Characterization of trypsin-and chymotrypsin-like genes in the midgut of the tsetse fly

Glossina morsitans morsitans (Diptera: Glossinidae)

Mikio YOSHIYAMA¹⁾, Minoru YAMAKAWA²⁾, Yuichi CHIGUSA³⁾
and Wendy C. GIBSON⁴⁾

¹⁾Animal Breeding and Reproduction Research Team, National Institute of Livestock
and Grassland Science, Ikenodai 2, Tsukuba, Ibaraki, 305–0901 Japan

²⁾Innate Immunity Laboratory, National Institute of Agrobiological Sciences,
Owashi 1–2, Tsukuba, Ibaraki, 305–8634 Japan

³⁾Department of Tropical Medicine and Parasitology, Dokkyo Medical University
School of Medicine, Mibu, Tochigi, 321–0293 Japan

⁴⁾School of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG U.K.

(Received: 27 October 2008; Accepted: 26 December 2008)

Abstract: Putative serine protease genes including trypsin and chymotrypsin were identified in the tsetse fly *Glossina morsitans morsitans* during an EST project. Trypsin and chymotrypsin are major digestive enzymes involved in the parasite-host interactions of vector insects. In this study, two genes designated Gmmtry1 and Gmmchy1 were further characterized during blood digestion and in responses against parasite infection. Both Gmmtry1 and Gmmchy1 mRNA was expressed at high levels in the posterior region of the midgut. Gmmchy1 transcript increased following an infectious blood meal suggesting chymotrypsin is regulated by the presence of the trypanosome during the early establishment of the parasite infection in the midgut. The possible roles of trypsin and chymotrypsin proteases in determining the refract-oriness of the tsetse fly against trypanosomes is also discussed.

Key words: Tsetse fly, trypsin, chymotrypsin, trypanosome, digestive enzymes

INTRODUCTION

Tsetse flies are medically important vectors of protozoan trypanosomes that cause African trypanosomiasis in humans and animals (Aksoy et al., 2003). The WHO report that at least 100,000 new cases of human African trypanosomiasis occur each year and 60 million people are at risk in 37 countries (WHO, 2001). The agents of Sleeping sickness, African trypanosomes rely on the tsetse fly (Diptera: Glossinidae) for successful transmission. The mammalian bloodstream form of trypanosomes rapidly differentiates into a procyclic form in the tsetse midgut and

the surfaces become covered with pro-cyclic proteins (Ruepp et al., 1997). The majority of ingested parasites are completely eliminated around day 3 after infection. A small portion of surviving parasites continues to proliferate and establish infections in the anterior region of the midgut of the tsetse fly (Gibson and Bailey, 2003). After establishment in the midgut, the trypanosomes migrate forward to the mouthparts or salivary glands where they differentiate into an infectious metacyclic form (Vickerman et al., 1988). Therefore, the successful establishment of a trypanosome infection in the tsetse midgut greatly relies on the early stage around 3 days after infection. Tsetse flies

are normally refractory to parasite transmission, even under ideal laboratory conditions infection rates are less than 20% (Moloo et al., 1992, 1994). In addition, the infection rate of the fly populations in the field fails to exceed 5% (Msangi et al., 1998).

Several factors, including sex of the tsetse fly, and presence of lectins, endosymbiotic bacteria and antimicrobial peptides play an important role in successful parasitic infection of the midgut (Maudlin and Ellis, 1985; Welburn and Maudlin, 1999; Hao et al., 2001; Hu and Aksoy, 2005; Hu and Aksoy, 2006).

Proteases are potentially important in the tsetse fly and trypanosome interactions. EP and GPEET procyclin, the major surface glycoproteins of procyclic forms of *Trypanosoma brucei brucei*, are truncated by serine proteases in the midgut of *Glossina morsitans morsitans*. Therefore, serine proteases have the potential to influence the tsetse fly and trypanosome interactions (Acosta-Serrano, 2001; Liniger et al., 2003, 2004).

