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Salivary gland transcripts of the kissing bug, *Panstrongylus chinai*, a vector of Chagas disease

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Abstract

The saliva of hematophagous arthropods injected during blood feeding contains potent pharmacologically active components to counteract the host hemostatic and inflammatory systems. In the present study, dominant salivary gland transcripts of *Panstrongylus chinai*, a vector of Chagas disease, were analyzed by sequencing randomly selected clones of the salivary gland cDNA library. This analysis showed that 56.5% of the isolated transcripts coded for putative secreted proteins, of which 73.7% coded for proteins belonging to the lipocalin family. The most abundant transcript of lipocalin family proteins was a homologue of pallidipin 2, an inhibitor of collagen-induced platelet aggregation of *Triatoma pallidipennis*. In addition, homologues of triafestin, an inhibitor of the kallikrein-kinin system of *T. infestans*, were identified as the dominant transcript. Other salivary transcripts encoding lipocalin family proteins had homology to triplatin (an inhibitor of platelet aggregation) and others with unknown function. Other than lipocalin family proteins, homologues of a Kazal-type serine protease inhibitor (putative anticoagulant), a hemolysin-like protein (unknown function), inositol polyphosphate 5-related protein (a regulator of membrane phosphoinositide), antigen 5-related protein (unknown function) and apyrase (platelet aggregation inhibitor) were identified.

Keywords

Panstrongylus chinai; Saliva; Transcriptome; Bioinformatics; cDNA library

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actatropica.2017.06.022>.

1. Introduction

Hematophagous arthropods have evolved a wide set of pharmacologically active molecules to counteract host hemostatic processes (Ribeiro, 1995). When probing in the host skin prior to blood sucking, they inject saliva containing various physiologically active agents such as vasodilators, anticoagulants and platelet aggregation inhibitors (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Valenzuela, 2004, 2005; Champagne, 2005). In addition, other bioactive agents, such as inhibitors of biogenic amine, complement system inhibitors and immunomodulators, have been identified in their saliva (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Valenzuela, 2004, 2005; Champagne, 2005). Since hematophagous arthropods have evolved their feeding strategy independently, each species has developed distinctive salivary components to overcome host defense mechanisms (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Valenzuela, 2004, 2005; Champagne, 2005). Therefore, salivary molecules of blood feeders have been extensively studied in various species to understand their universal and unique biological activities and to discover novel candidates for natural pharmacological agents (Ribeiro, 1995; Ribeiro and Francischetti, 2003; Valenzuela, 2004, 2005; Champagne, 2005).

Triatomine bugs, commonly known as kissing bugs, are blood sucking insects including the three major genera of the Chagas disease vector in Central and South America; genus *Rhodnius* in tribe Rhodniini and genera *Triatoma* and *Panstrongylus* in tribe Triatomini (Beard, 2005; Patterson et al., 2009; Schofield and Galvão, 2009). Of these, the genus *Panstrongylus* is composed of 13 species, and *Panstrongylus chinai* is known as a vector species of Chagas disease in Ecuador, Peru, and Venezuela (Abad-Franch et al., 2001; Grijalva et al., 2005, 2015; Patterson et al., 2009). Different from some other blood feeders such as mosquitoes, kissing bugs including *P. chinai* take 20–30 min for blood-sucking (Beard, 2005); however, they take blood efficiently regardless of host hemostatic and immune responses, suggesting their unique strategy to counteract host responses.

Salivary proteins, which are closely involved in their blood sucking strategy, have been explored in kissing bugs, especially in *Rhodnius prolixus*, which has characteristic cherry red-colored salivary glands (Champagne et al., 1995; Montfort et al., 2000; Ribeiro et al., 2004a; Andersen et al., 2005). Their functional analyses resulted in discovery of unique pharmacologically active components; for example, nitrophorin 2 (prolixin-S), one of the nitrophorins that are nitric oxide (NO)-binding heme proteins abundant in *R. prolixus*, was characterized to function both as an anticoagulant by the protein itself and a vasodilator by NO released from the protein (Champagne et al., 1995; Kaneko et al., 1999; Isawa et al., 2000; Ribeiro et al., 2004a). Different from *Rhodnius* species, salivary proteins of *Triatoma* and *Panstrongylus* species in the tribe Triatomini are colorless (Champagne et al., 1995). To identify the variations in blood sucking strategies in kissing bugs, salivary gland transcripts were recently extensively explored in *Triatoma* species, *T. brasiliensis* (Santos et al., 2007), *T. infestans* (Ribeiro et al., 2004a; Assumpção et al., 2008; Schwarz et al., 2014), *T. dimidiata* (Kato et al., 2010), *T. matogrossensis* (Assumpção et al., 2012) and *T. rubida* (Ribeiro et al., 2012). In addition, sialotranscriptome analysis was performed, more recently in a *Panstrongylus* species, *P. megistus* (Ribeiro et al., 2015). In the present study, to obtain further insight into the salivary biochemical and pharmacological complexity of triatomine

bugs, the salivary gland transcripts were analyzed in a *Panstrongylus* species, *P. chinai*, which is a vector of Chagas disease mainly distributed west of the Andean Cordillera in South America (Schofield and Galvão, 2009).

