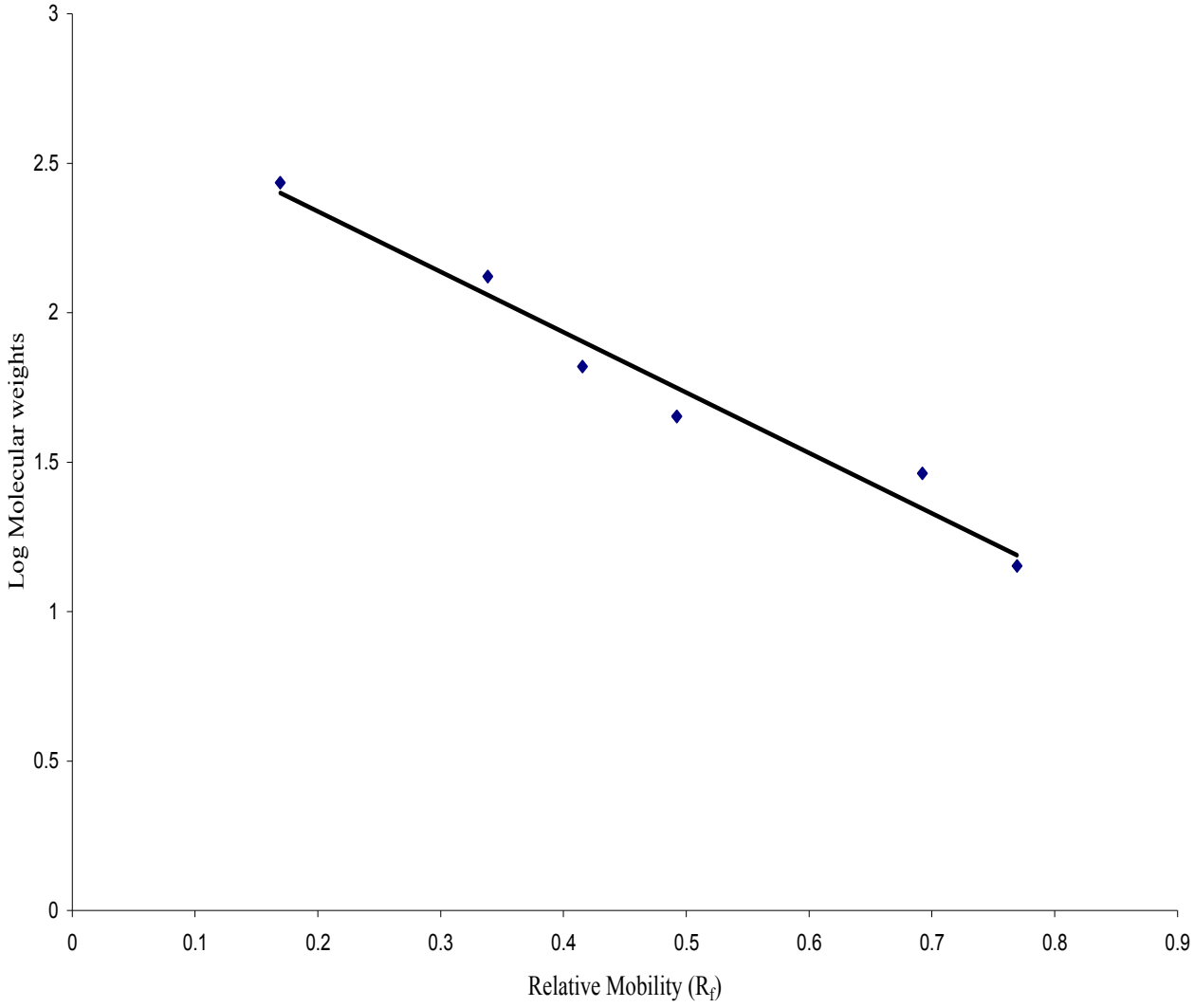

Figure 7: SDS-PAGE calibration standard curve for molecular weight estimation. The molecular weight standards used included; Albumin-66,000;Ovalbumin-45,000; Glyceraldehyde-3-phosphate dehygrogenase-36,000;Carbonicanhydrase-29,000; Trypsinogen-24,000; Trypsin inhibitor-20,100 and α-lactalbumin-14,200.

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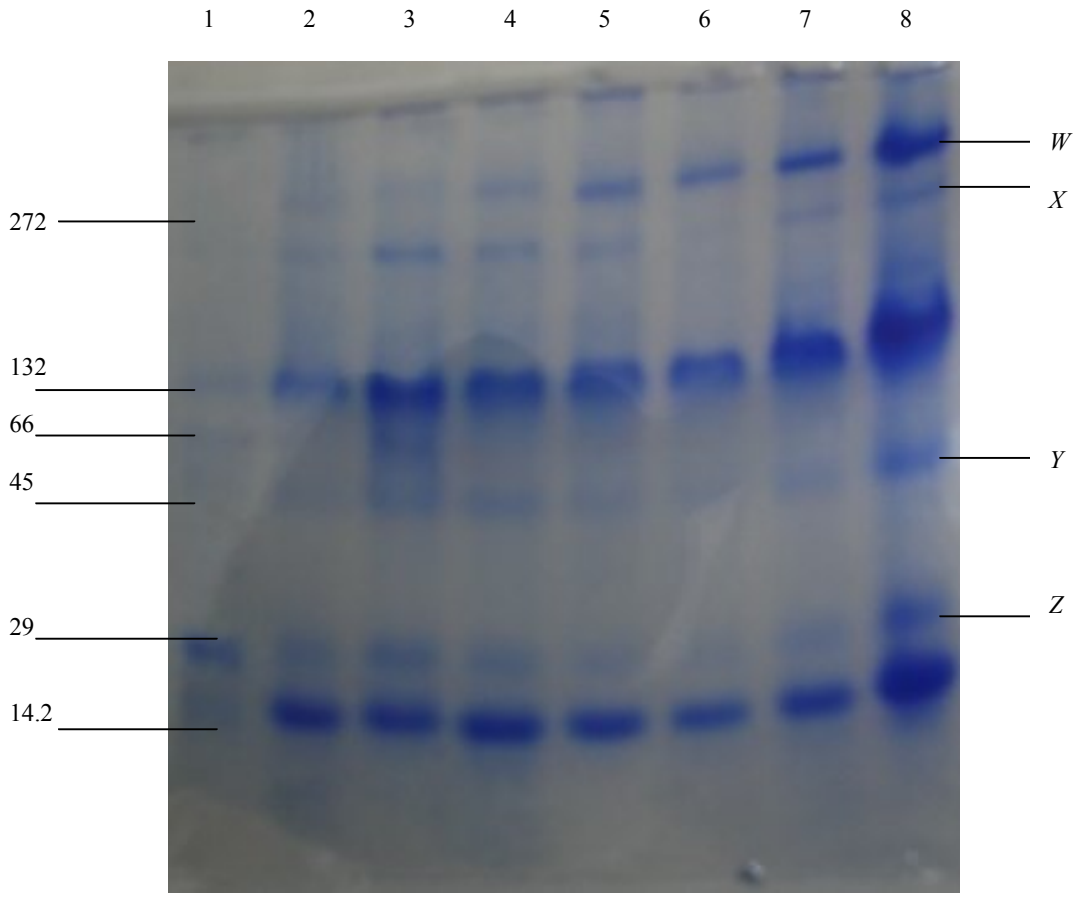

Figure8: A 5-15% Native PAGE of haemolymph samples collected at different time points after challenging the locusts with *T. brucei brucei.* Lane 1, native low molecular weight markers (50 µg) in kDa. Lane 2; 0 hour sample, lane 3; 6 hour sample (100 µg), lane 4; 18 hour sample (100 µg), lane 5; 24 hour sample (100 µg), lane 6; 30 hour sample (100 µg), lane 7; 42 hour sample (100µg), lane8; 48 hour sample (100µg). The gel was coomassie stained.

