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Redundancy of proteins in the salivary glands of *Panstrongylus megistus* secures prolonged procurement for blood meals☆

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ABSTRACT

Panstrongylus megistus, a vector for the Chagas disease parasite *Trypanosoma cruzi*, is a hematophagous bug widely distributed in South America. This ubiquitous triatomine is known to colonize different wild life habitats. Additionally, *P. megistus* synanthropy, preying upon mammals, birds, reptiles, and eventually being predators upon insect's hemolymph probably increases its ability to survive after prolonged fasting. It was suspected that the *P. megistus* mechanisms of adaptation to survival might include a salivary gland complex tool-box with a diversity of pharmacologically active proteins for obtaining blood meals. Herein we describe comprehensive proteome and transcriptome of the *P. megistus* salivary gland. The proteomic analysis led to the identification of 159 proteins, and the transcriptome revealed 47 complete cDNAs. A diversity of protein functions associated to blood feeding was identified. The most prevalent proteins were related to blood clotting, anti-platelet aggregation and anti-vasoconstriction activities, which correlate with the insect's ability to obtain meals from different sources. Moreover, a gene of resistance to insecticides was identified. These features augments the comprehension towards *P. megistus* enormous capacity to survive in adverse wild life-changing habitats.

1. Introduction

The cone-nosed bugs (Hemiptera: Reduviid) of the subfamily *Triatominae* are widespread vectors of *Trypanosoma cruzi* agent of Chagas disease in South America. *P. megistus* is historically important because it was the insect-transmitter of the protozoan parasite found in the blood of a child with an acute disease described by Dr. Carlos Chagas in 1909 [1]. *Triatoma infestans*, *Rhodnius prolixus* and *Panstrongylus megistus* used to be main vectors of *T. cruzi* to the human population that inhabits in the American continent. During the last 30 years there was a change with the successful insecticide dislodgment of *T. infestans* from human dwellings in the dry ecosystems. Consequently, the ubiquitous *P. megistus* inhabiting various ecosystems were not targeted by insecticides. Lately, the sylvatic *P. megistus* have invaded the peri-domicile [2,3] previously occupied by

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T. infestans, and now it has become an increasing threat to the health of the human populations in the hinterland. The wild-life *P. Megistus*, hiding away in ground burrows, birds nest, and crevices on tree barks, in different ecosystems are not susceptible to insecticide spraying and household infestations cannot be prevented. Nowadays, *P. megistus* are highly adapted to wild-life and to peri-domestic niches in a variety of ecosystems. With an enormous adaptability to different environments and synanthropic blood feeding, *P. megistus* have become the most important vector of Chagas disease in the Brazilian territory [4].

Interestingly, many authors have suggested that *P. megistus* blood-sucking habit appears to be a recent acquisition, because they share some morphological resemblances with both plant-sucking triatomines. In addition, *P. megistus* appears to have undergone morphological modifications over last two centuries, since species from different geographical areas appear to have major differences in the contents of salivary glands bioamines [5,6]. The evidences have suggested that the alimentary habits of this insect has changed over time, and thus the possibility exists that some factors involved in food uptake and digestion could explain its ability to survive in different habitats [7].

Regardless of its high epidemiologic importance in the transmission of Chagas disease, there is a paucity of data and information on the *P. megistus* salivary glands protein contents. For example, it is recognized that to counteract imposing difficulties towards the acquisition of a blood meal, *R. prolixus* [8], *T. infestans*[9], *T. brasiliensis*[10] and *T. pallidipennis*[11] have accumulated in their salivary glands several proteins involved in vasoconstriction, anti-platelet aggregation and coagulation. It was reported that such functional proteins appears to a much lesser extent in *P. megistus* salivary glands [7]. Differently, it was shown that the *P. megistus* saliva has a high content of anti-complement [12] and anticoagulant functions [13]. In this regard, there is additional interest to investigate this species salivary glands because the insect´s behave suggest possible variations in the mechanisms associated to preventing blood loss during its bite.

In this study we identified 159 proteins in the salivary proteome from *P. megistus*, of which 28 proteins have known blood-feeding activities. Also, we show sequences of 47 transcripts obtained from a cDNA library from *P. megistus* salivary glands, which were co-validated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique. Furthermore, we propose that a notable redundancy of pharmacologically active proteins in the *P. megistus* salivary glands is associated with the ubiquitous behavior of this hematophagous cone-nose bug.

