Proteomic and immunochemical study of the tsetse fly *Glossina morsitans morsitans* midgut and salivary gland

by

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Abstract

The work presented in this thesis describes a protein microchemical analysis of proteins of the salivary glands and midgut of *Glossina morsitans morsitans*, an important vector of African trypanosomes, the parasites that cause African sleeping sickness. A suite of protein chemical and immunochemical techniques was used to identify important tsetse molecules. In the salivary glands, N-terminal sequence analysis and mass spectrometry was used to identify four major soluble proteins. Two proteins with no known function were identified as tsetse salivary gland protein 1 (Tsal 1) and tsetse salivary gland protein 2 (Tsal 2). One protein was identified as tsetse salivary gland growth factor 1 (TSGF-1) and one as tsetse antigen 5 (TAg 5), a member of a large family of anti-hemostatic proteins. Several immunogenic molecules in tsetse saliva were also identified. Antibodies in feeder rabbit blood were used to probe for antigenic salivary proteins from three species of tsetse. Between three and five antigens of different masses were recognized on immunoblots from one-dimensional gels. One antigen was identified as Tsal2 and one as TAg5. The other molecules could not be identified.

Protein microchemical techniques were also used to identify proteins in the midguts of *G. m. morsitans*. Surprisingly, it was found that the most abundant protein in midguts of teneral (unfed) *Glossina morsitans morsitans* was a 60 kDa molecular chaperone of bacterial origin, from the endosymbiont *Wigglesworthia glossinidia*. Comparative two-dimensional gel electrophoresis and immunoblotting revealed that this protein was localized to the bacteriome and not the distal portion of the tsetse midgut.

Whole midguts were used as immunogen to generate a number of different anti-tsetse monoclonal antibodies in a strategy designed to select antibodies that could interfere

with parasite transmission. Surface reactive monoclonal antibodies were selected by immunofluorescence screening on tissue sections of tsetse midguts. One antibody, 4A2, demonstrated unusual, distinct, strong and reproducible immunostaining of a structure in the midgut lumen localized to the peritrophic matrix. Two-dimensional gel electrophoresis, immunoblotting and mass spectrometry were used to identify the antigen as tsetse protein Pro2, a proventriculin. The anti-Pro2 antibody was used in tsetse membrane feeding experiments at Yale University in attempts to influence the trypanosome life cycle in the fly. The results of these experiments were equivocal, indicating that much more extensive testing will be required to determine if interference with the peritrophic matrix is a useful approach for trypanosomiasis control.

Differential expression profiles of midgut proteins were determined for wild type and "salmon" mutant *G. m. morsitans* that differ in their ability to transmit trypanosomes. Isotope coded affinity tag (ICAT) technology and two-dimensional gel electrophoresis coupled to tandem mass spectrometry were used to show that 26 proteins were differentially regulated. Notably, tsetse EP-repeat protein, an unusual extended polypeptide structure with lectin-like activity, was shown to be upregulated in the high-transmitting "salmon" mutant. In addition, a trypsin-like serine protease, two *G. m. morsitans* proteins with unknown function, and a tsetse growth factor were upregulated. These proteins may influence the trypanosome life cycle in tsetse and could form the basis of future research for control of African sleeping sickness.

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Chapter 1. Introduction

African trypanosomiasis

A major epidemic is sweeping through sub-Saharan Africa where half a million people are currently afflicted with and will most likely die from African trypanosomiasis (Smith *et al.* 1998). African sleeping sickness in humans is caused by the protozoan parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* which are transmitted by tsetse flies (genus *Glossina*). Although this vector-parasite relationship was documented in 1895 (Bruce 1895), research over the past century (Vickerman 1997) has not produced a single effective vaccine. This is largely due to the fact that African trypanosomes have developed an excellent defense mechanism that allows the blood stream form parasites to express different glycoprotein surface coats during the course of an infection (Barry 1997) (Van der Ploeg *et al.* 1982) (Thon *et al.* 1989). The parasites' exquisite defense systems are aided by its developing tolerance to the few, antiquated drugs used to treat the disease (Matovu *et al.* 2001) and to civil unrest and socioeconomic limitations that prevent implementation of disease control programs. It was soon found following Bruce's discovery that two organisms were responsible, defining West and East African sleeping sicknesses (Dutton 1902).

When a human is inoculated by trypanosomes during an infected tsetse bloodmeal the protozoan parasites establish an infection in the bloodstream and although the humoral immune response by the host is able to clear most of the parasites, a small population of trypanosomes survive by using a mechanism called antigenic variation. The host then

recognizes these trypanosomes as though they were a new pathogen and another primary antibody response is generated. Previous memory immune effector cells are not specific for the newly expressed surface antigen of the new sub-population.

Initial symptoms are flu-like; these include headache, irregular fever, swollen lymph nodes and aching joints. They are easily overlooked or mistaken as either flu or malaria. Symptoms become much more serious when the parasites cross the blood-brain barrier and enter the cerebral spinal fluid and neural tissue. At this stage the "sleeping sickness" traits responsible for the name of the disease become obvious and include; a semi-conscious or extremely irregular sleep pattern, loss of coordination, motor control and mental stamina eventually leading to mental disorientation. Patients eventually slip into a coma. If left untreated, a patient with the acute disease (*T. b. rhodesiense*) will die within 6 to 12 months, whereas in the chronic form (*T. b. gambiense*) the deteriorating disease can persist for 5-20 years before the host succumbs and dies (Welburn *et al.* 2001).

Treatment is dependant on a small number of very old drugs. If the disease is surveyed regularly and recognized in the patient early, suramin which is administered intravenously and used to treat *T. b. rhodesiense* and pentamidine which is injected intramuscularly and used to treat *T. b. gambiense* are both effective even though they were developed 85 years ago (Hide 1999). Late stage treatment has historically been dependant upon melarsoprol, a 55 year-old anti-freeze and arsenic-based drug that causes encephalopathy and death in 5-15% of patients (Pepin and Milord 1994). The most recent drug in this arsenal is also the black sheep. The drug is an ornithine metabolism inhibitor called difluoromethylornithine (DMFO) which blocks polyamine biosynthesis, causing an impairment in cellular division. Eflornithine is an early-stage drug that has low human

toxicity and high efficacy against all stages of *T. b. gambiense* infection (di Bari *et al.* 1986) (McCann *et al.* 1987) (Louis *et al.* 2003). The drug, developed more than 30 years ago, was originally tested in clinical trials as an anti-cancer reagent, which was later abandoned. It was, however, discovered to be highly effective against sleeping sickness (Bacchi *et al.* 1980). In 1990, the manufacturer Marion Merrell Dow was granted marketing approval and Eflornithine officially became the first new drug for the treatment of trypanosomiasis in over 40 years (Coyne 2001). Five years later when Aventis became the patent owner and production was discontinued because of the labour intensive and highly toxic manufacturing process. Soon after it was discovered that topical application of the drug inhibited the growth of facial hair in women (Balfour and McClellan 2001). A market for Eflornithine was born in beauty salons. Eflornithine remains a profitable product for newest patent holder Bristol-Myers and is currently available for the treatment of trypanosomiasis (Sjoerdsma and Schechter 1999).

Trypanosomiasis prevention and control are achieved mainly by surveillance, drug treatment and tsetse control. Surveillance provides an estimation of the prevalence of infection and acts as an early warning system for potential epidemics of this endemic disease. Mobile teams must be deployed to screen for *gambiense* disease because of the more mild nature of this disease. Due to the fact that the symptoms are easily mistaken for the flu or malaria, many patients will not seek help and the disease would go un-noticed in absence of surveillance. The more acute, *rhodesiense* disease has more devastating effects initially, which can help to motivate the patient or their families to seek help from rural health facilities.

Diagnosis of trypanosomiasis can be facilitated by serological tests including the direct card agglutination trypanosomiasis test (CATT) (Magnus *et al.* 1978) for *T. b. gambiense* and the indirect immunofluorescence test (IFT) for *T. b. rhodesiense*. Positive serological screens are confirmed by a number of microscopic detection methods including blood smears to identify motile trypanosomes in blood, lymph node aspiration to demonstrate motile trypanosomes in node material and cerebral spinal fluid (CSF) to observe if motile trypanosomes have crossed the blood-brain barrier. Recent epidemics have occurred in areas that have experienced both decline in surveillance and increase in population movement. These are largely due to civil unrest (WHO 1986).

Major efforts to control trypanosomiasis focus on insect control through trapping and insecticides. Recently, the sterile insect technique has shown promise for the future (IAEA 2001). The most environmentally friendly methods are trapping and insecticide treated screens. The traps have a simple design and are easy to transport and set up thus making them, arguably, the most efficacious method of fly control. The traps take advantage of the fact that tsetse are attracted to large objects that contrast the landscape and to particular odours and gases such as carbon dioxide (Challier and Laveissiere 1973). Blue is a particularly tsetse-attractive colour; blue traps are often contrasted with black (Green 1988). Tsetse will move toward the direction of light so the black contrast can be used to steer tsetse to the entrance of the trap. Another trap design uses insecticide impregnated in the fabric of the trap (Kupper *et al.* 1982). This configuration relies less on tsetse entering the trap because simply landing on the outside of the trap will potentially kill the insect. Traps are generally considered to be effective over an area of approximately 50 m^2 which is the average visual range of the fly. These methods are used to control the disease at the endemic

level (Rozendaal 1997). In epidemic situations, more drastic, costly and less environmentally friendly techniques are required such as ground and aerial spraying with insecticide. The method involves spraying the resting places of tsetse including the base of shrubs and other shaded and protected areas. A basic requirement of an insecticide is that it must retain activity for approximately two months, as this is the maximum duration of the pupal stage. It is the emerging flies that will be killed (Jordan 1993). Insecticides used have included formulations of dichlorodiphenyltrichloroethane (DTT) and more recently, synthetic pyrethroids that have desirable properties such as low mammalian toxicity and rapid biodegradability (WHO 1986).

The tsetse life cycle results in only a small number of offspring. A significant investment is made for the birth of each F1, implicating that a particular tsetse population will not survive if the mortality rate can be sustained above natural levels. Nevill (1997a) has calculated that an increase in mortality of females of 4 % per day will cause extinction of a tsetse population. Therefore, it should be possible to eradicate tsetse in specific areas. However, these attempts are susceptible to failure due to invasion of tsetse populations from adjacent regions. Multiple countries must collaborate to prevent re-invasion from adjacent regions (Nevill 1997a). An essential part of a control program is to understand the biology and ecology of the *Glossina* species involved. Particularly important is an understanding of their movement, density and distribution thus necessitating the use of trapping methods to monitor the situation as the control effort progresses.

The sterile insect technique (SIT) involves breeding of millions of male *Glossina* that are irradiated (sterilized) prior to their release into regions that have previously been intensely trapped and sprayed with insecticide to drive the population as low as possible.

The flies are then released in regular intervals, driving a population of infertile males into the region. Due to the low reproductive rate and the fact the female tsetse mate only once, females remain unfertilized and do not produce offspring. The method has been shown to work in Zanzibar where *Glossina austeni* has been successfully eradicated. The eradication effort required the release of over 60 000 irradiated male flies per week. In one year (1995-1996) a total of 5.5 million sterile males were released. To produce so many males a massive colony was established in which over 700 000 female flies were reared (Dyck *et al.* 1997).

Tsetse biology

Tsetse flies are members of the order Diptera, residing within the superfamily *Hippoboscoidea* , and family *Glossinidae* which is monogenic. *Glossina* is comprised of 31 members, 23 species and 8 sub-species. The species can be categorized into three subgenera, *Austenia* (*G. fusca* group), *Nemorrhina* (*G. palpalis* group) and *Glossina* (*G. morsitans* group). Recently morphological and genetic evidence has proposed that a fourth subgenus, *Machadomyia*, be included because of significant differences in *Glossina austeni* from other members of the *morsitans* group in which it as historically been placed (Gooding *et al.* 2002). Approximately nine species of either the *G. palpalis* or the *G. morsitans* group are able to transmit sleeping sickness (Jordan 1993; Leak 1999). The nearest living relative of the tsetse fly is the blood feeding louse fly of the *Hippoboscoidea* family. Tsetse are robust insects that range in length from 6-14 mm and resemble the average house fly except for forward-pointing mouthparts (proboscis) and a characteristic wing venation, including a hatchet shaped cell (discal medial cell) in the centre of the wings that are folded back over

the abdomen when at rest. Tsetse flies spend a majority of their time at rest in shaded areas and often hide in tree trunks and at the base of shrubs or between roots. The flies search for food only briefly during the day and therefore reside close to food sources, which include riverbanks and forest trails where mammals bathe and consume water. Blood is the sole source of food for tsetse. Some species are less specific about their desired bloodmeal sources and are therefore considered a more dangerous species. Tsetse flies gain energy for flight through the partial breakdown of the amino acid proline, acquired from the bloodmeal (Leak 1998).

Tsetse Life Cycle

Tsetse reproduce by adenotrophic viviparity. Characteristically, mature larvae are deposited one at a time. This is a distinguishing feature of the *Glossina* species. The female tsetse retains a single egg that feeds on "milk" from modified accessory glands until the egg hatches and a third larval stage is deposited approximately 10 days after fertilization on moist soil or sandy and shaded areas. The larva immediately buries itself and becomes a pupa. The fly emerges in a temperature and moisture dependant fashion after 22-60 days. Females mate only once and can produce a larva every ten days depositing a maximum of approximately eight to ten offspring. Tsetse flies make a large investment in each offspring thereby reducing juvenile mortality (Leak 1998). Eggs develop sequentially in the female, alternating between the two right and the two left ovaries and after the female is approximately nine days old, the first egg passes into the uterus from one of the ovaries. However, the female is able to mate only a few days after emergence prior to release of the

eggs from the ovaries. After nine to ten days, there is the second ovulation from one of the other ovaries, and this cycle continues during the life of the female tsetse (Huebner *et al.* 1975). The egg is fertilized in the uterus by a sperm from the spermatheca (gained during earlier mating with a male). After three and one half days of development in the egg, the first instar larva breaks out of the egg case by using specialized structures within the tsetse reproductive tract (Davey 1974). The young larva develops in the female's uterus by feeding from modified accessory or tsetse milk glands that secrete a protein and lipid rich food source for the larvae. It will pass through two molts to finally reach the third instar before being larviposited by the female into soft, shaded, moist soil. The larva then excrete the metabolic waste products gained and burrows into the soil. Once the pupa is buried the skin hardens and forms a dark puparium. This is a temperature-dependent process that ranges from 20 days (at 30 °C) to 47 days (at 20 °C). The entire life cycle from egg fertilization to newly emerged adult takes approximately 48 days, with 18 of those days spent maturing within the female's uterus. The pupae stage is most susceptible to mortality since optimal environmental conditions such as the evaporation rate and the degree of compaction of the soil must be met. Most tsetse species mate on or near the host animal. *Glossina* females have been shown to produce species-specific cuticular hydrocarbons that induce a copulatory response from males of the same species. During the one to two hour mating session a spermatophore is formed within the female's uterus using the reproductive secretions from the adult male. At the climax of copulation the male ejaculates sperm into the spermatophore and within a few hours, the sperm moves from the spermatophore up the paired spermathecal ducts into the paired spermathecae. Although females will only mate once, males are able to mate numerous times with multiple females (Jordan 1993).

Trypanosome biology

Sleeping sickness can manifest as either a chronic or acute disease, depending on the subspecies of trypanosome. *Trypanosoma brucei brucei* causes sleeping sickness in humans. *T. brucei gambiense* causes chronic disease in West and Central Africa from Senegal to Sudan to Angola and Zaire in the south. Three *Glossina* species, all in the *palpalis* group, act as vectors for *T. b. gambiense*: *G. palpalis, G. fuscipes* and *G. tachinoides*. *Trypanosoma brucei rhodesiense,* which develops a much more acute infection, is endemic to an area that spans the east African savanna and woodlands and extends south to Botswana, Zimbabwe and Mozambique (WHO 1998). *T. b. rhodesiense* can be spread by four species *of Glossina*, all in the *morsitans* group (*Glossina morsitans morsitans; G. morsitans centralis, G. swynnertoni* and *G. pallidipes*). Trypanosomiasis is not a disease limited to humans. *Trypanosoma vivax* and *T. congolense* are very important parasites of domestic animals, mainly cattle, because they cause Nagana (animal trypanosomiasis). About ten million square kilometers of Africa is affected by trypanosomiasis, which limits farming, thereby significantly affecting regional economies. Cattle farming that does occur in tsetse-infected areas usually requires the cattle to be constantly treated with insecticides. In addition to the pathogens of cattle, *Trypanosoma simiae* is an important parasite of swine (Moloo *et al.* 1992).

The African trypanosome is a eukaryote but it has a number of biological processes and characteristics that make it one of the most unique eukaryotes known. It has a unusual RNA editing process, unique RNA structures (mini-exons) and very dynamic metabolic processes that drastically change during its life cycle. The most famous trypanosome trait is

its ability to evade mammalian immune responses by antigenic variation (Cross 1990). An important obstacle for trypanosomes is the immune response of the hosts in which they invade including both humans and tsetse (Hao *et al.* 2001). The parasite uses a clever mechanism of antigenic variation that protects the species from annihilation by the mammalian host and is often considered to be one of the most sophisticated strategies devised by a protozoan parasite to evade an immune response. The bloodstream form trypanosome is indeed very antigenic, which is the characteristic that is largely responsible for the symptoms shown by infected patients. In most cases a significant and largely effective antibody response is mounted against the antigenic structures (the variant surface glycoproteins; VSG) on the surface of the parasite. During the course of an infection the number of parasites in the bloodstream fluctuates with cyclic waves of symptomatic and asymptomatic periods and years can pass before the patient finally succumbs to the disease and dies. The waves of fever and cycles in parasite infestation correlate with the number of parasites in the blood. Although a strong immune response with markedly high levels of immunoglobulin and B lymphocyte proliferation is mounted, a few trypanosomes in the total population evade the response using antigenic variation allowing them to establish a new generation of parasites (Borst and Fairlamb 1998). The parasite accomplishes this by changing the expression of the VSG surface coat that surrounds the bloodstream forms of the parasite (Cross 1975) (Borst and Cross 1982). Escape from the immune response depends upon the ability to express a new VSG. To successfully accomplish this the parasite must have four components; 1. a large repertoire of surface antigens, 2. a mechanism for switching the antigen expression in a fraction of the population before the parasite is neutralized by antibodies, 3. the ability to express the antigen in a specific order to maintain population

homogeneity, and 4. the ability to maintain nutrient uptake in combination with VSG expression (Borst *et al.* 1998). Surface coat switching occurs continuously and spontaneously at a low level and each trypanosome encodes approximately 1000 different VSG genes ensuring that at least a small population is able to escape the antibody response. Each VSG glycoprotein is approximately 65 kD and 500 amino acids in length containing three domains. The N-terminus contains a signal peptide for transport through the ER and to the plasma membrane. This is cleaved from the mature protein. The C-terminal hydrophobic tail contains a recognition signal for attachment of a glycolipid anchor. When the anchor is attached the C-terminal 20 amino acids are cleaved off. The VSG glycolipid anchor consists of ethanolamine, a glycan structure containing several mannose moieties, a glucosamine and a phosphoinositol that is linked to a 1,2-dimyristoylglycerol that becomes buried in the plasma membrane. The glycolipid moiety is known as the cross-reacting determinant (CRD) that is recognized by antibodies that can react with all VSG variants, but only once the VSG has been released from the membrane surface (Shak *et al.* 1988). A surface coat is encoded by only one gene, called the expression-linked copy, that is transcribed in one of approximately 20 telomeric expression sites. Switching of the expressed gene has been demonstrated to occur by one of the following two methods (Rudenko *et al.* 1998; Rudenko 2000): A silent VSG gene can be inserted or copied into the expression site by DNA rearrangements or the expression can be controlled at the level of transcription by expression site activation or inactivation. The expression sites are very similar and have polycistronic transcription modules of 40-60 kb which include several expression site-associated genes (ESAGs) in addition to the expressed VSG gene (Borst *et al.* 1998). At the beginning of the infective phase, VSGs are produced by metacyclic parasites in the insect's salivary glands. A

subset of the repertoire (about 12) of the VSGs are produced and this remains during the first wave of parasitemia in the mammal. The whole repertoire is then open to expression and there is a preferred general order of expression. The parasite must shed its entire coat in order to survive the developing immune response against its original VSG. A trypanosome- and VSG-specific phospholipase C has been found in bloodstream forms. Due to the fact that all VSGs have the same attachment structure, only this one enzyme may be needed for rapid and complete cleavage and subsequent coat removal; Borst *et al.* 1996; Cross 1996).

These parasites are flagellated protist parasites that are members of the kinetoplastid group of protozoa. They have a nucleus, endoplasmic reticulum, Golgi apparatus and a set of subcellular organelles such as a mitochondrion, lysosomes and microbodies. The typical shape of the trypanosome is maintained by the presence of sub-pellicular microtubuli that lay just underneath the trypanosome's plasma membrane. The flagellum runs along the entire body of the cell and is attached by an undulating membrane and enters the body of the cell at the flagellar pocket region, the sole site of endocytosis. The group is distinguished by its large, Giemsa-staining structure, the kinetoplast, which is separate from the nucleus and is always located at the base of the flagellum or basal body (Simpson 1986). It often varies in position relative to the nucleus during different lifecycle stages (Vickerman 1985). The kinetoplast morphology changes with metabolism; bloodstream form, slender trypomastigoes have a simple and small mitochondrion, with few cristae that are short and tubular; bloodstream short-stumpy forms have a more elaborate mitochondrion; fly midgut forms have an elaborate array of plate-like cristae and the mitochondrion extends both anteriorally and posteriorally from kinetoplast; fly metacyclic forms undergo a degeneration of mitochondrion in preparation for life in the nutrient rich mammalian host (Vickerman *et al.*

1988). The kinetoplast is a DNA-rich structure that is equivalent to the mitochondrial DNA of most other eukaryotic cells but makes up a very much greater proportion of the DNA of the cell than does the single circle mitochondrial DNA of other cells. Although the kinetoplast DNA is much more elaborate and is a greater proportion of the cell's total DNA, it does not code for any more RNAs than other mitochondrial DNAs (Simpson 1986). In fact, some of the tRNAs of the kinetoplast are not encoded in this DNA and have to be imported from the cytoplasm which is not the case with mammalian mitochondria. The kinetoplast is very complex and contains 20-50 copies of a 22 kb maxi circle DNA that is comparable to mitochondrial DNA. In addition, there are up to 10,0000 1kb mini-circles that form a single network of concatenated circles (Fairlamb *et al.* 1975) (Simpson 1986).

The trypanosome must survive in different environments during different stages of its life cycle. The environment between hosts changes drastically with large differences in temperature, pH, host immune effectors and carbon sources. Like all living organisms, trypanosomes generate ATP as an energy carrier, which is mainly produced by the oxidation of carbohydrates using glycolysis and/or the more efficient tricarboxylic acid (TCA) cycle. Kinetoplastid protozoa compartmentalize the first seven enzymes of glycolysis and two enzymes of glycerol metabolism in a microbody, the glycosome, which is not present in any other eukaryotic group. The glycosome is a member of the peroxisome family (Martin and Borst 2003). While in its mammalian host, *Trypanosoma brucei* spp. depend entirely on glucose for ATP generation. Under aerobic conditions, most of the glucose is metabolized to pyruvate (Helfert *et al.* 2001). In the mammalian bloodstream there is an abundance of oxygen and glucose while the opposite is true in the insect gut or haemolymph. Insect form *T. brucei* spp. have a full complement of TCA and glycolysis enzymes, which is not

surprising since nutrients are not abundant in the strictly hematophagous insect and care must be taken to extract as much energy as possible from the given carbon sources. Normally, ATP production by oxidative phosphorylation is sensitive to cyanide. Cyanide reacts with cytochromes a/a_3 preventing the transfer of an electron to oxygen but oxidative phosphorylation in the insect gut forms of trypanosomes is not fully sensitive to cyanide. Trypanosomes can produce cytochrome O, which is insensitive. The forms of *T. brucei* spp. in the mammalian bloodstream use only inefficient glycolysis because of the abundance of available nutrients. Glycolysis produces much less ATP than the TCA cycle, and therefore respiration is 50 times that of a normal mammalian cell and in the bloodstream, *T. brucei* uses 10 times the amount of fuel as it does in the insect gut (Parsons *et al.* 2001). In trypanosomes, dihydroxyacetone phosphate metabolism is required to maintain oxidation of NADH and depends on an FAD-containing dehydrogenase coupled with a copper containing oxidase, known as the glycerophosphate oxidase system. Because trypanosomes use this unique system it has become the target of trypanocidal drugs. Suramin and salicylhydroxamic acid, a chelating agent that binds the copper in the trypanosome glycerophosphate oxidase system, take advantage of some of the unusual features of trypanosome biochemistry. These drugs take advantage of the fact that this is a metabolic pathway that mammals do not use (Ryley 1956) (Parsons *et al.* 2001).