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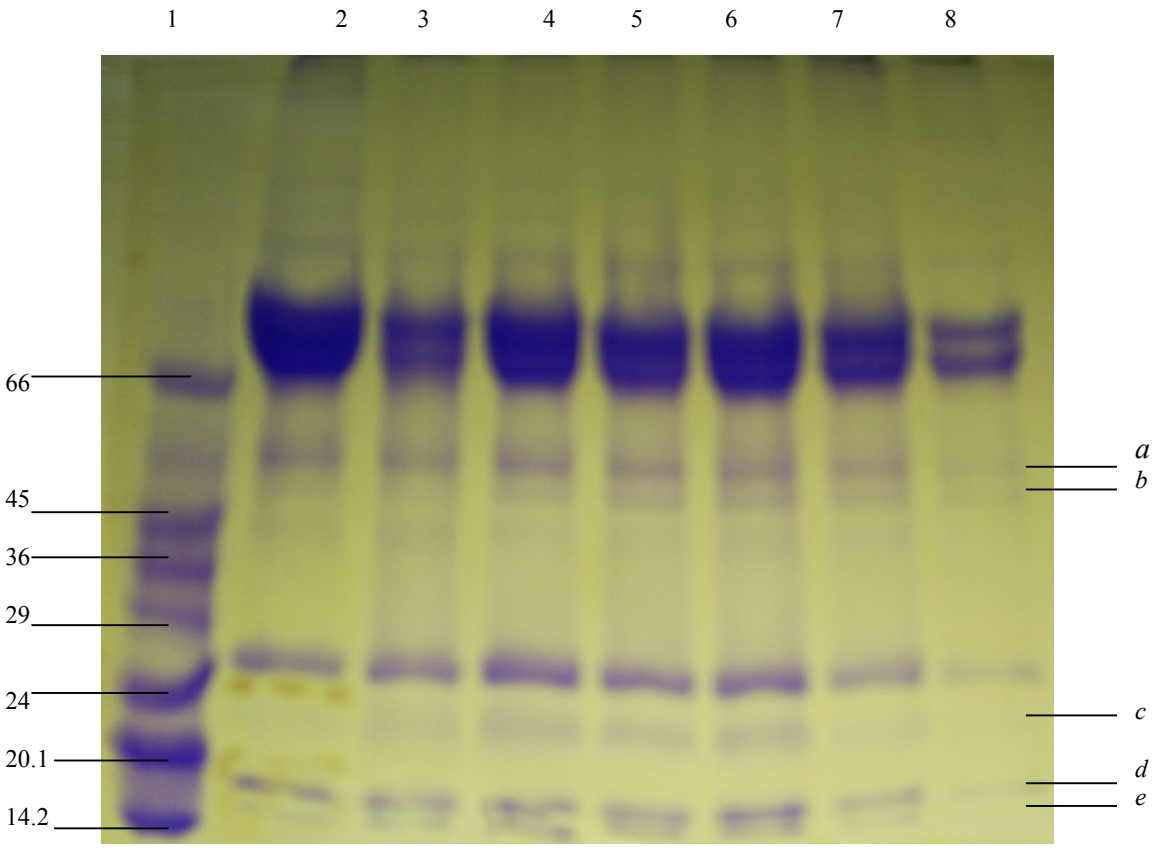

Figure 9: A 5-15% SDS-PAGE of haemolymph samples collected at different time points following *T. brucei brucei* challenge. Lane 1; Molecular weight markers (50 µg) in kDa, lane 2; 0 hour sample (100µg), lane 3; 6 hour sample (100µg), lane 4; 18 hour sample (100µg), lane 5; 24 hour sample (100µg), lane 6; 30 hour sample (100µg), lane7; 42 hour sample (100µg), lane 8; 48 hour sample (100µg). The gel was coomassie stained.

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4.1.2.2 ***Two-dimensional gel electrophoresis***

To improve precision of the resolution, the haemolymph polypeptides were analysed using two-dimensional polyacrylamide gel electrophoresis followed by silver staining. In order to determine the isoelectric points of the various proteins, a standard curve (Figure 10) was constructed using proteins of known isoelectric points (Figure 11) run on a 12 % SDS-PAGE . A comparison was made between haemolymph peptides from the control (Figure 12) and those collected from the immune challenged locusts. Haemolymph samples collected at 0 hour before challenging the locusts with *T. brucei brucei* and 18 hour, 24 hour and 48 hours (Figures 13, 14, and 15, respectively) following the parasite inoculation were compared. A 2 D-differential time course study for 0, 18, 24 and 48 hours post inoculation indicated that most of the changes in protein expression occurred 18 hours after *T. brucei brucei* challenge (Figure 13). Using the 0 hour sample as the control, five polypeptides (*a, b, c, d*, and *e*) were enhanced, induced or reduced with the 18 hour sample expressing all the proteins. After estimating the isoelectric points of these spots, they were found to fall approximately between 5.6 to 6.2 and the molecular weights ranging between 16 kDa to 54 kDa. Two polypeptides (*b* and *d*) were found to be induced 18 hours post infection, with polypeptide *c* being induced after 6 hour following the challenge. Protein *a*, and *d* were constitutively present in all of the samples with the only difference being their intensity which increased with the time of induction peaking at 18 hours and decreasing thereafter.

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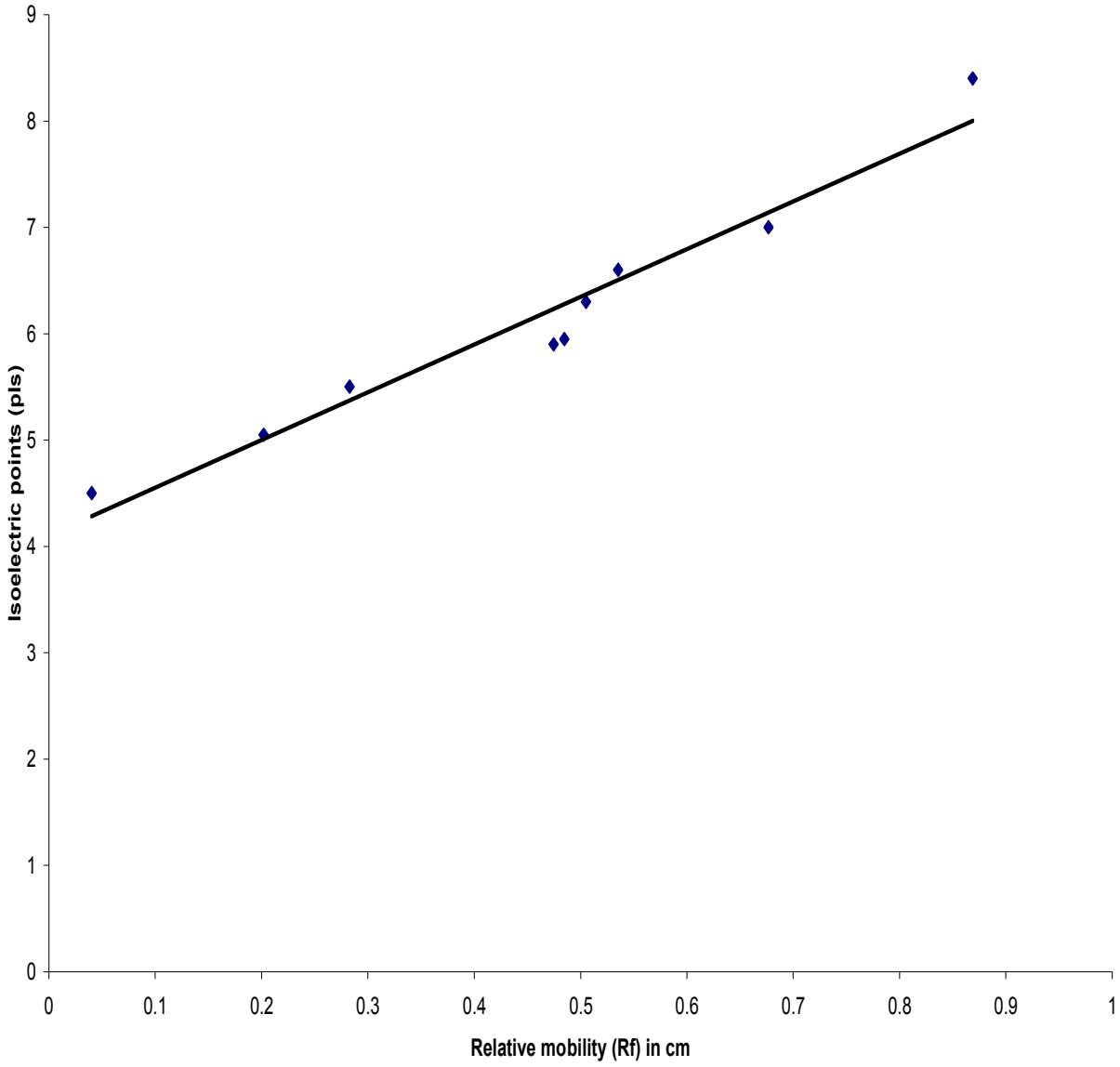