2. Materials and methods

2.1. *Triatomines and salivary glands collection*

Adult *P. megistus* were captured in dry savannah-like ecosystem were brought to our laboratory and reared with controlled temperature of 28 °C, 70% relative humidity and 12:12 h light/dark photoperiod. Fifty pairs of salivary glands were dissected from adult insects after 3, 5 and 7 days post blood-feeding in mice and placed in TRIZOL reagent (Invitrogen). For the mass spectrometry experiment we collected 50 pairs of salivary glands of adults after 3,5 and 7 days after blood-feeding. The salivary glands were punctured and centrifuged at 14.000 ×*g* for 5 min, and the supernatant was collected and immediately lyophilized and stored at −80 °C.

2.2. *cDNA library construction and transcripts analysis*

To isolated the mRNA and construct the cDNA library we used the technical procedures described on the manufacturer's protocol with some adaptations [9], using Micro-fast Track™ mRNA isolation (Invitrogen) and SMART cDNA library construction (Clontech) kits. After obtaining the phages with expected insert cells were platted on LB/MgSO4 plates with X-gal/IPTG with 15 μl cDNA library unamplified. Among 90% of the recombinants were white colonies, which were picked-up and transferred individually to 50 μl of pure water. The cDNA that was amplified with different primers sets [14] were sequenced unidirectionally with PT2F3 primer and DYEnamic ET DyeTerminator Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA) using MegaBACE 1000 sequencer (Amersham Biosciences, Little Chalfont, UK). The transcripts were subjected to analysis with BLAST algorithm [15], which results were dated January, 2008. ClustalW [16], CDD [17], and TREEview software[18] were used. The transcripts were translated using the BioEdit program and the resulting polypeptide sequences were subject to phylogenetics analysis. The statistical neighbor-joining bootstrap phylogenies tests were done with de MEGA4 package [19] and Peptide Signal predicted [20].

2.3. *Mass spectrometry by LC-MS*

The *P. megistus* lyophilized salivary gland proteins were diluted in 200 μl of HPLC grade pure-water (Sigma), and 10 μl of the solution was dried-out in a speed vac and redisolved in 40 μl 400 mM NH_4HCO_3 containing 8 M urea. The sample was then reduced with 5 mM dithiothreitol (DTT) for 15 min at 50 °C, alkylated with iodacetamide 100 mM for 15 min at room temperature, and protected from the light. The sample was diluted to 1 M urea final concentration and mixed with 4 μg of trypsin (Modified sequencing grade trypsin, Promega), and the digestion proceeded overnight at 37 °C. The samples that were desalted with a lab manufactured reverse phase ziptip (POROS R2-50 resin, Applied Biosystem) [21] and submitted to fractionation in a strong-cation exchange ziptip (POROS HS50, Applied Biosystems, Framingan, MA) were eluted with an increasing concentration of NaCl (0, 10, 20, 40, 60, 80, 100, and 150, 200 and 500 mM) [22]. After desalinization, each fraction was submitted to liquid chromatography mass spectrometry (LC-MS) analysis. The peptides were dissolved in 10 μl 0.05% formic acid and separated in a nanoHPLC system (1D plus, Eksigent, Dublin, CA) and analyzed by an eletrospray linear ion trap mass spectrometer (LTQ XL™, ThermoFisher Scientific, San Jose, CA) as previously described [22]. At first the raw data were converted to DTA files by BioWorks version 3.3.1 software (ThermoFisher Scientific) with the following parameters: threshold 10 counts, at least 15 ions and precursor mass range from 600 to 3500 Da. The DTA files were submitted to analysis using TurboSequest (Bioworks 3.3.1, ThermoFisher Scientific, San Jose, CA) [23] algorithm against the NCBInr database (downloaded on February