RNA editing of trypanosomes (kinetoplastids) is a distinctive form of mRNA maturation that involves posttranscriptional deletion and insertion of uridine residues in mitochondrial transcripts (Alfonzo *et al.* 1997). This process occurs only in the mitochondria and is required because the mRNA that is transcribed often either has too many or too few uridine nucleotides incorporated into the sequence and they must be processed before a

functional polypeptide of the correct sequence can be produced. As mentioned previously, the mitochondrial DNA is folded into minicircles and maxicircles. The minicircles encode guide RNAs (gRNA) and the maxicircles encode the genes that require editing prior to becoming functional (Simpson *et al.* 2000). These guide RNA molecules bind to the pre-mRNA at the specific sites to be edited (Simpson *et al.* 2003). Guide RNAs are approximately 60 kb in length and complementary to short stretches of the pre-mRNA molecule (Kable *et al.* 1996). The gRNAs consist of three main regions: a 5' anchor sequence that hybridizes to the mRNA and identifies the editing site, a central guiding sequence that directs the mRNA editing to become its complement (using Watson-Crick and G:U pairing), and a 3' oligo(U) tail that may tether the upstream, purine-rich pre-mRNA (Simpson *et al.* 2000). This editing occurs at multiple sites, contributing to over half of the protein-coding residues of some mRNAs. The process progresses 3' to 5' on the pre-mRNA. The RNA editing activity is catalyzed by a complex containing seven major polypeptides, including two ligases (Cruz-Reyes and Sollner-Webb 1996; Cruz-Reyes *et al.* 1998; Cruz-Reyes *et al.* 2001).

Trypanosome Life Cycle

The life cycle of the trypanosome involves multiple developmental stages in both mammals and tsetse (Vickerman *et al.* 1988) (see figure 1.4). In mammals, such as pigs, cattle and humans, the blood stream form of the trypanosome is covered by the VSG coat. The life cycle continues within the fly after ingestion of parasites in an infected bloodmeal.

As the trypanosomes differentiate in the tsetse midgut to procyclic forms, the VSG coat is lost and a set of lipid-anchored glycoproteins known as the procyclins forms a new surface coat (Roditi *et al.* 1989). The trypanosomes eventually migrate from the tsetse midgut to the salivary glands, where they mature into mammal infective forms that express a new VSG coat (Vickerman *et al.* 1988). The final stages of parasite maturation require life cycle changes that involve both initial attachment to the salivary gland epithelium and a free-swimming stage in the salivary lumen. When the hematophagous tsetse fly feeds, mature metacyclic trypanosomes are injected from the insect salivary glands to local tissue sites in the host mammal where the parasites begin to proliferate, thus establishing infection that spreads to the host bloodstream.

Tsetse - Trypanosome interactions

The life cycle of the trypanosome is completely dependant upon the tsetse fly. The parasite lifecyle within tsetse is very complex and the trypanosome must survive within and traverse multiple tsetse tissues (Vickerman 1985; Vickerman *et al.* 1988). Despite the dependence on tsetse as a host, the flies are not easily infected although it has been shown that under the right conditions a single trypanosome can establish a midgut infection (Maudlin and Welburn 1989; Maudlin and Welburn 1994). To establish an infection in tsetse, the parasite must cope with a barrage of host defences that are in place to prevent trypanosome infection. When tsetse collected from the foci of a major epidemic were examined for mature parasites in the salivary gland, less than 1 % of the flies had a detectable infection (Okoth and Kapaata 1986). This is in contrast to the fact that when trypanosomes

are exposed to a susceptible host a single parasite is capable of establishing an infection in tsetse and eventually becomes infective for humans (Maudlin and Welburn 1989). It was also demonstrated shortly afterwards that this low infection rate was not due to the fact that the wild tsetse may not have had the opportunity to feed on an infected host. The infection rates of wild field-caught tsetse and of puparia collected from the same field population that were fed on *T. congolense*-infected rabbits upon emergence revealed that the infection rate was not significantly increased (Maudlin 1991). The degree of infection is dependant on the refractoriness of the tsetse fly, for example a more refractory fly strain is less susceptible to infection. The degree of refractoriness has been demonstrated to be a maternally inherited trait and both highly susceptible and highly refractory strains can be bred (Maudlin 1982; Moloo *et al.* 1998). The nature of refractoriness, although not molecularly defined, indicates that molecular interactions between tsetse and trypanosome are important in the transmission of African sleeping sickness. In addition, salivary gland homogenates have also been shown to influence the growth, maturation and transmission of trypanosomes. Homogenized salivary gland tissue has been shown to initiate the transformation *in vitro* of procyclic trypanosomes into VSG-expressing forms that become infective for mice (Cunningham and Honigberg 1977; Cunningham and Taylor 1979). In addition, the proventriculus, a specialized structure of the midgut, is also a likely source of molecules that influence the maturation and establishment of trypanosomes (Van Den Abbeele *et al.* 1995).

To date, no specific parasite host receptor-ligand pair has been fully elucidated. The best-characterized class of tsetse molecules that may interact with trypanosomes and influence their development are the polysaccharide-binding lectins. In 1981 a landmark paper was published that showed triatomine insect lectins could specifically interact with

Trypanosoma cruzi species (Pereira *et al.* 1981). Since then midguts of a number of tsetse species have been explored for the presence of lectins. Two molecules have been observed to date, one 26 kDa, the other 29 kDa (Grubhoffer *et al.* 1994; Grubhoffer *et al.* 1997). Experiments that take advantage of the specific binding characteristics of lectins have shown that these molecules may be at least partially responsible for the susceptibility of tsetse to trypanosome infection. When tsetse were fed with trypanosome-inoculated blood containing specific lectin-inhibitory polysaccharides a 100 % infection rate was observed with certain sugars (Maudlin and Welburn 1987; Maudlin and Welburn 1988). Welburn (unpublished) has claimed that procyclins are capable of inducing the same effect as inhibitory sugars when fed with trypanosome inoculated bloodmeals, implicating that the major trypanosome surface molecule of insect form trypanosomes is a receptor for the apoptotic lectin(s). This phenomenon has been documented in other vector-borne disease models, including the *Leishmania*-sandfly system in which feeding of inhibitory sugars and lipophosphoglycan (major surface molecule of promastigotes) also produce significantly stronger infections, likely by making the lectin unavailable to impart its parasite lethal effects (Volf *et al.* 1998; Ham *et al.* 1991). The binding specificity of the procyclins has not been localized to the carbohydrate moiety of the GPI anchor (branched poly N-acetyllactosamine with a sialic acid terminus) or to the N-linked sugars present on the distal ends of the long extended structure of the procyclins. It is possible that the GPI sugar is sterically hindered from specific binding because it is at the base of a very rigid, linear charged polypeptide that extends away from the surface and by the fact the GPI sugar is next to the plasma membrane (Ferguson 1999). In a recent study using MALDI-TOF mass spectrometry it was shown that procyclins are quantitatively cleaved at the N-terminal (distal) end when tsetse by proteases present in the

midgut (protease origin was not determined and could be due to tsetse, symbiont or trypanosome polypeptides) (Acosta-Serrano *et al.* 2001). All procyclins except EP2 and GPEET have N-glycan (Man5-GlcNAc) in the N-terminal domain (Acosta-Serrano *et al.* 1999). The location of the N-linked sugar site is distal enough that all EP isoforms except EP-3-4, which retains an intact N-terminal glycan in the tsetse midgut, are cleaved off (Acosta-Serrano *et al.* 2001). EP protein 2-1 is not cleaved but does not contain a N-linked glycan even in its full-length form. Could tsetse have evolved a system to allow a portion of its surface membrane to be cleaved and act essentially as a mop or sponge cleaning up trypanocidal lectins? This would not be entirely surprising since it is believed that trypanosomes cleave procyclins with its own protease similar to the phospholipase GPI release mechanism to shed its VSG coat in the mammalian bloodstream (Butikofer *et al.* 2001; Rolin *et al.* 1996).

Unfortunately molecules of tsetse and trypanosome that specifically interact have yet to be described in molecular detail. In one of the early attempts to identify procyclin-binding molecules from tsetse, researchers in Belgium allowed live trypanosomes to briefly swim in medium containing biotinylated vector protein homogenates (Van Den Abbeele *et al.* 1996). Although a number of bands were seen on immunoblots when the trypanosomes were lysed and run on SDS-PAGE gels, no identifications were made.

Chapter 2. Identification and partial characterization of proteins in the salivary glands of the tsetse, *Glossina morsitans morsitans.*

1. Introduction

Molecules expressed in the tsetse salivary gland likely play both direct and indirect roles in the growth, maturation and transmission of trypanosomes. Homogenized salivary gland tissue has been shown to initiate the *in vitro* transformation of procyclic (midgut form) trypanosomes into VSG-expressing metacyclic salivary forms that are infective for mice (Cunningham and Honigberg, 1977; (Cunningham and Taylor 1979). In addition to the tsetse salivary glands, another site anterior to the midgut, the proventriculus, may also be a source of molecules that influence the maturation of trypanosomes (Van Den Abbeele *et al.* 1995). Specific molecules involved in interactions between tsetse and trypanosomes are unknown. What is known is that tsetse salivary gland extracts contain an anti-thrombin, anti-coagulant activity (Lester 1926) and platelet anti-aggregation activity (Mant and Parker 1981) that would both be involved in prevention of hemostasis. Tsetse saliva also contains molecules that cause immediate and delayed-type cutaneous hypersensitivity (Ellis *et al.* 1986). The anti-coagulant and immunoreactive molecules likely participate in the formation of a hematoma at the inoculation or feeding site. This would allow the fly to obtain a bloodmeal since clotting and inflammatory responses would be inhibited or minimized, thus allowing effective blood flow. In addition it has been shown in a similar vector borne disease model, Leishmaniasis, that the insect mouthparts penetrate the skin and bypass innate host defenses and the saliva contains potent vasodilators, blood clotting inhibitors and immunomodulating factors (Ribeiro 1995). One recently described molecule enhances

Leishmania amazonensis infection of mice by modulating interleukin-10 production (Norsworthy *et al.* 2004). In stark contrast to the observation that saliva co-injected with the parasites causes enhanced infection, it has also been shown that previous exposure to immunogenic components of the salivary gland can actually decrease the infection rate. This is likely due to the neutralization of the immunosuppressing and modulating factors that can no longer act to provide a favourable entry site at the bite wound since the immune system was previously primed for challenge. In mice, immunization with sandfly salivary glands provides immunoprotection from *Leishmania major* infection (Valenzuela *et al.* 2001). When the sandfly feeds on a previously challenged host, the vector's saliva enters at the bite site and effectively boosts the acquired immune system. Immune response against secreted molecules of the salivary gland can prevent parasite infection by interfering with essential immunosuppressive activity of the saliva. It is also possible that the presence of a more rapid immune response by memory immune-effector cells directly at the bite site inhibits parasite escape from the bite site. The bite site of a naïve host is normally immunosuppressed by the vector saliva. The role of insect vector saliva has in part been determined and sandflies share effector molecule activity with tsetse (Ellis *et al.* 1986).

Despite their importance for tsetse feeding and trypanosome transmission, only a few tsetse salivary molecules have been biochemically identified. A tsetse salivary gland thrombin inhibitor has been isolated (Cappello *et al.* 1996) and its cDNA characterized (Cappello *et al.* 1998). In addition, mRNAs encoding two growth factor-like proteins (TSGF-1 and TSGF-2) have been hypothesized to have platelet anti-aggregating activity (Li and Aksoy 2000). Two other mRNAs encoding proteins with no known function (Tsal1 and Tsal2) have been identified using differential expression screening of tsetse tissues, although

scanner (UMAX Astra 3400, Fremont, CA). The images were stored and manipulated in TIFF format using Photoshop™ 5.5 graphic software (Adobe Systems Inc., San Jose, CA).

2.8. Rabbit and human sera collection.

The tsetse colony at the University of Alberta in Edmonton BC Canada is maintained using lop-eared rabbits (Nash *et al.* 1966) as a bloodmeal source for a number of tsetse species including *G. m. morsitans, G. m. submorsitans, G. m. centralis, G. p. palpalis, G. p. gambiense* and *G. swynnertoni*. The rabbits were born on the following dates; Rabbit A, June 14, 1999; rabbit B, Nov. 28, 1999; rabbit C, June 14, 1999; rabbit D, March 26, 2002. Rabbits were used as feeders after they reached six months of age and served approximately two to three years service. The animals were exposed to flies every other day for two weeks following a rest period of three to four weeks. Six rabbits were used for each meal to feed approximately 1600 flies or approximately 270 flies per rabbit per feed. Rabbit sera was collected at the termination of its use as a feeder. The serum was allowed to clot and the red cells removed by centrifugation, sera was stored at -20 °C, shipped on dry ice and aliquoted and stored at -20 °C, fresh sera was used for each experiment.

The human sera used in the analysis was donated by professor Ron H. Gooding, who agreed to have samples of his serum, that had previously been collected at the University of Alberta, used in immunoblot experiments at the University of Victoria. Gooding was first exposed to tsetse in 1972 and was occasionally bitten by *G. m. morsitans* over the course of the following year. From mid-1973 until recently (2004) Gooding has been bitten by all of the aforementioned flies at a rate of approximately three to four fly bloodmeals per week.

2.9. 1-D and 2-D gel immunoblotting and protein transfer for N-terminal sequencing.

Protein transfer to membranes was used for a number of experiments. Blotted PDVF membranes were submitted for gas phase Edman degradation N-terminal sequencing, analysis of immunogenic antigen in salivary glands by 1-D gel blotting and for identification of important antigen by 2-D gel immunoblot coupled to proteolytic digestion and tandem mass spectrometric (ESI-Quadrupole-Time of Flight) and MALDI-ToF analysis. Electroblotting onto BioTrace ™ polyvinylidene (PVDF) membrane (Pall Corporation, Ann Arbor, MI) was performed as described by Beecroft *et al.* (1993). The proteins were transferred from the 1-D Bio-Rad mini-gels to the PVDF for 30 min at 90 V, with an ice pack in the transfer buffer outer chamber. One-dimensional gel transfers used Frementas, pre-stained 10 kDa ladder molecular weight standards to quality control the protein transfer process. The transfer of the proteins to the PVDF from the large format 2-D gel was accomplished one at a time 8 mA overnight at room temperature. All electroblotted gels were stained with Coomassie Blue G-250 to visualize the completeness of protein transfer to membrane. Samples to be submitted for N-Terminal or amino acid analysis were submitted on PVDF. The membrane was stained post-transfer with GelCode Blue (Pierce Chemical Company, Rockford, IL) and just as the target band started to appear the membrane was washed with water and the band excised with a scalpel. Antibodies for immunoblots were as for a number of experiments. Feeder rabbit sera was probed against salivary gland tissue separated by 1-D SDS-PAGE gel (0.75mm thickness). In these experiments one-half salivary gland equivalent was separated per lane. The primary antibody was feeder rabbit sera used at a dilution of 1:10,000 in 3 % skim milk powder 0.1 % Tween-20. The second

antibody, anti-mouse IgG/IgM-horseradish peroxidase conjugate (Caltag Laboratories, South San Francisco, CA), was diluted 1:50,000 goat. SuperSignal Dura chemiluminescence substrate (Pierce Chemical Company, Rockford, IL) was used for detection of the HRPO conjugated antibody. After development of autoluminograms (Kodak Biomax MR film), proteins were stained on the PVDF membrane with GelCode® Blue. The exposed film was then superimposed on the stained PVDF membrane to reveal the precise location of the immunoreactive protein bands in relationship to the entire protein profile. In a second immunoblot experiment human sera was probed against salivary gland tissue that had been separated by 1-D gel. Again, one-half salivary gland equivalent was separated per lane. The first antibody was human sera diluted 1:5,000 in 3 % skim milk powder 0.1 % Tween-20. The second antibody, a goat anti-human IgG-horseradish peroxidase conjugate (Caltag Laboratories, South San Francisco, CA), was diluted 1:25,000. The third immunoblot analysis probed tsetse feeder rabbit sera against salivary gland tissue that had been separated by 2-D gel. Ten pairs of glands were separated by 2-D gel electrophoresis and the primary antibody used was feeder rabbit sera diluted 1:10,000 in 3 % skim milk powder 0.1 % Tween-20. The second antibody, goat anti-mouse IgG/IgM-horseradish peroxidase conjugate (Caltag Laboratories, South San Francisco, CA), was diluted 1:50,000.

2.10. Tryptic digestion of protein.

Protein bands or spots of interest were cored from gels using a scalpel or four millimeter plastic straws. They were either transferred to 1.5 mL Eppendorf microcentrifuge tubes that had been previously rinsed with 50 % methanol to remove any contaminants prior

to tryptic digestion. Alternatively they were transferred to 96 well sterile tissue culture plates (one spot per well in 10 μL of 20 % w/v ammonium sulphate) for storage at -20 °C. For analysis by mass spectrometry, 2-D protein spots were de-stained (50 % v/v methanol/ 5 % v/v acetic acid), reduced with 10 mM DTT and alkylated with 100 mM iodoacetamide as described by Kinter and Sherman (2000). Following reduction and alkylation, protein spots were digested overnight at 37 °C with 20 ng/μL modified porcine sequence grade trypsin (Promega, Madison, WI) according to the manufacturer's directions. Peptides were extracted from the gel pieces using one wash with 30 μL of 50 mM ammonium bicarbonate and two x 30 μL elutions with 50 % (v/v) acetonitrile and 5 % (v/v) formic acid. The resulting pooled eluates were reduced to a final volume of 20 μL in a vacuum centrifuge prior to analysis by mass spectrometry.

2.11. Nanospray-MS/MS.

Peptides were desalted using glass capillary needles (Protana Inc., Staermosegaardsvej, Denmark) that had been packed with C18 resin and were extracted into sample needles using 1.0 μL 50 % (v/v) methanol/ 1 % (v/v) formic acid. Nanospray electrospray ionisation (ESI) was used to introduce ions into a PE-SCIEX Q-STR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA). Peptide fragmentation data searching was performed using the Mascot MS/MS Ions Search algorithm (Matrix Science; London, UK: http://www.matrixscience.com/).

2.12. MALDI-TOF mass spectrometry.

Peptides from each trypsin-digested sample were desalted using a ZipTip (C18 resin; P10, Millipore Corporation, Bedford, MA). For each sample, 1.0 μL of the desalted peptide mixture was mixed (1:1) with the matrix α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) and spotted onto a Voyager, 100 position, stainless steel MALDI plate (Applied Biosystems, Foster City, CA). An Applied Biosystems Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA) running in delayed extraction, reflectron mode was used to acquire MALDI-TOF data. Selected peptide masses were submitted to MS-Fit (Protein Prospector software package; San Francisco, CA: http://prospector.ucsf.edu/) and Mascot (Matrix Science, London, UK: http://www.matrixscience.com/) for database searching and determination of peptide mass maps.

2.13. **De novo** *sequencing of ESI-MS/MS spectra.*

Mass spectrometric fragmentation data was manually sequenced and inspected to confirm software-generated sequences and to confirm fragment ion spectrum search results following guidelines based on Kinter and Sherman (2001). All peptides examined were doubly charged peptides generated from tryptic digestion.

3. Results

3.1. Solubilization and fractionation of tsetse salivary gland proteins.

Salivary glands from teneral flies were treated to produce two fractions: PBS extracted salivary gland fraction "A" (SGA) which probably contained soluble lumen and duct contents and loosely bound peripheral membrane proteins, and salivary gland fraction "B" (SGB) which contained material not extracted by PBS treatment. The proteins in SGA and SGB were separated by SDS-PAGE (Figure 2.1). SGA contained four major PBS-soluble extractable proteins with apparent molecular masses of 56, 48, 46, and 29 kDa (Figure 2.1, panel A, lane 2; proteins labeled 1-4 respectively). Less abundant proteins were seen at 150, 74, 63 and 29.5 kDa. In contrast, SGB contained a wide range of proteins (Figure 2.1, panel B, lane 2) of varying masses presumably representing intracellular and membrane bound proteins that were not extracted in PBS.

3.2. Reverse-phase HPLC separation of soluble salivary gland proteins.

Proteins from 50 μL of SGA were separated using reverse-phase HPLC (Figure 2.2). Detection at 230 nm revealed 10 significant fractions (Figure 2.2, panel A) that were subsequently separated by 1-D SDS-PAGE (Figure 2.2, panel B). Fractions 1 through 4 did not contain significant amounts of proteins (not shown). Fractions 5 through 9 contained major, well-resolved proteins at 56, 48, 46, 29 and 18 kDa (fractions/lanes 5-9, respectively).

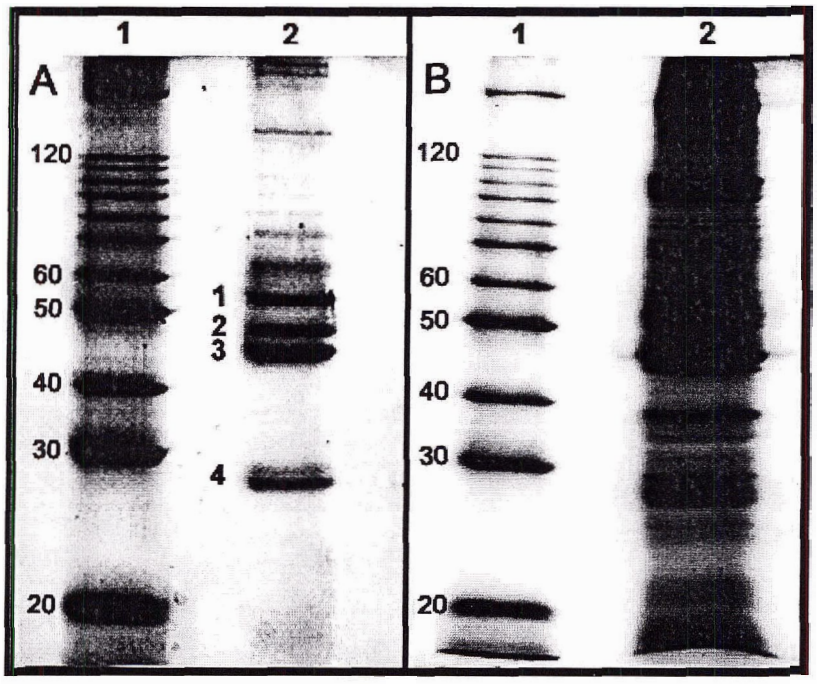

Figure 2.1. One-dimensional polyacrylamide gel profiles of proteins from salivary glands of teneral *G. m. morsitans*. Proteins were separated using a 10% gel and were stained using colloidal CBB. Panel A, lane 1, molecular mass standards (10 kDa ladder). Panel A, lane 2, 10 μL salivary gland fraction A (SGA). Panel B, lane 1, 10 kDa ladder. The standard masses are indicated. Panel B, lane 2, 1 μL Salivary Gland Fraction B (SGB). One-half gland equivalent was used per lane. Numbers 1-4 indicate the major proteins identified, 1) TSGF-1, 2) Tsal2, 3) Tsal1, 4) TAg5.

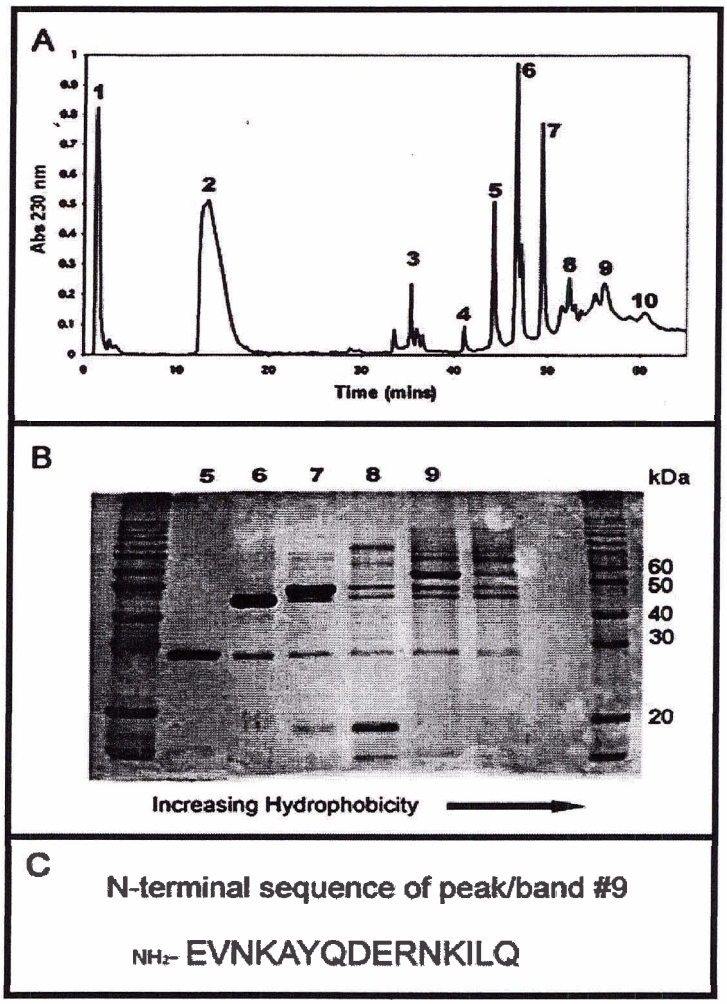

Figure 2.2. Separation of *G. m. morsitans* salivary proteins by HPLC and analysis by gel electrophoresis and N-terminal sequencing. Panel A, proteins from SGA were separated by HPLC using a C8 reverse-phase column. Panel B, isolated fractions (5-9) of increasing hydrophobicity shown in panel A were separated using a 10% acrylamide 1D-gel. Proteins were detected by staining with colloidal Coomassie Brilliant Blue G-250. Panel C, the N-terminal sequence of the major band in lane 9 of panel B was obtained after blotting of proteins to Immobilon-P™ and staining with GelCode Blue™. The protein was identified, using this sequence, as tsetse salivary gland growth factor-1 (TSGF-1).