Figure 10: Two dimensional acrylamide gel electrophoresis standard curve for isoelectric points determination. The protein standards used included; Conalbumin-76,000 and a pI-6.3. BSA-66,200; pI-5.5. Actin-43,000; pI-5.1. Glyceraldehyde phosphate dehydrogenase-36,000; pI-8.5. Carbonic anhydrase-31,000; pI-6.0. Soybean trypsin inhibitor-21,500; pI-4.5 and Myoglobin-17,500; pI-7.0.

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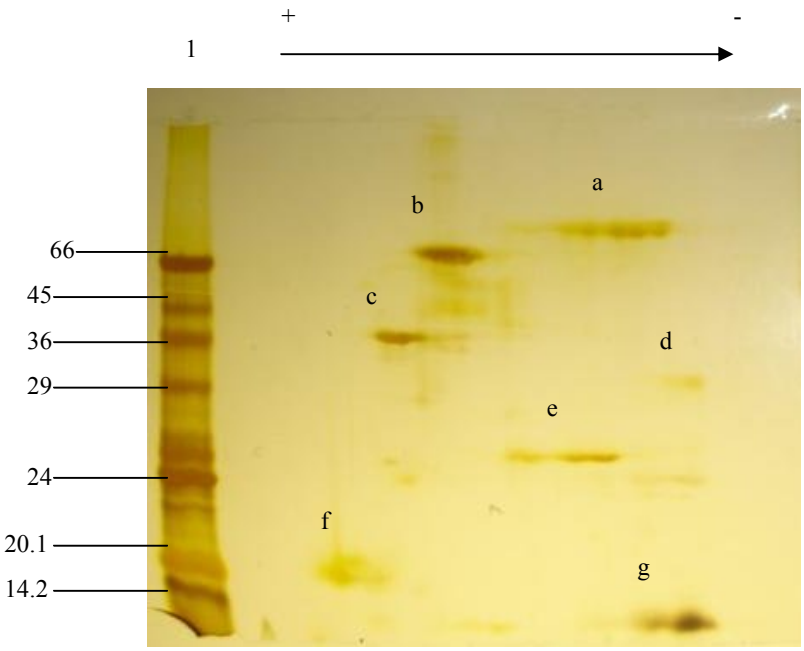

Figure 11: 12% SDS-PAGE for 2D Standards (Bio-Rad). 50µg sample of 2-D SDS-PAGE standards were separated through isoelectric focusing in a 7cm acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, then loaded onto a 12% SDS- PAGE slab gel. Lane 1; Molecular weight markers (15µg) in kDa. (a). Conalbumin, (b). Albumin, (c). Actin, (d). Glyceraldehyde phosphate dehydrogenase, (e). Carbonic anhydrase, (f). Trypsin inhibitor and (g). Myoglobin. The gel was silver stained.

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-Basic end

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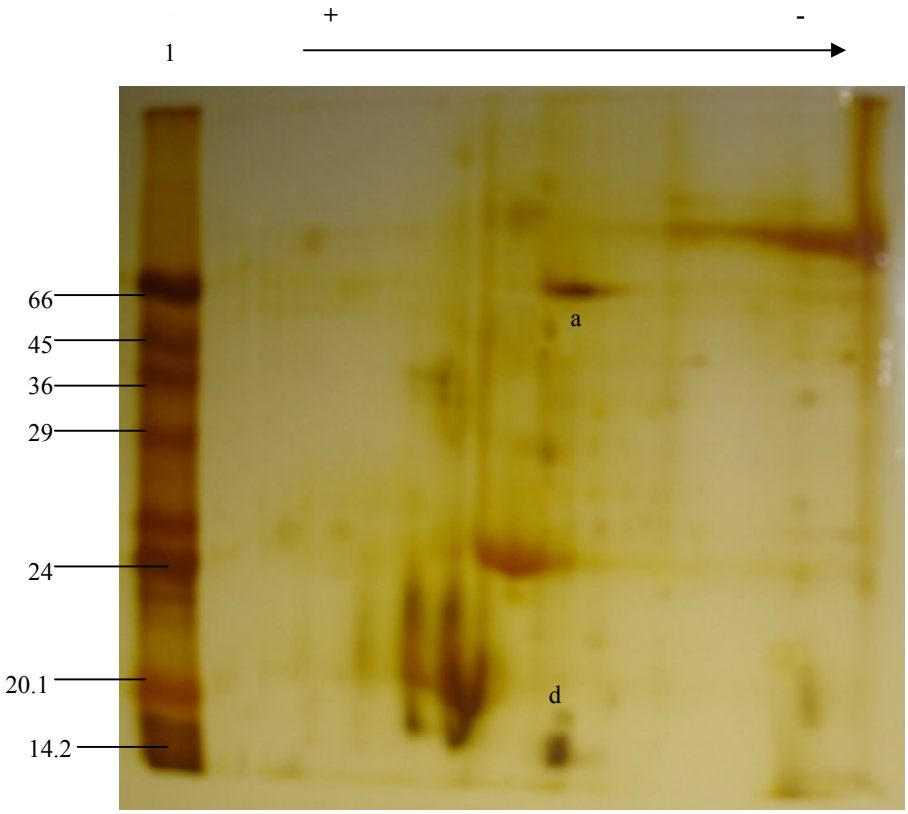

Figure12: 2D PAGE for 0hour haemolymph sample collected after challenging the locusts with *T. brucei brucei.*100µg of the sample was separated by isoelectric focusing on a 7cm acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, then loaded onto a 12 % SDS-PAGE slab gel. Lane 1: Molecular Weight Markers (15µg) in kDa. The gel was silver stained.

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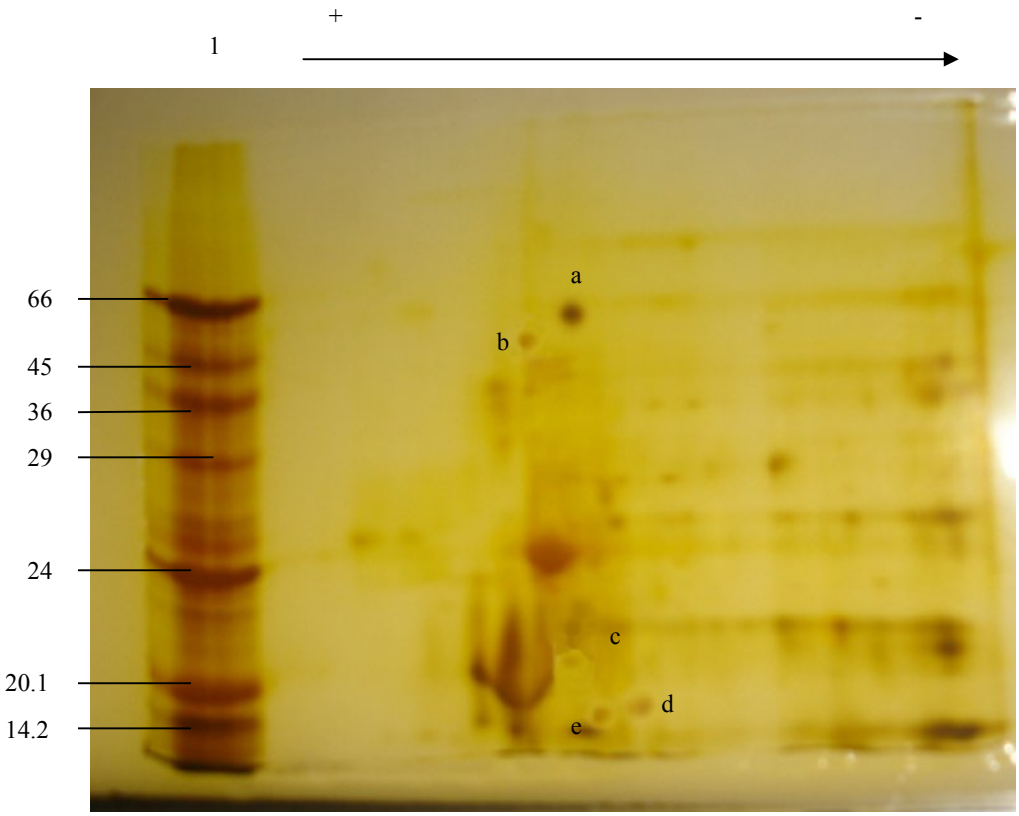