14, 2007), and considering 2.0 Da as precursor ion tolerance, cysteine carbamidomethylation and methionine oxidation as fixed and variable modifications, respectively. After the database search analyses the following filters were applied: distinct peptides (for exclusion of redundant hits); DCn≥0.1; protein probability≤1×10^{-3}; and Xcorr≥1.5, 2.2, and 2.7 for singly, doubly, and triply charged peptides, respectively. Then we performed a second analysis with another database which included (*i*) *Triatoma, Panstrongylus* and *Rhodnius* protein sequences from GenBank (downloaded on November 15, 2007); (*ii*) translated transcripts from the cDNA libraries from *P. megistus, R. brethesi*, *R. robustus* and *T. infestans*[9]; (*iii*) sequences from matches from the first analysis against NCBInr; and (*iv*) randomly generated protein sequences (100 K), used as false-positive entries (for calculating the false-discovery rate). The false-discovery rate was calculated to be 1.8% [22].

3. Results and discussion

3.1. ESTs sequences

The unidirectional sequencing of 47 transcripts of *P. megistus* cDNA library showed the presence of expected housekeeping genes such as elongation factors, ribosomal proteins, and translation initiation factors (Supplementary Table 1). We also found several new transcripts that matched described proteins with unknown function; one of these transcripts (GenBank accession number: 57657798) showing a signal peptide appears to indicate that a putative protein is probably secreted in the salivary gland lumen possibly during the blood feeding (Supplmentary Table 1). Besides, we found two secreted peptides previously described in *T. brasiliensis*[10] and *T. infestans*[9], and a hypothetical protein was also found with similarity to a *T. infestans* derived peptide. Altogether, the transcripts deduced proteins with blood-feeding functions comprised mostly lipocalins, pallidipins, triabins and triatins (Table 1).

3.2. LC-MS analysis

Proteome technology has been used to generate information for understanding the complexity of the salivary secreted proteins that play a role in the insect adaption to blood-feeding [8]. The *T. infestans* salivary glands proteome was carried out with basis on two-dimensional gel electrophoresis (2-DE) and mass spectrometry approach [9,24]. Additionally, the comparison of results obtained by liquid chromatography and mass spectrometry with those from cDNA libraries has generated complementary knowledge about the insect's salivary gland proteins [25]. The data available from several salivary proteomes [8–10] corroborate the findings of the cDNA library sequencing. Herein, we found 20 proteins that matched with *P. megistus* transcriptome analysis.

The *T. infestans* proteomic analysis showed 200 spots in 2-DE and 58 proteins were identified, and 34 of the identified proteins were related to lipocalins and apyrases [24]. Herein we have identified 159 proteins from *P. megistus* salivary gland proteome (Supplementary Table 2). As expected, the majority of these sequences (64.5%) bear similarities to saliva proteins from other insects, mostly hemiptera. Also, almost all identified saliva proteins were matched using the translated cDNA sequences from *P. megistus*, *T. infestans* and *R. robustus* salivary glands. Among 28 identified sequences from proteins known to be from insect´s saliva, 20 matched with *P. megistus* translated cDNA sequences. Additionally, there were housekeeping proteins, such as ribosomal proteins, actin, and metabolic enzymes. These intracellular proteins appear in the saliva as apocrine secretions [26]. Consistently with our EST data, we found 28 proteins with function similarities to those of pallidipin, lipocalins, infestin (non-classical Kazal type of thrombin inhibitor) and triatin transcripts. Also, novel proteins present in the *P. megistus* proteome are described in Table 2.

3.3. Features of the identified proteins

3.3.1. Lipocalins

The lipocalins were the most abundant protein-encoding transcripts, which comprise almost 39% of the saliva proteins involved in blood-feeding [27]. Here, we described the presence of 10 lipocalins, including lipocalin 1 and 4, and lipocalin-like Tin66 in the transcriptome experiment. Regarding to the LC-MS part we found 14 proteins similar to triatomine (*T. infestans* and *P. megistus*) salivary lipocalins, such as lipocalin, lipocalin1, lipocalin 4, lipocalin like tin66 that were identified. Lipocalins belong to a group of small proteins with different functions, many of which are related to transport of small hydrophobic molecules, such as histamine and nitric-oxide. Their tertiary structure is highly conserved with eight-stranded antiparallel β-barrels forming a cavity with an intern ligand-binding site [27,28]. Lipocalins were also predominant proteins in transcriptomes of *R. prolixus*[8] and of *T. infestans*[9], attaining 84% and 55% of the predicted secreted proteins, respectively.

