Molecular Characterization of Serine Proteases from both First and Third Larval Instars Of *Chrysomya megacephala*

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Abstract: The analysis of excretory/secretory products from third larval instar of *C. megacephala* using SDS-gel electrophoresis produces a band at 16KDa, band between 16KDa and 23KDa, and a broad band between 23 and 45KDa. The PCR product produced bands at 573 bp for trypsin of both first and third instars of *C. megacephala* and 715 bp for chymotrypsin of both instars. Comparisons with other dipterans trypsin and chymotrypsin showed that all the *C. megacephala* sequences have identity with other dipterans sequences.
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1. Introduction:

Blowflies are distributed worldwide and cause medical problems and losses to the animal industry (Zumpt, 1965; Greenberg, 1971, 1973; Kuhlhorn, 1983; Ghandour, 1988). Norris (1965) gave a comprehensive review on the bionomics of blowflies. Among blowflies, the oriental latrine fly *Chrysomya megacephala* (Fabricius) is one of the most common blowflies in Egypt (Gabre, 1994) and its range is expanding (Greenberg, 1988; Wells, 1991; Tomberlin *et al*., 2001). Greenberg (1971, 1973) reported that this species is among the most dangerous dipteran vectors of enteric pathogens. In Malaysia, *C. megacephala* is the dominant vector of helminthes parasite eggs (Sulaiman *et al*., 1988, 1989). On the positive side, *C. megacephala* is an important pollinator of mango in Australia (Anderson *et al*., 1982), and in Taiwan, farmers increase *C. megacephala* population to increase pollination of mango (Hu *et al*., 1995).

Proteolytic enzymes are a major component of the digestive process of parasites and are presumed to be released to interact with host tissues (Rhoads and Fetterer, 1997). Parasitic and microbial organisms utilize the digestive actions of proteases on proteins of cells, tissues and organs for the purposes of invasion and migration in host tissues.

The serine proteases are the dominant class of proteolytic enzymes in many insect species (Applebaum, 1985; Terra and Ferreira, 1994; Terra *et al.*, 1996). The parasitic insects of mammals have used the serine proteases in establishing suitable environments upon or within their hosts (Bowles *et al*., 1988; Sandeman *et al.*, 1990). Commonly, the same group of proteases is used for nutrient digestion within and without the gut (Casu *et al.*, 1994, Bowles VM *et al*., 1990).

These proteases are thought to be potential targets for vaccines or pesticides that disrupt establishment of larval stages on or within the host, and interfere with the digestion of food in the gut of larval and adult stages (Tellam and Bowles, 1997).

Serine protease (SPs) constitutes one of the largest families' enzymes in the animal kingdom. They play important roles in dietary protein digestion, blood clotting, immune responses, signal transduction, hormone activation, inflammation, and development [Herrero *et al*., 2005; O'Connell *et al* 2006]. SPs are characterized by an a.,ctive site termed the catalytictriad, which contains H, D, and S amino acids. The serine residue at the active site participates in the formation of a transient acylenzyme intermediate between the substrate and the protease (Rawlings and Barrett, 1994). Trypsin and chymotrypsin are among the best-characterized serine proteinases in the insect's digestive system (Sudeshna *et al.*, 2000).

Numerous clinical reports have been published that describe the outstanding effects of maggot therapy, most notably on debridement, cleansing, disinfection and healing of indolent wounds, many of which have previously failed to respond to conventional treatment (Baer, 1931, Weil *et al*., 1933; Thomas *et al*., 1996; Johnson *et al*., 1999; Thomas and Jones, 2000; Graninger *et al*., 2002; Sherman, 2003; Sherman *et al*., 2004). Current day maggot therapy, with its multi-action approach to wound cleansing and healing, is highly successful**.** Enzymes can be produced from any living organism, either by extracting them from their cells or by recovering them from cell exudates**.** The molecules involved in the beneficial effects of maggots are believed to be contained in their excretions/secretions (ES).

Antibiotic resistance has become a global public-health problem, thus it is imperative that new antibiotics continue to be developed.

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response, which is the principal defense system for the majority of living organisms, and are found among all classes of life ranging from prokaryotes to humans (Herrero *et al*. ,2005; O'Connell *et al*., 2006). They represent a new family of antibiotics that have stimulated research and clinical interest (Rawlings and Barrett 1994).