2. Materials and methods

2.1. *Panstrongylus chinai* salivary glands

Panstrongylus chinai were reared in an insectary room at Hokkaido University in Japan and maintained at 26 ± 2 °C with uncontrolled humidity. They were fed at two-week intervals on rats. Salivary glands were dissected from fourth and fifth-instar nymphs after 2 weeks of feeding, transferred to an RNAlater reagent (Ambion, Austin, TX) and stored at -20° C until mRNA extraction.

2.2. Construction of a salivary gland cDNA library

Panstrongylus chinai salivary gland mRNA was isolated from 22 sets of the salivary glands using a Micro FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). The PCR-based cDNA library was prepared following the instructions for the SMART cDNA library construction kit (BD-Clontech, Palo Alto, CA) with some modifications (Valenzuela et al., 2004). The quality of the cDNA was checked by agarose gel electrophoresis and the absence of smaller fragments derived from degraded mRNA was confirmed. The obtained cDNA library was fractionated using a Chromaspin 1000 column (BD-Clontech) into small (approximately 400–800 bp), medium (approximately 800–1200 bp) and large (> 1200 bp) transcripts based on their electrophoresis pro-files on a 1.1% agarose gel. Pooled fractions were ligated into Lambda TriplEx2 vector (BD-Clontech) and packaged into lambda phage (Stratagene, La Jolla, CA).

2.3. Sequence analysis of the cDNA library

Single, isolated phage plaques were picked from the plate using sterile wooden sticks and placed into 50 μ l of water. Amplification of cDNA was performed in a volume of 20 μ l using a pair of primers, PT2F1 (5'-AAG TAC TCT AGC AAT TGT GAG C-3') and PT2R1 (5'-CTC TTC GCT ATT ACG CCA GCT G-3'), Premix Taq (Takara Bio, Shiga, Japan) and 4 μ l of phage suspension as templates. After an initial denaturation at 75 °C for 3 min and following 95 °C for 4 min, PCR amplification was performed with 33 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and polymerization (72 °C, 1 min 30 s). PCR products were cleaned using Multiscreen PCR cleaning plates (Millipore Corporation, Bedford, MA), and the cleaned PCR product was suspended in 50 μ l of water. The products were submitted to Macrogen Inc. (Seoul, Korea) for sequence analyses with a PT2F3 primer (5'-TCT CGG GAA GCG CGC CAT TGT-3').

2.4. Bioinformatics

Expressed sequence tags (ESTs) were trimmed of primer and vector sequences and clustered. The ESTs were grouped based on nucleotide homology of 95% identity over 100 residues using the BLASTn algorithm (Altschul et al., 1997). The assembly of the ESTs into transcript contigs was done using the CAP3 algorithm, generating a consensus sequence (Huang, 1992). Contigs and singletons (contig containing only one sequence) were

compared using BLASTx or BLASTn (Altschul et al., 1997) of the non-redundant (NR) protein database of the National Center of Biological Information (NCBI), the gene ontology database (GO) (Ashburner et al., 2000), and the Conserved Domains Database (CDD) that include all Pfam (Bateman and Birney, 2000), SMART (Schultz et al., 1998) and COG protein domains in the NCBI (Marchler-Bauer et al., 2002). Additionally, contigs were compared using BLASTn (Altschul et al., 1997) to custom databases of mitochondrial (mit-pla) and rRNA (rrna) nucleotide sequences. Identification of putative secreted proteins was conducted using SignalP server (Bendtsen et al., 2004).

2.5. Phylogenetic analysis

The sequences that had homologies with secreted proteins by BLASTx analyses were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using Molecular Evolutionary Genetics Analysis (MEGA) version 5.2 (Tamura et al., 2011). Phylogenetic trees by the neighbor-joining method were constructed with the distance algorithms available in the MEGA package. Bootstrap values were determined on 1000 replicates of the data sets.

3. Results and discussion

3.1. Sequence analysis of the *P. chinai* salivary gland cDNA library

A Panstrongylus chinai salivary gland cDNA library was constructed and sequence analysis was performed on 1152 randomly selected clones. As a result, 773 high-quality sequences were obtained and classified into 302 contigs. Three categories of expressed genes were derived from the manual annotation of the contigs: secreted, housekeeping and unknown. The putative secreted category comprised 437 clones (56.5%) in 64 contigs. High ratios of transcripts encoding secreted proteins were reported in other triatomine bugs (Ribeiro et al., 2004a, 2012, 2015; Santos et al., 2007; Assumpção et al., 2008, 2011, 2012; Kato et al., 2010; Santiago et al., 2016). The housekeeping category had 155 clones (20.1%) in 111 contigs, and the category of “unknowns” comprised 181 clones (23.4%) in 127 contigs.

3.2. Housekeeping genes

The contigs of housekeeping genes (111 gene contigs with 155 sequences in total) were divided into 17 subgroups on the basis of their possible function (Table 1). The largest subgroup was associated with “translation, ribosomal structure and biogenesis” (50 sequences in 33 contigs), and followed by “energy production and conversion” (24 sequences in 11 contigs), “posttranslational modification, protein turnover, chaperones” (11 sequences in 8 contigs), and “signal transduction mechanisms” (11 sequences in 8 contigs). Twenty-three sequences in 17 contigs, which represent conserved proteins with unknown function were classified as “unknown conserved”. Other sequences identified with homology to housekeeping genes were associated with cell structure, transport, metabolism, and signal transduction.