Also belonging to the lipocalin family, there were eight cDNA sequences of pallidipin, which is an inhibitor of collagen-induced platelet aggregation [27]. Consistently, the LC-MS results showed 5 pallidipin-like proteins, which were confirmed by the *P. megistus* transcriptome. The pallidipin first described in *T. pallidipennis*[29] was shown to inhibit the platelet activation. Differently from the majority of lipocalins, pallidipin has no binding sites for small molecules. Also, other sequences related to lipocalins, such as triabin and triatin were found in the salivary gland of *P. Megistus*. Additionally, the *P. megistus* showed a thrombin inhibitor previously described in *T. pallidipennis*[30]. Interestingly, several sequences from *P. megistus* matched triatin from *T. infestans*.

The phylogenetic studies have shown that the lipocalin complex stems from the divergence between primary structures, which appear to result from a rapid molecular evolution rate and a possible occurrence of gene duplication [31]. In this study we pursued alignment of the lipocalins, aiming at the construction of a neighbor-joining phylogenetic-tree (Fig. 1). We observed that *Panstrongylus sp.* lipocalins are placed apart from those obtained from *Rhodnius sp.* On the other hand, *P. megistus* triabin and pallidipin appear to be closely related to those from *T. infestans*, *T. brasiliensis* and *T. pallidipennis*. Contrastingly, the *P. megistus* triatin appears to be different from *T. infestans* and *T. brasiliensis* clusters, as shown by sequence alignments (Fig. 2). Such divergence may be explained by a species-independent pallidipin allele acquisition over time.

Table 1 – Transcripts description and function in *Panstrongylus megistus* salivary glands.

Transcripts	Sequences	GenBank	Number of Clusters	Function
Lipocalin	Pm36	FG589957	7	Transport
	Pm03	FG589926		
	Pm06	FG589928		
	Pm44	FG589965		
	Pm10	FG589932		
	Pm33	FG589954		
	Pm37	FG589958		
Lipocalin 1	Pm28	FG589949	1	Transport
Lipocalin 4	Pm07	FG589929	1	Transport
Lipocalin-like Tin66	Pm23	FG589944	1	Transport
Pallidipin 2	Pm22	FG589943	3	Antiplatelet activity
	Pm15	FG589936		
	Pm08	FG589930		
Pallidipin-like salivary lipocalin	Pm39	FG589960	5	Antiplatelet activity
	Pm41	FG589962		
	Pm30	FG589951		
	Pm26	FG589947		
	Pm38	FG589959		
Secreted kazal-type proteinase inhibitor	Pm47	FG589968	1	Vasodilator
Triabin-like lipocalin 4 a	Pm14	FG589935	1	Anticoagulation activity
Trialysin allele			1	Pore-forming activity
Triatin	Pm46	FG589967	2	Pore-forming activity
Triatin-like salivary lipocalin	Pm12	FG589933	3	Pore-forming activity
	Pm09	FG589931		

3.3.2. *Kazal domains-containing peptides*

Vasodilation is a process that increases blood flow and maintains the blood circulation, and it is essential to the insect's feeding success. The Kazal domains are present in several vasodilation proteins, such as vasotab from *Hybomitra bimaculata*[32], rhodniin from *R. prolixus*[33] and infestin from *T. infestans*[34]. Additionally, Kazal domains proteins also have anticoagulant (rhodniin, and infestin 1 and 2) function, which depends on a conserved cysteine residues pattern, showing tridimensional conformation [35]. Infestin has two non-classical Kazal-type domains present in six sequences coded by single gene [34], which underwent mutation and tandem duplication from a common ancestor, and post- transcriptional processing [36]. However, its post-transcriptional mechanism remains unknown. Interestingly, the rhodniin protein, which is a thrombin specific inhibitor, shows an association rate two- or three-fold higher than other Kazal domains-containing proteins [33].

Table 2 – Proteins with blood-feeding functions found in *Panstrongylus megistus* proteome.