An EST gene discovery project to identify putative protease genes including trypsin and chymotrypsin from the midgut of adult *G. m. morsitans* was conducted by Lehane et al. (2003). Previous studies reported that two serine protease genes of the tsetse fly are constitutively expressed (Yan et al., 2001). In addition, the lectin-trypsin complex was isolated from *Glossina* species and shown to stimulate the transformation of the bloodstream form trypanosome into the procyclic forms *in vitro* (Abubakar et al., 2006; Osir et al., 1995). Here we report further characterization of the trypsin and chymotrypsin genes from *G. m. morsitans* (named *Gmmtry1*, *Gmmchy1*), their expression profiles during blood digestion and responses against parasitic infection.

MATERIALS AND METHODS

Tsetse flies

Glossina m. morsitans was originally es-

tablished from puparia collected in Zimbabwe. The colony is kept in the insectary at Liverpool School of Tropical Medicine at $24 \pm 2^\circ\text{C}$ and 55–60% relative humidity. Flies receive defibrinated horse blood by an artificial membrane feeding system (Moloo, 1971). To survey expression profiles during parasite establishment, flies were fed blood containing $1 \times 10^6/\text{ml}$ of bloodstream form *T. b. brucei* TSW196. Flies fed on the infected meal were subsequently maintained on defibrinated sterile blood. Midguts were microscopically dissected at designated times.

Gene contig analysis and Bioinformatics

Genes encoding putative trypsin and chymotrypsin were retrieved from GeneDB (<http://www.genedb.org/genedb/glossina/index.jsp>), and a Blast search performed (<http://www.ncbi.nlm.nih.gov/BLAST/>). Signal peptide sequences and protein domains were searched for using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) and Pfam (<http://www.sanger.ac.uk/Software/Pfam/>), respectively. Sequences were aligned by GENETYX-MAC ver 9.0 (Software Development).

RT-PCR

To determine tissue specific expression profiles, three parts of the midgut and the fat body were dissected from flies 24 h after feeding. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. After DNaseI treatment for 1 h at 37°C , first strand cDNA was synthesized from $1 \mu\text{g}$ of total RNA using a First Strand cDNA Synthesis kit (Promega) with oligodT primers. Gene specific primers, *Gmmtry1*QF; 5'-TGC AGC TAC CAT GCC CAA GGG-3', *Gmmtry1*QR; 5'-ATT GCG CCT TGT AAG CAA TGG-3', *Gmmchy1*QF; 5'-GGT TAC GTA CCC TGT ACG GCG-3', and *Gmmchy1*QR; 5'-CAA GAT GAA TCC TGC AAC ACA AAT-3' were used. The *Gmm* GAPDH clone (*Gmm2682*) from the

Table 1. Trypsin and chymotrypsin-like genes identified from an EST library.

Contig	Ortholog	Acc. No.	E value	Pfam	E value	Sequence length (bp)
Gmm3293	Dp GA14937-PA	EAL26142	3e-45	Trypsin	1.1e-06	1,061
Gmm3216	De delta trypsin	AAA83243	2e-38	Trypsin	9e-15	1,093
Gmm3061	Dm alpha trypsin	AAF58659	8e-39			979
Gmm3354	Gm trypsin-like	AAF91346	1e-73	Trypsin	4.9e-17	896
Gmm3108	Gm trypsin-like	AAF91346	2e-121	Trypsin	1.4e-74	1,140
Gmm3359	Gm trypsin-like	AAF91346	2e-42			1,016
Gmm2697	Gm trypsin-like	AAF91346	4e-59			579
Gmm3340	Gm trypsin-like	AAF91346	6e-34			915
Gmm3330	Gm chymotryp.	AAF91345	1e-66			936
Gmm3362	Gm chymotryp	AAF91345	5e-39			1,085
Gmm3230	Gm chymotryp.	AAF91345	3e-121	Trypsin	1.3e-38	993
Gmm7528	Dm GRAAL2	CAC35209	7e-54	Scav. receptor	9.4e-25	563
Gmm3310	De Eta trypsin	AAA83237	1e-21			980
Gmm2574	Gm Pro3	AAN52278	3e-56			938
Gmm2542	Gm Cathepsin B	AAK07477	1e-89			571
Gmm2539	Gm Cathepsin B	AAK07477	3e-95	papain	3.3e-20	584
Gmm7163	Dm easter	AAF55170	6e-61			473
Gmm7162	Dp GA18526	EAL27739	5e-37			483

EST was used as a reference gene. *GmmGAPDH* specific primers designed for PCR were GmmGAPDHF; 5'-CTC ATT CGT CGG TGT TCG ATG-3' and GmmGAPDHR; 5'-GCG TTG AAC AGC AAT ATT TCC CT-3'.