3.3. N-terminal sequencing of isolated salivary gland proteins.

Gas-phase N-terminal microsequencing of the proteins in the reverse-phase HPLC fractions in liquid phase (acetonitrile and water) and the four protein bands transferred to PVDF blotting membrane was attempted in several experiments. The PVDF-immobilized major 56 kDa protein in fraction/lane 9 (Figure 2.2, panels A and B) yielded a 15 amino acid sequence (Figure 2.2, panel C). This sequence perfectly matched the predicted N-terminal 15 amino acid sequence of tsetse salivary gland growth factor 1 (TSGF-1). None of the other three proteins yielded any N-terminal sequence. However, micro-amino acid analysis indicated that sufficient levels of protein were present in each sample (data not shown), suggesting that their N-termini may have been blocked.

3.4. Major protein identification by mass spectrometry.

Peptide mass mapping was performed on each of the four major soluble proteins from the gel shown in Figure 2.1 (Lane 2) and with the corresponding protein spots taken from the 2-D gel (Figure 2.3, arrows) to ensure protein purity and to correlate the apparent molecular masses of the intact, undigested proteins with those of the 1-D gel bands. For each set of peptides, the NCBI non-redundant database was searched using the MS Fit and Mascot algorithms and in all cases positive protein identifications were made. Protein 1 was Tsetse Salivary Gland Growth Factor-1 (TSGF-1), Proteins 2 and 3 were identified as Tsetse Salivary Protein-1 (TSAL1) and Tsetse Salivary Protein-2 (TSAL-2) respectively and Protein

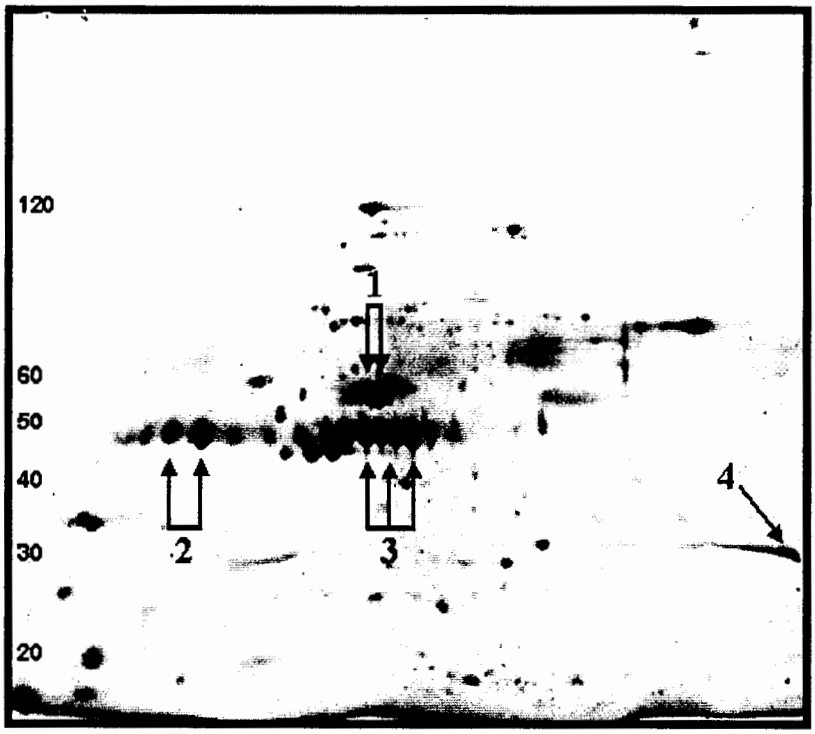

Figure 2.3. Two-dimensional polyacrylamide gel profile of the *G. m. morsitans* salivary gland proteome. Twenty-five pairs of salivary glands from teneral *G. m. morsitans* were solubilized in highly denaturing "urea-mix" and the proteins were separated using the ISO-DALT multiple 2-D gel system. First dimension gels contained wide range (pH 3-10) ampholines and the second dimension gels were 5-15% acrylamide gradients. Proteins were stained with colloidal CBB G-250. Gels are shown with decreasing molecular weights from top to bottom and the acidic end to the left, according to Cartesian coordinates. Positions of molecular mass standards (10 kDa ladder; run simultaneously on a separate gel) are shown on the right side of the figure. The arrows indicate protein spots used for mass spectrometric identification.

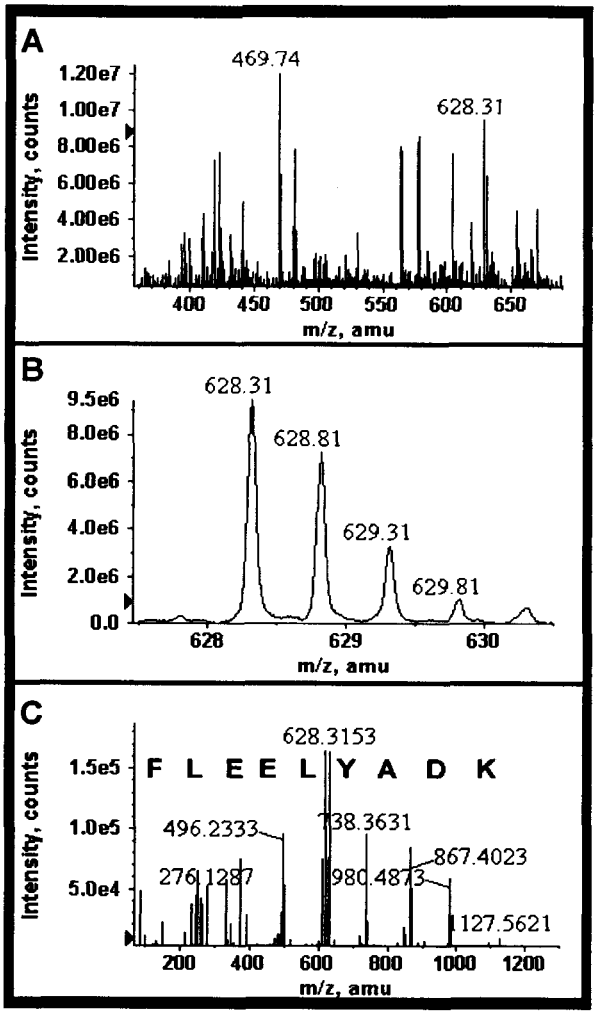

Figure 2.4. Tandem mass spectrometric identification of salivary gland TSGF-1. Panel A contains the survey scan spectra from a tryptic digest of a 2D-gel separated protein spot, previously digested with trypsin. The target parent ion at 628.31m/z is a doubly charged species representing a tryptic peptide with mass 1252.62 Da. Panel B reveals that after closer inspection the parent ion of 628.3m/z is indeed a doubly charged species as the isotopic envelop is separated by ½ mass unit (m/z, z =2). Panel C shows the fragmentation or product ion spectrum after collisionally induced dissociation (CID). The unfragmented parent ion component of the spectrum is visible at 628.31 m/z. The y-ion series is indicated

Table 2.1. Mass spectrometric identification of soluble salivary gland proteins from Teneral *Glossina morsitans morsitans*

Protein[a]	Accession number[b]	Mass (kDa)	pI[d]	Mass map Coverage %[e]	Peptide sequences[f]
TSGF1	AF140521	56.6	5.52	17	^{215}QFLEELYADK^{224}
TSAL1	AF259958	45.6	5.06	36	^{164}NINGAAVNYR^{174} ^{243}LSTYDYANIVPQYK^{256}
TSAL2	AF259959	43.9	6.95	31	^{249}DIYDGNIWR^{257}
TAg5	AF259957	28.9	8.58	38	^{118}YAGQNLAELGR^{128} ^{129}SGGPPPDYR^{137} ^{54}YQSALLDAHNK^{64}

[a] Identified by peptide mass mapping (MALDI-TOF mass spectrometry), peptide sequencing (Q-TOF mass spectrometry) and database searching.

[b] In the NCBI non-redundant database.

[c] Predicted from the translated protein sequence.

[d] Predicted from the translated protein sequence.

[e] Percentage of the protein sequence covered by peptide masses obtained by MALDI-TOF mass spectrometry.

[f] Unique signature sequences obtained by fragmentation of selected peptides (Q-TOF mass spectrometry). The position of the peptide sequences in the target protein are shown as superscripts.

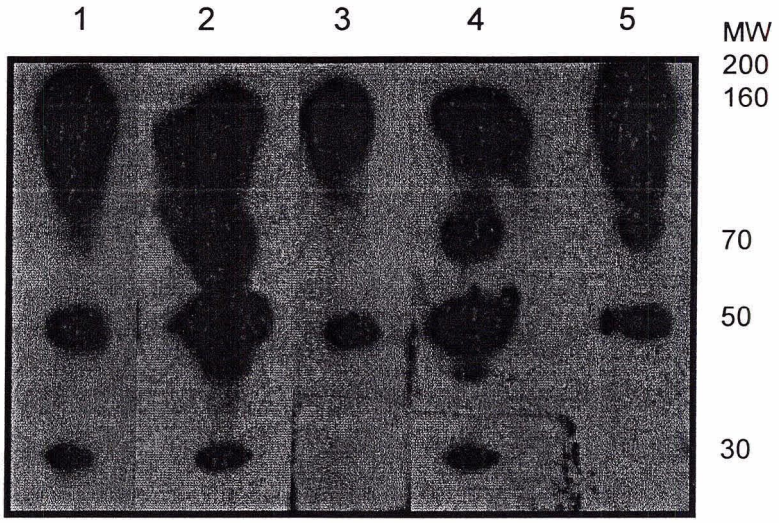

Figure 2.5. Identification of immunogenic molecules in tsetse salivary glands (1 pair per lane) using tsetse colony feeder rabbit sera. 12.5% SDS-PAGE gel at 10 mA stack, 20 mA separation gel, transferred to PVDF membrane for immunoblot analysis. All rabbit sera (1° antibody) diluted 1:10,000. Second antibody (goat anti-rabbit IgG HRPO) was diluted 1:50,000. Lane 1, feeder rabbit A serum. lane 2, rabbit B: lane 3, rabbit C: lane 4, rabbit D: lane 5, rabbit E. Fermentas™ mw ladder indicated in kDa on the right side of the blot.

4 was Tsetse Antigen–5 (TAg5). Some biochemical details of these proteins and summarized mass spectrometric data used in their identification are shown in Table 2.1. To increase the confidence in the initial protein identifications determined by peptide mass mapping, peptides from the digested 1-D gel protein bands and 2-D gel spots were also analyzed by tandem mass spectrometry. From the survey scans, selected peptides were chosen for fragmentation and primary structure determination. The NCBI non-redundant database was searched with the resulting peptide sequences using the Mascot MS/MS Ions search algorithm. Signature peptide sequences were obtained with all four proteins (Table 2.1) allowing an unequivocal identification of each of these molecules.

3.5. Analysis of immunogenic salivary gland molecules: feeder rabbit and human sera.

Tsetse fly salivary glands from three species of fly: *G. m. morsitans, G. p. palpalis,* and *G. pallidipes* were screened for the presence of immunogenic molecules. The proteins were identified in immunoblots using polyclonal sera from tsetse colony feeder rabbits as a probe for antigenic salivary gland proteins that had been separated by one-dimensional SDS-PAGE and blotted onto PVDF membrane. Human sera from exposed (to tsetse feeding) and non-exposed individuals were also analyzed for immunoreactivity. Figure 2.5 shows the results of immunoblots of tsetse salivary glands using tsetse feeder rabbit sera as probes. All rabbit sera (1^{o} antibody) were diluted 1:10,000 and the second antibody, goat anti-rabbit (IgG HRPO) was diluted 1:50,000. Lane 1, feeder rabbit A sera. This rabbit serum contains antibodies specific for antigens of three different molecular masses in the 1-D gel separated immunoblot. The molecular masses resemble those described by (Ellis *et al.* 1986) of 160,

48, and 28 kDa. Lane 2, feeder rabbit B serum. A similar staining pattern was seen as with the first rabbit serum with an additional band appearing at approximately 70 kDa. Lane 3, feeder rabbit C serum. This serum detected the fewest salivary proteins with only two proteins being detected at 160 and 48 kDa. Lane 4, feeder rabbit D serum. This serum detected the same proteins as feeder rabbit B serum. Lane 5, feeder rabbit E serum. This serum recognized at least three species (50 70 and 160 kDa) with an indication that there may be a higher molecular weight species in the poorly separated upper region of the gel (at least 200 kDa). Lane 6 (not shown), negative control rabbit serum (naïve lopp-eared rabbit serum sample from UVic Animal Care Unit) produced no bands on the one-dimensional immunoblot. Human and tsetse colony feeder rabbit immunoblotted with *G. m. morsitans* salivary glands results are shown in Figure 2.6. Lane 1, feeder rabbit A sera recognizes three species (see also lane 1, Figure 2.5). Lane 2 contains the serum from a human donor who fed tsetse on his arms and legs over a period of years. The serum contained antibodies specific for two molecules at approximately 50 and 200 kDa, a unique staining pattern when compared to the rabbit antisera. Lane 3 contains serum from a non-exposed human negative control in which no specific antibodies were present.

Western blot activity of tsetse feeder rabbit A antiserum against multiple species of tsetse salivary glands is shown in Figure 2.7. Lane 1. *G. m. morsitans*, again rabbit A's serum stains three species of molecules 160, 48, and 28 kDa. Lane 2. *G. pallidipes* and lane 3, *G. palpalis.* These two species react similarly yet are different from *G. m. morsitans.* Antigenic salivary gland molecules were identified using one- and two-dimensional gel immunoblots, and either in-gel or on-PVDF digestion and tandem mass spectrometric analysis (see Figure 2.8). Whole salivary glands (10 pairs per gel) were separated on two 2-

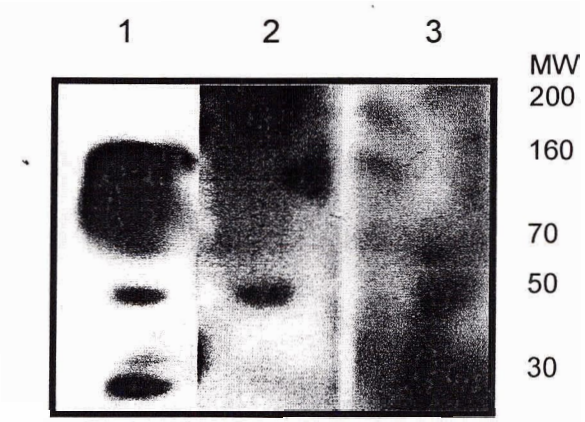

Figure 2.6. Immuno blot analysis of human serum and tsetse colony feeder rabbit serum against *G. m. morsitans* salivary glands. Lane 1 "Dave" feeder rabbit serum, lane 2 "Ron" serum exposed human tsetse, lane 3 "Terry" negative control human serum.

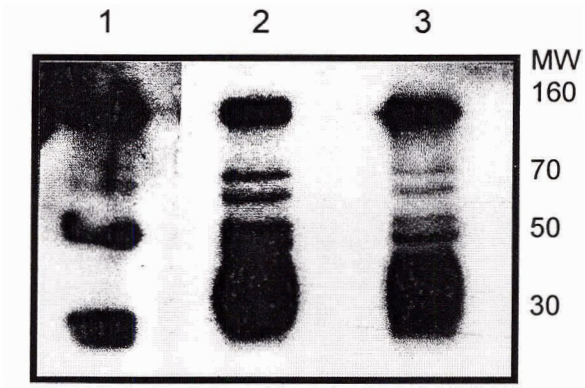

Figure 2.7. Immunoblot analysis of feeder rabbit "Dave" serum against multiple species of tsetse salivary glands. Lane 1 *G. m. morsitans*. Lane 2, *G. pallidipes*. Lane 3, *G. austeni*. Lane 4 (not shown), Fermentas 10 kDa molecular weight ladder, pre-stained is shown on the right side of the blot.

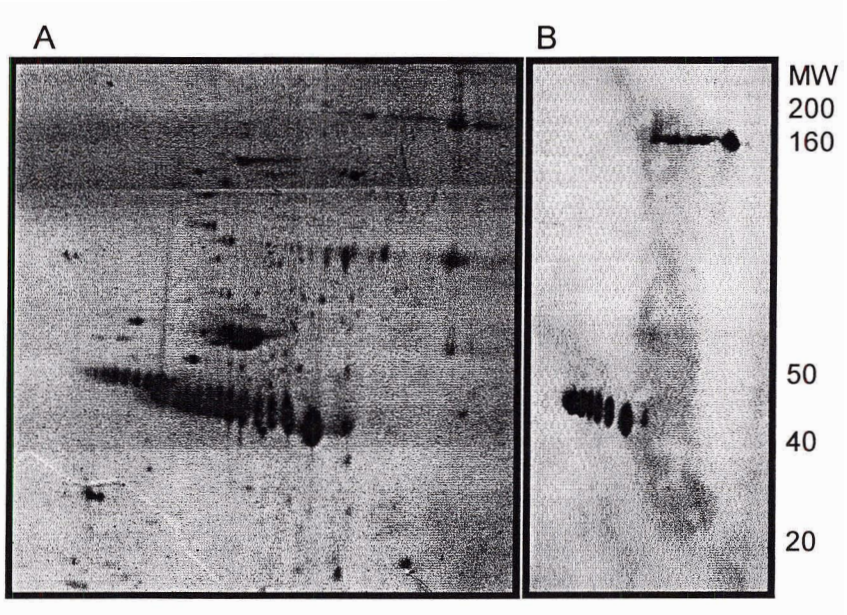

Figure 2.8. Identification of antigenic salivary gland molecules using 2-D gel electrophoresis and immunoblot analysis. Whole salivary glands (10 pairs per gel) were separated on two 2-D gels (IEF pH 3-10, DALT 10-16.5%), one gel stained with CCB and the other transferred to PVDF and blotted with tsetse feeder rabbit "Dave" serum. Panel A, CCB stained gel. Panel B, PVDF blot stained with feeder rabbit serum.

D gels (IEF pH 3-10, DALT 10-16.5%), one gel stained with CCB and the other transferred to PVDF and blotted with tsetse feeder rabbit "Dave" serum. Panel A. CCB stained gel. Panel B. PVDF blot stained with feeder rabbit sera located the area on the stained gel which was excised, digest and analyzed by tandem mass spectrometry.

4. Discussion

4.1. Identification of major molecules in the salivary glands of tsetse G. m. morsitans

Tsetse flies utilize blood as food and as may be expected, given their wide geographical distribution in Africa, feed upon a wide variety of hosts. Remarkably, some tsetse feed on all vertebrates they encounter, including mammals, birds, reptiles and even fish (Weitz 1956). Tsetse flies are the world's most promiscuous hematophagous insects (http://ufbir.ifas.ufl.edu/chap). Thus it is not surprising that tsetse have evolved efficient mechanisms for blood feeding. Tsetse salivary glands contain molecules involved in prevention of blood clotting, including a thrombin inhibitor (Lester and Lloyd, 1926; Cappello *et al.*, 1996; 1998) and platelet anti-aggregating activities (Mant and Parker, 1981; Li and Aksoy, 2000) that probably aid in the formation of hematomas thus increasing the blood available at the feeding site. Salivary glands also contain immunomodulatory molecules that are involved in hypersensitivity reactions (Ellis *et al.*, 1986) although these molecules have not been identified. Whether such inflammatory mediators play a role in trypanosome establishment in the host is unknown. All of these molecules are present in the

saliva prior to injection into the bite site. For this reason we focused on the identification of soluble salivary proteins of teneral *G. m. morsitans*.

Few proteins (10 in total, 4 of which were abundant) were observed in stained 1-D gels of the soluble salivary gland extracts (SGA). These soluble molecules were probably either free in the salivary lumen or ducts or were loosely bound peripheral proteins. They are likely injected into the host upon feeding. Proteins of approximately the same apparent molecular mass were present in the 1–D gels of the non-soluble material (SGB) and although these were not shown to be related to the four major secreted proteins, it is possible that they were membrane bound or internal forms of the same molecules ultimately destined to be secreted. Separation of these proteins by 2-D gel electrophoresis and their analysis by mass spectrometry may determine if this is the case.

The four major soluble molecules in SGA were separated by reverse-phase HPLC and 1-D and 2-D gel electrophoresis and were identified using mass spectrometry. Despite the fact that the NCBI database contained only 16 unique tsetse sequences and only seven of these were designated as from the salivary gland, the search algorithms were successful in identifying all four of the proteins. All four salivary proteins predicted by differential screening of cDNAs (Li and Aksoy, 2000; Li *et al.*, 2001) were identified by us by protein microchemical techniques, implying that the mRNA levels and protein levels were related. This direct correlation is not always the case (Anderson and Seilhamer, 1997; Goodlet and Aebersold, 2001), however, a subset of secreted proteins was shown to have a stronger correlation between RNA message and protein product (Anderson and Seilhamer, 1997). Although the mRNAs encoding the four soluble salivary proteins were not strictly quantified, the high proportion of the sequences in the cDNA library and relative ease of detection of

transcripts by northern blotting (Li and Aksoy, 2000) implies that they were abundant species. This would be expected for messages encoding molecules destined for secretion in large quantities.

The theoretical mass and pI of TSGF-1 matched the 1-D gel migration position and 2-D gel coordinates of Protein 1. Sequence analysis of the translated protein gene product of *tsgf-1* revealed extensive similarities to both insect- and mollusk-derived growth factors and the presence of adenosine-deaminase motifs (Li and Aksoy, 2000). This activity has been associated with modulation of vasodilation and platelet aggregation in mosquitoes (Ribeiro *et al.* 2001). An intriguing possibility is that preferential expression of tsetse adenosine deaminase activity in salivary glands may allow survival of African trypanosomes in this tissue since these parasites are purine scavengers and lack adenosine deaminase (Ogbunude *et al.* 1985). A related protein, TSGF-2, also predicted from a cDNA sequence by Li and Aksoy (2000), was not found to be a major soluble protein in salivary extracts of the teneral *G. m. morsitans* that we used. These authors reported that TSGF-2 was expressed in much lower quantities than TSGF-1 when tested by western blotting and in one tsetse species (*G. austeni*) this protein was absent. The *Tsgf-2* DNA sequence does not appear to encode any potential signal peptide and thus the TSGF-2 protein may not be secreted into the salivary gland lumen. The putative TSGF-1 protein sequence contained a predicted N-terminal hydrophobic signal peptide, a feature suggestive of secreted proteins. Our successful N-terminal sequencing of the mature TSGF-1 protein showed that the signal peptide had been removed as expected and as originally predicted by Li and Aksoy (2000).

Proteins 2 and 3 were identified as TSAL-1 and TSAL-2. As with TSGF-1, these proteins were predicted to have N-terminal signal sequences. Database searching with both

Tsal1 and *Tsal2* DNA sequences and sequences of their translated protein products failed to find any significant sequence matches to molecules from other organisms. In addition, searching of ProDom, a protein domain database (http://protein.toulouse.inra.fr/prodom/doc/prodom.html) failed to identify any of the common sequence motifs. We were unable to retrieve any information suggestive of possible functions for these molecules.

Protein 4 was identified as Tsetse Antigen–5 (TAg-5) a protein predicted from a cDNA sequence, *Tag5* (Li *et al.*, 2001). The mass and very basic pI (8.58) values were again confirmed by positions of the Coomassie Blue stained protein on both 1-D and 2-D gels. The translated protein sequence for the predicted protein had similarity to the CAP (Crisp-Antigen 5, Plant pathogenesis protein) family of proteins (Li *et al.*, 2001). Members of this family of proteins have been identified in a number of diverse organisms and share a core sequence of about 200 amino acids that have been hypothesised to have a variety of functions. In hornets and wasps (Fang 1988) and in fire ants (Hoffman 1995) Antigen 5 is a major venom allergen that causes allergic reactions. The TAg-5 protein sequence is closely related to proteins Agr and Agr2 expressed in the proventriculus and midgut of *Drosophila melanogaster* and LuloAG5 which is expressed in the salivary gland of the sandfly *Lutzomyia* (Li *et al.*, 2001). The salivary gland tissue was the primary site for *TAg5* gene expression although it was also found to be weakly expressed in the tsetse proventriculus and midgut (Li *et al.*, 2001), which is supported by our direct protein microchemical analysis. TAg-5 may interfere with hemostasis or play a role in local tissue reactions in the host, but these are only hypotheses based on observations of related molecules in other organisms.

A role for salivary proteins in facilitating tsetse feeding on host blood seems well founded since molecules involved in preventing hemostasis or in modulation of hematoma formation have been identified. These are tsetse thrombin inhibitors (Cappello *et al.*, 1996) and TSGF-1 and TSGF-2 which probably have adenosine deaminase activity (Li and Aksoy, 2000) and thus would perturb mechanisms involved in vasodilation and platelet aggregation (Ribeiro *et al.*, 2001). What is not clear is the role that salivary proteins may play in trypanosome-tsetse interactions or in the mammalian host at the site where saliva and parasites are injected. One can postulate a role for TSGF-1 and TSGF-2 growth factor-like activity in influencing parasite differentiation and maturation in the salivary gland although this has not been tested. It is probable that all four of the major soluble salivary proteins are injected into the host in tsetse saliva. In addition to involvement in modulating hemostasis, some of them may trigger mechanisms that influence localization and establishment of bloodstream forms of trypanosomes after metacyclic forms are injected, perhaps at the inflammatory lesion known as the chancre (Akol 1986). A candidate for this type of activity is TAg-5, which by analogy with other members of the CAP family of proteins, may cause hypersensitivity reactions which have been described in rabbits injected with tsetse saliva (Ellis *et al.*, 1986).