3.3. Putative secreted proteins

The transcripts coding for secreted proteins were further analyzed using the BLASTx algorithm for comparison to the NCBI non-redundant protein database. Table 2 shows the

classification of transcripts coding for putative secreted proteins in *P. chinai* salivary glands. Remarkably, out of 437 transcripts associated with putative secreted proteins, 322 transcripts (73.7%) coded for the lipocalin family of proteins. Other transcripts encoding for secreted proteins were homologous to kazal-domain peptides, hemolysin, inositol polyphosphate 5-phosphatase, antigen 5-related protein, apyrase, trypsin, heme-binding protein, triplatin, and others (Table 2). Lipocalins, the most abundant transcripts in this cDNA library were also reported to be present in the salivary glands of ticks (Mans, 2005; Mans and Ribeiro, 2008; Mans et al., 2008), but not in the saliva of mosquitoes and sand flies (Valenzuela et al., 2003, 2004; Ribeiro et al., 2004b, 2007; Arcà et al., 2005, 2007; Anderson et al., 2006; Kato et al., 2006, 2013; Calvo et al., 2009; Hostomská et al., 2009; Rohoušová et al., 2012; Chagas et al., 2013; de Moura et al., 2013). A high percentage of secreted lipocalins were also reported in other *Triatoma* and *Panstrongylus* species: 55.0% in *T. infestans* (Assumpção et al., 2008), 93.8% in *T. brasiliensis* (Santos et al., 2007), 89.9% in *T. dimidiata* (Kato et al., 2010), 53.0% in *T. matogrossensis* (Assumpção et al., 2012), 65.3% in *T. rubida* (Ribeiro et al., 2012) and 87.2% in *P. megistus* (Ribeiro et al., 2015).

3.3.1. Lipocalins—Of the transcripts coding for lipocalin-like molecules from this cDNA library, homologues of pallidipin 2, an inhibitor of collagen-induced platelet aggregation in *T. pallidipennis* saliva (Noeske-Jungblut et al., 1994; Haendler et al., 1995), were the most abundant transcripts accounting for 47.2% from the transcripts coding for this family of proteins (Table 3, and Table 1 in Data in Brief). The second most abundant transcript (36.3%) was similar to triatin-like salivary lipocalin identified in the saliva of *T. dimidiata* (Kato et al., 2010). Other transcripts had homologies to molecules similar to salivary lipocalin from *T. dimidiata* (7.7%), lipocalin-like TiLipo37 from *T. infestans* (5.0%), and molecules similar to pallidipin-like salivary lipocalin from *T. dimidiata* (1.9%) (Table 1 in Data in Brief). Phylogenetic analysis of *P. chinai* salivary lipocalins together with other representative triatomine lipocalins resulted in the formation of five major clades (Fig. 1, Table 1 in Data in Brief). Further characteristics of *P. chinai* lipocalin-like clades are described below.

3.3.1.1. Pc lipocalin clade I: This group is the most abundant transcript in *P. chinai* salivary glands, and is composed of 152 clones in 17 contigs. The molecules showed a similarity to pallidipin 2, an inhibitor of collagen-induced platelet aggregation, identified in *T. pallidipennis* saliva (Noeske-Jungblut et al., 1994; Haendler et al., 1995). Sixteen contigs (Pc02, Pc05-Pc08, Pc10-Pc12, Pc14 and Pc16-Pc22) showed the highest homology to pallidipin 2 while Pc03 was homologous with Td42, a molecule similar to pallidipin 2, in *T. dimidiata* saliva with unknown function. The sequences of Pc05, Pc06, and Pc17 were truncated in the 5' region. In the alignment of the encoding proteins with pallidipin 2, all six cysteine residues were conserved, suggesting a similar protein structure based on the disulfide bonds (Fig. 1 in Data in Brief). Phylogenetic analysis resulted in the formation of four distinct subclades; the first containing Pc11, Pc19, Pc20 and Pc22 that were closer to pallidipin 2, the second containing Pc02 and Pc08, the third containing Pc07 and Pc21, and the fourth containing Pc10, Pc12, Pc14, Pc16 and Pc18 (Fig. 1, and Fig. 1 in Data in Brief).

3.3.1.2. Pc lipocalin clade II.: This clade consisted of 6 clones in 3 contigs (Pc85, Pc156 and Pc173), and had homology with a lipocalin Td38 in *T. dimidiata* saliva with unknown function. The sequence of Pc173 was truncated in the 5' region. Alignment of the encoding proteins with Td38 showed that all six cysteine residues were conserved (Fig. 2 in Data in Brief).