Real-time quantitative PCR

First strand cDNA synthesis and gene specific primers were employed as described above. PCR reactions were performed in a final volume of 20 μ l with the LightCycler[®] 480 system (Roche) using Fast Start Reaction Mix SYBR Green1 (Roche). Cycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 60°C for 5 s and 72°C for 10 s. Serial dilutions of the pGEM[®]-T Easy vector containing the *GmmGAPDH* gene were used as a standard curve. The expression levels were quantified by the second derivative maximum method of LightCycler Software (Roche). Transcript levels were normalized with the GAPDH

gene as an internal control and the mean $\pm$ standard deviations were calculated from the three independent experiments. The data were compared using the Student's *t*-test. Self-cleared flies were defined as those which had had a trypanosome-infected blood but which had no microscopically detectable trypanosomes in the midgut. Flies fed normal blood only were defined as controls.

Trypsin and chymotrypsin enzyme assays

Teneral *G. m. morsitans* were fed by artificial membrane. At designated times midguts were dissected and individually stored in 100 μ l of PBS. For trypsin activity, 1 mM N α -benzoyl-DL-arginine p-nitroanilide (BapNA Sigma) was used. For chymotrypsin activity, 0.5 mM of N-succinyl-ala-ala-pro-phe-p-nitroanilide substrate (AAPF Sigma) was used. The substrates were dissolved in dimethylsulphoxide (DMSO) at 100 mM Tris-HCl buffer (pH 8.0) immediately prior to assay,

The EST gene discovery project from adult tsetse flies reported 18 cDNAs that

code for serine proteases in the midgut (Table 1) (Lehane et al., 2003). By Blast search, Gmm3354, Gmm3108, Gmm3359, Gmm2697 and Gmm3340 showed high homology with *Glossina* trypsin-like serine protease precursor (Yan et al., 2001; acc. no. AAF91346) and Gmm3330, Gmm3362 and Gmm3230 showed similarity with *Glossina* chymotrypsin-like serine protease precursor (Yan et al., 2001; acc. no. AAF91345). Gmm3293 showed strong similarity with *Drosophila pseudoobscura* trypsin-like serine protease (acc. no. XM_001361526). Gmm2574 is identical to *GmPro3*, a serine protease gene previously reported by Hao and Aksoy (2002). Gmm7528 showed a high homology with *GRAAL*, a multi-domain serine protease with an immune function from *Drosophila* (Munier et al., 2004).

Analysis by SignalP indicated the presence of putative signal peptide sequences from Gmm3293, Gmm3216, Gmm3362 and Gmm3230. Two of them, Gmm3293 and Gmm3230, were designated as *Gmmtry1* (acc. no. EU589384) and *Gmmchy1* (acc. no. EU589385), respectively. *Gmmtry1* showed the highest homology to the *Drosophila* trypsin-like gene (GA14937-PA) with 63% identity, whereas *Gmmchy1* showed 82% identity with *Glossina* chymotrypsin-like gene (AF252868).

The deduced amino acids sequences were aligned and showed structural similarities with trypsins and chymotrypsins from other insects (Fig. 1 A, B). The highly conserved catalytic triad for trypsins (H, D, S marked with an *) and cleavage site of putative activation peptides (noted with an arrow) are conserved between R and I (Kraut, 1977). Moreover, the D and G residues that confer trypsin specificity (represented + in Fig. 2A) are also present in *Gmmtry1* (Stroud et al., 1974; Yan et al., 2001). Whereas, the amino acid residues involved in chymotrypsin specificity (noted by +) are present in *Gmmchy1* (Vizioli et al., 2001). The G residue in the substrate

Fig. 2. (A) *Gmmtry1* and *Gmmchy1* transcription during blood meal digestion. Real-time quantitative PCR was performed on RNAs isolated at the designated times after feeding (unfed, 24 h, 48 h) from male midguts. Gray bar indicates *Gmmtry1*; Black bar indicates *Gmmchy1*. Results are presented with bars indicating the mean $\pm$ standard deviation. The GAPDH gene was used as an internal control.