In the present study the excretory/secretory products of third larval instar of *C. megacephala* were analyzed by using gel electrophoresis. The cDNA was completely sequenced encoding a trypsin-like and a chymotrypsin-like proteinase and their respective genomic DNA from first and third larval instars of *C. megacephala*. In addition, the predicted protein sequences used for extensive phylogenetic comparison with Dipteran trypsins and chymotrypsins. The aim of this study is to ensure the production of serine proteases especially trypsin and chymotrypsin by larvae of *C.megacephala* which may lead us in the near future to extract and use these proteolytic enzymes in maggot therapy.

2- Material and Methods

2.1. Rearing of insect

The laboratory colony of *C. megacephala* used in this study was established in the Department of Entomology, Faculty of Science, Helwan University**.** *C. megacephala* was reared according to the method of Gaber *et al*., 2005.

Adults from the stock colony of *C. megacephala* were kept in cages (38 × 38 × 56 cm) at 25 ± 3 °C, 14 hrs photoperiod and 60–70% R.H. The cages were made with a wooden floor, a glass roof, and wire gauze on three of the sides. The fourth side was wooden with a circular hole fitted with a cloth sleeve to facilitate daily feeding, cleaning of the cage, and removal of eggs. Adults were supplied daily with granular sucrose, water, and beef meat (beef hereafter).Water was supplied by dipping a piece of cotton as a wick in a bottle filled with water, and the beef was provided in a Petri- dish. Egg batches were removed daily and transferred to a fresh piece of beef placed in a rearing enamel bowl (35 cm in diameter) covered with muslin secured with a rubber band. At the prepupal stage, dry autoclaved sawdust was added to the bowl as a medium for pupation. Pupae were sieved from the sawdust and transferred to adult cages described above for adult emergence.

2.2. Collection of larval secretions

Native excretions/secretions (nES) were collected by incubating third-instar larvae of *C.megacephala* in a small quantity (100 larvae per 1ml) of sterile Milli-Q ultrapure water (Millipore UK Ltd., Hertfordshire, UK) for 1 h at 30 °C in darkness. The sterile liquid was siphoned from the containers and centrifuged at 10,000 × *g* for 5 min to remove particulate material, after which the supernatant was retained for testing.

2.3. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The individual components of ES products were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). ES products were concentrated by ultra- filtration followed by precipitation in 80 % (v/v) ice-cold acetone. The precipitate was redissolved in reducing sample buffer and boiled for 5 minutes prior to electrophoresis on 12 % polyacrylamide gel. Proteins bands were detected by staining with 0.25 % Coomassie brilliant blue R250 in 25 % ethanol, 10% acetic acid followed by distaining in the same solvent without the Coomassie stain. Proteinase components were examined after separation on substrate gels.

2.4. Polymerase chain reaction (PCR)

Degenerate oligonucleotide primers were synthesized using a Gene Assembler Plus oligonucleotide synthesizer (Pharmacia). Design of the forward PCR primers was based on the amino-terminal amino acid sequences of purified *Chrysomya bezziana* trypsin (F1) or chymotrypsin (F2). The sequences of the reverse primers were based on conserved amino acids surrounding the active site residue Ser-195 (Elvin *et al*., 1993). In order to limit primer redundancy, three reverse primers, termed R1, R2 and R3 were used (Elvin *et al*., 1993, 1994).

The sequences of the primers are as follows:

F1 (trypsin): 5-AT (TCA) GGNAA (TC) TT (TC) CCNTGGCA (AG) GT-3:

F2 (chymotrypsin): 5-CCNGGNCA (AG) TT (TC) CCNTA (TC) CA (AG) G-3;

R1:-5-A(GA)NGGNCCNCCNGA(AG)TCNCC-3;

R2: 5-A(GA)NGGNCCNCC(AG)CT(AG)TCNCC-3;

R3: 5-A(GA)NGGNCCNCCNGT(AG)TCNCC-3.