Figure 13: 2D PAGE for 18 hour haemolymph sample collected after challenging the locusts with *T. brucei brucei.*100µg of the sample was separated using isoelectric focusing in 7cm acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, then loaded onto a 12% SDS-PAGE slab gel. Lane 1; Molecular Weight Marker (15µg) in kDa. The gel was silver stained.

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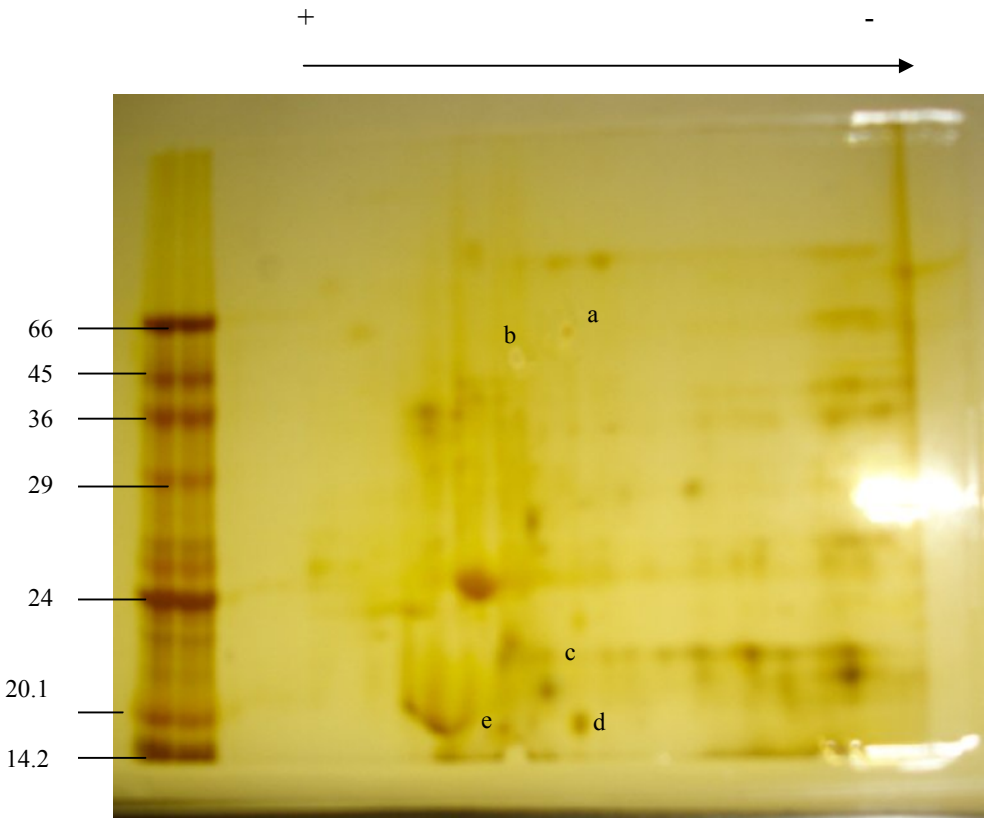

Figure 14:2D PAGE for 24 hour haemolymph sample collected after challenging the locusts with *T. brucei brucei*.100µg of the sample was separated using isoelectric focusing in a 7cm IPG acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, then loaded onto a 12% SDS-PAGE slab gel. Lane 1; Molecular Weight Marker (15µg) in kDa. The gel was silver stained.

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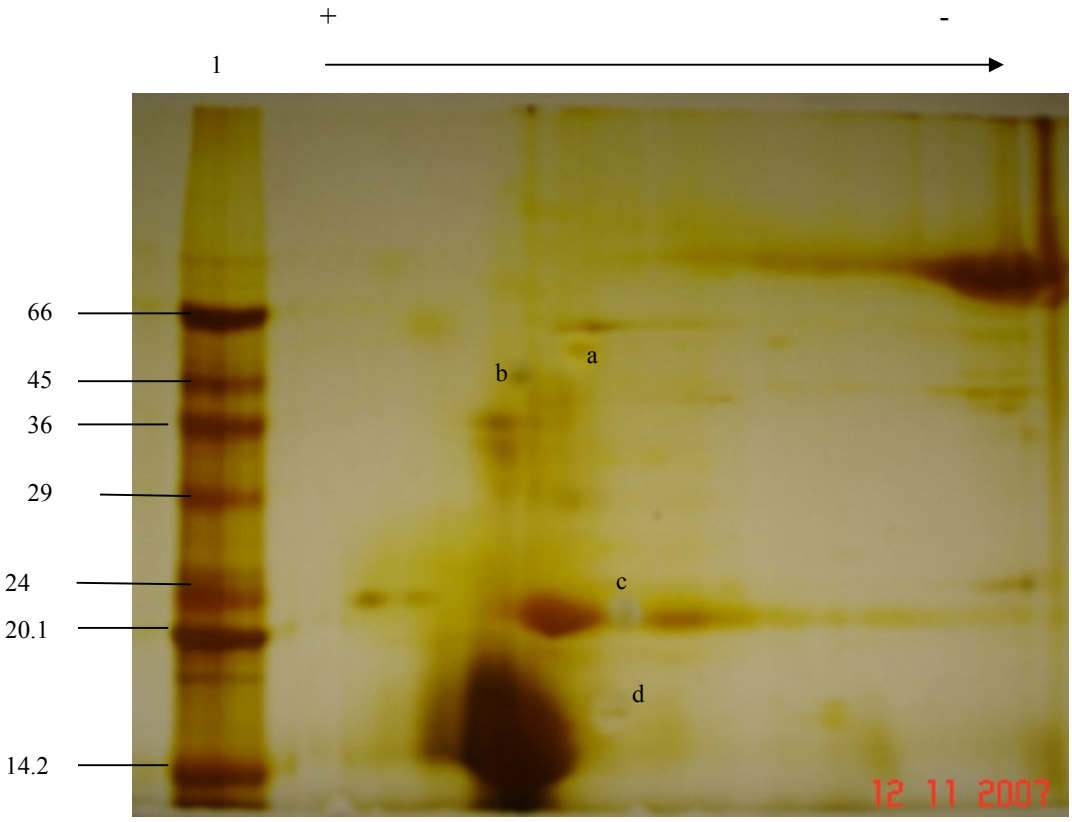

Figure 15: 2D PAGE for 48 hour haemolymph sample collected after challenging the locusts with *T. brucei brucei.* 100µg of the sample was separated through isoelectric focusing in a 7cm acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, then loaded onto a 12% SDS-PAGE slab gel. Lane 1; Molecular Weight Marker (15µg) in kDa.