(B) Expression of *Gmmtry1* and *Gmmchy1* in the fat body and different parts of the midgut by RT-PCR. The male midgut was divided into 3 parts. Lane 1; Fat body, Lane 2; Anterior midgut containing the proventriculus (blood free portion), Lane 3; Mid midgut (portion containing red undigested blood), Lane 4; Posterior midgut (portion containing blackish digested blood). GAPDH gene was used as an internal control.

binding pocket is replaced by S at position 250, which is characteristic of chymotrypsins (Kraut, 1977). All sequences contain six spatially conserved cysteine residues that may form three disulfide bridges (marked by C).

Expression after blood feeding and location

of *Gmmtry1* and *Gmmchy1*

Total RNA was isolated at 24 h and 48 h following a blood meal and analyzed by real-time quantitative PCR for *Gmmtry1* and *Gmmchy1* genes. Expression of *Gmmtry1* mRNA was stable at low levels until 48 h post blood meal (PBM), while *Gmmchy1* was slightly detectable in unfed midguts, and dramatically increased at 24 h and 48 h PBM (Fig. 2A). A previous study showed that *Gsp2* and *Gsp1* genes are expressed at high levels in the teneral tsetse fly and their expression levels only vary less than 20% by 48 h PBM (Yan et al., 2001). To further confirm the tissue specific distribution and location of transcription in the midgut, semi-quantitative RT-PCR was conducted on RNA from specific parts of the midgut and the fat body (Fig. 2B). Midguts were divided into three part: the anterior midgut including the proventriculus (blood free region), mid midgut (the rest of the center region) and posterior midgut (blackened digested blood present). Both *Gmmtry1* and *Gmmchy1* mRNA was completely absent in the fat body and anterior midgut. In contrast, both genes were expressed at high levels in the mid midgut and posterior midgut. Hao and Aksoy (2002) reported that putative serine protease, *GmPro3*, is preferentially expressed in the proventriculus.

Trypsin and chymotrypsin enzyme assays in the midgut

The expression profiles of trypsin and chymotrypsin at the protein level were studied by assays for trypsin and chymotrypsin activity in the midgut. Assays followed the methods previously reported by Ramalho-Ortigao et al., (2003). Individual midguts were dissected 6, 24, 48 and 72 h PBM. The equivalent of 1/10 of a single midgut was incubated with BAPNA or AAPF substrates and the colorimetric formation measured after 30 min at 405 nm. As shown in Fig. 3A, trypsin activity was detectable at very low levels even in the unfed midgut. The

Fig. 3. *Glossina morsitans morsitans* midgut trypsin and chymotrypsin activity. (A) Trypsin assays were performed on midguts dissected at various times after a blood meal (6 h, 24 h, 48 h and 72 h) and mixed with 1 mM BAPNA. (B) Chymotrypsin assays were carried out with AAPF as a substrate. Measurements were performed at 405 nm with the end point at 30 min. try and chy in the right lane indicate the positive controls (1 mg/ml).

activity increased sharply at 6 h PBM followed by a reduction at 48 h PBM. At 72 h PBM, the activity returned to slightly higher levels. Similarly, chymotrypsin activity was also evaluated (Fig. 3B). Chymotrypsin activity was undetectable in the unfed midguts of both females and males. At 6 h PBM, activity reached a maximum and then gradually decreased until 48 h PBM. Enzyme activity is relatively higher in midguts from females than males throughout all times except for 48 h PBM.

Gene expression during parasite establishment

Fig. 4. Analysis of transcriptional levels of *Gmmtry1* and *Gmmchy1* in trypanosome infected and self cleared midguts of male flies. Results were normalized using the GAPDH gene as an internal control. Gray bar indicates *Gmmtry1*; Black bar indicates *Gmmchy1*. C, control flies; I, infected flies; S/C, self-cleared flies; 2, 2 days; 8, 8 days; 12, 12 days. Range bars represent the standard deviation and asterisks indicate significant differences compared to the controls ($P < 0.05$).