3.3.1.3. Pc lipocalin clade III.: This clade consisted of 25 clones in 6 contigs (Pc56, Pc57, Pc64, Pc70, Pc103 and Pc150), and had a homology with triafestin-1, an inhibitor of kallikrein-kinin system activation in *T. infestans* saliva (Isawa et al., 2007). Two contigs (Pc56 and Pc57) and Pc70 showed the highest homology to Td26 and Td40 from *T. dimidiata* saliva, respectively, the function of which are unknown. On the other hand, two contigs (Pc64 and Pc103) and Pc150 were homologous with salivary lipocalin and triabin-like lipocalin 4a precursor from *T. infestans*, respectively, neither of which have identified functions. The sequence of Pc56 was truncated in the 5' region. Alignment of the encoding proteins with triafestin-1 showed that the GXW motif, which is often observed in lipocalins, was conserved in 4 contigs (Pc57, Pc64, Pc70 and Pc150), and all six cysteine residues were located at the corresponding positions in all molecules (Fig. 3 in Data in Brief). Phylogenetic analysis resulted in the formation of close (Pc56, Pc57, Pc70 and Pc150) and relatively distinct (Pc64 and Pc103) subclades to triafestin-1 (Fig. 1, and Fig. 3 in Data in Brief).

3.3.1.4. Pc lipocalin clade IV.: This clade consisted of 117 clones in 6 contigs (Pc04, Pc13, Pc15, Pc58, Pc59 and Pc226), and had homology with Td18, a salivary protein from *T. dimidiata* with unknown function (Kato et al., 2010). The sequences of Pc04 and Pc226 were truncated in the 5' region. In the alignment of the encoding proteins with Td18, the GXW motif was conserved, and all six cysteine residues were located at the corresponding positions in all molecules (Fig. 4 in Data in Brief). Phylogenetic analysis resulted in the formation of close (Pc13 and Pc15) and distinct (Pc58 and Pc59) subclades to Td18 (Fig. 1, and Fig. 4 in Data in Brief).

3.3.1.5. Pc lipocalin clade V.: This clade consisted of 16 clones in 3 contigs (Pc41, Pc42 and Pc43), and had homology with lipocalin-like TiLipo37 from *T. infestans* saliva with unknown function. Pc41 and Pc42 showed the highest homology to lipocalin-like TiLipo37, while Pc43 was homologous with salivary lipocalin from *T. brasiliensis* with unknown function. The GXW motif was conserved, and all six cysteine residues were located at the corresponding positions in all molecules (Fig. 5 in Data in Brief). Phylogenetic analysis resulted in the formation of distinct subclades (Pc41, Pc42 and Pc43) to lipocalin-like TiLipo37 (Fig. 1, and Fig. 5 in Data in Brief).

3.3.1.6. Other lipocalins.: Two contigs (Pc122 and Pc301) and Pc123 had homologies with Td24 and Td23 from *T. dimidiata* saliva with unknown function, respectively (Table 3). On the other hand, Pc124 and Pc276 were homologous with salivary lipocalin and triabin-like salivary lipocalin from *T. infestans*, respectively, neither of which have known functions, and Pc188 showed homology with salivary lipocalin 1 from *T. brasiliensis* with unknown function (Table 3).

3.3.2. Kazal-type serine protease inhibitor—Thirty four clones in 3 contigs (Pc76, Pc77 and Pc266) contained a Kazal domain in the sequences. Kazal-type serine protease inhibitors are small proteins, typically 40–60 amino acids in length, with characteristic sequences $CX_{1-6}CX_{6-9}PVCGX_{8-15}CX_{3-6}CX_{7-16}C$, in which X_n are integral numbers of amino acid residues. Each set of cysteine residues, 1st and 5th, 2nd and 4th, and 3rd and 6th, forms a disulfide bond, resulting in a characteristic three-dimensional structure (Rimphanitchayakit and Tassanakajon, 2010). Kazal-type serine protease inhibitors identified in mosquito and leech saliva and in the midgut of kissing bugs have been characterized to work as anticoagulants during blood feeding (Friedrich et al., 1993; Campos et al., 2002; Nowak and Schrör, 2007; Santos et al., 2007; Rimphanitchayakit and Tassanakajon, 2010; Watanabe et al., 2010, 2011). Although homologous proteins to Kazal-type serine protease inhibitors have been identified in the saliva of kissing bugs (Santos et al., 2007; Assumpção et al., 2008, 2012; Schwarz et al., 2014; Ribeiro et al., 2015; Santiago et al., 2016), their bioactivities have not yet been characterized. In the *P. chinai* salivary gland transcripts coding for secretory proteins, a higher ratio of this molecule (7.8%) was identified when compared to other triatomine bugs (0.5–2.8%) (Santos et al., 2007; Assumpção et al., 2008, 2012; Schwarz et al., 2014; Ribeiro et al., 2015; Santiago et al., 2016), suggesting its important role in blood feeding in this species. The sequence of Pc76 was truncated in the 5' region. Alignment of the encoding proteins with Kazal-type serine protease inhibitor-like protein from *T. infestans* showed that all six cysteine residues and PVCG sequences were conserved in Pc77 and Pc266 (Fig. S1).