The abundance of the four cDNAs transcripts has been found to vary in different species of tsetse which exhibit widely ranging abilities to transmit trypanosomes (Li *et al.*, 2001). It remains to be seen whether differences in transcript abundance correspond to varying levels of protein expression in the salivary glands and whether these proteins influence vector competence. It is thus of considerable interest that immunization with a salivary protein isolated from phlebotamine sandflies protected mice from infection with

Leishmania major (Valenzuela *et al.* 2001). Since we are now able to isolate the soluble tsetse salivary gland proteins in reasonable quantities, we plan to test them for their influence on cyclical development of trypanosomes.

4.2. Major antigenic molecules of the salivary gland

Live rabbits are routinely used as food source for blood feeding insects (Jordan *et al.* 1968). Tsetse culture can be facilitated in this fashion as well as via a synthetic membrane feeding system. As was discussed earlier, tsetse saliva contains molecules that facilitate tsetse feeding on blood. During the feed, immunomodulating molecules are injected into the bite site, ensuring painless, free flow of blood. In a Leishmaniasis model, the mouthparts of the sandfly penetrate the skin, bypassing a major innate host defense. In addition, the sandfly saliva contains potent vasodilators, blood clotting inhibitors and immunomodulating factors (Ribeiro 1995). Saliva enhances *Leishmania amazonensis* infection of mice by modulating interelukin-10 production (Norsworthy *et al.* 2004). Normally a penetrating puncture wound like that caused by the proboscis of the fly, would immediately be recognized by the host's innate immune system and a response would be initiated. Blood clotting itself is a very rapid response that would interfere with blood feeding and could harm the insect. It has been demonstrated that when fractions of saliva are used to immunize a mammalian host, it became refractory to Leishmania infection by a sand fly bite. Valenzuela (2001) examined saliva from the sand fly carrier of *Leishmania major* and identified a protein, SP15, an immunogenic target of natural immune responses in mice. A DNA vaccine was used to immunize mice, which were later injected with a mixture of *L. major* and fly saliva. The

resulting infection was significantly milder compared to infection in a control group of mice that were not vaccinated. The immunized mice had much smaller skin lesions and the infections were cleared within six weeks. Non-vaccinated mice developed large skin ulcers and were not able to eliminate the parasite. The authors also vaccinated knockout mice incapable of producing antibodies. These mice were still protected by the vaccine, suggesting that T cells play a role in protection (Valenzuela *et al.* 2001). The results demonstrate that a vaccine containing a component of insect vector saliva can protect mice from the severe symptoms associated with cutaneous leishmaniasis. The same laboratory has used a similar approach to stimulate immunoprotection in guinea pigs from the Lyme disease (*Borrelia burgdorferi).* Repeated exposure to non-infected ticks caused the guinea pigs to become immune. When the animals were exposed to infected ticks the insects did not feed as long and showed reduced fecundity (Gorman 2003) (Valenzuela *et al.* 2002).

In my work with tsetse approximately five different molecular weight antigens could be detected in salivary glands. Different feeder rabbits produced antibodies that recognized a spectrum of immunogenic molecules of 160, 70, 50 and 28 kDa. The immune human serum recognized a very heavy species of molecule of greater than 200 kDa that could not be identified. Two of the salivary gland molecules could be identified. One of these was Tsal2 a molecule with unknown function and the other TAg-5, which is similar to other known allergens from a number of species. The rabbit and human sera both contain antibodies that are specific for a higher molecular weight species at 160-200 kDa. The antigen could not be identified. The CCB stained region of all 2-D gels was an extremely faint, unresolved protein streak that yielded few detectable peptides after digestion and extraction of in-gel digests. The peptides that were analyzed gave poor fragmentation data thus no sequence data was

acquired. The lowest molecular weight species of salivary antigen was found to be Tsetse antigen five (TAg-5). This molecule is known to be similar to an immunogenic molecule in other insect vector species. Antigen 5 is a member of the CAP family of secreted proteins found in a diverse range of organisms. This family contains an important core sequence of approximately 200 amino acids that provides the diverse range of activity found in this set of molecules. Antigen 5 has been identified as one of the major venom allergens of ants and wasps (Fang 1988) and in plants they have been shown to be induced in response to infection by viral, bacterial and fungal pathogens. They and are involved in local acute-phase defense responses and are known as pathogenesis related proteins. Two related cDNAs have been characterized from Drosophila, the first gene (Antigen 5 related, *agr*) is primarily expressed in the proventriculus of larva and adult and as such may be a constituent of the peritrophic matrix (Kovalick *et al.* 1998). The second gene (Antigen 5 related 2, *agr2*) is expressed in posterior midgut cells in adults and may be involved in mediating immune reactions in the gut (Megraw *et al.* 1998). Can these salivary gland molecules act in similar fashion to those found in sandfly saliva that are able to inhibit Leishmania parasite growth? The molecules identified in this study are prime candidates for future immunization / trypanosome infections in mice.

Chapter 3. Identification and partial characterization of proteins in the midgut of the tsetse *Glossina morsitans morsitans.*

1. Introduction

After the tsetse ingests a trypanosome-infected bloodmeal, the parasite must establish as a midgut-adapted procyclic trypanosome. This transformation is necessary for trypanosome growth and survival. Establishment depends on factors produced by and present in the tsetse midgut. Experiments performed by Nguu et al (1996) demonstrated that the mammalian bloodstream form (BSF) trypanosomes could be transformed into procyclic forms *in vitro* when incubated with uncharacterised tsetse midgut homogenates. Less than a dozen tsetse midgut proteins have been biochemically described. A number of different midgut proteins influence parasite survival, including lectins, proteolytic enzymes and trypanolysins (Welburn and Gibson 1989); (Imbuga *et al.* 1992). Evidence exists that demonstrates midgut molecules act as effectors during the developmental stages of vector-borne parasites in related systems. Anti-*Anopheles* midgut monoclonal antibodies, when fed to mosquitoes in an artificial membrane feeding system, significantly reduced vector competence and survival (Lal *et al.* 2001). In this Chapter, the midgut proteome was analyzed in an effort to identify some of these important proteins in a biologically relevant context.

The first set of experiments partially characterized major tsetse midgut molecules using protein microchemical techniques. The second set focused on the derivation of monoclonal antibodies to probe for targets that could possibly disrupt trypanosome establishment in the tsetse midgut. *Glossina morsitans* midguts were used as immunogen to

generate a panel of mouse monoclonal antibodies. One anti-midgut mAb, 4A2, was tested in a transmission blocking assay at Yale University in the Department of Medicine under the supervision of Dr. Serap Aksoy. The final experiment employed isotope-coded affinity tag (ICAT) differential protein expression technology as well as 2-dimensional gel electrophoresis to analyze the differences between two strains of *Glossina m. morsitans* that exhibit a significant difference in their susceptibility to trypanosome infection. To enhance detection of important membrane and low abundance proteins that are difficult to analyze with traditional two-dimensional gel electrophoresis-based approaches, we have used a cleavable ICAT reagent employing the basic methodology of (Gygi *et al.* 1999). Two-dimensional gel electrophoresis (2DGE) (O'Farrell *et al.* 1977) was used as a complementary analytical validation method to ICAT analysis, by providing supportive quantification evidence and potentially increasing the number of peptides available for identification. Although not as quantitative as ICAT, 2DGE is still the most powerful orthogonal (pI and mw) two dimensional separation technique routinely employed and has an advantage over the ICAT methodology in that it is not exclusive to cysteine-containing proteins. Using these techniques together provides a powerful set of tools which employed with customized database searching identified 208 unique protein. Of these, at least 25 appear to differentially regulated (see Appendix II) including, tsetse EP-repeat protein, which was shown to be upregulated in the salmon eye-coloured mutant. Other important tsetse regulated proteins include, trypsin-like serine protease, two *G. m. m. morsitans* proteins, Tsal1 and Tsal2 with unknown function, a tsetse growth factor and proventriculin2 (Pro2) a molecule found in the lining of the tsetse midgut within the peritrophic matrix.

2. Experimental Procedure

2.1. One- and Two-dimensional gel electrophoretic analysis of tsetse midgut proteins.

SDS-PAGE (1-D and 2-D) protocols were previously described in Chapter Two. For 1-D gels, midgut tissues (0.5 midgut equivalent) were resuspended in 25 µl of 1X Laemmli sample buffer (Laemmli 1970) and loaded onto 10 % acrylamide, 0.75 mm 1-D sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) minigels. High-resolution 2-D SDS-PAGE was performed using the ISO-DALT multiple 2-D system (Anderson and Anderson 1978) as previously described (Anderson *et al.* 1985) and in Chapter two). Five midguts, five bacteriomes and five midguts with bacteriomes removed were separately solubilized in 30 µL of urea mix (9 M urea, 4 % NP-40 (v/v), 2 % Pharmalyte 3-10 ampholines (v/v), 2 % DTT (w/v)) and mechanically disrupted using a 1 mL syringe equipped with a 21 G needle. Samples were processed as in (Materials and Methods Chapter Two).

2.2 Immunoblotting

Immunoblotting was performed as previously described (Beecroft *et al.* 1993) except that a 1:500 dilution of primary antibody (mouse monoclonal anti-GroEL; SPA-807, Stressgen Biotechnologies, Victoria, BC) and a 1:50,000 dilution of secondary antibody (goat anti-mouse IgG/IgM-horseradish peroxidase conjugate; Caltag Laboratories, San Francisco, CA were used, followed by addition of SuperSignal Dura chemiluminescence substrate

(Pierce Chemical Company, Rockford, IL). After development of autoluminograms (Kodak Biomax MR film), proteins were stained on the PVDF membrane with GelCode® Blue. The exposed film was then superimposed on the stained PVDF membrane to reveal the precise location of the immunoreactive protein bands in relationship to the entire protein profile.

2.3. Mass spectrometry

Identification of molecules by MALDI-TOF and liquid-chromatography tandem mass spectrometry (Quadrupole / time of flight: QSTAR) was performed as outlined in Materials and Methods Chapter Two.

2.4. Antigen preparation.

Midguts, stored in 1 mL cryovials, were taken from storage at –70 °C. The tube was transferred directly to a bucket of ice to keep the tissue at approximately 0 °C. Each step was performed on ice, including sonication. Midguts were sonicated in phosphate-buffered saline (PBS) or distilled water using a narrow probe, Sonifier Cell Disruptor W 185E (Heat Systems-Ultrasonics, Inc, Plainview, NY, USA.) 5 × 5 seconds. PBS was used as buffer for immunizations and water for enzyme-linked immunosorbent assay (ELISA). The amount of tissue used in each experiment depended on the application and details are provided below. The individual guts were removed from pooled tissue that had been stored in lots of 5 to 20 guts per cryotube. For immunization the PBS sonicated tissue was mixed with an equal volume of Freund's complete or incomplete adjuvant.

2.5. Mouse immunization.

For the initial immunization, one midgut was used. Midguts were solubilized PBS by sonication. The midgut homogenate was added to an equal volume of Freund's complete adjuvant (GibcoBRL Burlington, ON) in a 3 mL syringe and was mixed to a homogenous paste with the aid of a bench top sonicator. One-hundred μL of this solution were injected intraperitoneally (IP) into each of two female BALB/c mice. The first booster immunization was given three weeks post-primary injection in 50 % Freund's incomplete antigen. A second boost was given three weeks after the first boost. A blood sample was taken from the mouse's tail vein ten days after the second boost to determine antibody titre by indirect ELISA. After the test bleed sample was assessed it was determined to proceed with the fusion (see Figure 3.4). A final intravenous (IV) boost was given three weeks after the second boost and three days before the fusion.

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used for a number of assays. Antibody titers were determined from mouse test bleeds prior to hybridoma production. Hybridoma monoclonal antibody (mAb) screening on the immunizing antigen was also performed using ELISA. Titration analysis post-fusion to test for activity of the hybridomas for mAb isotyping were also determined using ELISA. Monoclonal antibodies in hybridoma supernatants and polyclonal mouse test-sera were screened for binding to the immunizing antigen mixture by indirect ELISA (Tolson *et al.* 1989) using antigen midgut tissue that had been disrupted by sonication in distilled

water. Tsetse tissues were sonicated on ice using a narrow probe and a Sonifier Cell Disruptor W 185E (Heat Systems-Ultrasonics, Inc, Plainview, NY, USA). Tissue lysates were dried onto flat-bottom ELISA plates (Falcon 3915 PRO-BIND™ Assay Plates, Becton-Dickinson, Oxnard, CA). Isotyping of mAbs was performed using hybridoma tissue culture supernatants and an antigen-capture ELISA kit (American Qualex, La Mirada, CA) according to instructions supplied by the manufacturer. Hybridomas were picked into ten 96-well microtitre tissue culture plates (Costar, Cambridge MA, USA). After three days the clones were fed 100 µL of 10 % hybridoma medium and ELISA screening was performed the following day. The ELISA assay plates were coated with 0.15 midgut equivalent per well. The midguts were sonicated in 50 mL water and 100 µL of this mixture was loaded into each well and dried overnight in a 37° C incubator. The plates were blocked with 3 % skim milk 0.05 % Tween-20 (Sigma-Aldrich St. Louis MO) for one hour at 37 °C followed by three quick washes with phosphate buffered saline (PBS) 0.05 % Tween-20 (PBST). A total of 125 µL of each hybridoma supernatant were screened on five plates using two hybridoma supernatants per well (this method was chosen to limit the use of scarce and valuable tissue) and positive results would be resolved later after two rounds of ELISA to remove non-specific and stick antibody-secreting clones

2.7. Myeloma - lymphocyte fusion (hybridoma production)

Derivation of hybridomas was performed as previously described (Beecroft et al 1986) using ClonaCell-HY™ (StemCell, Vancouver BC, CA) for single step selection and cloning (Pearson et al 1980).

2.8. Cryopreservation of hybridomas

Twenty mL of freezing medium (90 % FBS, 10 % DMSO) was filter sterilized using a 0.2 μm syringe filter. Cells from a healthy rapidly dividing cell culture were centrifuged (50 mL of culture) at 1000 x g for five min at room temperature and the supernatant removed and discarded. The hybridomas were resuspend by tapping the tube and the cells mixed in 6 mL of freezing medium. One mL of the cell suspension was added to each of 6 labeled freezing vials. The vials were placed into freezing head of Nitrogen tanks and allowed to slowly cool overnight. The frozen vials were sunk in liquid nitrogen and catalogued. Original cultures were maintained at a small volume until it has been verified that the frozen cells could be recovered.

2.9. Immunoblotting of anti-midgut mAbs for hybridoma clone screening.

Five tsetse midguts were solubilized in 300 μL of Laemmli buffer (5 / 300 = 0.017 guts / μL). One-quarter of a gut was used for each lane of the gel (0.25 gut = 0.017 gut/ μL x X μL, X = 14.7 μL). 1-D and 2-D gels and immunoblots were performed as described in Materials and Methods in Chapter 2.

2.10. Midgut tissue thin section preparation and immunofluorescence screening.

The methacrylate embedded tissue was prepared as outlined in the Kulzer Histo-Technique description using the Technovit 7100 kit (Heraeus Kulzer, Germany). The slides

were dried in a slide drying hot plate and then inspected with an inverted microscope for the presence of tissue. To screen monoclonal antibodies with these sections, the slides were first fixed with 5 % skim milk powder in PBS 0.1 % Tween 20 (PBST) for one hour at 37 °C and then washed with PBST 3 x 5 min and 3 x 10 min. The tissue culture supernatants were used "neat" and 175 µL were pipetted onto each section taking care that the volume was not so much as to spill into the next section of the slide. The primary antibodies were allowed to incubate on the slides for 1 hour at 37° C inside of a humidified chamber to prevent fluid loss and then washed with PBST 3 x 5 min and 3 x 10 min. An equal mixture of two Alexafluor FITC-conjugated goat anti mouse antibodies (one anti-IgG and the other anti-IgM) were used as secondary antibodies. Each antibody was diluted 200 fold in 5 % skim milk powder in PBST and incubated 1 hour at 37 °C. The slides were then washed with PBST 3 x 5 min and 3 x 10 min before examining the stained slides with a Zeiss UV microscope. Images were captured with a digital camera at 100X magnification.

2.11. PEG enrichment and concentration of mAb 4A2 from murine ascites fluid.

Five mL ascites fluid was centrifuged at 400 x g for 6 min to remove insoluble material. An equal volume of 80 mM potassium phosphate pH 7.0 was added to the ascites supernatant for a total volume of 10 mL. To this solution a 50 % solution of PEG-8000 (in 80 mM potassium phosphate buffer pH 7.0) was added dropwise using a burette and stir plate and magnetic stir bar until the final concentration of PEG in the mixture reached 15 %. This sample was centrifuged at 3000-5000 x g for 30 min at room temperature. This was accomplished using a Beckman J2-HC centrifuge with a JA-17 rotor using round bottom

tubes. The supernatant was decanted from the pellet and set aside for testing. The pellet was resuspended in 10 mL phosphate buffer at (2X the original antibody sample volume). To this mixture a 50 % PEG solution was again added using a burette until the final PEG concentration reached 13 %. This mixture was centrifuged at 3000 x g for 30 mins at room temperature. The supernatant was decanted and set aside for testing and the pellet resuspended in PBS using an adequate volume to completely resuspended the pellet. This solution was dialyzed (14,000 mw cutoff) against 6 L PBS for 48 h. The buffer was changed once after 24 h. The optical density at 280 nm was used to estimate protein concentration with 1.4 OD_{280} = 1.0 mg/mL.

2.12. Tsetse and Trypanosome Cultures

The *G. m. morsitans* flies used were colonized at the Yale University insectary (23± 1 °C, 55-60 % relative humidity). They were originally obtained from Bristol University (Hao *et al.* 2001) and occasionally supplemented by flies from the International Atomic Energy Agency (IAEA) Seibersdorf Austria. The Yale tsetse colony was maintained on the artificial membrane feeding system (Moloo *et al.* 1999). For parasite infection, tsetse were fed a laboratory strain of *T. b. brucei* (YTAT1.1).

2.13. Tsetse infection assay and parasitemia determination

To investigate the killing effect of a purified monoclonal antibody on trypanosome infected tsetse the 4A2 mAb was fed to tsetse with trypanosome inoculated blood meals.

Three groups of two-day-old teneral flies were set up according to feeding diet. These included a test monoclonal, a control monoclonal and a control group (PBS only). A fresh infective blood (defibrinated bovine) meal containing 2 x 10^6 Ytat1.1 trypanosome parasites/mL culture stage was prepared and divided into the three test groups and supplemented with 0.06 M D-L glucosamine. The two monoclonal antibodies were prepared at a 10 mg/mL concentration and diluted 1 in 50 in the blood meals. This monoclonal regime was given at the initial blood feed and each successive feed for both groups for the entire 10 day trial period. Tsetse were fed every other day. At the end of 10 days, the period for midgut -stage parasites flies, flies were dissected in sterile 1x PBS under a dissecting microscope. Parasitemia was scored by counting the number of living trypanosomes using a compound microscope. A positive score of parasitemia meant an infection rate of one parasite/field and a negative score indicated zero parasites/field. A minimum of 20 fields/slide/fly were viewed before a score was assigned. The trial for the purified test monoclonal antibody was repeated following the same parasite, antibody and glucosamine feeding regimes. The antibody diet was given on all feeding days.

2.14. Midgut Protein homogenate preparation and quantitation

Forty wild type and 41 salmon mutant midguts were thawed and solubilized in 500 µL of 2 % SDS in 25 mM ammonium bicarbonate (ABC) by vortex mixing. The volume was subsequently brought up to 1 mL by adding a saturated urea solution in 25 mM ammonium bicarbonate (ABC). Tissue homogenates were quantified with a BioRad protein assay kit using instructions provided by the manufacturer.

2.15. High resolution 2-D gel electrophoresis

a) Isoelectric Focusing (IEF)

Three hundred µg of total protein were added to an appropriate volume of rehydration buffer the protein mixture was dispensed evenly across the trough in a reswelling tray. An IPG Immobiline Drystrip pH 3-10 NL (Amersham Biosciences Inc. Baie d'Urfé, Québec) was placed along the lane and covered with mineral to prevent evaporation during a 12 h rehydration. After rehydration the strips were focused with an Ettan IPGphor isoelectric focusing apparatus (Amersham Biosciences Inc. Baie d'Urfé, Québec). Focusing was performed at 500 V for 1 h followed by 1000 V for another hour and finally 8000 V for 8 hours and 20 min for a total of 65000 Vh for complete rehydration and focusing.

b) Slab gel electrophoresis

The IPG strips, pH 3-10 (non-linear) were removed from the Ettan IPGphor (Amersham Biosciences Inc. Baie d'Urfé, Québec) equilibrated in SDS buffer and just prior to use, the dithiothreitol concentration was adjusted to 1% (w/v). The IPG strips were placed in individual plastic tubes and equilibrated for 15 min at room temperature. After incubation the reduced strips were rapidly transferred to another tube containing 10 mL SDS equilibration buffer and 2.5% (w/v) iodoacetamide. They were incubated for an additional 15 min at room temperature. The reduced and alkylated IPG strips were transferred to the top of gradient slab gels and sealed in place with agarose.

2.16. Gel staining with Colloidal Coomassie Brilliant Blue and Sypro Ruby

Acrylamide gels were stained with either Colloidal Coomassie Brilliant Blue (CCB) (SERVA Blue G, research grade; SERVA Electrophoresis, Heidelberg, DRG) or Sypro Ruby (Molecular Probes, Eugene OR). CCB 2-D gels were stained as follows: gels were agitated gently in fixative (50 % v/v ethanol, 3 % v/v ortho phosphoric acid) for 24 h at room temperature, washed three x 30 min in distilled water and allowed to equilibrate in Neuhoff solution (16% w/v ammonium sulphate, 25% v/v methanol, 5% v/v ortho phosphoric acid) (Neuhoff *et al.* 1988) for one hour with gentle agitation. One gram of Coomassie Brilliant Blue (CBB) G-250 was sprinkled into the Neuhoff solution and staining continued for 2-3 days. When dark protein spots were visible, gels were either scanned and protein spots cored or the intact gels were transferred into a 20 % (w/v) ammonium sulphate solution for storage at 4 °C (Neuhoff *et al.* 1988). Sypro Ruby gels were processed in the following manner: Gels were fixed overnight using a solution of 10 % methanol, 6 % acetic acid and stained overnight in Sypro Ruby stain (Molecular Probes, Eugene OR). Gels were destained 3 x 15 min with fixative and stored in the same solution.

2.17. 2-D gel imaging and robotic spot picking

The stained gels were imaged using a proXPRESS gel scanner (PerkinElmer Life and Analytical Sciences, Inc. Boston, MA, USA). Sypro Ruby stained gels used top illumination with a solid black bottom tray and green acrylic sheet for flat field acquisition. Images were

acquired with an excitation of 480 nm and emission wavelength of 620 nm at a resolution of 100 μm to allow optimal interface with the ProPic robot.

For Coomassie Brilliant Blue G-250 stained gels, the flat field was acquired using bottom illumination with the solid black tray removed and a white light diffuser plate. A 530 nm emission filter was used for acquisition without an excitation filter and the resolution was set to 100 μm. The ProXPRESS scanned gels were analyzed using Progenesis software (Non-Linear Dynamics, Newcastle UK) and spot picking was performed on the Investigator ProPic (Genomic Solutions, Ann Arbor, Michigan). Excised spots were placed into 96 well output plates.

2.18. Robotic in-gel digestion of excised protein spots

The Investigator ProGest (Genomic Solutions, Ann Arbor, Michigan) robotic digestor was used for automated in-gel protein digestion of protein spots in 96 well format. For analysis by mass spectrometry, cored protein spots were de-stained (50 % v/v methanol, 5 % v/v acetic acid), reduced with 10 mM dithiothreitol (DTT) and alkylated with 100 mM iodoacetamide. Following reduction and alkylation, protein spots were digested for 4 h at 37 ^{o}C with 20 ng/L modified porcine sequence grade trypsin (Promega, Madison, WI) according to the manufacturer's directions. Peptides were extracted from the gel pieces using 30 μL of 50 mM ammonium bicarbonate followed by two 30 μL aliquots of a 50 % (v/v) acetonitrile, 5 % (v/v) formic acid solution. The three extractions were pooled and reduced to 20 μL in a vacuum centrifuge prior to analysis by mass spectrometry.

2.19. ICAT Labeling.

Both wild-type and salmon mutant midgut protein samples were reduced, labeled, digested and chromatographed in accordance with the C_0/C_9 cleavable ICAT protocol (Applied Biosystems, Framingham) with some modification. The sample protein concentrations were determined using a modified Bradford protein assay (Bio-Rad, Hercules, CA): 100 μg of control and experimental samples were labeled with 1 unit of light and heavy ICAT reagent respectively and dissolved in 350 μL 25mM ammonium bicarbonate pH 8.2 containing 20% acetonitrile (ACN). Labeling was allowed to proceed at 37 °C for 2 h followed by dilution of the samples to bring the urea concentration below 1.5 M, at which point 40 μg of sequencing grade modified trypsin (Promega, Madison, WI) were added. Tryptic digestion was allowed to occur at 37 °C for 18 h at which point the reaction was quenched by adjusting the pH to 2.9 in preparation for off-line strong cation exchange (SCX) fractionation.