Transcripts of several genes such as defensin and serine protease increase in the mosquito midgut after infection with *Plasmodium* (Dimopoulos et al., 1997). We examined *Gmmtry1* and *Gmmchy1* up-regulation in response to parasite infection. To investigate immune responsiveness, flies were fed blood containing the bloodstream form trypanosome (1×10^6 /ml). Flies were dissected 2, 8, 12 days PBM and midgut parasite infection scored as either infected or self-cleared. The transcription levels of *Gmmtry1* and *Gmmchy1* on the above days after infection were compared by real-time quantitative PCR (Fig. 4). Although *Gmmtry1* showed no differences in transcription levels at all times assayed, the expression level of *Gmmchy1* was significantly higher in infected midguts than the controls at all times tested ($P < 0.05$). Up-regulation of *Gmmchy1* was statistically significant in day 2, 8, 12 infected midguts compared against the controls.

DISCUSSION

We identified and characterized puta-

tive trypsin and chymotrypsin genes from the tsetse fly *G. m. morsitans*. A total of 18 trypsin and chymotrypsin-like genes were identified from an EST library. *Gmmtry1* and *Gmmchy1* were subjected to further characterization and a high similarity to other insects such as mosquito and sand fly were observed. *Glossina* serine protease genes *Gsp1* (chymotrypsin-like gene) and *Gsp2* (trypsin-like gene) are expressed at high levels in the teneral tsetse fly and remain stable until 48 h PBM suggesting post translational regulation. Both *Gsp1* and *Gsp2* are encoded by one or possibly two loci in the tsetse genome (Yan et al., 2001). *Gmmtry1* mRNA is constitutively expressed upon blood digestion during the digestive cycle. On the other hand, *Gmmchy1* transcripts rapidly increased in response to a blood meal. Although the transcriptional induction of attacin and defensin as well as nitric oxide synthase (NOS) occurs in the pre-ventriculus in response to an immune challenge (Hao et al., 2003), expression of *Gmmtry1* and *Gmmchy1* was observed mainly in the posterior midgut (Fig. 3B). The trypsin activities of *Gsp1* and the chymotrypsin activities of *Gsp2* reached peak levels approximately 24 h after bloodmeal, but the functions for parasite infections were not investigated (Yan et al., 2001). *Gmmtry1* mRNA was constitutively expressed at a low levels after blood digestion and enzyme activity of trypsin increased suggesting other multiple trypsins were involved with digestion during the blood-meal process.

Insect vector midgut proteases may have a direct effect on the parasitic infection and be regulated by the presence of the parasite. Borovsky and Schlein (1987) investigated the possible role of trypsin and chymotrypsin proteases in determining susceptibility of sand flies to certain species of *Leishmania*. In *Phlebotomus papatasi*, a specific component of the trypsin-like activity presents the survival of *Leishmania donovanni* (Borovsky and Schlein, 1987). In *Aedes aegypti*, midgut

trypsin has been shown to activate *Plasmodium* chitinase to traverse the peritrophic membrane (Shahabuddin et al., 1996; Vinetz et al., 2000). In addition, midgut trypsin expression is affected by infection with *Plasmodium* in *Anopheles gambiae* (Bonnet et al., 2001). Real-time quantitative PCR indicated significant differences in *Gmmchy1* expression between control and infected midguts. Because transcription of *Gmmchy1* was up-regulated as parasites transformed to the procyclic form in the midgut from 2 to 8 days after an infectious meal, it is possible that *Gmmchy1* is induced in the presence of trypanosome infections. No significant differences were observed in *Gmmchy1* expression between infected and self-cleared after an infectious meal. A possible explanation is that trypanosome debris may remain in the midgut and cause continuous induction of *Gmmchy1*. The mosquito trypsin-like serine protease, *Sp22D*, consisting of a chitin binding domain, low-density lipoprotein receptor and scavenger receptor cysteine-rich domains also shows an immune response (Danielli et al., 2000). It remains to be seen if the increase in *Gmmchy1* transcripts in infected midguts simply reflects the stimulation of the digestion process by the presence of parasites in the blood meal.