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4.1.3 Effects of sugars on induced proteins

To examine the effects of sugars on the induction of proteins, the locusts were challenged with *T. brucei brucei* followed immediately by sugars (D-glucosamine, N-acetylglucosamine, D-glucose and D-galactose). Haemolymph samples were collected after 18 hours and upon protein quantification, D-glucosamine was found to have the highest inhibitory effect followed by N-acetylglucosamine, D-glucose while D-galactose had the least inhibitory effect on the induction of proteins (Figure 16). This time point was chosen because protein induction following the parasite inoculation was found to peak at about 18 hours. To confirm this, a bioassay was carried out to ascertain the extent of parasite lysis in each sample. Lysis increased with the protein concentration with D-galactose showing the highest activity at all concentrations (Figure 17). After two hours of incubation, 80% lysis was observed in 0.5 mg/ml of protein in the haemolymph sample collected after 18 hours following D-galactose treatment. For the other samples, the level of trypanolytic activity from the highest was in D-glucose treated sample (60%) followed by N-acetylglucosamine (30%) and D-glucosamine treated sample had the least activity of approximately 10%.

Following the sugar treatment, the polypeptides were compared using a SDS-PAGE (Fig. 18). Polypeptide *a*, (65.8 kDa) was slightly suppressed in the presence of D-glucosamine as compared to D-galactose treatment. Band *b* which is a polypeptide of approximately 43.7 kDa was found to be suppressed in the presence of N-acetyl glucosamine and almost absent in the presence of D-glucosamine. Polypeptide *c*, molecular weight 27.5 kDa was absent in the presence of D-glusosamine with induction being slightly suppressed following D-galactose treatment. Induction of band *d*, molecular weight 20.4 kDa was highly suppressed in the presence of D-glucosamine and N-acetyl glucosamine but unaffected in the presence of D-galactose and D-glucose. Band *e* with a molecular weight of approximately 14 kDa was found to be unaffected by any of the sugar treatment.

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After challenging the locusts with *T. brucei brucei*, the sugars; D-glucosamine, N-acetyl glucosamine, D-glucose and D-galactose, D-glucosamine at a concentration of 500mM was found to have the highest inhibition on protein induction with D-galactose having the least effect. With protein induction peaking after 18 hour, samples collected at this point following D-glucosamine and D-galactose treatment were analysed using 2D-PAGE. Analysis of the two gels indicated that the peptides were constitutively induced with the only difference being their intensity. Compared to D-galactose (Figure 20), induction was highly suppressed in the presence of D-glucosamine (Figure 19). Profiling of the polypeptides in the insects haemolymph samples collected after 18 hours following the trypanosomes and the sugar challenge indicated that the spots which only differed in intensity, had their isoelectric points falling approximately between 5.4 and 7.8 and molecular weights ranging from 14 kDa to 65 kDa.

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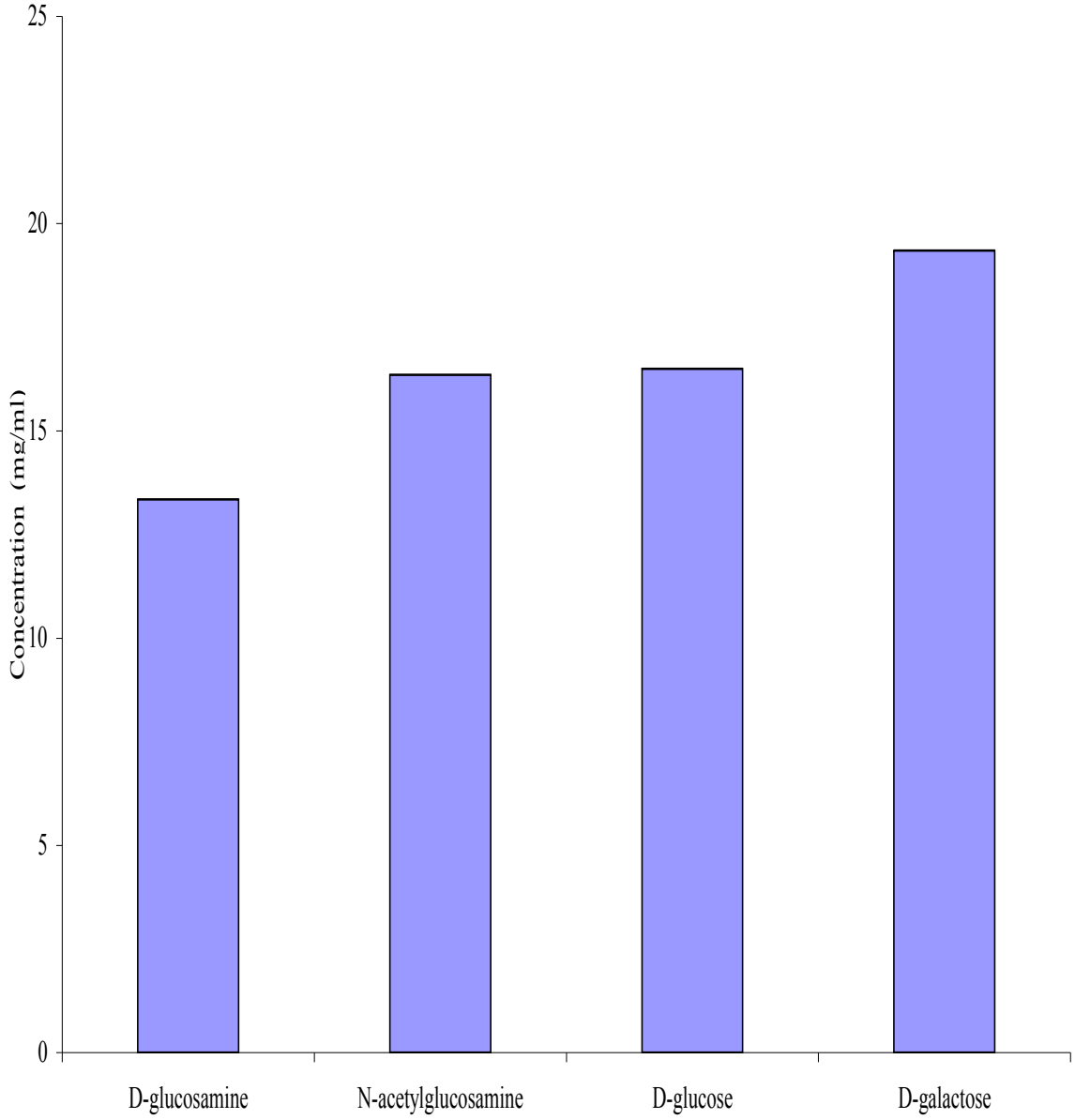

Figure 16: Protein concentrations for the various haemolymph samples collected after *T. brucei brucei* challenge followed by sugars (D-glucosamine, D-galactose, D-glucose, and N-acetylglucosamine).