R1 and R2 reverse primers were used to amplify trypsin cDNAs, whilst all three reverse primers were used to amplify chymotrypsin cDNAs.cDNA was prepared from mRNA isolated from homogenates of first and third instar larvae of *Chrysomya megacephala.*

Briefly, total RNA was extracted from larvae using TRIZOL (Life Technologies, Grand Is., and

NY). MRNA was purified from total RNA by affinity chromatography on an oligodT affinity column (Invitrogen, Groningen, the Netherlands) and double stranded cDNA was synthesized using a riboclone cDNA synthesis kit (Promega, Madison, WI). PCRs were conducted in 100 _l volumes containing 3 mM MgCl2, 0.25 mM of each dNTP, 100 pmol of each primer, 2.5 unit *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 5 ng DNA, through 40 thermocycles (2 min, 94 °C; 1 min, 50°C and 2 min, 72 °C).

2.4- Sequence and phylogenetic analyses

Sequences were carried out in the RNA sequencing facility at Jenagen Labs Corporation, Germany. Searches of databases were carried out using BLAST programs at the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/).

3-Results

i. Total protein

The total protein measured from native excretory/secretory product of 100 larvae per I ml distilled water was 0.9 mg.

Ii.Gel electrophoresis

The analysis of native excretory/secretory (100 larvae/1ml or 0.9mg protein) products from third larval instar of *Chrysomya megacephala* produces a single band at 16KDa,a band between 16KDa and 23KDa and a broad band between 23KDa and 45KDa (Fig. 1).

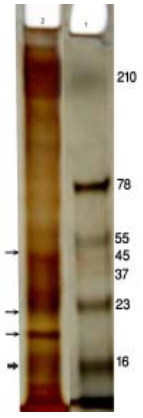

Fig. 1: Electrophoretic pattern using SDS-gel electrophoresis. Lane (1) representing marker and lane (2) representing native excretory/secretory product produced from third larval instars of *Chrysomya megacephala*

iii. RT-PCR product

The F1 forward primer and R1 or R2 reverse primers were used to amplify *Chrysomya megacephala* trypsin cDNAs, and the F2 forward primer and all three reverse primers were used to amplify *Chrysomya megacephala* chymotrypsin cDNAs. The amplified product resulting from PCR using cDNAs isolated from both first and third larval instars of *Chrysomya megacephala* produce bands between 500bp and 715bp (Fig. 2). For trypsin of both instars the amplified PCR product is at 573bp while for chymotrypsin of both instars the amplified PCR product is at 715bp (Fig. 2, Table 1).

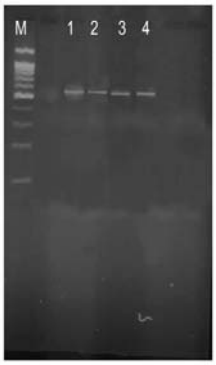

Fig. 2: Electrophoretic pattern showing rt-pcr product.
Lane (M) representing marker,
Lane (1) representing trypsin of first larval instar
Lane (2) representing trypsin of third larval instar
Lane (3) representing chymotrypsin of first larval instar Lane (4) representing chymotrypsin of third larval instar

Table 1: Showing molecular weight bands for lanes 1-4 against 100bp ladder

Molecular weight	Lane 1	Lane 2	Lane 3	Lane 4
900				
800				
			715	715
700				
600				
	573	573		
500				
400				
300				
200				
100				

BLAST Results

An overview of the database sequences aligned to the query sequence of the studied species using BLAST showed that sequence of chymotrypsin from both first and third larval instars of *Chrysomya megacephala* has 84% identity with *Drosophila melanogaster* 211000022280419