Trypanosomes expressing procyclin become visible approximately 3 h PBM (Gibson and Bailey, 2003). During this stage, the surface glycoproteins of the procyclic forms of *T. brucei brucei* are clipped off by proteolytic enzymes in the midgut of the tsetse fly (Acosta-Serrano et al., 2001; Liniger et al., 2003; Liniger et al., 2004). Therefore, tsetse fly trypsin and chymotrypsin likely trim the glycoproteins from the surface of the trypanosome and play a key role in mechanisms of midgut defense against parasites. Trypsins are also able to kill transitional stage *Leishmania amastigotes* in the midgut of *P. papatasi* (Pimenta et al., 1997). High concentrations of trypsin enzymes result in complete lysis of trypanosomes (Imbuga

et al., 1992). These studies predicted that trypsin directly eliminates the parasites by proteolytic activity in the midgut. Alternatively, the inhibition of parasite development and survival by trypsin and chymotrypsin may reflect a disturbance in the parasites surface coat rather than directly killing the parasites.

Because a large loss of developing parasites occurs predominantly during early establishment in the midgut (Gibson and Bailey, 2003), only a limited number of tsetse flies are able to transmit trypanosome parasites. Several tsetse fly immune factors, including midgut proteases (Imbuga et al., 1992; Osir et al., 1995), lectins (Maudlin and Welburn, 1987), antimicrobial peptides (Boulanger et al., 2002; Hao et al., 2001; Hu and Aksoy, 2005; Hu and Aksoy, 2006), reactive oxygen intermediates (Hao et al., 2003) and antioxidants (MacLeod et al., 2007) are known to respond to establishment of parasites in the midgut.

Although the specific mechanisms of tsetse fly trypsin and chymotrypsin remain to be elucidated, these proteases may cause surface disturbances of parasites in the early stages of infection. Our gene expression analysis suggests that *Gmmtry1* might not be involved with parasite elimination. A possible explanation may be that a certain trypsin and/or chymotrypsin in the tsetse serine protease gene family contributes to cleavage of the surface proteins of the procyclic form and facilitate subsequent attack by the anti-trypanosomal factors described above.

Further research is needed on the direct effect of trypsin and chymotrypsin on the parasite membrane as well as on the expression analysis in response to Gram-negative and Gram-positive bacteria. Recently para-genetic transformation by bacterial symbionts has been developed (Aksoy, 2003; Rio et al., 2004). Using this technique, expression of recombinant proteins may reveal their roles in *Trypanosoma* survival in the tsetse fly midgut.

ACKNOWLEDGEMENTS

We thank Dr. Hiromitsu Tanaka for his technical support. This work was supported by a grant from the Research Fellowships of the Japan Society for the Promotion of Science.