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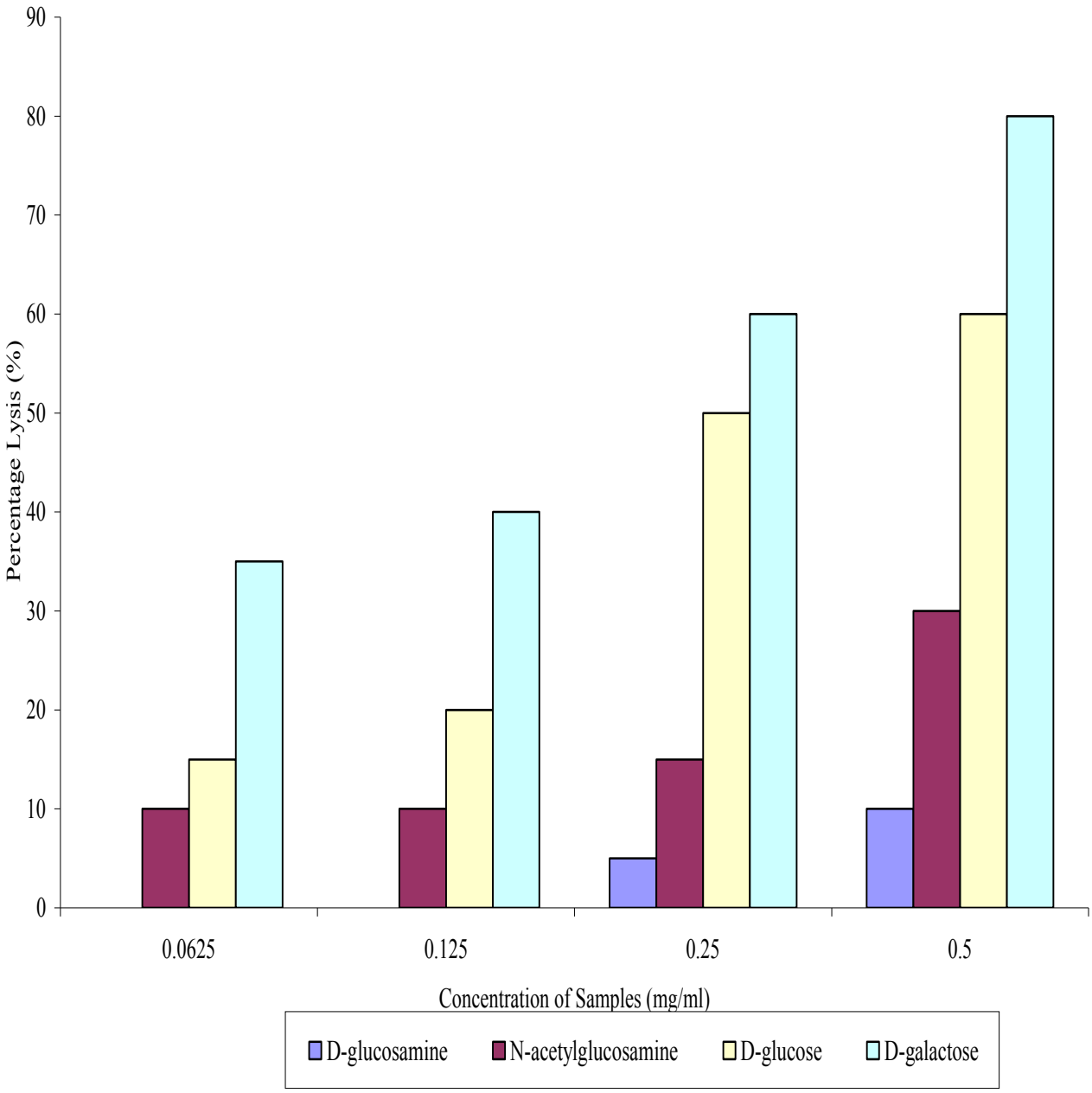

Figure 17: *In vitro* lysis assay**:** haemolymph samples collected after challenging the insects with *T. brucei brucei* followed by sugars (D-glucose, D-galactose and D- glucosamine).

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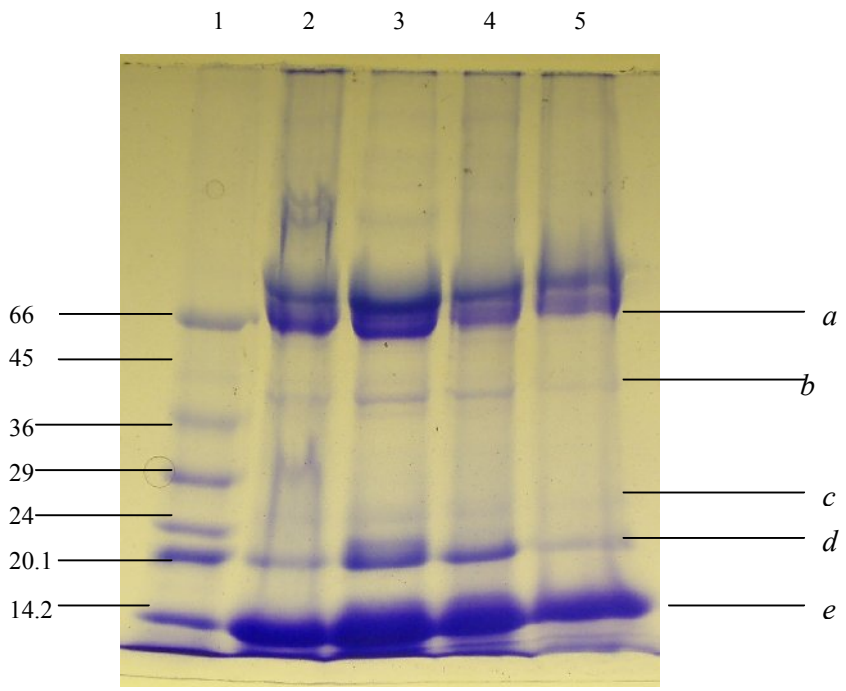

Figure 18: 5-15% SDS –PAGE of haemolymph collected at different time points after challenging the locusts with *T. brucei brucei* followed by sugars (D-glucose, D-galactose and D-glucosamine). Lane 1; Molecular weight markers (50 µg) in kDa, lane 2; D-glucose (100µg), lane 3; D-galactose (100µg), lane 4; N-acetyl glucosamine (100µg) and lane 5; D-glucosamine (100µg). The gel was coomassie stained.

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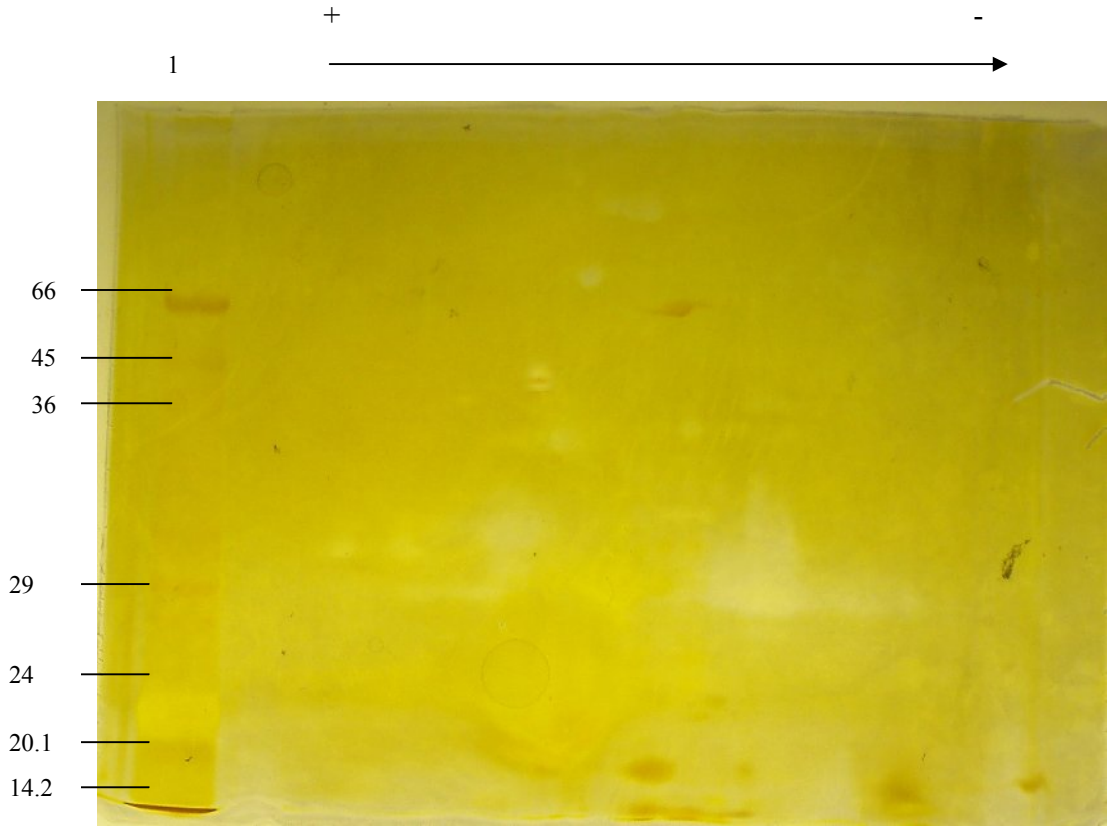

Figure 19: 2D PAGE for haemolymph sample collected 18 hours after challenging the locusts with *T. brucei brucei* followed by D-glucosamine. 100µg of the sample protein was resolved through isoelectric focusing in a 7cm acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, followed by separation on a 12% SDS-PAGE slab gel. Lane 1; molecular weight marker (15µg). The gel was silver stained.