3.3.3. Hemolysin-like protein—Thirty-four clones in 3 contigs (Pc60, Pc61 and Pc118) shared homology with a hemolysin-like protein identified in *T. infestans* saliva, of which the function is not known (Tables 2 and 3). The sequence of Pc76 was truncated in the 5' region. Hemolysin is a pore-forming toxin identified in microorganisms (Gouaux, 1998; Melton et al., 2004; Wassenaar, 2005). The role of this protein in the saliva of triatomine bugs is not known; however, it is speculated that the protein may act as a cytolytic protein, causing erythrocyte lysis and aiding in the early steps of the digestion process (Assumpção et al., 2008). Alternatively, it may work as an antimicrobial peptide in the saliva. The putative amino acid sequence of Pc60 showed a high homology with *T. infestans* hemolysin-like protein with similar molecular weight, whereas Pc61 showed lower similarity and lacked its C-terminal region (Fig. S2).

3.3.4. Inositol polyphosphate 5-phosphatase-like protein—Twenty clones in 5 contigs (Pc24, Pc29, Pc31, Pc32 and Pc292) shared homology with an inositol polyphosphate 5-phosphatase (IP5P)-like protein identified in *T. infestans* saliva (Tables 2 and 3). The sequence of Pc292 was truncated in the 5' region. Although the role of salivary IP5Ps remains unclear, the enzyme is well-conserved in kissing bugs, suggesting that it plays an important role in the blood sucking process of this insect (Ribeiro et al., 2004a, 2012, 2015; Andersen and Ribeiro, 2006; Santos et al., 2007; Assumpção et al., 2008, 2011, 2012; Kato et al., 2010; Schwarz et al., 2014; Santiago et al., 2016). In the alignment of the encoding proteins with IP5P-like protein identified in *T. infestans* saliva, two cysteine residues were located at the corresponding positions in all molecules (Fig. S3). Phylogenetic analysis resulted in two subclades; three contigs (Pc29, Pc31 and Pc32) showed a closer

relationship to IP5P-like protein from *T. infestans* saliva whereas a singleton (Pc24) composed a separate subclade (Fig. S3).

3.3.5. Antigen 5-related protein—Thirteen clones in 2 contigs (Pc80 and Pc294) shared homology with an antigen 5-related protein identified in *T. infestans* saliva (Tables 2 and 3). This family of proteins belongs to the cysteine-rich secretory proteins (CRISPs) that are related to venom allergens in social wasps and ants (Lu et al., 1993; Hoffman, 1993; King and Spangfort, 2000). Transcripts coding for this family of protein have been identified in the salivary glands of blood sucking insects including kissing bugs (Ribeiro et al., 2004a, 2012, 2015; Santos et al., 2007; Assumpção et al., 2008, 2011, 2012; Kato et al., 2010; Schwarz et al., 2014; Santiago et al., 2016). Salivary antigen 5 family proteins from kissing bugs were shown to inhibit collagen-induced platelet aggregation and neutrophil oxidative burst (Assumpção et al., 2013). Alignment and phylogenetic analysis of antigen 5-related proteins from various species indicated that *P. chinai* salivary antigen 5-related proteins had closer relationships with those from kissing bugs than those from saliva of other blood sucking arthropods such as mosquitoes and sand flies (Fig. S4).

3.3.6. Apyrase-like protein—Five clones in 3 contigs (Pc158, Pc159 and Pc270) shared homology with a 79 kDa salivary apyrase in *T. infestans* (Tables 2 and 3). Apyrase is a member of the nucleoside triphosphate-diphosphohydrolase family present in a variety of organisms. Salivary apyrase has been identified in a variety of blood-sucking arthropods and functions to hydrolyze ADP resulting in inhibition of ADP-induced platelet aggregation when injected into the host (Ribeiro and Francischetti, 2003; Faudry et al., 2004).

3.3.7. Trypsin-like protein—Two clones in a contig Pc97 showed homology with a serine pro-tease in the saliva of *Creontiades dilutus*, phytophagous Hemiptera, and a trypsin-like protein in *T. infestans* saliva (Tables 2 and 3). In phytophagous insects, salivary trypsin-like proteins injected into plant tissue are shown to play important roles in extra-intestinal digestion to facilitate efficient nutrition intake in the gut (Zhu et al., 2003). Although the biological function of this enzyme in blood sucking arthropods has yet to be determined, it is speculated that this protein is related to digestion and absorption of nutrients from host blood. Alignment of these three proteins showed that all nine cysteine residues were conserved among them (Fig. S5).

3.3.8. Heme-binding protein—A singleton Pc237 shared homology with Td76 from *T. dimidiata* saliva with unknown function (Tables 2 and 3, Fig. S6). The homologues were reported in the salivary glands of *T. brasiliensis* and *T. dimidiata* (Santos et al., 2007; Kato et al., 2010) and in the hemolymph and oocytes of *R. prolixus* (Paiva-Silva et al., 2002). The roles of these salivary proteins for blood-feeding remains to be clarified, but it may function as a vasodilator by the effect of nitric oxide potentially associated with heme binding on the salivary protein as reported in nitrophorin 2 (prolixin-S), a heme-binding protein of *R. prolixus* saliva (Champagne et al., 1995; Yuda et al., 1997; Kaneko et al., 1999).