(gb|AABU01002389.1) (Celniker *et al*., 2007), *Drosophila ananassae* strain TSC#14024-0371.13 (gb|AAPP01004053.1|) (Clark *et al*.,2007), *Drosophila melanogaster* mitochondrion (ref|NC_001709.1|) (Lewis *et al*., 1995), *Drosophila grimshawi* strain TSC#15287-2541.00 Ctg01_3398 (gb|AAPT01003399.1|) (Clark *et al*.,2007), *Drosophila mojavensis* strain TSC#15081-1352.22 Ctg01_3824 (gb|AAPU01003823.1|) (Clark *et al*.,2007), *Drosophila virilis* strain TSC#15010-1051.87 Ctg01_17602 (gb|AANI01017563.1|) (Clark *et al*.,2007),83% identity with *Drosophila mojavensis* strain TSC#15081-1352.22 Ctg01_1947 (gb|AAPU01001946.1|) (Clark *et al*.,2007), *Drosophila virilis* strain TSC#15010-1051.87 Ctg01_7082 (gb|AANI01007064.1|) (Clark *et al*.,2007), *Drosophila mojavensis* strain TSC#15081-1352.22 Ctg01_5844 (gb|AAPU01005843.1|) (Clark *et al*.,2007), *Drosophila virilis* strain TSC#15010-1051.87 Ctg01_4771 (gb|AANI01004754.1|) (Clark *et al*.,2007) , *Drosophila mojavensis* strain TSC#15081-1352.22 Ctg01_3366 (gb|AAPU01003365.1|) (Clark *et al*.,2007), *Drosophila ananassae* strain TSC#14024-0371.13 Ctg01_16291 (gb|AAPP01016269.1|)(Clark *et al*.,2007), 85% identity with *Drosophila willistoni* strain TSC#14030-0811.24 1099000011229 (gb|AAQB01011230.1|) (Clark *et al*.,2007), 82% identity with *Drosophila virilis* strain TSC#15010-1051.87 Ctg01_18064 (gb|AANI01018023.1|) (Clark *et al*.,2007), *Drosophila mojavensis* strain TSC#15081-1352.22 Ctg01_4387 (gb|AAPU01004386.1|) (Clark *et al*.,2007), 86% identity with *Drosophila willistoni* strain TSC#14030-0811.24 1099000002653 (gb|AAQB01002654.1|) (Clark *et al*.,2007), 80% identity with *Drosophila virilis* strain TSC#15010-1051.87 Ctg01_17027 (gb|AANI01016988.1|) (Clark *et al*.,2007),87% identity with *Drosophila willistoni* strain TSC#14030-0811.24 1099000006770 (gb|AAQB01006771.1|) (Clark *et al*.,2007), 74% identity with *Nasonia vitripennis* genomic contig, reference assembly (based on Nvit_1.0 SCAFFOLD916)(ref|NW_001820656.1|NviUn_WGA916_1), 75% identity with Nasonia vitripennis genomic contig, reference assembly (based on Nvit_1.0 SCAFFOLD423) (ref|NW_001818170.1|NviUn_WGA423_1), 73% identity with Nasonia vitripennis genomic contig, reference assembly (based on Nvit_1.0 SCAFFOLD2404) (=ref|NW_001816131.1|NviUn_WGA2404_1) Table 2).

The sequence of trypsin from both instars of *C. megacephala* has100% identity with *Chrysomya bezziana* clone CbSp22 serine protease K4.1/F1R2 mRNA, partial cds (AF302489.1) (Murshani *et al*.,2001),99% identity with *Chrysomya bezziana* clone CbSp20 serine protease K6.1/F1R1 mRNA, partial cds (AF302487.1) (Murshani *et al*.,2001),99% identity with *Chrysomya bezziana* clone CbSp13 serine protease K6.2/F1R2 mRNA, partial cds (AF302480.1) (Murshani *et al*.,2001),98% identity with *Chrysomya bezziana* clone CbSp14 serine protease K4.1/F1R2 mRNA, partial cds (AF302481.1) (Muharsini *et al*.,2001),95% identity with *Chrysomya bezziana* clone CbSp15 serine protease K17/F1R2 mRNA, partial (AF302482.1) (Murshani *et al*.,2001) and *Chrysomya bezziana* clone CbSp1 serine protease K14/F1R2 mRNA, partial cds (AF302468.1) (Murshani *et al*.,2001),94% identity with *Chrysomya bezziana* clone CbSp10 serine protease K10/F1R1 mRNA, partial cds (AF302477.1) (Murshani *et al*.,2001),93% identity with *Chrysomya bezziana* clone CbSp12 serine protease K8/F1R1 mRNA, partial cds (AF302479.1) (Murshani *et al*.,2001),93% identity with *Chrysomya bezziana* clone CbSp19 serine protease K16/F1R2 mRNA, partial cds (AF302486.1) (Murshani *et al*.,2001),91% identity with *Chrysomya bezziana* clone CbSp21 serine protease K2.2/F1R2 mRNA, partial cds (AF302488.1) (Murshani *et al*.,2001) (Table 3,).

Table 2 Sequences producing significant alignments using blast between chymotrypsin of both first and third larval instars of *C.megacephala* and other Dipterans sequences.