REFERENCES

- Abubakar, L. U., Bulimo, W. D., Mulaa, F. J. and Osir, E. O. 2006. Molecular characterization of a tsetse fly midgut proteolytic lectin that mediates differentiation of African trypanosomes. *Insect. Biochem. Mol. Biol.*, 36: 344–352.
- Acosta-Serrano, A., Vassella, E., Liniger, M., Kunz Renggli, C., Brun, R., Roditi, I. and Englund, P. T. 2001. The surface coat of procyclic *Trypanosoma brucei*: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. *Proc. Natl. Acad. Sci. USA*, 98: 1513–1518.
- Aksoy, S. 2003. Control of tsetse flies and trypanosomes using molecular genetics. *Vet. Parasitol.*, 115: 125–145.
- Aksoy, S., Gibson, W. C. and Lehane, M. J. 2003. Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. *Adv. Parasitol.*, 53: 1–83.
- Bonnet, S., Prevot, G., Jacques, J. C., Boudin, C. and Bourgouin, C. 2001. Transcripts of the malaria vector *Anopheles gambiae* that are differentially regulated in the midgut upon exposure to invasive stages of *Plasmodium falciparum*. *Cell. Microbiol.*, 3: 449–458.
- Borovsky, D. and Schlein, Y. 1987. Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. *Med. Vet. Entomol.*, 1: 235–242.
- Boulanger, N., Munks, R. J., Hamilton, J. V., Vovelle, F., Brun, R., Lehane, M. J. and Bulet, P. 2002. Epithelial innate immunity. A novel antimicrobial peptide with antiparasitic activity in the blood-sucking insect *Stomoxys calcitrans*. *J. Biol. Chem.*, 277: 49921–49926.
- Danielli, A., Loukeris, T. G., Lagueux, M., Muller, H. M., Richman, A. and Kafatos, F. C. 2000. A modular chitin-binding protease associated with hemocytes and hemolymph in the mosquito *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA*, 97: 7136–7141.
- Dimopoulos, G., Richman, A., Muller, H. M. and Kafatos, F. C. 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. Natl. Acad. Sci. USA*, 94: 11508–11513.
- Gibson, W. and Bailey, M. 2003. The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosomes. *Kinetoplastid Biol. Dis.*, 2: 1.
- Hao, Z. and Aksoy, S. 2002. Proventriculus-specific cDNAs characterized from the tsetse, *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol.*, 32: 1663–1671.
- Hao, Z., Kasumba, I. and Aksoy, S. 2003. Proventriculus (cardia) plays a crucial role in immunity in tsetse fly (Diptera: Glossinidae). *Insect Biochem. Mol. Biol.*, 33: 1155–1164.
- Hao, Z., Kasumba, I., Lehane, M. J., Gibson, W. C., Kwon, J. and Aksoy, S. 2001. Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. *Proc. Natl. Acad. Sci. USA*, 98: 12648–12653.
- Hu, C. and Aksoy, S. 2006. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. *Mol. Microbiol.*, 60: 1194–1204.
- Hu, Y. and Aksoy, S. 2005. An antimicrobial peptide with trypanocidal activity characterized from *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol.*, 35: 105–115.
- Imbuga, M. O., Osir, E. O., Labongo, V. L., Darji, N. and Otieno, L. H. 1992. Studies on tsetse midgut factors that induce differentiation of blood-stream *Trypanosoma brucei brucei* in vitro. *Parasitol. Res.*, 78: 10–15.
- Kraut, J. 1977. Serine proteases: structure and mechanism of catalysis. *Annu. Rev. Biochem.*, 46: 331–358.
- Lehane, M. J., Aksoy, S., Gibson, W., Kerhornou, A., Berriman, M., Hamilton, J., Soares, M. B., Bonaldo, M. F., Lehane, S. and Hall, N. 2003. Adult midgut expressed sequence tags from the tsetse fly *Glossina morsitans morsitans* and expression analysis of putative immune response genes. *Genome Biol.*, 4: R63.
- Liniger, M., Acosta-Serrano, A., Van Den Abbeele, J., Kunz Renggli, C., Brun, R., Englund, P. T. and Roditi, I. 2003. Cleavage of trypanosome surface