2.20. Cation Exchange Chromatography.

To remove non-peptide material and to simplify the complexity of the ICAT labeled samples, off-line SCX fractionation was performed prior to the avidin cleanup. This was carried out on a Beckman System Gold micro-flow HPLC system. Buffer A consisted of 5 mM potassium dihydrogen phosphate pH 3.0 in 25 % ACN, while buffer B consisted of 5 mM potassium dihydrogen phosphate, 350 mM KCl pH 3.0 in 25 % ACN. The sample was diluted with 3 mL of cation exchange loading buffer (100 % buffer A) and adjusted to pH 3.0 with concentrated HCl. Samples were then loaded onto the 4.6 x 100 mm Polysulfoethyl A column

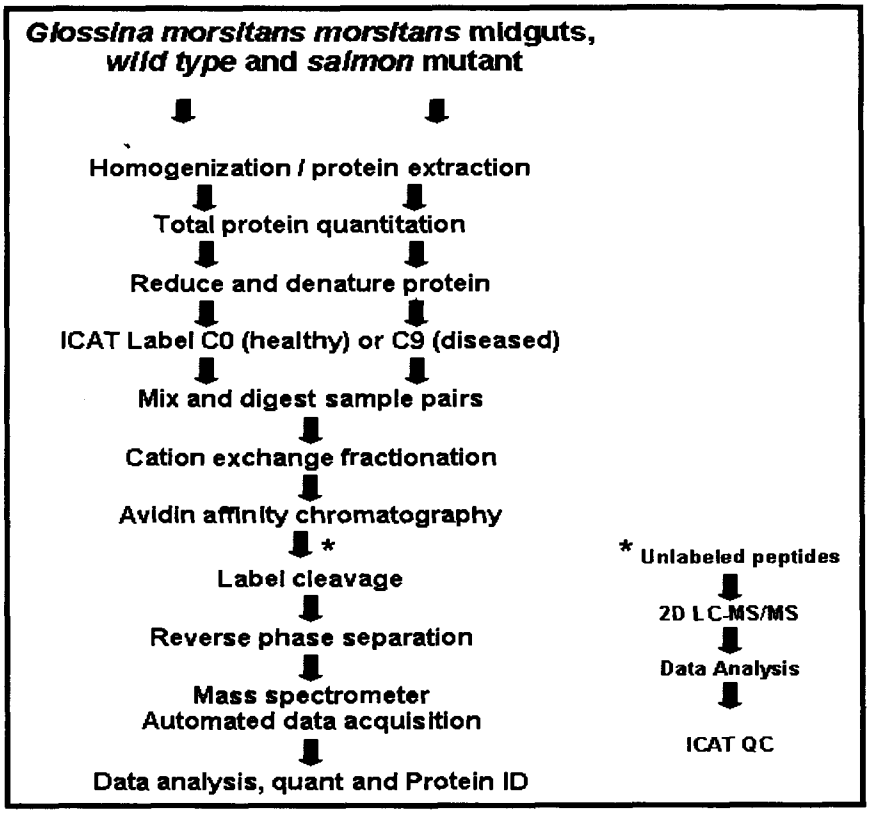

Figure 3.1. ICAT analysis flow chart. Midguts were isolated from wild type and salmon-eyed mutants tsetse flies (*Glossina morsitans morsitans*). Each tissue sample was homogenized and proteins solubilized, quantified, reduced, denatured and ICAT labeled separately. Mutant tissues were derivatized with the ^{13}C-heavy isotope ICAT label (C9) while wild type tissues were derivatized with the light ICAT label (C0). The mutants and wt preparations were then combined and digested with trypsin. The resulting peptides were separated by cation exchange chromatography (SCX), generating an addition ten fraction. Each SCX fraction was Avidin purified to separate cysteine (Avidin eluate) and non-cysteine containing peptides (Avidin wash). Each fraction (Avidin eluate and Avidin wash) was the subjected to LC-MS/MS analysis. Cys-containing, Avidin eluate MS/MS data was processed with ProICAT™ software (Applied Biosystems) for identification and quantitation of light and heavy ICAT peptide pairs. Non-Cys-containing LC MS/MS data was processed with proID™ for identification of avidin flow-through peptides (Applied Biosystems).

(PolyLC, Columbia MD) by means of serial injection via a 1000 µL sample loop. Peptide elution was facilitated using the following gradient: 0-20 % B in 5 min, 20-80 % B in 60 min, 80-100 % B in 5 min and 100 % B for 5 min. The SCX column was cleaned by injecting 1 mL of 1 M KCl over the column to remove any bound trypsin. All samples were collected in 1.5 mL microfuge tubes (washed first with 100 % methanol) at a rate of 0.5 mL/min and frozen at -80 °C for further clean up by avidin affinity chromatography.

2.21 Avidin affinity chromatography.

Each SCX fraction was passed over an avidin affinity column according to the $C_0/_{C9}$ cleavable ICAT protocol (Applied Biosystems, Framingham). The ICAT labeled peptides were then acid cleaved according to the ABI published protocol, dried in a rotary Speed Vac concentrator and stored at -20 °C for further analysis by mass spectrometry.

2.22. Mass spectrometry analysis by nano LC-MS/MS.

All MS analysis on the samples was performed on a MDS SCIEX API QSTAR Pulsar in positive ion mode (Applied Biosystems MDS SCIEX, Concord, Ontario, Canada). Liquid chromatography was performed with an LC Packings Famos autosampler, Switchos, and Ultimate dual gradient solvent delivery system (LC Packings, San Francisco, CA). The Switchos was fitted with a 5 mm x 300 µm 100 Å PepMap C18 µ-precolumn trapping cartridge and a 75 µ x 15cm 100Å PepMap C18 separation column (LC Packings, San Francisco, CA). All samples were dissolved in 5 % ACN, 3 % formic acid solution. A flow

rate of 150 nL/min was used for all experiments. Ions were introduced into the mass spectrometer using a 10 μ New Objective silica tip PicoTip™ emitter (New Objective, Woburn, MA) coupled to a Protana nanospray source (Proxeon, Denmark). The independent data acquisition (IDA) method used for data acquisition employed a keratin/trypsin exclusion list in order to minimize potentially contaminating peptides.

2.23. Data Analysis

Data acquired from the ICAT IDA experiments were analyzed by ProICAT software version 1.0 SP3 (Applied Biosystems MDS SCIEX, Concord, Ontario, Canada) while data acquired from the LC-MS/MS experiments using the 2-D gel spots were analyzed by ProID software version 1.0 SP3 (Applied Biosystems MDS SCIEX, Concord, Ontario, Canada). All peptide sequences were analyzed manually for confirmation of automated IDs (see also Materials and Methods Chapter Two, *de novo* sequencing of tryptic peptides). Spotfire DecisionSite™ for Functional Genomics (Summerville, MA) was used for ICAT expression data analysis. The software uses high-impact visualization to examine the expression and annotation dimensions of the ICAT data and performs a wide array of numerical analyses allowing identification and categorization of important protein expression patterns.

3. Results

3.1. Proteomic analysis of major tsetse midgut proteins.

Solubilisation of whole midgut and separation of the midgut proteome by 1-D gel electrophoresis resolved proteins of widely divergent apparent molecular masses (Figure 3.1, lane 1). Major proteins of 26, 28, 32-34, 42, 48, 52, 58-60 and 78 kDa were the most intensely Coomassie blue stained species. High-resolution 2-D gel electrophoretic analysis of midgut proteins (Figure 3.2) also revealed major protein spots of 26, 28, 32-34 and 58-60 kDa. In addition, relatively abundant lower molecular mass spots of 15 and 18 kDa that were observed in the 2-D gel were not resolved in the non-gradient 1-D gel. The major 42 and 48 kDa proteins observed as major bands in the 1-D gel were not present on the 2-D gel because 1-D gel bands were comprised of multiple proteins with the same approximate molecular mass but with different isoelectric points. Additionally the 1-D gel bands may contain proteins that are not separable by conventional isoelectric focusing. The most abundant proteins on the 2-D gel were a set of slightly acidic spots at 60 kDa (Figure 3.2). Five peptides with matching sequences, equivalent to 8% coverage of the protein, identified a GroEL-like chaperonin of the weevil, *Sitophilus oryzae*, (NCBI accession number gi|7443844) that and again as a SymL protein (symbionin) from the aphid *Myzus persicae* (NCBI accession number gi|2754808). These insect-derived sequences were expressed by the endosymbiotic bacteria *S. oryzae* principal endocytobiote (SOPE) and *Buchnera aphidicola,* respectively and suggested that the symbionts of tsetse could be the source of the 60 kDa tsetse midgut protein.

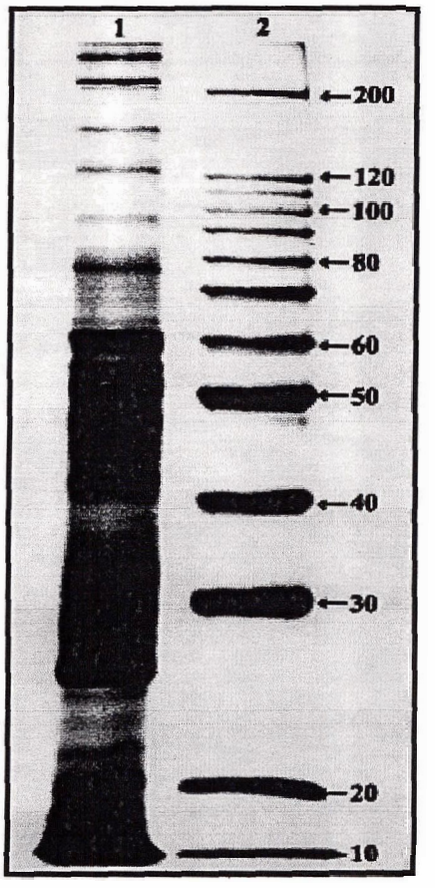

Figure 3.2. SDS-PAGE analysis of whole midgut from teneral *Glossina morsitans morsitans*. A single midgut was solubilized in 40 µL of Laemmli buffer and 20 µL of this mixture was separated in a 10% gel. Proteins were stained using colloidal Coomassie Brilliant Blue G-250. Lane 1, midgut lysate (1/2 gut total); Lane 2, molecular weight standards (10kDa ladder). (previously published in Haines et al. 2002).

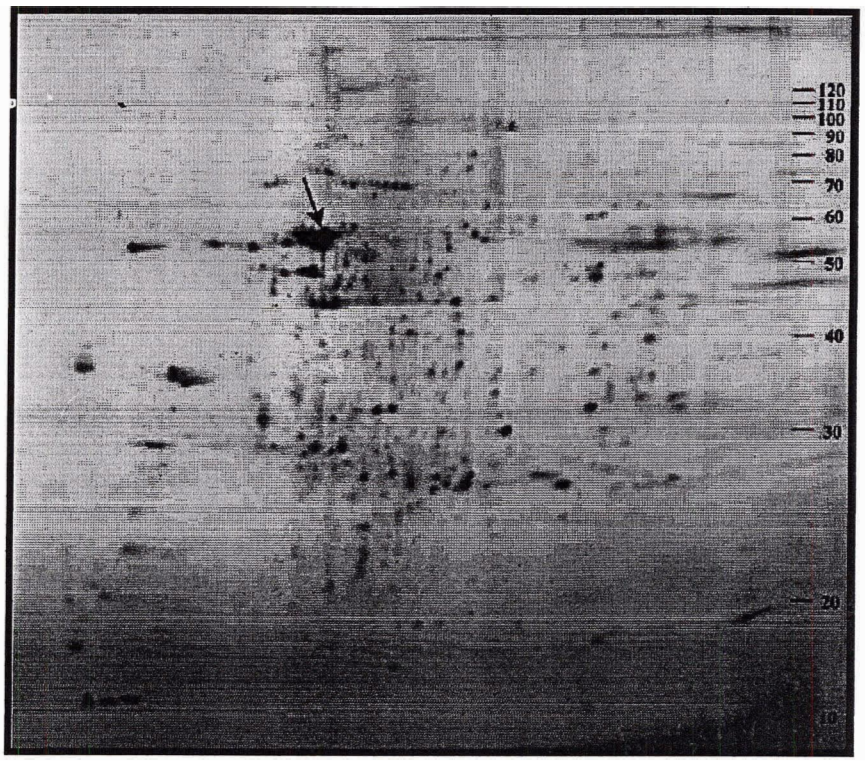

Figure 3.3. 2-D gel analysis of whole midgut from teneral *G. m. morsitans*. Midgut proteins were stained using colloidal Coomassie Brilliant Blue G-250. The arrow indicates the major 60 kDa protein, GroEL, identified by mass spectrometry. Wide range ampholytes, pH 3-10, were used in the first dimension IEF and a 10-16.5% gradient SDS-PAGE gel in the second dimension. The gel is shown with the basic end to the right. (previously published in Haines et al. 2002).

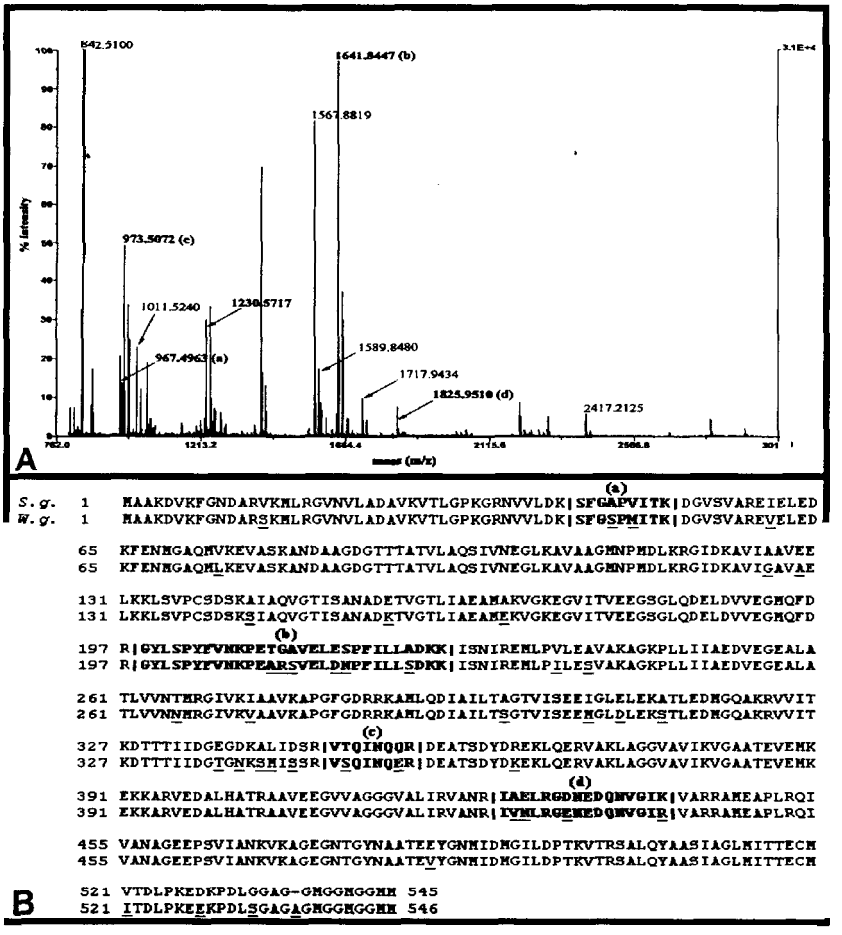

Figure 3.4. Peptide mass spectrum of the trypsin digested 60 kDa protein from teneral *G. m. morsitans* midgut with correlation to the masses of the amino acid sequences of GroEL for *S. glossinidius* and *W. glossinidia.* Panel A: MALDI-ToF MS peptide mass fingerprint (spectrum) with 30% coverage of the total protein sequence. Peptides labelled a,b,c and d, are unique to *W. glossinidia.* Panel B: Comparison of amino acid sequences of GroEL from *S. glossinidius* and *W. glossinidia.* Amino acid differences are underlined and unique *W. glossinidia* peptides observed in the MALDI-TOF spectrum are shown in bold (a, b, c and d) and correspond to the peaks designated in the spectrum in panel A. (previously published in Haines et al 2002)

To determine the exact origin of the 60 kDa GroEL-like midgut chaperonin, peptide mass fingerprinting was performed and the results compared to predicted tryptic peptide sequences of the 60 kDa chaperonin sequences of both *S. glossinidius* and *W. glossinidia.* The results are shown in Figure 3.3. Twenty-three of the 73 peptide masses used to search the NCBI nr.7.1.2001 database identified bacterial GroEL with 30% coverage (Mascot; Matrix Science, London, UK). Four of the peptide masses were unique to the *W. glossinidia* peptide fingerprint (designated a, b, c, and d in Figure 3.3), unequivocally determining that the GroEL-like molecule was from this endosymbiont.

3.2. Mouse immunization, cell fusion and monoclonal antibody screening

Approximately 2500 hybridoma clones were obtained in each experiment (both midgut and salivary gland fusions). ELISA was used as a tool for immune testing of immunized mice and for screening of supernatants from hybridoma clones. An ELISA using mouse immune sera (see Figure 3.4) determined that the animals had generated a strong immune response and were ready for lymphocyte harvest. A total of 960 hybridomas from the midgut fusion were picked into sterile 96-well tissue culture plates and supernatants were screened by ELISA on the immunizing antigen. After rounds of screening one clone, 4A2 was selected for further study. Summary of the anti-midgut monoclonal antibody production results can be found in appendix III. Examples of hybridoma 4A2 anti-midgut monoclonal antibody 1-D and 2-D gel Western blot activity can be seen in Figures 3.10 and 3.6 respectively.

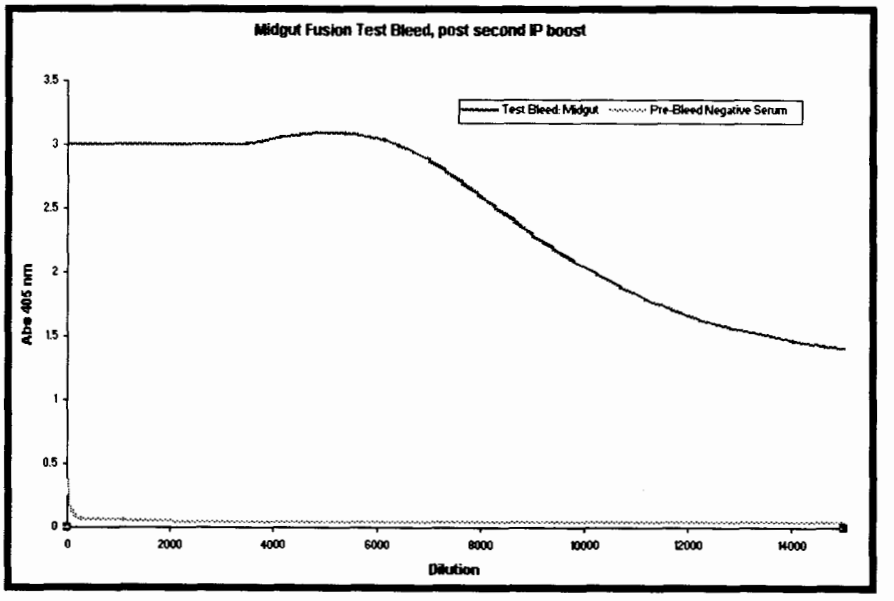

Figure 3.5. Indirect ELISA titration of test bleed serum from a midgut immunized mouse. Mouse serum was titrated against whole midgut from teneral *G. m. morsitans*. Whole midguts were sonicated in water and 1/8th midgut was dried down in each well. Serial 2-fold dilutions of serum were performed. Antibodies were detected with a goat anti-mouse second antibody coupled to alkaline phosphatase and visualized using PNPP as substrate. Absorbance was read at 405nm.

3.3. Immunofluorescence on tsetse midgut tissue sections.

Although all midgut clones were screened by immunofluorescence on thick sections of midgut tissue, only one clone was specific to a structure in the midgut. A subsequent fusion produced additional hybridomas that were also specific to this structure and all of these clones reacted with a molecule of similar molecular mass (18-23 kDa) on immunoblotted 1-D SDS-
PAGE gels. Figure 3.5 shows the immunofluorescence of anti-midgut monoclonal antibody 4A2 on thick sections of tsetse midgut tissue.

3.4. Midgut antigen identification by 2-D gel electrophoresis and mass spectrometry.

To identify the molecule(s) recognized by the anti-midgut monoclonal antibody 4A2 (see Figure 3.5) within the complex mixture of proteins, whole midgut homogenate proteins (same sample used in the BALB/c immunization procedure) were separated by 2-D gel electrophoresis, an immunoblotted with mAb 4A2. The staining pattern and position on the developed photographic film matched exactly to an acidic low molecular weight and poorly resolving protein species on the corresponding CCB stained gel (see Figure 3.6). This spot was then excised and identified by tandem mass spectrometry (MS/MS) as tsetse *Glossina morsitans morsitans* Pro2 (Proventriculin 2). Pro2 is produced in the proventriculus of the tsetse, a specialized structure at the anterior base of the midgut (see Figure 3.7).

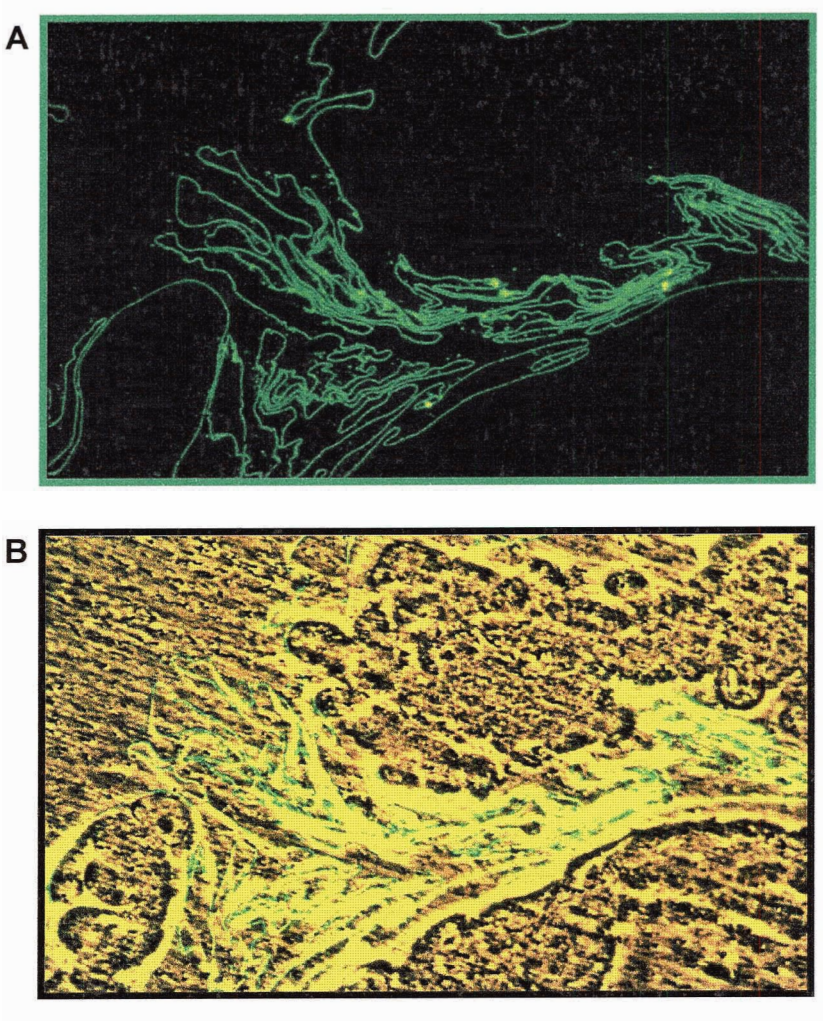

Figure 3.6. Immunofluorescence on methacrylate embedded tsetse midgut tissue using an anti-Pro2 (4A2) mouse monoclonal antibody. Tissues were cut at 0.6 mm and dried onto treated glass slides. Second antibody was Alexafluor-488 labeled goat-anti-mouse FITC. Tissues were observed at 630 X magnification. Panel A, Pro2 protein of the peritrophic matrix is stained with antibody and visualized by excitation with UV at 440 nm. Panel B, a phase contrast image to highlight the tissue being stained in Panel A. Embedded-section screening and imaging performed with Angela Jackson.