Proteins/transcript sequence code	Specie	Number of isoforms	Function
79 kDa salivary apyrase	*Ti*	1	Antiplatelet aggregation
Glutathione transferase	*Ae*	1	Detoxification
Infestin 1–7 precursor	*Ti*	1	Anticoagulant
Kazal-type proteinase inhibitor/Pm47	*Ti; Tb*	2	Vasodilator
Lipocalin/Pm36	*Ti; Pm*	8	Transport
Lipocalin 1/Pm28	*Pm*	1	Transport
Lipocalin 4/Pm07	*Pm*	1	Transport
Lipocalin-like Tin66/Pm23	*Pm*	1	Transport
Pallidipin-like lipocalin/Pm39	*Pm*	2	Antiplatelet activity
Pallidipin 2/Pm22	*Pm*	3	Antiplatelet activity
Triabin-like lipocalin 4a/Pm14	*Pm*	1	Anticoagulation activity
Trialysin allele	*Ti*	1	Pore-forming activity
Triatin-like salivary lipocalin/Pm12	*Pm*	2	Pore-forming activity
Salivary trypsin	*Ti; Tb*	4	Amidolytic activity

*Ae: *Anopheles dirus*, *Ti*: *Triatoma infestans*, *Pm*: *Panstrongylus megistus*, *Tb*: *Triatoma brasiliensis*.

In our study, transcript sequences from the *P. megistus* salivary glands revealed 40% identity with a small *T. infestans* Kazal domains-containing proteinase inhibitor, and 33% identity with a *T. brasiliensis* secreted peptide, showing vasodilator function similar to vasotab protein from the horse fly *H. bimaculata*[32]. However, we did not find conserved cysteine residues described in a similar protein sequence from *T. infestans*[9].

3.3.3. *Trialysin*

Trialysin is a 22 kDa protein with the ability to form pores in biological membranes of prokaryotes and eukaryotes. Interestingly, its function is similar to those found in various classes of lytic molecules [37,38]. The trialysin is functionally similar to the antimicrobial bacterial toxins with amphypatic motif in the N-terminal region, forming an α-helical structure with positive charges in one side and hydrophobic amino acids on the other side [37].

The alignment of *P. megistus* trialysin cDNA transcript with other related species showed 59% similarity with trialysin from *T. infestans*. Its sequence shows a shared conserved motif comprising a small region in pro- and pre-peptide. *T. infestans*