Accession	Description	Max. score	Total score	E value	Max. ident.
gb|AABU01002389.1|	Drosophila melanogaster 211000022280419, w... 0.0	710	710	0.0	84%
gb|AAPP01004053.1|	Drosophila ananassae strain TSC#14024-0371...0.0	704	704	0.0	84%
ref|NC_001709.1|	Drosophila melanogaster mitochondrion, compl...0.0	693	693	0.0	84%
gb|AAPT01003399.1|	Drosophila grimshawi strain TSC#15287-2541... 0.0	689	689	0.0	84%
gb|AAPU01003823.1|	Drosophila mojavensis strain TSC#15081-135...0.0	678	678	0.0	83%
gb|AAPU01001946.1|	Drosophila mojavensis strain TSC#15081-135... 0.0	673	673	0.0	84%
gb|AANI01017563.1|	Drosophila virilis strain TSC#15010-1051.8...0.0	673	673	0.0	83%
gb|AANI01007064.1|	Drosophila virilis strain TSC#15010-1051.8...0.0	667	667	0.0	85%
gb|AAQB01011230.1|	Drosophila willistoni strain TSC#14030-081... 0.0	665	665	0.0	83%
gb|AAPU01005843.1|	Drosophila mojavensis strain TSC#15081-135... 0.0	662	662	0.0	83%
gb|AANI01004754.1|	Drosophila virilis strain TSC#15010-1051.8...0.0	656	656	0.0	82%
gb|AANI01018023.1|	Drosophila virilis strain TSC#15010-1051.8...7e-171	608	608	0.0	83%

Accession	Description	Max. score	Total score	E value	Max. ident.
gb|AAPU01003365.1|	Drosophila mojavensis strain TSC#15081-135...2e-146	527	527	0.0	86%
gb|AAQB01002654.1|	Drosophila willistoni strain TSC#14030-081... 2e-136	494	494	0.0	85%
gb|AAPU01004386.1|	Drosophila mojavensis strain TSC#15081-135...1e-114	422	422	0.0	80%
gb|AANI01016988.1|	Drosophila virilis strain TSC#15010-1051.8...4e-108	399	399	0.0	80%
gb|AAPP01016269.1|	Drosophila ananassae strain TSC#14024-0371...2e-106	394	394	0.0	83%
gb|AAQB01006771.1|	Drosophila willistoni strain TSC#14030-081...5e-93	350	350	0.0	87%
ref|NW_001820656.1|NviUn_WGA916_1	Nasonia vitripennis genomic...5e-63	250	250	0.0	74%
ref|NW_001818170.1|NviUn_WGA423_1	Nasonia vitripennis genomic...1e-49	206	206	0.0	75%
ref|NW_001816131.1|NviUn_WGA2404_1	Nasonia vitripennis genomi...8e-41	176	176	0.0	73%

Table 3: Sequences producing significant alignments using blast between trypsin of both first and third larval instars of *C. megacephala* and other Dipterans sequences

Accession	Description	Max score	Total score	E value	Max ident	Links
AF302489.1	Chrysomya bezziana clone CbSp22 serine protease K4.1/F1R2 mRNA, partial cds	970	970	0.0	100%	
AF302487.1	Chrysomya bezziana clone CbSp20 serine protease K6.1/F1R1 mRNA, partial cds	929	929	0.0	99%	
AF302480.1	Chrysomya bezziana clone CbSp13 serine protease K6.2/F1R2 mRNA, partial cds	929	929	0.0	99%	
AF302481.1	Chrysomya bezziana clone CbSp14 serine protease K4.1/F1R2 mRNA, partial cds	926	926	0.0	98%	
AF302482.1	Chrysomya bezziana clone CbSp15 serine protease K17/F1R2 mRNA, partial cds	826	826	0.0	95%	
AF302468.1	Chrysomya bezziana clone CbSp1 serine protease K14/F1R2 mRNA, partial cds	824	824	0.0	95%	
AF302477.1	Chrysomya bezziana clone CbSp10 serine protease K10/F1R1 mRNA, partial cds	769	769	0.0	94%	
AF302479.1	Chrysomya bezziana clone CbSp12 serine protease K8/F1R1 mRNA, partial cds	767	767	0.0	93%	
AF302486.1	Chrysomya bezziana clone CbSp19 serine protease K16/F1R2 mRNA, partial cds	760	760	0.0	93%	
AF302488.1	Chrysomya bezziana clone CbSp21 serine protease K2.2/F1R2 mRNA, partial cds	719	719	0.0	91%	