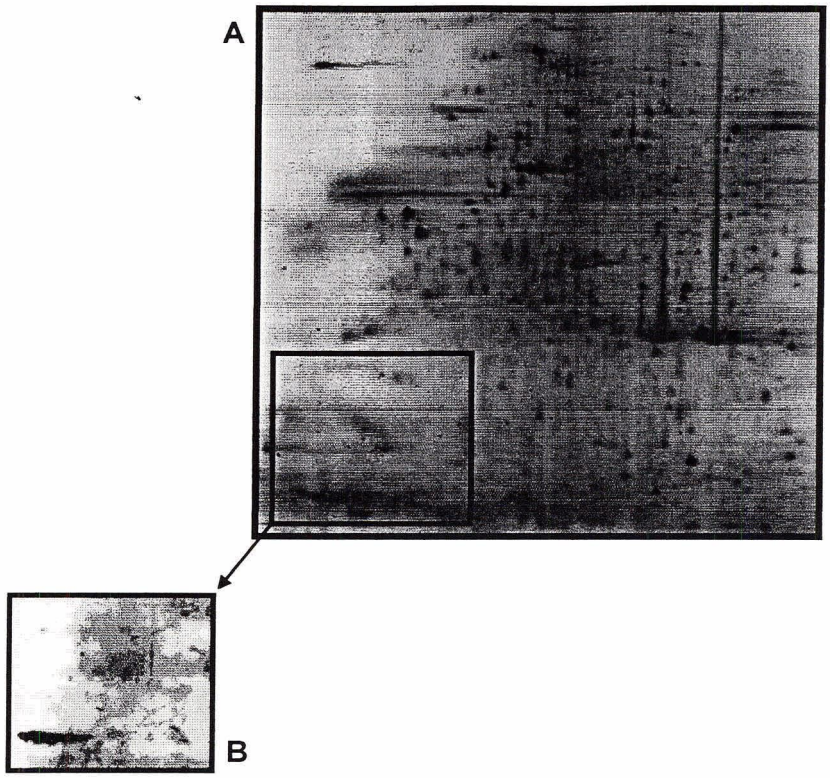

Figure 3.7. Identification of the specific antigen recognized by mAb 4A2. Five midguts per gel were separated by the Iso-Dalt 2-D gel electrophoresis system using wide range ampholytes (pH 3-10). Two gels were run separately, one gel was used for Colloidal Coomassie Blue (CCB) staining and the other used to transfer the proteins onto BioTrace ™ polyvinylidene (PVDF) membrane (Pall Corporation, Ann Arbor, MI) for immunoblotting as previously described (Beecroft et al., 1993). Panel A. Colloidal CBB G 250 stained gel, Panel B, A scale 1:1 section of PVDF immunoblot of the gel in Panel A indicating the band stained by the 4A2 mAb. This band was excised from the stained gel and subjected to tryptic digestion and MS/MS analysis. The protein was identified as tsetse *Glossina m. morsitans* Proventriculin-2 (*Gm*Pro2).

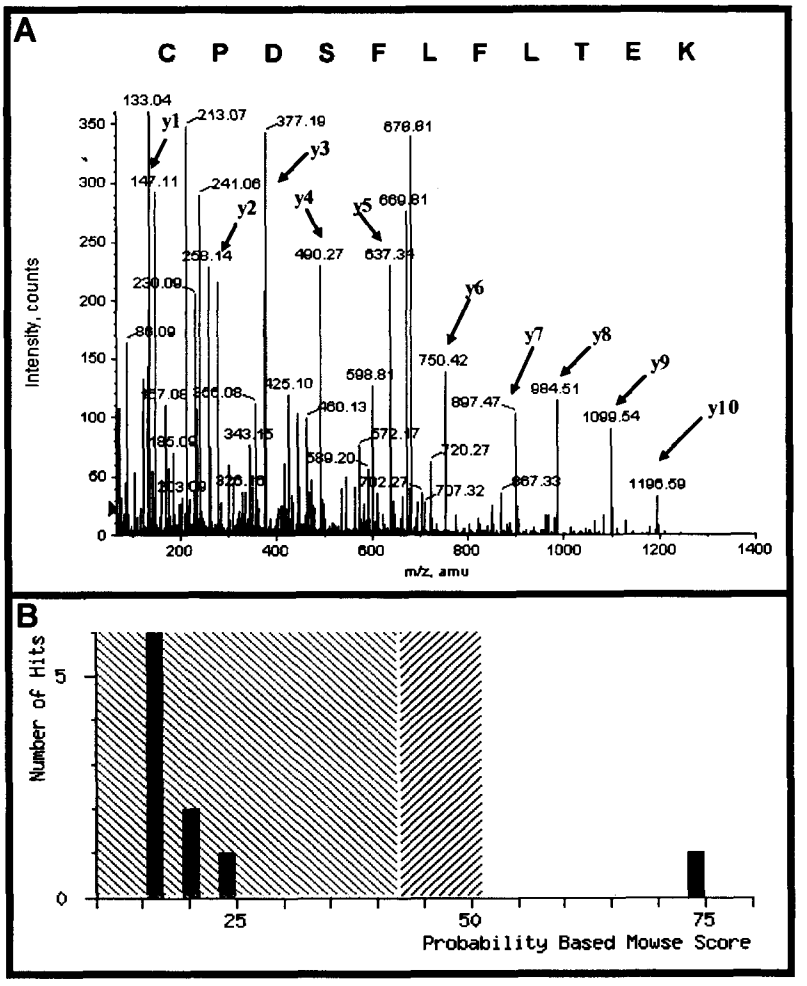

Figure 3.8. Identification of the specific antigen recognized by mAb 4A2 using tandem mass spectrometry. Panel A, the product ion spectrum of a 678.81doubly charged ion from a tryptic peptide of the 4A2 protein. The primary amino acid sequence is indicated above the y-ion fragment series indicated with arrows. Panel B, fragment ion masses were searched against the NCBI non-redundant database using the Mascot search algorithm. The ions score (-10*Log(P) is based on the probability that the observed match is a random event. In this search, individual ions scores > 42 indicate peptides with significant homology and individual ions scores > 51 indicate identity or extensive homology ($p<0.05$). The peptide fragmentation (sequence) and database search identified tsetse *Gm*Pro2.

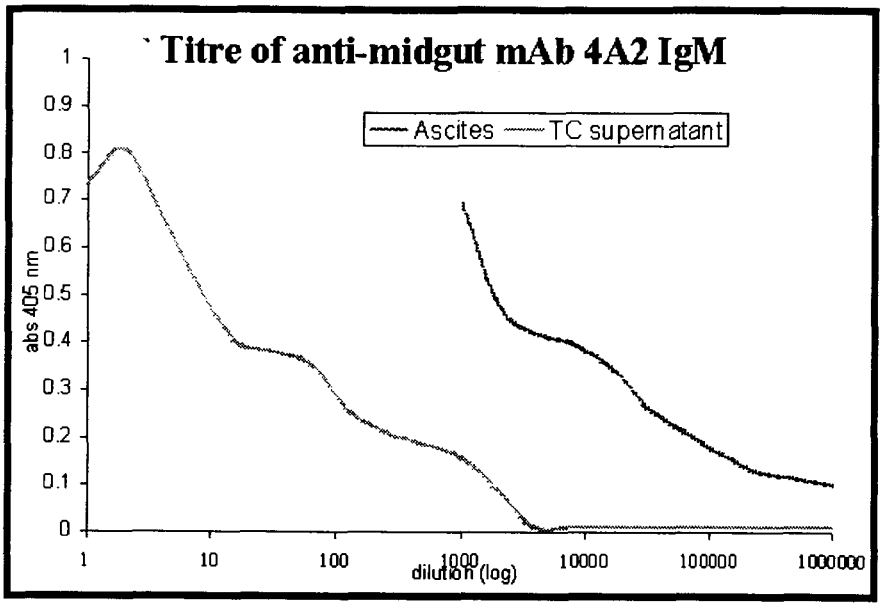

Figure 3.9. Indirect ELISA titration of 4A2 ascites fluid and monoclonal antibody tissue culture supernatant. Both fluids were titrated against whole midgut from teneral *G. m. morsitans*. Whole midgut was sonicated in water and 1/8th midgut was dried down in each well. Serial 2-fold dilutions of ascites and tissue culture supernatants were titrated. Antibodies were detected with a goat anti-mouse second antibody coupled to alkaline phosphatase and visualized using PNPP as substrate. Absorbences were read at 405nm

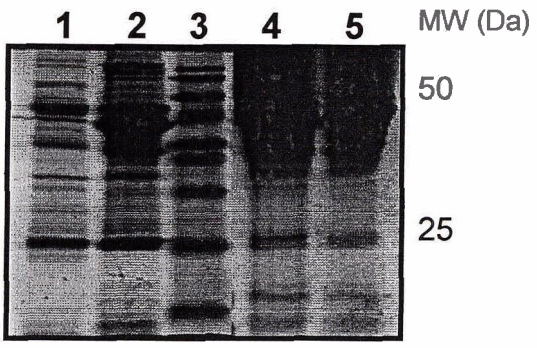

Figure 3.10. SDS-PAGE purity test of anti-Pro2 mAb pre- and post-ascites production and polyethylene glycol enrichment. SDS-PAGE 12.5% separating 4% stacking gel, 10 mA stacking potential, 20 mA separation, gel was stained with CCB. Lane 1, 4A2 PEG pellet, 0.25 uL. Lane 2, 4A2 ascites, 0.5 uL. Lane 3, 10kDa MW ladder. Lane 4, PEG supernatant "1", 10 uL. Lane 5, PEG supernatant "2", 5 uL. Experiment with assistance from Angela Jackson.

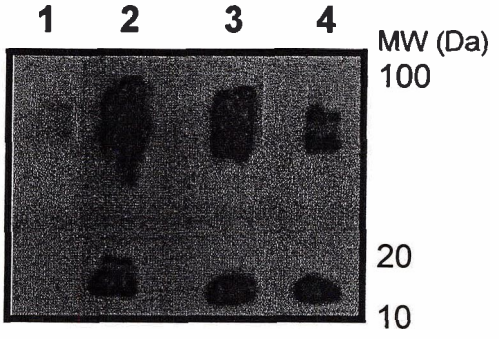

Figure 3.11. Immunoblot activity of anti-Pro2 mAb pre- and post ascites production and polyethylene glycol enrichment (10 second exposure). One gut was solubilized in 80 µL Laemmli buffer and 20 µL were loaded into each of four lanes. Lane 1, negative control mAb CP11 (anti simiae mouse IgM) 1:3 dilution. Lane 2 Tissue culture supernatant mAb 4A2 1:3 dilution. Lane 3, PEG enriched anti-Pro2 mAb, 1:5000 dilution. Lane 4, anti-Pro2 ascites pre-PEG enrichment 1:5000 dilution. Secondary antibody, goat anti-mouse 1gG/IgM, was diluted 1:1000 (Caltag Laboratories, Ont, Can). SDS-PAGE was 12.5% separating 4% stacking gel, 10 mA stacking potential 20 mA separation. Experiment with assistance from Angela Jackson.

3.5. Purification of mAb from murine ascites fluid.

Ascites fluid from the 4A2 hybridoma were produced commercially. The ascites fluid was titrated by ELISA to determine the activity of specific antibody and a titre of greater than 1/10, 000 was observed. This fluid was subjected to a gentle PEG precipitation to further enrich the antibody. The enrichment of mAb 4A2 was monitored with SDS-PAGE (Figure 3.9). Lane 1 contains the PEG enriched mAb 4A2. The abundant contaminating proteins in the 40 to 50 kDa region have been depleted leaving immunoglobulin chains at 25 kDa and 50 kDa. Lane 2 contains 4A2 ascites fluid prior to PEG enrichment revealing the significant non-immunoglobulin content in the ascites fluid. Lane 3 contains a 10 kDa MW ladder. Lane 4 contains the first supernatant of the PEG enrichment protocol. Although some heavy chain and light chain appear to be present, a great deal of contaminating protein appears to have been left in the supernatant. Lane 5 contains the supernatant from the second stage PEG precipitation of the experiment and again contains a large amount of contaminant proteins.

After purification and desalting, the activity of the mAb 4A2 was assessed using Western blotting on midgut tissue. The immunoblot activity of the anti-Pro2 mAb is shown in Figure 3.10. The PEG enriched mAb was clearly still active at a 1/5000 dilution and was thus suitable for further experiments in tsetse.

3.6. Tsetse and trypanosome culture and tsetse infection assay

In the initial trial, tsetse that were fed the test anti-peritrophin mAb 4A2 demonstrated a 0.0 % trypanosome infection rate. In contrast the tsetse that did not receive the antibody

Trial A	n	Parasite	MG+ve	MG-ve	Total Mortality	% Mortality	Final % Infection
Control - PBS	50	YTat 1.1	14	24	12	24.0	36.8
Test - 4A2	150	YTat 1.1	0	111	39	26.0	0.0

Trial B	n	Parasite	MG+ve	MG-ve	Total Mortality	% Mortality	Final % Infection
Control - PBS	60	YTat 1.1	15	30	15	20.0	25.0
Control - mAb	50	Rump 503	4	35	11	22.0	8.0
Test - 4A2	80	YTat 1.1	24	42	9	20.0	30.0

Table 3.1. Summary of results, transmission blocking assay (membrane feeding). In the first trial *Glossina m. morsitans* were divided into a test group with n=150 and a control group with n= 50 flies. The control group were fed trypanosome inoculated sheep's blood containing 2% N-acetylglucosamine and mock antibody (PBS). The test group received trypanosome inoculated sheep's blood containing 2% N-acetylglucosamine and 30 μg/mL anti-Pro2 mouse monoclonal antibody. Percent mortality and infection were recorded for the first experiment only. Tsetse midguts were dissected after 14 days and scored for presence of parasites in the tissue.

The second trial was carried out with $3x10^6$ of *T. b. brucei* ytat 1.1 parasites in the mAb 4A2 test group while the negative control mAb group was fed and equivalent number of *T. b. brucei* RUMP 503. The control PBS group was treated as outlined in Trial 1.

experienced a 28 % midgut infection rate. To perform the infection / parasitemia assay (membrane feeding) *Glossina m. morsitans* were divided into a test group with n=150 and a control group with n= 50 flies. The test group was fed trypanosome-inoculated sheep's blood containing 2 % N-acetylglucosamine. and mock antibody (PBS). The test group received trypanosome inoculated sheep's blood containing 2% N-acetylglucosamine and 30 μg/mL anti-Pro2 mouse monoclonal antibody. Percent mortality and infection were recorded. Trypanosome midguts were dissected after 14 days and scored for presence of parasites in the tissue. Mortalities between the two groups were similar at 26% in the test group and 24% in the control group. The results of a second trial did not resemble the first. In the second experiment the test mAb, 4A2 did not appear to have an effect and a 36.4% infection was observed. In this experiment two controls were employed, the first, a PBS mock mAb produced a 33% infection and the second control, an anti-*Kudoa* thyrsites mAb (4H2, IgM), produced a 10.3% infection.

3.7. ICAT differential expression analysis of tsetse salmon mutant midgut tissue.

A flow chart depicting the ICAT analysis protocol is outlined in Figure 3.11. Mutant tissues were derivatized with the ^{13}C-heavy isotope ICAT label (C9) while wild type tissues were derivatized with the light ICAT label (C0) containing no ^{13}C. The ICAT labeled peptides were separated by cation exchange chromatography (SCX) (see Figure 3.12), generating an additional ten fractions. The avidin purified SCX fractions were then subjected to LC-MS/MS analysis. Cys-containing, avidin eluate MS/MS data were processed with ProICAT software for identification and quantitation of light and heavy ICAT peptide pairs.

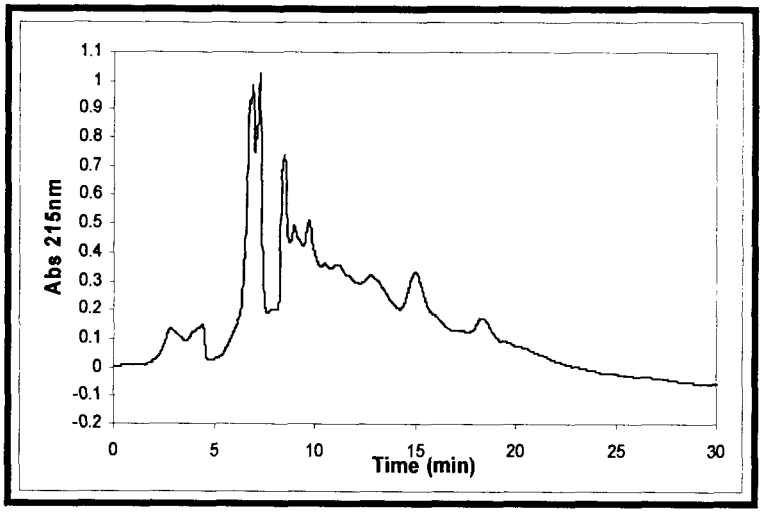

Figure 3.12. Strong cation exchange chromatographic fractionation of a labeled peptide mixture. Peptides (200 μg) were applied to a Beckman System Gold μ-flow HPLC fitted with a 4.6 x 100 mm Polysulfoethyl A column (PolyLC Columbia MD). Buffer A consisted of 5 mM potassium dihydrogen phosphate, pH 3.0 in 25% ACN, while buffer B consisted of 5 mM potassium dihydrogen phosphate, 350mM KCl, pH 3.0 in 25% ACN. The column was developed with the following gradient: 0-20% B in 5 min, 20-80% B in 60 min, 80-100% B in 5 min and 100% B for 5min.

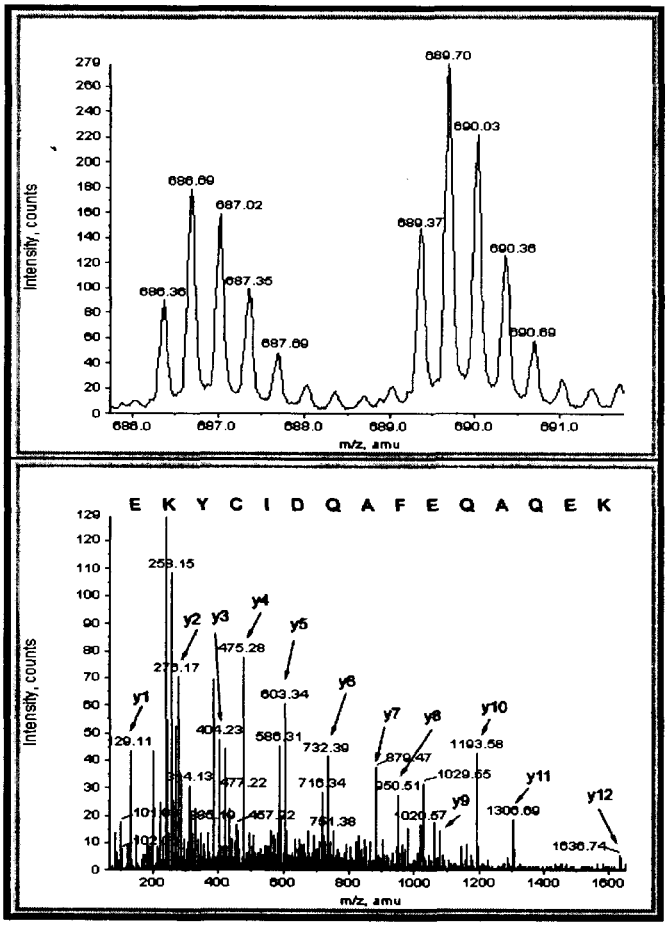

Figure 3.13. MS quantitation and identification evidence for tsetse EP repeat protein. Panel A, peptides of the same species differing by only a light- and heavy-ICAT reagent label of 9 amu, observed as a 4.5 amu. The peak heights differ by 1.56-fold indicating an upregulation in the mutant tsetse midgut. Panel B shows the CID fragmentation pattern of the above peptide providing a staggering array of fragment ions revealing the primary sequence of the parent peptide.

Peptides	#
total unique	226
confidence 90 - 100	103
confidence 75 - 89	36
confidence 74 - 50	87
Proteins	#
total unique	208
H:L > 1.33	19
H:L < 0.66	7
no quantification	63

Table 3.2. Overall number of peptides/proteins identified using ICAT technology. The number of identified proteins is indicated according to either their up- or down-regulation and sorted by the indicated threshold confidence intervals. Two different databases were searched, the NCBI non-redundant public database and the Celera Discovery Systems combined annotated database.

accession	name	species	P	C	S	H:L
trm|Q9NBA4	Tsal2 protein precursor	Glossina m. morsitans	2	92	20	3.20
gi|15239799	disease resistance protein	Arabidopsis thaliana	1	99	23	3.05
trm|Q9U7C6	Salivary gland growth factor-1	Glossina m. morsitans	1	99	26	2.47
gi|8927462	antigen 5 precursor	Glossina m. morsitans	1	93	16	2.43
gi|13399647	Dihydropyrimidine Dehydrogenase		1	98	25	2.37
trm|O45042	Putative protective antigen	Onchocerca volvulus	1	58	15	2.07
gi|15864611	tsetseEP protein	Glossina m. morsitans	6	99	18	1.93
gi|15242492	glycosyl hydrolase family 3	Arabidopsis thaliana	1	61	14	1.92
gi|8927464	Tsal1 protein precursor	Glossina m. morsitans	1	99	18	1.82
gi|24266649	Pro2	Glossina m. morsitans	2	99	23	1.63
trm|Q9NBC9	Trypsin-like serine protease	Glossina m. morsitans	2	99	21	1.49
emb|CAA31512	neurone-specific enolase	Homo sapiens	1	65	12	1.37
emb|CAA42754	myosin heavy chain	Drosophila melanogaster	1	99	23	1.33

accession	name	species	P	C	S	H:L
gi|22978624	hypothetical protein	Ralstonia metallidurans	1	62	15	0.66
gi|10177486	heat shock transcription factor	Arabidopsis thaliana	1	86	17	0.47
emb|CAA9770	DIP2	S.cerevisiae	1	73	15	0.43
dbj|BAB21073	P0501G01.2	Oryza sativa	1	55	10	0.23

accession	name	species	P	C	S	H:L
gi|14193259	ATP synthase beta subunit	C. Carsonella ruddii	1	81	16	1.11
emb|CAA7297	elongation factor Ef-Tu	Buchnera aphidicola	1	94	16	1.07
pir|S24650	Na+/K+-exchanging ATPase	Bufo marinus	2	98	15	1.06
emb|CAA42019	ribosomal protein S27	Rattus rattus	1	84	15	1.04
trm|Q9ZIV3	70 kDa heat shock protein	F. ferrugineum	1	57	20	1.02
prf|2020435A	Arg kinase	Procambarus clarkii	1	99	17	1.01
gi|11386885	Hexokinase 1	Nicotiana tabacum	1	81	18	1.01
rf|XP_143986.2	60S RIBOSOMAL PROTEIN L9	Mus musculus	1	76	17	0.98
pir|S29130	calreticulin (clone 8)	Xenopus laevis	1	93	14	0.97
dbj|BAA07160	beta-tublin	Haliotis discus	1	98	19	0.92
dbj|BAB62621	40S ribosomal protein S5	Oryza sativa	2	99	21	0.91
gi|11931708	cold shock protein	Ascovirus DpAV4	1	99	13	0.91
trm|Q962R1	Ribosomal protein S18	Spodoptera frugiperda	2	97	24	0.91

Table 3.3. Differentially expressed proteins are listed as a sample of the data generated in this study. Column P, the number of peptides identified for the given protein. Column C, the confidence of the database hit for the best representative peptide. Column S, the probability score for the matched sequence. Column H:L, the ratio of heavy to light peptides found for the identified protein. Ratios above one are upregulated in the diseased tissue, ratios less than one are down regulated in the diseased tissue. The H:L ratios indicated are for the best scoring peptide and may very from spectra in other Figures in this thesis (ie. when example ICAT peptide ion pair spectra are shown).

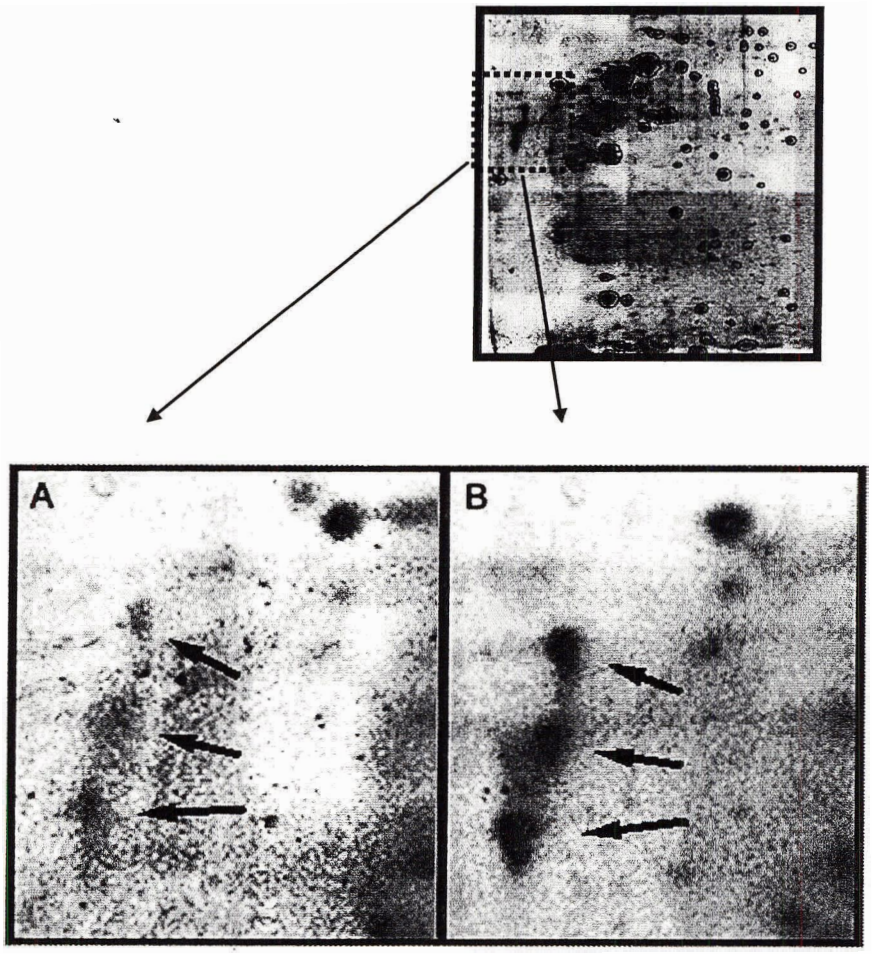

Figure 3.14. EP-repeat protein was identified by ICAT analysis its presence and regulation confirmed by 2D gel electrophoresis. Shown is a close-up of the region of the gel in which the EP-repeat protein resolves. Panel A shows EP-protein expression in wild type tissue while Panel B shows EP-protein expression in the midgut of the more trypanosome susceptible SE mutant fly.