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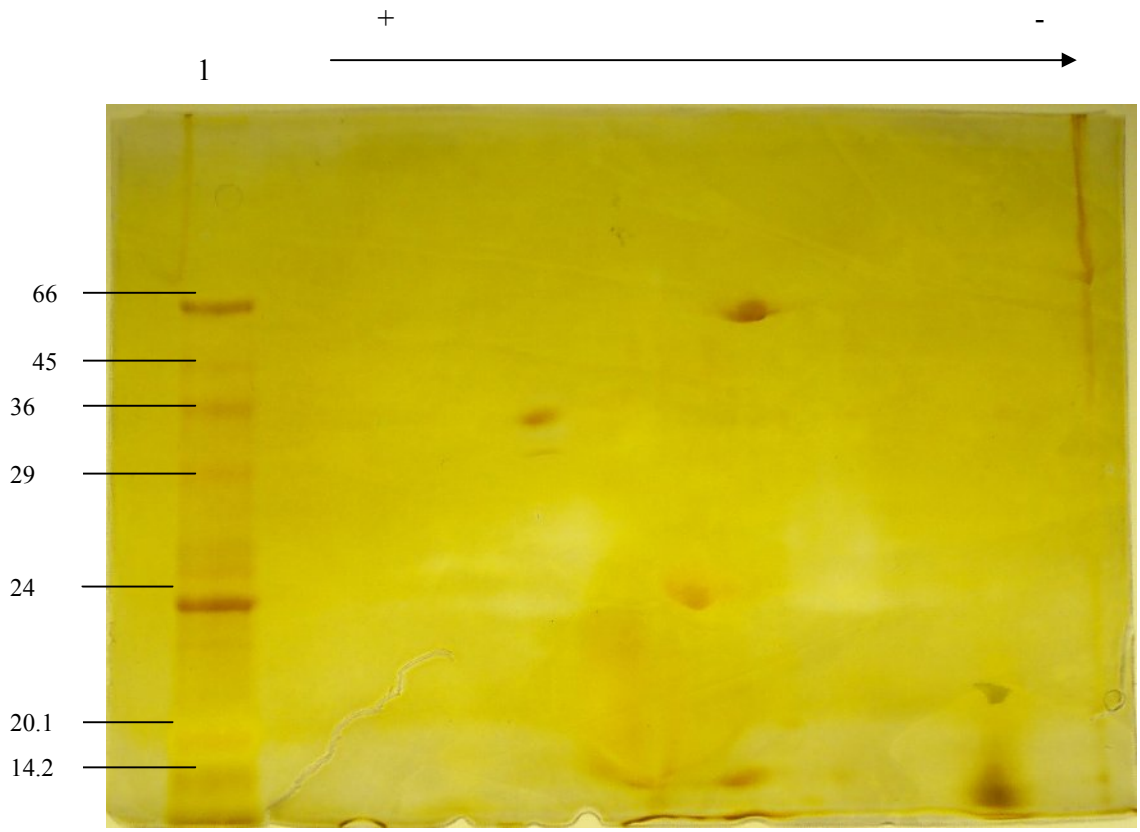

Figure 20: 2D PAGE for the haemolymph sample collected 18 hour after challenging the locusts with *T. brucei brucei* followed by D-galactose. 100µg of the sample protein was resolved through isoelectric focusing in a 7cm acrylamide tube gel with carrier ampholytes of a pH range of 5-7 and 3-10, followed by separation on a 12% SDS-PAGE uniform gel. Lane 1; molecular weight marker (15µg). The gel was silver stained.

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4.1.4 Immunological cross-reactivity with lectin-trypsin factor

Antibodies raised against *Glossina* proteolytic lectin were used in immunoblotting experiments to screen for the presence of a similar protein in each of the haemolymph samples collected at different time points. Following the transfer of the proteins onto a nitrocellulose membrane, no cross-reactivity was detected with samples from *Schistocerca gregaria* a non-haematophagous, non-vector of *T. brucei brucei* (figure not shown).

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4.2 DISCUSSION

Through co-evolution, parasites have managed to exploit vector-derived molecules for their survival (Wellburn and Maudlin, 1989). The success of vector-parasite interactions depends largely on the immune response of the insect vectors. Insects do not have the antigen-antibody complexes characteristic of the adaptive immunity of vertebrates, but have defense mechanisms, which rely on cellular and humoral components of their innate immunity, which has to be highly efficient if insects are to survive in hostile environments. During their development within the insect hosts, parasites undergo great morphological, physiological and biochemical changes and they must change their surface molecules that facilitate interactions with specific insect tissues essential for their survival, development and subsequent infectivity to the vertebrate host (Kaslow and Wellburn, 1996; Roditi and Liniger, 2002). The findings of this study show that immune response mounted in the haemolymph against *T. brucei brucei* in a non-haematophagous, non-vector insect, *Schistocerca gregaria* vary with time with the activity of the induced proteins peaking after 18 hours and being suppressed in the 48 hour sample. *In vitro* assays indicated that the 18 hour sample had the highest activity followed closely by the 6 hour sample. The 18 hour peak activity also coincided with the highest protein concentration in the induction profile after the challenge. Previous *in vitro* studies have revealed the presence of parasite agglutinins against the trypanosomatid flagellates *T. brucei* and *Leishmania hertigi* in the haemolymph of *Schistocerca gregaria* and *Periplaneta americana* (Ogoyi *et al*., 2003).

Within the insect vectors, parasites have specific locations in which to develop, with the African trypanosomes, the agents of human and animal trypanosomosis developing both in the digestive tract and the salivary glands without entering the haemolymph. In Dipteran insects, antimicrobial peptides (AMPs) are synthesized by the fat body and released into the haemolymph, but can also be expressed by the haemocytes and various epithelia especially the

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anterior part of the gut. Recently, several AMPs such as defensins, cecropins, attacins and diptericins were characterised in *Glossina morsitans morsitans* infected with bacteria or *T .brucei brucei* (Boulanger *et al*., 2002)**.** These immune proteins were found to be induced only during the first week following an infection, with diptericin being constitutively expressed and up regulated upon infective blood meal.

Biochemical approaches such as reverse-phase high performance liquid chromatography and mass spectrometry analysis, in association with *in vitro* antimicrobial assays have been used to study induction of AMPs in immune challenged insects (Bulet and Uttenweiler, 1999). Upon infection *per os* with bacteria or parasites, AMPs were detected locally in the tsetse gut, the main site of flagellate infections, and also systematically in the haemolymph, where no parasites are found (Boulanger *et al*., 2002). Whereas AMPs concentrations reach their peak around 24 hours after bacterial infections, the pattern of induction following parasite ingestion varies according to the developmental stage of the parasite suggesting a possible role for surface molecule variation of parasite in this induction (Boulanger *et al*., 2006). In the field, the rate of tsetse infection is quite low, suggesting the presence of different mechanism to control flies parasitism. Trypanosome infection regulation has been described mainly as lectin mediated (Kaslow and Welburn, 1996). Recent work has shown that the tsetse fly immune response to pathogens also involves different AMPs where a defensin, a cecropin and a partially characterized an attacin-like molecule produced after systemic bacterial infection and *per os* infection with bacteria or trypanosomes. (Boulanger *et al*., 2001). The AMPs of *Glossina morsitan* exhibit strong sequence similarities with those from *Drosophila melanogaster* and *Sarcophaga peregrina*. These AMPs are detected in the haemolymph only during the first days of *per os* infection with trypanosomes. The absence of these peptides is noticeable with time, a phenomenon which may reflect a variation in the trypanosome antigenicity during its complex migration from the gut lumen through to the

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salivary glands (Hao *et al*., 2001). The results of this study demonstrate the presence of parasite agglutinins in the haemolymph of *Schistocerca gregaria* with the highest activity being observed in the 18-hour sample. These results concur with previous work done using both the vector (tsetse fly) and non-vectors (*D. melanogaster* and *S. peregrina*), where the induced proteins were found to peak at about 12-48 hours. In this study, *Schistocerca gregaria* haemolymph induced proteins showed lectin-like properties due to the inhibition of their activity as well as induction by D-glucosamine. Previous *in vitro* studies revealed that the trypanosome-lysing factor was present in both the haemolymph and the midgut of *Schistocerca gregaria* but activity was much higher in the latter.