4-Disscussion:

The analysis of native excretory/secretory products from early third instar of *Chrysomya megacephala* produces a single band at 16 KDa, a band between 16 KDa and 23 KDa and a broad band between 23 KDa and 45 KDa. Insect trypsins have been characterized and purified from species of Coleoptera, Orthoptera, Lepidoptera and Diptera. Most insect trypsins are 20–30 kDa as determined by SDS-PAGE **(Terra and Ferreira, 1994)**. These insect trypsins are most active at alkaline pH, are not activated by calcium ions, and are sensitive to natural trypsin inhibitors **(Terra and Ferreira, 1994).** Recently, workers in Nottingham, UK, demonstrated *in vitro* a range of enzymes secreted by *Phaneicia sericata* larvae **(Chambers *et al.*, 2003)**. Four proteolytic enzymes, comprising two serine proteases, a metalloproteinase and an aspartyl proteinase, were detected, with molecular weights ranging from 20 to 40 kDa, with activity across a wide pH range.

In the present study the amplified product resulting from PCR using cDNA isolated from whole first and third instars of *Chrysomya megacephala* formed a band from about 500 bp to 715 bp. Chymotrypsin isolated from whole first and third instars of *Chrysomya megacephala* showed band at 715 bp while trypsin showed band at 573 bp. At the molecular level, an analysis of a cDNA library showed 40 clones of 115–810 bp representing putative digestion-associated proteins **(Pedra *et al*., 2003).** 12 clones to trypsin (533–810 bp) and 22 clones to chymotrypsin (115–806 bp) two other clones of 691 and 710 bp were classified to belong to different serine proteinases **(Kollien *et al*.,2004).**

Chymotrypsin of both first and third instars of *C.megacephala* (Cmchy) shows a 73-75% identity with chymotrypsins of *Nasonia* species and 80-87% with chymotrypsins of *Drosophila* species. Trypsin of both instars of *C.megacephala* (Cmtry) shows 91-100% identity with trypsins of *C.bezziana*.

As serine proteinases have different functions, gene families of trypsin and chymotrypsin have been described in many insects. In the noctuid moth *Mamestra configurata* and the lesser grain borer *Rhyzopertha dominica* eight and three genes encoding trypsin were identified, respectively **(Hegedus *et al*., 2003).**

Sequences of seven trypsin-like genes of a blood-sucking crustacean, the salmon louse *Lepeophtheirus salmonis*, show high similarities in their nucleotides and corresponding amino acid residues, differences ranging from one amino acid residue between trypsin types 1 *and 4 to 18 amino acid residues between types 2 and 7* ***(Johnson et al.*, 2002)**. A blood-sucking Diptera, *Haematobia irritans exigua*, possesses at least four genes encoding trypsin **(Elvin *et al*., 1993)**. In the malaria vector *Anopheles gambiae*, seven different genes encoding trypsins are clustered within 11 kb and two genes encoding chymotrypsin within 6 kb **(Vizioli *et al*., 2001)**. In the gut of female *Aedes aegypti* one constitutively transcribed early trypsin is augmented by two blood meal-induced late trypsins **(Barillas-Mury *et al*., 1995).**

Considering chymotrypsin, at least three and eight genes are expressed in larvae of the Lepidoptera *Helicoverpa zea*, *Agrotis ipsilon* and *Mamestra configurata*, respectively **(Hegedus *et al*., 2003).**

These numbers of trypsin and chymotrypsin encoding genes represent only a minimum number, since more than twenty genes encoding trypsin were identified during genome analysis of the fruit fly *Drosophila melanogaster* **(Wang *et al*., 1999).**

Graf and Briegel (1985) identified at least 20 different trypsin isozymes in midgut homogenates prepared from blood-fed female mosquito *(Aedes aegypti).*

In bloodsucking insects, such as mosquito or stable fly, *(Stomoxys calcitrans)* the vast majority (75%-85%) of proteolytic activity involved in blood meal digestion is due to serine proteases, representing both trypsin- and chymotrypsin-like activity **(Briegel and Lea 1975)**. **Muharsini *et al*., (2001)** using PCR analysis and subsequent cloning and sequencing identified 22 different sequences falling into two trypsin and four chymotrypsin families from larvae of *Chrysomya bezziana.*

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