Non-Cys-containing LC MS/MS data were processed with proID for identification of avidin flow-through peptides (Applied Biosystems). Quantitation and identification evidence for tsetse EP repeat protein is shown in Figure 3.13. Panel A shows peptides of the same species differing by only a light- and heavy-ICAT reagent label that are separated by 9 amu, this is observed as a 4.5 amu change in the spectra because of the peptide 2+ charge state. The peak heights differ by 1.56 fold indicating an up regulation in the salmon mutant. Panel B shows the fragmentation pattern of the peptide comprising this ICAT pair. Because peptides are preferentially broken along the amide of the peptide backbone, the fragment ions differ by the masses of specific amino acid residues indicated as the primary amino acid sequence given in Panel B as well as the y-ion fragment series in Figure 3.13. Identification of the series of fragments elucidates the primary sequence of the parent peptide. The overall number of peptides/proteins identified using ICAT technology is presented in Table 3.2. The number of identified proteins is indicated according to either their up- or down-regulation and sorted by threshold confidence levels of 50 and 90%. Two different databases were searched, the NCBI non-redundant public databases and the private Celera Discovery System database. The latter adds molecular function and biological processes parameters to the database entries, helping to simplify final data analysis by the researcher.

A partial list of differentially expressed proteins is presented in Table 3.3 and represent a sample of the data generated in this type of study. *Glossina m. morsitans* proteins identified and found to be significantly regulated include a tsetse serine protease, tsetse EP protein, Tsal2, Tsal1, Pro2 and tsetse salivary gland growth factor (see Table 3.3).

3.8. Differential expression analysis of salmon mutant midgut tissue by 2-D gel electrophoresis

Tsetse EP repeat protein was first identified by ICAT analysis and its presence and regulation was confirmed by 2-D gel electrophoresis (see Figures 3.13, 3.14 and Table 3.3). Shown in Figure 3.14 is a close-up of the region of the 2-D gel in which the EP repeat protein resolves. Panel A shows EP protein expression in wild type tissue while Panel B shows EP protein expression in the midgut of the tsetse salmon mutant. Progenesis software (Non-Linear Dynamics, Newcastle UK) determined the spot height, area and volume and used these values to indicate an approximate 2-fold up regulation for the best scoring peptide pair in the tsetse mutant midgut.

4. Discussion

4.1. Identification of major midgut proteins

After initial analysis the 2-D gels clearly revealed a 60 kDa molecule as the most abundant gel separable protein in tsetse midgut. Tandem mass spectrometric analysis and database searching using peptide fragmentation data obtained after in-gel tryptic digestion showed that a number of peptide sequences matched sequences in a GroEL-like protein isolated from the principal endosymbiotic bacterium of the weevil *Sitophilus oryzae* (Heddi *et al.* 1999). Was it possible that the major tsetse soluble midgut protein was related to a chaperone of bacteria? Two Gram-negative symbionts, the primary endosymbiont

Wigglesworthia glossinidia and the secondary symbiont *Sodalis glossinidius*, live within the tsetse midgut. Immunological characterization of these symbionts (isolated from non-teneral flies) showed that abundant proteins synthesized by both bacteria were chaperonins (Aksoy 1995).

All *Glossina* species harbour the obligate endosymbiont, *Wigglesworthia*, within the bacteriome, a specialized midgut organelle (Aksoy 1995; Aksoy *et al.* 1995). These bacteria provide B-complex vitamins essential for tsetse survival (Nogge 1980). The symbiont *Sodalis* is found in a range of host tissues both extracellularly and intracellularly (Aksoy *et al.* 1997; Dale and Maudlin 1999). Analysis of the *Sodalis* genome (Akman *et al.* 2001), suggests a similar role for *Sodalis* in the biosynthesis of cofactors and vitamin metabolites. MALDI-TOF mass spectrometry was used to determine the species of origin of the abundant 60 kDa protein in the tsetse midgut. Initially, the predicted masses of the tryptic peptides generated from an in-silico digest of GroEL sequences from both *Sodalis* and *Wigglesworthia* were determined. Comparison of these two mass lists revealed that there were a number of unique peptide masses that could be used to define the origin of the midgut molecule (see Figure 3.3). The GroEL from *Wigglesworthia glossinidia* was unambiguously identified by the presence of four unique peptides and is the predominant molecule within the midgut of *G. m. morsitans*. This result was supported by the fact that comparison of midgut (bacteriome free) and bacteriome tissue by 2-D gel electrophoresis revealed that only bacteriomes contained the 60 kDa protein (results were also published in Haines *et al* 2002). Bacteriome-free midguts contain *Sodalis*, but do not contain *Wigglesworthia.* Finally, an anti-GroEL monoclonal antibody produced by Lee Haines was used to confirm that the 60

kDa GroEL-like protein was present only in the bacteriome and not in the rest of the midgut, supporting that it originated from *Wigglesworthia* (data not shown).

GroEL (Hsp 60) is essential for cell survival and growth of *E. coli* (Fayet *et al.* 1989). There are a number of situations that could stimulate the overexpression of GroEL in such a system. The tsetse midgut is a very hostile environment and as a stress-induced chaperonin may be necessary for survival in the presence of an array of midgut proteases, significant changes in protein composition and fluctuations in temperature. Chaperones are known to direct protein folding across membranes, which could help facilitate the import of the large number host proteins required for survival. In addition, Haines et al (2002), have proposed that the endosymbiont may secrete the chaperone. GroEL has previously been shown to bind anti-bacterial peptides secreted by insects (Otvos 2000). A secreted GroEL could bind immobilize any anti-bacterial peptides or immunoproteins produced by the tsetse and protect the microbes from the immune system of the insect.

4.2. Monoclonal antibodies against tsetse midgut

Whole midguts were used as immunogen to generate a number of different monoclonal antibodies (mAb). One mAb demonstrated strong, clear and reproducible immunostaining of midgut tissue sections of what appeared to be on the lumenal surface (see Figure 3.5). The antigen was identified as tsetse protein Pro2, a proventriculin or protein of the peritrophic matrix localized to the peritrophic matrix (PM) that lines the tsetse midgut. This was supported by tissue-section immunofluorescence results (see Figure 3.5). Inspired by findings that anti-mosquito midgut antibodies were able to block the development of

Plasmodium falciparum and *P. vivax* in mosquitoes (Lal *et al.* 2001), it was decided to test this antibody in a transmission blocking experiment at Yale University in the laboratory of Dr. Serap Aksoy in the Medical School of Public Health and Epidemiology. The purified monoclonal antibody was taken to Yale University for the membrane feeding experiments. Tsetse that were fed the test anti-peritrophin antibody demonstrated a 0.0 % trypanosome infection rate indicating it had an effect on the establishment of trypanosomes in the midgut. Mortalities between the two groups were similar at 26 % in the test group and 24 % in the control group suggesting that the antibody itself was not causing insect mortality. The results of a second trial did support the findings of the first. In the second experiment the test mAb, 4A2 did not appear to have an effect (see Table 3.1). Unfortunately only the first experiment could be supervised directly. This was not the case for the second experiment. The procedure followed in the repeat experiments were unacceptable and a number of potential mistakes were made including use of unhealthy trypanosomes and in one case a different strain of parasite was used mid-experiment. This antibody will not be used in another similar trial due to the fact that a large number of resources must be used to ensure proper experimental quality control.

Even if antibody effectors could be identified, how could this be utilized for trypanosome control? The endosymbionts of tsetse may be the answer. It has been demonstrated that when bacteria engineered to produce a single chain antibody were introduced into the insect midgut that they could block parasite development (Yoshida *et al.* 2001). Much discussion and preliminary work has paved the way for such a system in tsetse (Cheng and Aksoy 1999; Aksoy 2000; Aksoy *et al.* 2002; Aksoy 2003) and all that has been missing, is the candidate target molecule for interference of tsetse-trypanosome interactions.

4.3. Differential expression analysis of two tsetse mutants

The present report details the first attempt to employ proteomics methods in elucidation of differentially expressed midgut proteins of two tsetse fly strains that exhibit significant differences in susceptibility to trypanosome infections. Figure 3.11 provides a brief description of the ICAT analytical method employed in this study. The major advantages of the method are that functionally important proteins are detected directly rather than inferred from transcript data and relative expression levels are quantitated. As expected, many of the identified molecules were constitutively expressed 'house-keeping' proteins. Table 3.2 provides summary data of the total number of peptides identified and total numbers of differentially expressed proteins identified at specified levels of confidence. Table 3.3 Shows an example of the ICAT data output. Both upregulated and down-regulated hits are presented as well as a number of unregulated, house-keeping proteins showing the ICAT chemistry was working well. (The full data set has been attached in Appendix IV). ProICAT confidence levels arise from a composite algorithm which measures the distances of the scores from the top scoring peptide to a group of random lower scoring peptides, together with the distance to the next possible identification call (personal communication Applied Biosystems, Foster City CA). Peptides where the top uninterpreted sequence scores are numerically close and specify different proteins, suffer reduced confidence levels. Consequently, peptide confidence data with values less than 90 may be significant but require further investigation for validation.

MS and MS/MS spectra were used to validate significant hits from ICAT analysis. Resulting spectra were examined for fragment ions of the y- and b-ion series. Predicted

sequences could be confirmed in most cases by fragment assignment, care was taken to assign unexpected ions in the spectrum due to neutral losses. ICAT results that identified unnamed, hypothetical or other unassigned proteins were investigated further by BLAST analyses of the sequences retrieved from the NCBI or Celera Discovery System combined species databases by ProICAT. ICAT analysis has provided identification of 226 different peptides representing 208 unique proteins with more than 25 species of proteins being differentially expressed in the tsetse salmon eye mutant. Of particular interest were a number of *Glossina m. morsitans* proteins that were found to be differentially expressed. These include a tsetse serine protease, tsetseEP protein, Tsal2 and Tsal1 (with unknown function), Pro2 and tsetse salivary gland growth factor 1 (see Table 3.3).

Tsetse EP-Repeat protein was determined to be expressed approximately 1.56 fold more in the tsetse mutant (this value is based on an average of the 6 peptides found for tsetseEP protein, the most confident peptide ICAT pair indicated an up regulation of 1.93-fold). EP procyclin has been shown to represent the major substrate for a stage-specific, membrane-associated trans-sialidase (Butikofer *et al.* 1997). The function of this molecule in tsetse is not known although the EP-repeat portion is an interesting motif because of the secondary structure imposed upon molecules containing such a repeat. EP repeats are found in a very diverse range of organisms in proteins that display a diverse range of activities (see Appendix V). No obvious roles have been suggested for the EP dipeptide. An EP repeat characteristically forms a polyproline type II helix structure (extended, rigid and linear) (Alaoui-Ismaili and Richardson 1996). The trypanosome itself also contains a famous GPI-anchored membrane protein, procyclin, that utilizes the EP repeat. The function is unclear although it has been postulated that the extended structures are used to hang functional

moieties, such as the distal N-linked carbohydrate of procyclin, away from the surface of the trypanosome (Pearson 2001). Although the function of the tsetse EP protein is not known it is clear from ICAT and 2D-gel analysis that this protein is upregulated in the more susceptible mutant strain of tsetse indicating the tsetse EP protein may influence, directly or indirectly, trypanosome maturation in the tsetse midgut. In view of the fact that the EP containing procyclins of the trypanosome have recently been shown to be quantitatively cleaved at the distal end of the protein by proteases present in the tsetse midgut (Acosta-Serrano *et al.* 2001). It is also interesting that a tsetse trypsin-like serine protease was also shown to be upregulated in the SE mutant midgut (see Table 3.3). What is yet to be determined is whether the protease is produced by the insect host, an endosymbiont or a trypanosome. Unpublished research has shown that the trypanocidal lectin present in the fly gut may bind procyclin (Welburn, unpublished), at the glycosylation site present at the distal (N-terminus) of the mature polypeptide. It is tempting to speculate that the parasites may cleave their own surface coat and that the cleavage product may be a diversionary tactic to protect the parasite from lethal effects of the lectin.

In addition to the EP protein a tsetse protein, referred to as Pro2, was also identified and found to be upregulated in the SE mutant. Pro2 is a protein found in the chitinous membrane of the tsetse midgut peritrophic matrix (PM). The significance of the latter structure in digestion and as barrier for pathogen transmission make it a very strong candidate to be explored for targets in an effort to control vector insects. Recently, monoclonal antibodies directed against the midgut components of *Anopheles gambiae* have been found to reduce plasmodium transmission (Lal *et al.*, 2001). Pro2 is a member of the peritrophin-15 family and is likely synthesized and secreted in the tsetse proventriculus, a

specialized structure at the base of the midgut that produces the chitinous lining of the tsetse midgut. The molecule contains a conserved chitin binding motif and contains no N-linked or predictable O-linked glycosylation sites (Hao and Aksoy 2002).

Four other *G. m. morsitans* proteins have been found to be regulated, salivary gland growth factor 1 (TSGF-1), tsetse antigen 5 (TAg5) and salivary proteins 1 and 2 (Tsal1 and Tsal2). The latter have unknown function and show little homology with any other proteins in the heterologous database. Elucidation of the role of Tsal2 could be a worthwhile undertaking as it is upregulated up to 3 fold in the tsetse mutant. Tsal1, Tsal2 and TAg5 mRNA transcripts have been shown to be expressed in multiple developmental stages while TSGF-1 is expressed only in adult tissues. Although these molecules were first described in salivary glands their expression has been shown in other tissues including the midgut (Hao and Aksoy 2002). The relationship between trypanosome susceptibility and differential Tsal1 and Tsal2 gene expression in different species of tsetse has previously been examined. It was determined that Tsal1 is downregulated in refractory species of tsetse. This observation is conversely supported by the present ICAT results in which in a more susceptible strain (the SE mutant) the Tsal1 protein is upregulated compared to the reference species. ICAT technology has provided high confidence, and in some cases validated identifications of potentially important molecules that now warrant further biochemical analyses.

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Appendix I

Summary of the final anti-midgut mAb production results

ID	ELISA	Western blot	Sections	Isotype
4D6	1.836	band at approx 25 kDa	y	M
2H6	0.361	weak band at approx 50 kDa	y	G2b
1B3	2.382	n/a	y	M
2C4	2.445	multiple bands across entire mw range	y	G2b
2EE6	0.464	strong band at 50 kDa	y	G2b
4D2	0.611	band at 25 kDa	n	G1
4F4	0.306	weak band at 50 kDa	n	G1
4H5	0.541	weak band approx 25 kDa	n	G2b
10G10	0.862	two bands approx 25 and 50 kDa	n	M
10H6	0.769	two bands 50 and >100 kDa	y	G
2EE8	1.926	band at approx 25 kDa	y	M/G1
5F2	2.024	band(s) >100 kDa	y	M
4A2	1.567	single band at 25 kDa	y	M

ID = Clone designate,

ELISA (with immunizing midgut antigen) = relative signal strength

Western blot = description of activity.

Fluorescence on midgut thin sections. All fluorescence appeared to stain the PM similar to the activity of the anti-Pro2 mAb, 4A2.

Appendix II

Summary of ICAT differential expression results

H:L ratio reported for most confident scoring peptide, C = Confidence, S = Score, H:L = Expression Ratio (Test : Control)

accession_id	name	species	peptide	C	S	H:L
gi|17266328	1-aminocyclopropanecarboxylic acid synthase	Medicago truncatula	LVPVICESANNFK	62	12	1.2351538
trm|Q8ISF5	1MDa_1 protein	Caenorhabditis elegans	ENIGDICEIDGEK	95	21	1.1304316
gi|17986316	3-OXOACYL- REDUCTASE	Brucella melitensis	VNCVAPGAILNER	52	13	1.2759598
gi|133897	40S ribosomal protein S27A		ECPGENCGAGVFOAAHEDR	99	17	0.9101892
trm|Q9ZIV3	70 kDa heat shock protein (Fragment)	Flavobacterium ferrugineum	CLKPCERALK	57	20	1.0237633
spt|Q9CMW1	Acetyl-coenzyme A synthetase	Pasteurella multocida	CAVEQGGALAIK	56	18	0.8033226
gi|15559050	ADP-ATP translocator	Ethmostigmus rubripes	GOVDCFVR	67	15	1.6959368
trm|Q95VX4	ADP-ATP translocator	Ethmostigmus rubripes	GMVDCFVR	51	12	0.7973293
trm|Q7P029	Alcohol dehydrogenase class III	Chromobacterium violaceum	VMRAALECCHK	79	16	
gi|1490628	An4a		CGDLOAAQQVVNR	99	12	0.8612817
gi|17565150	ankyrin and patatin family member (5V188)	Caenorhabditis elegans	LADVVALCERCR	99	15	0.9914133
trm|O22460	Anthocyanidin synthase	Callistephus chinensis	EKYCNDIASGK	85	16	1.2186048
gi|8927462	antigen 5 precursor	Glossina morsitans morsitans	SCGSSAQDCK	93	16	2.4314584
prf|2020435A	Arg kinase	Procambarus clarkii	LGFLTFCPTNLGTTIR	99	17	1.0087598
gi|14193259	ATP synthase beta subunit	Candidatus Carsonella ruddii	IIDLLCPFLK	81	16	1.1101389
gi|15791494	ATP synthase F1 sector gamma subunit	Campylobacter jejuni	GLCGGFNIKTLK	61	7	
emb|CAA73233.1	ATP synthase, gamma subunit	Drosophila melanogaster	GLCGAVHTGVAR	99	21	1.1935857
gb|AAH08028.2	ATP5A1 protein	Homo sapiens	LYCIYVAIGQK	92	14	1.1583242
gb|AAH08028.2	ATP5A1 protein	Homo sapiens	KLYCIYVAIGQK	92	15	0.8604307
rf|NP_635500.1	ATP-dependent DNA ligase	Saccharomonospora sp.	CPDGAAGACFFQK	63	13	
gi|15234555	BEL1-like homeobox 2 protein (BLH2)	Arabidopsis thaliana	CLKDAVAAQLK	59	18	0.793197
gi|1066143	beta-tublin	Haliotis discus	NOOAACDPR	99	22	0.9150076
dbj|BAA07160.1	beta-tublin	Haliotis discus	TAVCDIPPR	98	19	
gi|16974673	beta-tubulin 4Q	Saimiri sciureus	NOOTACDPR	86	15	0.5190656
spt|P36569	Biotin synthase	Serratia marcescens	VCSGGIVGLGETVRDR	78	16	1.0863351
dbj|BAB20634.1	Cad89D	Drosophila melanogaster	RGNNTEVLCPR	61	12	
pir|S29130	calreticulin (clone 8)	Xenopus laevis	HEQNIDCGGGYVK	93	14	0.974697
emb|CAA74392.1	catalase 2	Caenorhabditis elegans	LGPNYIQLPVNCPYR	67	16	1.0335783
trm|P90628	Cathepsin L-like protease	Leishmania major	CGISWSGSSKSICQYV	60	10	
gi|20129741	CG1845-PA	Drosophila melanogaster	ARLLCELVR	65	19	1.0151483
trm|Q8INW6	CG31794-PD	Drosophila melanogaster	DGFPYCEPDYHNLFSPR	99	19	0.9181247
trm|Q8IQQ3	CG32171-PD	Drosophila melanogaster	CHLSLVDK	74	19	0.9354873
trm|Q8IPE8	CG4389-PB	Drosophila melanogaster	FINEAVLCLEEK	99	23	1.0722444
gi|24642084	CG5548-PA	Drosophila melanogaster	HEYLTCEYEDYVLR	99	18	1.1079336
gi|21357643	CG7470-PA	Drosophila melanogaster	LASIVEQVAECHLEGR	99	18	

accession_id	name	species	peptide	C	S	H:L
trm|Q9VQR2	CG8844 protein (GM03559P) (RE65754p)	Drosophila melanogaster	RVPTIDQCYTDDAVCR	99	21	
gi|13399647	Chain A, Dihydropyrimidine Dehydrogenase		CADAPCQK	98	25	2.3655893
gb|AAK96899.1	CH-rich interacting match of PLAG1	Mus musculus	CPLCMHSALDMTR	59	14	0.9353277
rf|ZP_00009794.	Succinyl-CoA synthetase, beta subunit	Rhodopseudomonas palustris	CDVIAEGVVAAVK	99	20	1.1427051
rf|ZP_00056375.	COG0468: RecA/RadA recombinase	Magnetospirillum magnetotacticur	GGTCAFVDAEHALDPSYARK	56	12	
rf|ZP_00004472.	Pseudouridylate synthases, 23S RNA-specific	Rhodobacter sphaeroides	CAWMALSPITGR	64	10	
gi|13358055	conserved hypothetical	Ureaplasma urealyticum	TFSLADHGLICINCESKIINK	66	12	1.4375707
trm|Q89U31	Conserved hypothetical protein	Bradyrhizobium japonicum	MEFCVHGPR	57	10	0.9859059
gi|10177486	heat shock transcription factor	Arabidopsis thaliana	FMEFKCR	86	17	0.4666334
emb|CAA35210.1	Cu-Zn superoxide dismutase	Drosophila melanogaster	AVCVINGDAK	94	16	1.204574
emb|CAA35210.1	Cu-Zn superoxide dismutase	Drosophila melanogaster	IGCGVIGIAK	90	16	1.0980713
gi|117781	Cytochrome C2		GCLACHNPEKK	93	14	1.1467868
gi|10441333	cytosolic branched-chain amino acid aminotransfe	Ovis aries	VREOFGSGTACVVCPVSTILYK	93	8	0.8380317
emb|CAA97700.1	DIP2	Saccharomyces cerevisiae	IWGLDFGDCHK	73	15	0.4295243
gi|15239799	disease resistance protein (TIR-NBS-LRR class),	Arabidopsis thaliana	TALEILCLYAFK	99	23	3.0545186
emb|CAB65993.1	dJ101K10.3 (novel protein)	Homo sapiens	YDCYLQR	98	10	1.0583502
gi|24585709	Elongation factor 2b CG2238-PA	Drosophila melanogaster	SFCOYILDPIYK	99	23	0.6386312
emb|CAA72977.1	elongation factor Ef-Tu	Buchnera aphidicola	TICTGVEMFR	94	16	1.0667265
trm|Q9U065	Elongation factor-1 alpha (Fragment)	Therevidae gen. sp. 6	SGDAAIVNLVPSKPLCVES	80	14	0.6406056
trm|Q7YEU6	Endonuclease	Saccharomyces servazzii	KIMIVSNCK	56	12	
rf|XP_319782.1	ENSANGP00000005182	Anopheles gambiae	VGLTNYAAAYCTGLLVAR	63	14	0.9535411
rf|XP_317139.1	ENSANGP00000006697	Anopheles gambiae	GPVISMVEDLFLYSCVER	99	7	0.9181247
rf|XP_313023.1	ENSANGP00000011707	Anopheles gambiae	GFELFCAQSFAK	99	23	0.8832404
rf|XP_313349.1	ENSANGP00000011832	Anopheles gambiae	CFIVGADNVGSR	56	19	
rf|XP_314689.1	ENSANGP00000013096	Anopheles gambiae	VGNHFLQVCK	99	19	1.0735238
gi|31196857	ENSANGP00000013737	Anopheles gambiae	SSPQPPEKVCOPR	93	16	2.371407
rf|XP_314051.1	ENSANGP00000015684	Anopheles gambiae	INAFAYLECSAK	99	20	0.8534049
rf|XP_320284.1	ENSANGP00000016663	Anopheles gambiae	SSISSAKSPCLSR	81	8	0.7278481
rf|XP_321803.1	ENSANGP00000016829	Anopheles gambiae	CWLFAALNCIR	99	19	
rf|XP_311308.1	ENSANGP00000017680	Anopheles gambiae	CSLSFLSAWR	55	14	0.9571212
rf|XP_310377.1	ENSANGP00000018909	Anopheles gambiae	VCTLSITDAGDSDIIRSLPEAQA	55	10	
rf|XP_313917.1	ENSANGP00000021862	Anopheles gambiae	YGIDCTNIAEDNIMDVADFEK	97	13	0.8413671
gi|31238695	ENSANGP00000023803	Anopheles gambiae	COALAQLLTEQNFPAIGIHR	78	13	1.0223004
gb|AAA51461.1	epidermal growth factor receptor type III	Drosophila melanogaster	EICYEVLGLLDK	99	16	0.8162194
rf|NP_187219.2	expressed protein	Arabidopsis thaliana	GFTCSLLEIGINLEMHLR	94	10	1.527079
accession_id	**name**	**species**	**peptide**	**C**	**S**	**H:L**