3.3.9. Triplatin-binding protein—A singleton Pc01 had homology with triplatin from *T. infestans* saliva although the 5' region was truncated. Triplatin was identified as a collagen-induced platelet aggregation inhibitor (Morita et al., 2006).

3.3.10. Other putative secreted proteins—Singletons Pc75 and Pc134 shared homology with salivary proteins MYS3 from *R. prolixus* and MYS2 from *T. brasiliensis*, respectively (Table 3). A singleton Pc115 was homologous to the glutathione peroxidase 6-like protein of *Cimex lectularius*. A contig, Pc09, did not have marked similarity to known proteins.

4. Conclusions

The present study identified dominant transcripts containing a variety of lipocalin in the salivary glands of *P. chinai*, a vector of Chagas disease in Ecuador and Peru (Grijalva et al., 2005, 2015). Most *P. chinai* salivary components were similar to those of *Triatoma* species when compared to those of *Rhodnius* species (Ribeiro et al., 2004a, 2012, 2015; Santos et al., 2007; Assumpção et al., 2008, 2011, 2012; Kato et al., 2010; Schwarz et al., 2014; Santiago et al., 2016), corresponding with morphology and phylogenetic relationships of mitochondrial DNAs (García et al., 2001; Schofield and Galvão, 2009; Justi et al., 2014). In addition, salivary proteins of *Panstrongylus* species are colorless as observed in *Triatoma* species, whereas those of *Rhodnius* species are cherry red due to dominant components of heme-binding proteins in the saliva, which is indicative of common feeding strategies in *Panstrongylus* and *Triatoma* species. Salivary proteins homologous to procalin, which was identified as the major allergen of *T. protracta* saliva, were abundant in *Triatoma* species; e.g., approximately 60% in *T. dimidiata* (Kato et al., 2010; Assumpção et al., 2012). On the other hand, the most abundant salivary protein in *P. chinai* was a homologue of pallidipin 2, an inhibitor of collagen-induced platelet aggregation of *T. pallidipennis*, accounting for about 50% of transcripts encoding secreted proteins, suggesting diverse feeding strategies among kissing bugs.

In the *P. chinai* salivary gland transcripts, a higher ratio of transcripts encoding a Kazal domain-containing protein was identified when compared to other kissing bugs (Santos et al., 2007; Assumpção et al., 2008, 2012; Schwarz et al., 2014; Ribeiro et al., 2015; Santiago et al., 2016). Several Kazal-type serine protease inhibitors containing 2–8 Kazal domains were identified from midgut of kissing bugs; rhodniin from *R. prolixus* (Friedrich et al., 1993), infestin from *T. infestans* (Campos et al., 2002), brasiliensin from *T. brasiliensis* (Araujo et al., 2007) and dipetalogastin from *Dipetalogaster maximus* (Mende et al., 1999), and were characterized to inhibit several enzymes such as thrombin, trypsin and chymotrypsin (Rimphanitchayakit and Tassanakajon, 2010). On the other hand, Kazal-type serine protease inhibitors identified from saliva of kissing bugs contain only one Kazal domain and have not been characterized (Santos et al., 2007; Assumpção et al., 2008, 2012; Schwarz et al., 2014; Ribeiro et al., 2015; Santiago et al., 2016). Functional analysis will reveal enzymatic properties and contribute to further study on the target specificity of these enzymes.