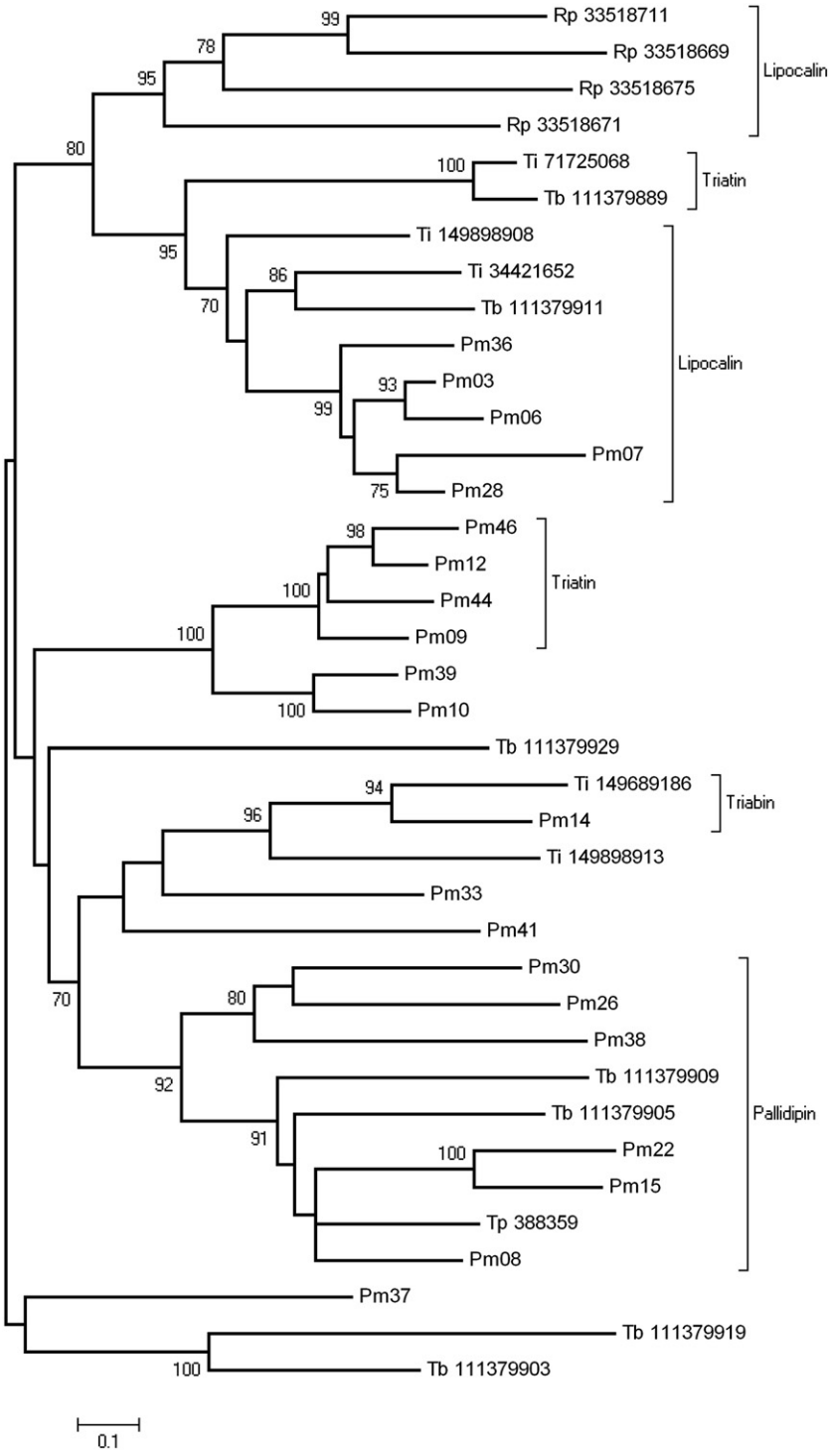

Fig. 1 – Dendogram showing convergent evolution aspects of several lipocalin family proteins from the saliva of *Panstrongylus megistus* (Pm), *Rhodnius prolixus* (Rp), *Triatoma infestans* (Ti), *Triatoma brasiliensis* (Tb) and *Triatoma pallidipennis* (Tp). The analysis of sequences from nonreduntant protein database of the National Center for Biotechnology Information (NCBI) were made, which are represented by the first letters of gender and specie followed by its GenBank gi number. The numbers in the dendogram nodes indicate percent bootstrap support for the phylogeny. The bar (bottom) indicates 10% amino acid divergence in the sequences. The dendogram was constructed with MEGA4 package and cut-off 70 was observed.

trialysin accumulates in the salivary gland with a pro-peptide in the N-terminal that prevents its activation, and thus inhibiting its lytic activity. Interestingly, during saliva secretion a proteolytic activity cleaves the pro-peptide thus forming a mature protein with affinity to cellular membrane. When cleavage takes place, a mature protein conformation change takes place in the N-terminal portion enhancing its pore-forming activity [38].