- glycoproteins by alkaline trypsin-like enzyme(s) in the midgut of *Glossina morsitans*. *Int. J. Parasitol.*, 33: 1319–1328.
- Liniger, M., Urwyler, S., Studer, E., Oberle, M., Renggli, C. K. and Roditi, I. 2004. Role of the N-terminal domains of EP and GPEET procyclins in membrane targeting and the establishment of midgut infections by *Trypanosoma brucei*. *Mol. Biochem. Parasitol.*, 137: 247–251.
- MacLeod, E. T., Maudlin, I., Darby, A. C. and Welburn, S. C. 2007. Antioxidants promote establishment of trypanosome infections in tsetse. *Parasitology*, 134: 827–831.
- Maudlin, I. and Ellis, D. S. 1985. Association between intracellular rickettsial-like infections of midgut cells and susceptibility to trypanosome infection in *Glossina* spp. *Z. Parasitenkd.*, 71: 683–687.
- Maudlin, I. and Welburn, S. C. 1987. Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans*. *Trop. Med. Parasitol.*, 38: 167–170.
- Moloo, S. K. 1971. An artificial feeding technique for *Glossina*. *Parasitology*, 63: 507–512.
- Moloo, S. K., Kabata, J. M. and Sabwa, C. L. 1994. A study on the maturation of procyclic *Trypanosoma brucei brucei* in *Glossina morsitans centralis* and *G. brevipalpis*. *Med. Vet. Entomol.*, 8: 369–374.
- Moloo, S. K., Sabwa, C. L. and Kabata, J. M. 1992. Vector competence of *Glossina pallidipes* and *G. morsitans centralis* for *Trypanosoma vivax*, *T. congolense* and *T. b. brucei*. *Acta Trop.*, 51: 271–280.
- Msangi, A. R., Whitaker, C. J. and Lehane, M. J. 1998. Factors influencing the prevalence of trypanosome infection of *Glossina pallidipes* on the Ruvu flood plain of Eastern Tanzania. *Acta Trop.*, 70: 143–155.
- Munier, A. I., Medzhitov, R., Janeway, C. A., Jr., Doucet, D., Capovilla, M. and Lagueux, M. 2004. *graa1*: a *Drosophila* gene coding for several mosaic serine proteases. *Insect Biochem. Mol. Biol.*, 34: 1025–1035.
- Osir, E. O., Abubakar, L. and Imbuga, M. O. 1995. Purification and characterization of a midgut lectin-trypsin complex from the tsetse fly *Glossina longipennis*. *Parasitol. Res.*, 81: 276–281.
- Pimenta, P. F., Modi, G. B., Pereira, S. T., Shahabuddin, M. and Sacks, D. L. 1997. A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sand fly midgut. *Parasitology*, 115: 359–369.
- Ramallo-Ortigao, J. M., Kamhawi, S., Rowton, E. D., Ribeiro, J. M. and Valenzuela, J. G. 2003. Cloning and characterization of trypsin- and chymotrypsin-like proteases from the midgut of the sand fly vector *Phlebotomus papatasi*. *Insect Biochem. Mol. Biol.*, 33: 163–171.
- Rio, R. V., Hu, Y. and Aksoy, S. 2004. Strategies of the home-team: symbioses exploited for vector-borne disease control. *Trends Microbiol.*, 12: 325–336.
- Ruepp, S., Furger, A., Kurath, U., Renggli, C. K., Hemphill, A., Brun, R. and Roditi, I. 1997. Survival of *Trypanosoma brucei* in the tsetse fly is enhanced by the expression of specific forms of procyclin. *J. Cell Biol.*, 137: 1369–1379.
- Shahabuddin, M., Lemos, F. J., Kaslow, D. C. and Jacobs-Lorena, M. 1996. Antibody-mediated inhibition of *Aedes aegypti* midgut trypsins blocks sporogonic development of *Plasmodium galinaceum*. *Infect. Immun.*, 64: 739–743.
- Stroud, R. M., Kay, L. M. and Dickerson, R. E. 1974. The structure of bovine trypsin: electron density maps of the inhibited enzyme at 5 Angstrom and at 2–7 Angstrom resolution. *J. Mol. Biol.*, 83: 185–208.
- Vickerman, K., Tetley, L., Hendry, K. A. and Turner, C. M. 1988. Biology of African trypanosomes in the tsetse fly. *Biol. Cell.*, 64: 109–119.
- Vinetz, J. M., Valenzuela, J. G., Specht, C. A., Aravind, L., Langer, R. C., Ribeiro, J. M. and Kaslow, D. C. 2000. Chitinases of the avian malaria parasite *Plasmodium gallinaceum*, a class of enzymes necessary for parasite invasion of the mosquito midgut. *J. Biol. Chem.*, 275: 10331–10341.
- Vizioli, J., Catteruccia, F., della Torre, A., Reckmann, I. and Muller, H. M. 2001. Blood digestion in the malaria mosquito *Anopheles gambiae*: molecular cloning and biochemical characterization of two inducible chymotrypsins. *Eur. J. Biochem.*, 268: 4027–4035.
- Welburn, S. C. and Maudlin, I. 1999. Tsetse-trypanosome interactions: rites of passage. *Parasitol. Today*, 15: 399–403.
- WHO. 2001. Scientific Working Group on African Trypanosomiasis (Sleeping Sickness). WHO/Tropical Disease Research Unit, Geneva.
- Yan, J., Cheng, Q., Li, C. B. and Aksoy, S. 2001. Molecular characterization of two serine proteases expressed in gut tissue of the African trypanosome vector, *Glossina morsitans morsitans*. *Insect Mol. Biol.*, 10: 47–56.