In conclusion, the most abundant proteins of *P. chinai* saliva were identified in this study. The result will provide further insights into the evolution of salivary components in blood sucking arthropods. In addition, cDNAs and recombinant proteins prepared from these transcripts will result in the discovery of novel pharmacologically active compounds, as well as the development of biomarkers following exposure to *P. chinai*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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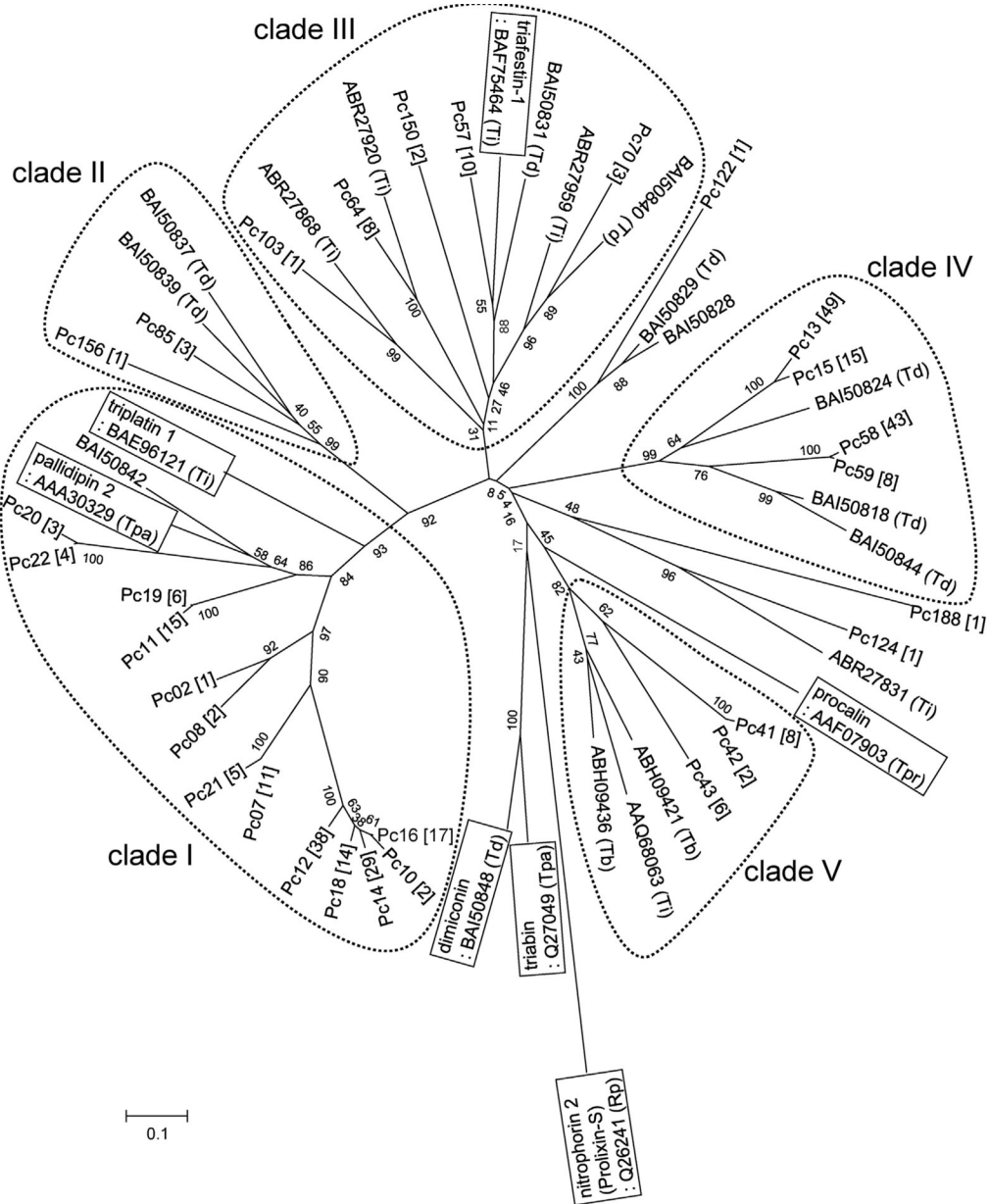


Fig. 1. Phylogenetic tree of the *Panstrongylus chinai* salivary lipocalins with those from other triatomine bugs. The sequences of *P. chinai* lipocalin obtained in the present study were aligned with representative triatomine lipocalin sequences obtained from the non-redundant protein database, and a phylogenetic tree was constructed. The number of sequences in each contig was shown in square brackets. The sequences from the database are represented by “GenBank accession number (abbreviation of the species name)”. Td, *Triatoma dimidiata*; Ti, *T. infestans*; Tb, *T. brasiliensis*; Tpa, *T. pallidipennis*; Tpr, *T. protracta*; Rp, *Rhodnius prolixus*. The scale bar represents 0.1% divergence. Bootstrap values are shown around branches.

Table 1Functional classification of housekeeping genes expressed in *Panstrongylus chinai* salivary glands.

Type of transcripts	Clusters	Sequences	% Sequences
Translation, ribosomal structure and biogenesis	33	50	32.3
Energy production and conversion	11	24	15.5
Posttranslational modification, protein turnover, chaperones	8	11	7.1
Signal transduction mechanisms	8	11	7.1
Cytoskeleton	6	6	3.9
Intracellular trafficking, secretion, and vesicular transport	4	6	3.9
Inorganic ion transport and metabolism	4	4	2.6
RNA processing and modification	4	4	2.6
Amino acid transport and metabolism	3	3	1.9
Carbohydrate transport and metabolism	3	3	1.9
Transcription	3	3	1.9
Cell cycle control, cell division, chromosome partitioning	2	2	1.3
Cell wall/membrane/envelope biogenesis	2	2	1.3
Chromatin structure and dynamics	1	1	0.6
Lipid transport and metabolism	1	1	0.6
Nuclear structure, Intracellular trafficking, secretion, and vesicular transport	1	1	0.6
Unknown conserved	17	23	14.9
Total	111	155	100.0

Table 2

Classification of transcripts coding for putative secreted proteins in *Panstrongylus chinai* salivary glands.

Type of transcripts	Clusters	Sequences	% Sequences
Lipocalin family	41	322	73.7
Kazal peptide	3	34	7.8
Hemolysin	3	34	7.8
Inositol polyphosphate 5-phosphatase	5	20	4.6
Antigen 5-related	2	13	3.0
Apyrase	3	5	1.1
Trypsin	1	2	0.5
Heme-binding protein	1	1	0.2
Triplatin	1	1	0.2
Others	4	5	1.1
Total	64	437	100.0

Table 3

Most abundant salivary gland transcripts from *Panstrongylus chinai*.