Further analysis of the various haemolymph samples using two dimensional PAGE led to the identification of several peptides which differed in isoelectric points, molecular weights and intensity. In addition there were peptides which were found to be induced with time following *T. brucei brucei* challenge whereas others were upregulated or downregulated with time. This is in agreement with previous work where AMPs induction increased with the time of infection and decreasing thereafter to levels which could not be detected in the haemolymph (Hao *et al*., 2001).

In previous *in vitro* studies, antibodies raised against the protein (*Gpl*) were used in immunoblotting experiments to check for the presence of a similar protein in several members of the *Glossina spp* and other non-vector insects. However, no cross-reactivity was detected with midgut extracts prepared from non *Glossina* haematophagous insects (sandflies, mosquitoes or stable flies) (Osir *et al*., 1995). *Glossina* proteolytic lectin is one of the induced peptides among the members of the *Glossina spp*. Tsetse flies, which are haematophagous are the only known vectors of African trypanosomosis, a debilitating disease affecting humans and other vertebrates**.** Following immunoblotting, no cross reactivity with antibodies raised against *Glossina* proteolytic lectin (*Gpl*) was detected within the haemolymph samples from

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Schistocerca gregaria. Previous studies showed the absence of cross reactivity of antibodies raised against *Gpl* with crude homogenates from sandflies, mosquitoes and stable flies which are haematophagous and non-vectors of *T. brucei brucei*. Lack of cross-reaction now with *Schistocerca gregaria*, a non-haematophagous, non-Dipteran insect concurs with the previously done work, an indication that the induced proteins from *S. gregaria* have no homology to *Gpl*. This is a possible explanation for the fact that members of the *Glossina* species are the only known vectors of trypanosomes (Stiles *et al*., 1990). Studies have shown that this proteolytic lectin is the active molecule that transforms the bloodstream form of trypanosomes to the procyclic forms (Abubakar *et al*., 2003).

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CHAPTER FIVE

CONCLUSION AND RECOMEDATIONS

5.1 Conclusion

The discovery of AMPs has increased the understanding of basic insect immunity and also their role in conjunction with lectins, proteolytic cascades, digestive enzymes and peritrophic matrix in regulating and controlling parasite development. The induced haemolymph peptides have potential in the development of new strategies to control arthropod–borne diseases, such as those caused by flagellate parasites. Future prospects for the control of African trypanosomosis involve tsetse vector manipulation (Askoy *et al*., 2001) and the use of symbiotic microorganisms, which include: *Wiggleworthia spp*, *Sodalis spp*, and *Wolbachia spp*, can be exploited to express foreign gene products. Since the endosymbionts live in close proximity to the developing trypanosomes, antipathogenic gene products introduced and expressed in these cells could adversely affect trypanosome transmission.

Detailed understandings of vector-parasite association remain scarce and the use of AMPs, agglutinins, lectins and trypanolytic proteins remain a viable possibility (Stiles *et al*., 1990; Ingram, 1998). The results of this study are an indication that further advances in this field could augment the more conventional and ongoing vector control approaches in a bid to contain trypanosomosis. *Schistocerca gregaria* induced peptides if well studied will form an important platform for the understanding of the tsetse-trypanosome interaction with the potential of being exploited to combat arthropod transmitted diseases .

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5.2 Recommendations

Further investigations on the differentially induced proteins in the haemolymph of S. gregaria should focus on the identification of individual proteins by mass spectrometry and subsequent validation of the roles the proteins identified by functional genomic approaches .

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APPENDICES

BUFFERS

Phosphate Buffer

0.05M $NaH_2Po_4.2H_2o$

0.09M Na_2HPo_4

0.07M Nacl

Volume adjusted to 1000ml with distilled to attain a pH of 8.0.

Resolving gel Buffer

1.875M Tris-HCl, pH adjusted to 8.8 with 10% HCl

Stacking gel Buffer

0.6M Tris-HCl, pH adjusted to 6.8 with 10% HCl

SDS-Reservoir Buffer

25mM Tris-HCl

192mM Glycine

0.1% SDS

Volume adjusted to 1000ml and a pH of 8.3

SDS-Sample Buffer

0.0625M Tris-HCl (pH 6.8)

10% SDS

2% ß-mercaptoethanol

10% Glycerol

0.05% Bromophenol Blue

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2D Solubilization Buffer

9M Urea

4% NP-40

0.5% Triton X-100

1.6% Ampholine (pH 5-7)

0.4% Ampholine (pH 3-10)

20mM DTT

2D Equilibration Buffer

6M Urea

2% SDS

375mM Tris-HCl (pH 8.8)

20% Glycerol

2% DTT

2D Sample Overlay Buffer

7M urea

1% Ampholine i.e. 0.8% v/v pH 5-7

0.2% v/v pH 3-10

0.05% Bromophenol Blue (Stock solution)

Stock Acrylamide

30% w/v Acrylamide

0.8% w/v Bisacrylamide

Volume adjusted with distilled water and stored at 4^{o}c

Isoelectric Focusing Gel

9.2M urea

4% Acrylamide

2% NP-40

10% APS

TEMED

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MINI GELS FOR SDS-PAGE

To prepare a 5, 10, 12, 15 and 20% SDS-resolving Gels

	5%	10%	12%	15%	20%
Acrylamide (30% T)	0.467ml	0.934ml	1.12ml	1.4ml	1.867ml
1.875M Tris (pH 8.8)	560µl	560µl	560µl	560µl	560µl
Water	1.734ml	1.267ml	1.082ml	0.8ml	0.333ml
SDS (10%)	30µl	30µl	30µl	30µl	30µl
APS (10%)	25µl	25µl	25µl	25µl	25µl
TEMED	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
Total volume	2.8ml	2.8ml	2.8ml	2.8ml	2.8ml

To prepare a 4% SDS- Stacking Gel

Acrylamide (30% T)…….0.34ml
0.6M Tris HCl (pH 6.8)….....0.25ml
Water.....1.875ml
SDS (10%).....25 µl
APS (10%).....12.5 µl
TEMED.....2.5 µl
Total volume 2.5ml

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Silver Staining

	REAGENTS	TIME
PROTEIN FIXATION	Washing with 10% Acetic acid, 50% Methanol	1-2 Minutes
	Washing with 50% Methanol using a shaker	20 Minutes
	Rinsing in distilled water	5 Minutes
	Washing with 50% Methanol while shaking	20 Minutes
	Incubating with 2.5% Glutaraldehyde (0.5ml Glutaraldehyde in 20ml of distilled water)	30 Minutes
REACTION	Soln. A= 0.4g $AgNO_3$ in 1.2ml of distilled water. Soln. B= 1.0ml of 2M NaOH, 0.8ml NH_4OH adjusted to 20ml with distilled water	Titrate solution B with A.
DEVELOPMENT	1.25ml of 1% citrate, 62.5µl of 38% formalin adjusted to 125ml with distilled water	Until reasonable band development is achieved.
STOPPING REACTION	5% Acetic acid (2mls of acetic acid in 40mls of distilled water)	-5 Minutes
STORAGE	7% Acetic acid (3.5mls of acetic aid in 50mls distilled water)	Indefinitely

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