gi|18403408	expressed protein	Arabidopsis thaliana	LCTNNCGFFGSAATMNMCSKC	55	12	
rf|NP_197653.2	F-box protein family	Arabidopsis thaliana	LSIQKNENDPSCVTLWTDCVAF	55	12	
gi|120165	Fimbrin	Gallus gallus	IENCNYAVELGK	99	18	0.8791782
gi|15603875	GcpE	Pasteurella multocida	GINFIACPTCSRQEFDVIGTVNA	86	13	
gi|17861704	GH23483p	Drosophila melanogaster	GVVISCGDHTVOGR	99	17	1.3183248
gi|29246546	GLP_384_11857_7355	Giardia lamblia ATCC 50803	ACSLQQRDPCSNLR	93	20	0.8150498
emb|CAA72173.1	glutamate dehydrogenase (NAD(P)+)	Drosophila melanogaster	CACVDVPFGGAK	93	16	0.6854555
gi|1174958	Glycoprotein GX precursor	Bovine herpesvirus 1	NASLECNSRPR	94	12	
gi|15242492	glycosyl hydrolase family 3	Arabidopsis thaliana	FCNAGLSIKAR	61	14	1.9197206
trm|Q8I5N5	GTP-binding protein, putative	Plasmodium falciparum	VILCVNKCESYK	99	14	1.1222217
dbj|BAC99562.1	haloacid dehalogenase-like hydrolase-like protein	Oryza sativa	CVSAAAGAGGGGRER	93	20	0.8477846
spt|P02828	Heat shock protein 83 (HSP 82)	Drosophila melanogaster	FHTSASGDDFCSLADYVSR	99	20	0.929864
gi|33152405	hemolysin activation/secretion protein	Haemophilus ducreyi 35000HP	VCLPYK	53	15	1.0872798
gi|11386885	Hexokinase 1	Nicotiana tabacum	CGTPDAKLK	81	18	1.0060242
gi|15673900	homoserine O-succinyltransferase	Lactococcus lactis subsp. lactis	LCGIYKSSVEQPK	97	14	0.9079561
spt|Q8PS69	Hydroxylamine reductase (EC 1.7.-.-) (Hybrid-clust	Methanosarcina mazei	ALPENTVILTAGCAK	97	13	
rf|NP_850052.1	hypothetical protein	Arabidopsis thaliana	CPTCKAVGFVLCR	99	11	1.8914515
trm|Q7SBI2	Hypothetical protein	Neurospora crassa	VTVLLQILKECK	92	17	0.9393024
gi|22979269	hypothetical protein	Ralstonia metallidurans	MLCTHK	52	10	0.9262102
gi|15601327	hypothetical protein	Vibrio cholerae	VCADDLCELER	83	10	0.8926618
gi|13812187	hypothetical protein	Guillardia theta	NKCCSVFISSESIIIK	98	10	0.8906734
rf|NP_456243.1	hypothetical protein	Salmonella enterica serovar Typh	SHKCDAHHVFMIFYYSFMI	58	7	0.8413671
gi|15218428	hypothetical protein	Arabidopsis thaliana	VQELONVCEAIK	99	15	0.8216271
gi|22978624	hypothetical protein	Ralstonia metallidurans	CPCLAARVPSTSR	62	15	0.6569449
rf|NP_909689.1	hypothetical protein	Oryza sativa	GGCGAVGGGR	56	12	
gi|29829945	hypothetical protein	Streptomyces avermitilis MA-468(	LCVDDEAPPATR	59	15	
gi|22969589	hypothetical protein	Rhodospirillum rubrum	ALALGLCDEAGEDA	62	7	
gi|21233920	hypothetical protein	Proteus vulgaris	ANCPGTTTQCHK	82	14	
gi|23482033	hypothetical protein	Plasmodium yoelii yoelii	IADCVCPQECKCD	87	11	
trm|Q8IL25	Hypothetical protein	Plasmodium falciparum	KVLYLVGWAVC	59	11	
gi|23013196	hypothetical protein	Magnetospirillum magnetotacticur	AEEGCDOATDPR	88	11	
gi|22999033	hypothetical protein	Magnetococcus sp. MC-1	LAOTIAQDEASCVVAGOPR	72	8	
trm|Q88XI7	Hypothetical protein	Lactobacillus plantarum	CYGLQGIDGLRHR	55	15	
gi|23473160	hypothetical protein	Desulfovibrio desulfuricans G20	AVEDSGADOLSCINTLSGOAVI	69	12	

accession_id	name	species	peptide	C	S	H:L
gi|23137756	hypothetical protein	Cytophaga hutchinsonii	IAEGCDRPCSFCAIPVOR	63	9	
trm|Q95YC4	Hypothetical protein	Caenorhabditis elegans	KPVEAEKWTKPLNCHK	85	9	
spt|O66417	Hypothetical protein AA26	Aquifex aeolicus	LVPCYK	51	14	
trm|O67938	Hypothetical protein AQ_2194	Aquifex aeolicus	AACPLITK	61	17	
gi|11359957	hypothetical protein DKFZp434D1411.1 - human (f	Homo sapiens	LCIDCSGSHR	52	8	
pir|D71870	hypothetical protein jhp0928 - Helicobacter pylori (s	Helicobacter pylori	GQDNILSCTTDAR	99	14	
pir|A81870	hypothetical protein NMA1049 [imported] - Neisser	Neisseria meningitidis	GGILLKCAIGK	83	17	0.9237292
gi|21399832	hypothetical protein predicted by GeneMark	Bacillus anthracis A2012	RIINGIICLDWK	71	17	
trm|Q8PPF5	Hypothetical protein XAC0731	Xanthomonas axonopodis pv. citri	CTGACVHDK	64	18	
rf|XP_351175.1	hypothetical protein XP_351174	Homo sapiens	RASSGVELGDCK	92	9	1.2475145
rf|NP_910481.1	hypothetical protein~predicted by GeneMark.hmm	Oryza sativa	CNNQALLGLAR	53	15	0.99963
gi|22831284	hypothetical protein~similar to full-Length cDNA frc	Oryza sativa (japonica cultivar-grc	CLQSSRQTPAR	97	20	1.1417693
gi|109292	Ig lambda chain precursor - rabbit (fragment)	Oryctolagus cuniculus	LTCTLSSAHK	58	16	0.8639584
gi|11374664	isocitrate dehydrogenase (NADP) (EC 1.1.1.42), c	Homo sapiens	CATITPDEK	82	17	0.7875555
gi|1129167	J1590	Saccharomyces cerevisiae	CAIFYLALK	54	15	0.7859562
trm|Q61752	Kallikrein (Fragment)	Mus musculus	MLCAGEMGGGK	65	12	1.054136
dbj|BAA02796.1	KIAA0006	Homo sapiens	TLMGQCQDLRK	63	12	
dbj|BAA20763.2	KIAA0304 protein	Homo sapiens	QLHPGPCGLQAVSQR	57	16	0.8582117
dbj|BAA31587.2	KIAA0612 protein	Homo sapiens	VSCPSPHPSPEAR	57	10	0.9441822
gb|AAA28663.1	laminin B1 subunit precursor	Drosophila melanogaster	SSCYPATGNLLIGR	80	14	
dbj|BAB60867.1	large protein	Measles virus	AACSNIATTMAK	66	14	
gb|AAL39610.2	LD19356p	Drosophila melanogaster	IPELAINPLCER	98	20	1.0157264
trm|Q8STG9	LD29847p (DSec61alpha) (Preprotein translocase	Drosophila melanogaster	FLEVIKPFCSILPEIAKPER	92	13	0.964683
gi|18448482	luteinizing hormone beta subunit	Daubentonia madagascariensis	CGACRLSSSDCGGPR	64	13	
dbj|BAC65549.1	mKIAA0382 protein	Mus musculus	TGTGDIATCDSPR	57	17	1.1150581
dbj|BAC98074.1	mKIAA1016 protein	Mus musculus	VLVIPVCFREMK	58	8	1.180261
emb|CAA42754.1	myosin heavy chain	Drosophila melanogaster	NVQQVTNSIGALCK	99	23	1.3287896
gi|1079184	Na+/K+-exchanging ATPase (EC 3.6.3.9) alpha ch	Ctenocephalides felis	COELALGDVOSIR	99	22	1.613541
pir|S24650	Na+/K+-exchanging ATPase (EC 3.6.3.9) alpha-1	Bufo marinus	NLEAVETLGSTSTICSDK	98	15	1.0648247
pir|S24650	Na+/K+-exchanging ATPase (EC 3.6.3.9) alpha-1	Bufo marinus	IAGLCNRAVFPAGQENTPILK	56	11	
emb|CAA31512.1	neurone-specific enolase	Homo sapiens	ACNCLLLK	65	12	1.365286
emb|CAA35621.1	Nm23 protein	Homo sapiens	GDFCIQVGR	78	15	0.9342789
trm|Q93XG6	Nucleotide-binding leucine-rich-repeat protein 1	Oryza sativa	KTTACSALSPLSCFSNIR	67	10	0.7050348
pir|A45386	omega-conotoxin-sensitive calcium channel alpha-	Rattus norvegicus	LCRQGYTIR	92	10	

accession_id	name	species	peptide	C	S	H:L
emb|CAD41546.2	OSJNBb0091E11.16	Oryza sativa subsp. japonica	CHTCVQLKQPCKPHK	55	10	
gi|15289923	P0483G10.12	Oryza sativa (japonica cultivar-grc	OVMVGCLIAILAR	99	12	0.6743444
dbj|BAB21073.1	P0501G01.2	Oryza sativa subsp. japonica	SLSDHGPASCFK	55	10	0.23367
gi|1706258	Peptidyl-prolyl cis-trans isomerase (PPIase) (Rotar	Blattella germanica	VIPNFOCQGGDFTNHNGTGGK	64	11	0.9203722
trm|Q8LP45	Pleiotropic drug resistance like protein	Nicotiana tabacum	DISGIIKPCR	64	14	1.0585374
gi|9628750	polymerase processivity factor	Human herpesvirus 7	QRCGM	62	9	
gi|24266646	Pro1	Glossina morsitans morsitans	LNENALEODCPK	99	25	0.8216271
gi|24266649	Pro2	Glossina morsitans morsitans	RCPDSFLFLTEK	99	23	1.6259226
gi|24266649	Pro2	Glossina morsitans morsitans	CPDSFLFLTEK	99	20	1.4636155
pir|D71288	probable glutamate synthase (gltA) - syphilis spiroc	Treponema pallidum subsp. pallid	CLNCKTKPCVK	99	13	
gi|24586400	Proteasome alpha6 subunit CG18495-PA	Drosophila melanogaster	SGDCAVVATQK	61	12	
spt|Q99435	Protein kinase C-binding protein NELL2 precursor	Homo sapiens	GYDFCSER	85	10	
gi|19112119	protein kinase dsk1	Schizosaccharomyces pombe	IADLGNACWTRK	83	12	1.137832
gi|15217521	protein kinase -related	Arabidopsis thaliana	CAESFEKLDK	81	20	1.1199993
gi|12239342	proteinx0005	Homo sapiens	FCLVPSMEGVR	60	15	
dbj|BAB62621.1	putative 40S ribosomal protein S5	Oryza sativa subsp. japonica	AQCPLVER	75	15	0.9141791
dbj|BAB62621.1	putative 40S ribosomal protein S5	Oryza sativa subsp. japonica	TIAECLADELINAAK	99	21	0.7858809
gi|11931708	putative cold shock protein	Ascovirus DpAV4	TTCVLSSGRTHSSR	99	13	0.9138308
trm|Q9FBV4	Putative DNA-binding protein	Streptomyces coelicolor	GCLVGEVFLASAVR	99	10	0.8653949
gi|33599724	putative fumarylacetoacetate hydrolase family prot	Bordetella bronchiseptica	LGATLGLVLGRTACR	98	20	
gi|4090876	putative hemolin	Hyphantria cunea	CEVNNHVGQK	58	14	1.0579187
trm|O45042	Putative protective antigen	Onchocerca volvulus	ACKEHPDCYDQR	58	15	2.0674839
gi|17564330	putative protein, with 3 coiled coil domains, of bilat	Caenorhabditis elegans	LDEALQNSDKCQK	99	13	1.1651795
gi|27311287	putative reverse transcriptase	Oryza sativa (japonica cultivar-grc	GLGCGREGGGGAQTEPDPWK	79	13	
gi|1353135	Putative serine/threonine-protein kinase C41C4.4 i	Caenorhabditis elegans	AVISDFGLCKR	87	15	
trm|Q968A1	Putative zinc-metalloproteinase precursor	Glossina morsitans morsitans	IIEEISCIR	99	22	0.933531
gi|11260409	pyruvate kinase-like protein - Arabidopsis thaliana	Arabidopsis thaliana	VKASVIICFTSSGR	99	12	0.9110121
gi|17737369	Rab-protein 10 CG17060-PA	Drosophila melanogaster	AFCELAEAILDK	83	13	0.7853888
trm|Q8IGP0	RE56164p (Fragment)	Drosophila melanogaster	VSPCPEALNNAACNIR	80	11	
gb|AAL68340.2	RH06643p	Drosophila melanogaster	YGLNICR	91	21	1.1329799
trm|Q8IGC5	RH56418p	Drosophila melanogaster	EQELFFFHELSPGSCFFQPR	57	11	0.9801526
gi|15901834	ribosomal large subunit pseudouridine synthase, R	Streptococcus pneumoniae TIGR	QFSNKTSLAHCK	61	13	1.0103755
trm|Q962R1	Ribosomal protein S18	Spodoptera frugiperda	RAGECTEEEVEK	97	24	0.9130131

accession_id	name	species	peptide	C	S	H:L
emb|CAA42019.1	ribosomal protein S27	Rattus rattus	LTEGCSFR	84	15	1.0398839
gi|17647897	Ribosomal protein S5 CG8922-PA	Drosophila melanogaster	LTCSLOOK	79	16	
gi|23509211	rifin	Plasmodium falciparum 3D7	MKLLHYCK	60	14	
trm|Q9U7C6	Salivary gland growth factor-1 precursor	Glossina morsitans morsitans	CTNENGVQLLTFR	99	26	2.4723673
gi|17862964	SD05601p	Drosophila melanogaster	GYLGECGIR	68	14	0.8807775
gi|13235329	secreted portion of glycoprotein G-2	Human herpesvirus 2	ERCLPPQTPAAPSDLPR	80	20	0.9369973
gi|23479880	serine/threonine protein phosphatase PP5	Plasmodium yoelii yoelii	VFICHGGIPSK	97	14	1.0159445
emb|CAD24460.3	SI:dZ1C23.22 (TCR-alpha V segment II-70)	Danio rerio	DSALYFCAL	63	13	1.0625141
gi|20831583	similar to 40S ribosomal protein S2	Mus musculus	GCGQGRGR	59	14	
rf|XP_143986.2	similar to 60S RIBOSOMAL PROTEIN L9	Mus musculus	TVCSHVQNKIK	76	17	0.9771938
gi|27676758	similar to ADP,ATP carrier protein, liver isoform T2	Rattus norvegicus	GQGHTVLQCGGK	80	17	0.7384817
rf|XP_129490.2	similar to complement factor H precursor - mouse	Mus musculus	CVATDQLEKCR	80	13	0.8712479
gb|AAR10096.1	similar to Drosophila melanogaster CG1475	Drosophila yakuba	CEELNLSGHFYR	99	23	0.9142819
gb|AAR09822.1	similar to Drosophila melanogaster CG4800	Drosophila yakuba	LQECFAFGDKK	99	17	0.6670198
gb|AAR09756.1	similar to Drosophila melanogaster CG9091	Drosophila yakuba	STCAQCGYPAAK	99	22	0.8007705
gb|AAR09892.1	similar to Drosophila melanogaster Mlp60A	Drosophila yakuba	ALDSTNCTEHEK	99	23	1.01507
gb|AAR09737.1	similar to Drosophila melanogaster RpL9	Drosophila yakuba	TINSNQCVK	92	16	1.0267167
gb|AAR09673.1	similar to Drosophila melanogaster RpS12	Drosophila yakuba	LVTALCNEHQIPLIR	99	19	1.1934126
gb|AAR09795.1	similar to Drosophila melanogaster RpS17	Drosophila yakuba	ICEEVAIIPTKPLR	99	22	0.9949319
gi|27718477	similar to interleukin-1 receptor-associated kinase :	Homo sapiens	GVRGSAMAGPCGAR	58	12	
rf|XP_293261.2	similar to Sodium/hydrogen exchanger 3 (Na(+)/H(	Homo sapiens	LQCHPLDHILLAIKDISR	94	17	
gi|27678866	similar to tripartite motif protein TRIM5, isoform alp	Homo sapiens	CAQPHLOGEMGFK	83	18	
gi|477738	T-cell receptor alpha chain (V-J region, CD4+ clone	DH12, house-dust-mite-reactive)	YFCAVGAGGFKTIFGAGTR	71	12	1.7465981
dbj|BAA32974.1	T-plastin	Cricetulus griseus	LENCNYAVELGK	99	18	0.8231855
pir|S05988	translation elongation factor eEF-2 - fruit fly (Droso	Drosophila melanogaster	SFCMYILDPIYK	97	14	0.8517007
gi|15924261	tRNA pseudouridine 5S synthase	Staphylococcus aureus subsp. au	VICGKGTYIR	99	16	
trm|Q9NBC9	Trypsin-like serine protease precursor	Glossina morsitans morsitans	ETMVCGYATAK	99	21	1.4924483
trm|Q9NBC9	Trypsin-like serine protease precursor	Glossina morsitans morsitans	IKETMVCGYATAK	99	19	1.1569012
gi|8927464	Tsal1 protein precursor	Glossina morsitans morsitans	TELLLACTGAQNYFK	99	18	1.8179396
trm|Q9NBA4	Tsal2 protein precursor	Glossina morsitans morsitans	SPASENLCK	92	20	3.1960979
gi|8927466	Tsal2 protein precursor	Glossina morsitans morsitans	GTELLLACTGEGNGLK	98	16	

accession_id	name	species	peptide	C	S	H:L
gi|15864611	tsetseEP protein	Glossina morsitans morsitans	YCIDQAFEQAQEK	98	16	1.4305894
gi|15864611	tsetseEP protein	Glossina morsitans morsitans	EKYCIDQAFEQAQEK	99	27	1.3639864
trm|Q95P09	TsetseEP protein precursor	Glossina morsitans morsitans	FASDKCAQEGQNAR	99	21	1.9635997
trm|Q95P09	TsetseEP protein precursor	Glossina morsitans morsitans	CAQEGQNAREEETK	97	19	1.4465055
trm|Q95P09	TsetseEP protein precursor	Glossina morsitans morsitans	GLGACVAK	51	13	1.4324389
gi|18309217	two-component sensor histidine kinase	Clostridium perfringens	GNGLGIKICK	66	10	1.0650803
gi|15236885	ubiquitin-like protein (SMT3)	Arabidopsis thaliana	LONAYCDR	58	14	
gi|17555410	UNCoordinated locomotion UNC-86, POU protein	Caenorhabditis elegans	ALAHLKOPGVGSLSQSTICR	95	14	
rf|NP_078053.1	unique hypothetical membrane lipoprotein	Ureaplasma urealyticum	ICDDIEYTR	54	12	1.1103272
gi|15929366	Unknown (protein for IMAGE:3684087)	Homo sapiens	GVHLCQVLONHK	99	10	0.8162194
emb|CAA32638.1	unnamed protein product	Drosophila melanogaster	CSTIFINGK	99	18	1.1083823
dbj|BAC86017.1	unnamed protein product	Homo sapiens	CQPTNCSPAGPR	92	16	0.991033
gi|16552016	unnamed protein product	Homo sapiens	ESCEHLGDCYSR	93	11	0.9696219
gi|12853204	unnamed protein product	Mus musculus	QLPDAICRTR	56	11	0.8440078
dbj|BAB55222.1	unnamed protein product	Homo sapiens	CLKYLDSMGQK	99	9	0.6670198
emb|CAA35916.1	unnamed protein product	Rattus rattus	GCEVVVSGK	62	14	
gi|21757165	unnamed protein product	Homo sapiens	CCPQLCSCLCCK	59	8	
dbj|BAC85401.1	unnamed protein product	Homo sapiens	VSCKASGGTLR	57	8	
gi|18033165	VirB4	Agrobacterium tumefaciens	AGAITSRNFAGLVCFENFPVGC	63	8	0.9787257
trm|Q8LSN9	YZ1	Zea mays	CSTAENSSAPGR	57	9	1.0191309
gi|1786146	ZPT4-3	Petunia x hybrida	KYECLNCK	87	13	

Appendix III

Examples of proteins that contain at least three-tandem glutamic acid-proline dipeptide repeats.

Monocarboxylate transporter	transporter/exchanger	*Mus musculus*
26 kDa protein	uncharacterized	*Autographa californica virus*
Putative TPR-repeat protein	scaffold-protein-protein	*Arabidopsis thaliana*
Hypothetical protein	hypothetical	*Desulfitobacterium hafniense*
Conserved protein	uncharacterized	*Microbulbifer degradans*
Hypothetical protein T12D8.9	hypothetical	*Caenorhabditis elegans*
Protein conserved in bacteria	uncharacterized	*Rhodobacter sphaeroides*
Procyclin EP2	membrane surface	*Trypanosoma evansi*
CG15778-PA	hypothetical	*Drosophila melanogaster*
Zinc finger protein 219	uncharacterized	*Homo sapiens*
Proline-rich membrane protein	uncharacterized	*Bordetella parapertussis*
Amino acid transporter SLC	small molecule transport	*Canis familiaris*
Nucleolar protein 3	apoptosis repressor	*Homo sapiens*
GABA-A epsilon subunit	inhibitory neurotransmitter	*Rattus norvegicus*
TonB protein	nutrient acquisition	*Salmonella typhimurium*
GLP_243_18223_17171	hypothetical	*Giardia lamblia*
Muscle troponin-T	musculature control	*Salmo trutta*
Actin binding protein	cytoskeletal regulation	*Candida albicans*
Tfp pilus assembly protein	type V secretion	*Pseudomonas syringae*
Hypothetical protein	hypothetical	*Oryza sativa*
ENSANGP00000014855	hypothetical	*Anopheles gambiae*

Appendix IV

Abbreviations and Supplier List

1D	one dimensional
2D	two dimensional
BLAST	Basic Local Alignment Search algorithm for proteins
BSF	bloodstream forms
°C	Celsius
CBB	Coomassie brilliant Blue G-250 *reagent* (SERVA Blue G)
CCB	Colloidal Coomasie Blue *staining method*
cDNA	complementary deoxyribonucleic acid
CSF	cerebro spinal fluid
d	day
Da	Dalton
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBI	European Bioinformatics Institute
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EP	glutamic acid:proline dipeptide
FITC	fluorescein isothiocyanate
fm	femptomole
Gmm	*Glossina morsitans morsitans*
h	hour(s)
Hsp60 (GroEL)	heat shock protein 60
Ig	immunoglobulin
IPTG	isopropyl β-D-Thiogalactopyranoside
ISO-DALT	isoelectric focusing (first dimension) and separation by molecular weight (in Daltons; second dimension)
kDa	kilodaltons
M	molarity
mAb(s)	monoclonal antibodies
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
mg	milligram
min	minute(s)
µL	microliter
mM	millimolar
mol	mole
NCBI	National Center for Biotechnology Information
ng	nanogram

PBS	phosphate buffered saline
pI	isoelectric point
pmol	picomole
PVDF	polyvinylidene difluoride
Q-TOF	quadruple time-of-flight mass spectrometer
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
sec	second(s)
SIT	sterile insect technique
SwissProt	Swiss Institute of Bioinformatics Protein Database
TLCK	Tosyllysylchloromethyl ketone
TPCK	Tosylphenlanalylchloromethyl ketone
Tris	tris(hydroxymethyl)aminomethane
UV	ultra-violet
V	Volt
v/v	volume per volume
Vh	volt-hour
VSG	variant surface glycoprotein
w/v	weight per volume

List of Suppliers

Item	Supplier	Address
10 kDa molecular mass ladder	Gibco BRL®	Burlington, ON
15 ml polystyrene sterile screw cap tube	Sarstedt, Inc.	Newton, SC
Acrodisc™ syringe filter	Pall Gelman Laboratories	Ann Arbor, MI
α-cyano-4-hydroxycinnamic acid	Sigma-Aldrich Canada	Milwaukee, WI
Alexa™ Fluor 488	Molecular Probes	Eugene, OR
Bioanalyst Software	PE-SCIEX	Boston, MA
BioTrace™ (PVDF)	Pall Corporation	Ann Arbor, MI
ClonaCell HY™ System	StemCell Technologies Inc.	Vancouver, BC
DTT	Sigma-Aldrich Canada	Oakville, ON
Falcon 3915 PRO-BIND ELISA Plates	Becton Dickinson	Oxnard, CA
FITC conjugated mAbs	Caltag Laboratories	Burlingame, CA
GelCode™ Blue	Pierce Chemical Company	Rockford, IL
goat anti-mouse IgG/IgM-horseradish peroxidase	Caltag Laboratories	San Francisco, CA
isotyping ELISA kit	American Qualex	La Mirada, CA
MALDI internal peptide standards	Sigma Chemical Company	St. Louis, MO

MALDI Plate (stainless steel)	Applied Biosystems	Foster City, CA
Mass Spectrometer DE-STR	Applied Biosystems	Foster City, CA
Mini-Protean II one-dimension gel apparatus	Bio-Rad Laboratories	Hercules, CA
Nanosep MF filters	Pall Filtron Corp.	Northborough, MA
Perkin-Elmer gas-phase sequencer	Applied Biosystems	Foster City, CA
PE-SCIEX Q-STRi	Applied Biosystems	Foster City, CA
porcine trypsin (sequence grade)	Promega Corp.	Madison, WI
Q-TOF glass capillary needles	Protana Inc.	Staermosegaards, Denmark
Q-TOF mass spectrometer	Micromass	Beverly, MA
SERVA Blue G	SERVA Electrophoresis	Heidelberg, DRG
Speed Vac Concentrator	Savant	Hicksville, NY
SuperSignal Dura™ chemiluminescence substrate	Pierce Chemical Company	Rockford, IL