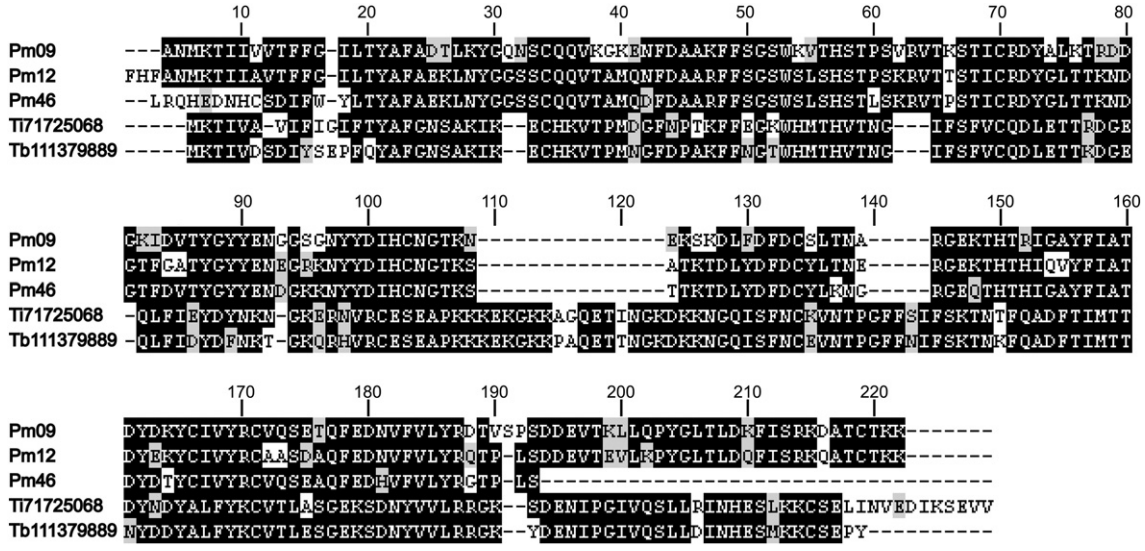

Fig. 2 – ClustalW alignment of triatin translated genes from *Triatoma infestans*, *Triatoma brasiliensis* and *Panstrongylus megistus*.

3.3.4. *Trypsin*

Several enzymes have been detected in *P. megistus* salivary glands proteome, which may play important roles during the insect's blood-feeding. We found four proteins similar to trypsin from *T. infestans* and *T. brasiliensis*. This enzyme is a serine protease similar to *T. infestans* triapsin, which is stored in the D2 salivary gland. Upon saliva ejection this enzyme with amidolytic function becomes active to participate in the blood-feeding process [39].

3.3.5. *Glutathione S-transferase*

The glutathione S-transferase (GST) belongs to a diverse family of enzymes. This enzyme can have multiple functions in cellular transporting, and protection against the oxidative stress. Interestingly, GST detoxicates and prevents the insect's death from insecticides. *T. infestans* GST is associated with resistance against DDT (1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane) [40]. In this study we show that the saliva of *P. megistus* has GST similar to that in the salivary glands of *Anopheles dirus*.

3.3.6. *Apyrase*

Platelet aggregation is essential for preventing the host's hemostasis. The insect's blood-feeding depends on the mechanism of evasion from the physical barrier posed by the platelet aggregation. Once activated, the platelet changes its conformation and releases ADP in the blood vessels, which activates and aggregates platelets. Apyrase is a nucleoside triphosphate–diphosphohydrolase capable to remove phosphate from ATP and ADP, consequently, inhibiting platelet aggregation. In the *P. megistus* saliva we found a 79 kDa apyrase, which is also present in *T. infestans*[9,24,41,42], *T. brasiliensis*[10] and in *R. prolixus*[8] salivary secretions.

4. Conclusions

Among over 130 species of triatomines we found the ubiquitous *P. megistus* with an increasing participation in a complex epidemiologic chain of transmission of the *T. cruzi* to the human population in Latin America. To play this role *P. megistus* has possibly gone through a convergent evolution, whereby it accumulated a great number of pharmacologically active proteins in its salivary glands. In this study we have undertaken transcriptomics and proteomics analyses to identify the main bioamines related to this insect blood-feeding ability. This species fastidious growth in captivity used to be a bottleneck to molecular and biochemical analyses of its salivary glands. In this study, we captured the wild life *P. megistus* that colonized slowly in the laboratory, and obtained the insect's salivary glands to construct a cDNA library. A total of 47 transcripts revealed main proteins with vasodilation, anti-clotting, anti-platelet aggregation activities. Additionally, *P. megistus* proteome revealed 159 proteins, including several that showed similarities with apyrase, lipocalins, thrombin inhibitor, trialysin, trypsin and glutathione S-transferase. Overall, 9/14 protein family transcripts (65%) were confirmed by proteomics. The main conclusion in this study is the demonstration of a complex salivary gland tool-box with elements capable to perform some functions associated with the insects' blood-feeding and resistance to insecticide. An important finding in this study is the demonstration of glutatione-S transferase gene, thus confirming field-studies reporting a substantial resistance of *P. megistus* to DTT and pyrethroids insecticides. The complementary techniques used in this study provide useful information, which are required for new insights necessary for curtailing the transmission of Chagas disease in several ecosystems. Further studies shall disclose the mechanism whereby some proteins influence the insect's behavior and adaptation to different environments, which makes *P. megistus* real threats to human health.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.04.028.

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