Cluster	No. of seq in cluster	pI	Mature Mw (kDa)	Cleavage position	Best match to NR protein database: Accession No.	E value	% identity	Accession No.
Pe13	49	4.83	18.7	18–19	Td18, similar to triatin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50824	8e-71	61	AB999669
Pe58	43	7.10	19.8	18–19	Td11, similar to triatin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50818	7e-83	66	AB999688
Pe12	38	8.44	19.3	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	2e-49	46	AB999668
Pe77	32	8.30	5.7	27–28	salivary kazal-type proteinase inhibitor (<i>Triatoma infestans</i>): ABR27896	3e-18	56	AB999695
Pe14	29	7.72	19.3	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	2e-49	45	AB999670
Pe16	17	8.44	20.2	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	5e-49	46	AB999672
Pe60	17	5.95	22.9	18–19	hemolysin-like secreted salivary protein 1 (<i>Triatoma infestans</i>): ABR27902	1e-75	56	AB999690
Pe61	16	5.07	18.8	20–21	salivary secreted protein (<i>Triatoma infestans</i>): ABR27926	4e-46	59	AB999691
Pe11	15	5.29	18.7	16–17	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	5e-80	64	AB999667
Pe15	15	5.31	20.1	18–19	Td18, similar to triatin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50824	3e-78	61	AB999671
Pe32	15	9.10	34.6	16–17	salivary inositol polyphosphate 5-phosphatase (<i>Triatoma infestans</i>): ABR27973	5e-146	68	AB999682
Pe18	14	5.68	19.4	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	6e-50	45	AB999674
Pe80	12	9.13	24.1	24–25	Td16, similar to antigen 5-like protein (<i>Triatoma dimidiata</i>): BAI50822	1e-79	55	AB999696
Pe07	11	4.94	19.2	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	2e-46	44	AB999663
Pe57	10	8.95	22.0	18–19	Td26, similar to salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50831	1e-75	57	AB999687
Pe41	8	8.66	18.6	18–19	lipocalin-like TILipo37 (<i>Triatoma infestans</i>): AAQ68063	7e-57	51	AB999683
Pe59	8	7.10	19.9	18–19	Td45, similar to pallidipin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50844	1e-81	64	AB999689
Pe64	8	9.19	19.2	15–16	salivary lipocalin (<i>Triatoma infestans</i>): ABR27920	3e-73	63	AB999692
Pe19	6	5.33	18.7	16–17	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	1e-79	64	AB999675
Pe43	6	8.65	18.5	18–19	salivary lipocalin (<i>Triatoma brasiliensis</i>): ABH09436	4e-52	50	AB999685
Pe21	5	4.95	19.2	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	4e-44	43	AB999677
Pe22	4	7.05	19.3	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	2e-60	55	AB999678
Pe20	3	7.72	19.2	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	8e-60	54	AB999676
Pe70	3	8.65	21.5	18–19	Td40, similar to triabin-like lipocalin 4a precursor (<i>Triatoma dimidiata</i>): BAI50840	3e-98	68	AB999693

Cluster	No. of seq. in cluster	pI	Mature Mw (kDa)	Cleavage position	Best match to NR protein database: Accession No.	E value	% identity	Accession No.
Pe85	3	5.14	22.4	16–17	Td38, similar to pallidipin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50839	1e-53	50	AB999697
Pe159 ^a	3				79 kDa salivary apyrase precursor (<i>Triatoma infestans</i>): CAE46445	2e-25	57	AB999709
Pe08	2	8.67	19.8	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	2e-62	53	AB999664
Pe09	2	8.90	6.7	20–21	No matches found			AB999665
Pe10	2	6.49	19.4	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	7e-49	46	AB999666
Pe31	2	9.23	34.2	16–17	salivary inositol polyphosphate 5-phosphatase (<i>Triatoma infestans</i>): ABR27973	1e-144	68	AB999681
Pe42	2	8.20	18.7	18–19	lipocalin-like TLipo37 (<i>Triatoma infestans</i>): AAQ68063	2e-57	52	AB999684
Pe97	2	5.74	34.3	18–19	serine protease (<i>Creona ades dilutus</i>): AAL15154	1e-66	45	AB999698
Pe150	2	8.44	21.9	16–17	triabin-like lipocalin 4a precursor (<i>Triatoma infestans</i>): ABR27959	4e-38	44	AB999706
Pe17 ^b	2				pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	1e-37	61	AB999673
Pe173 ^a	2				Td38, similar to pallidipin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50839	1e-04	47	AB999710
Pe02	1	8.97	19.1	18–19	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	7e-62	55	AB999658
Pe03	1	8.68	16.6	19–20	pallidipin 2 (<i>Triatoma pallidipennis</i>): AAA30329	3e-35	58	AB999659
Pe24	1	6.11	35.3	17–18	salivary inositol polyphosphate 5-phosphatase (<i>Rhodnius prolixus</i>): AAB08434	2e-78	47	AB999679
Pe29	1	8.93	34.5	16–17	salivary inositol polyphosphate 5-phosphatase (<i>Triatoma infestans</i>): ABR27973	2e-145	68	AB999680
Pe75	1	3.88	14.7	21–22	salivary protein MYS3 precursor (<i>Rhodnius prolixus</i>): AAQ20837	6e-11	31	AB999694
Pe103	1	9.42	21.7	18–19	salivary lipocalin (<i>Triatoma infestans</i>): ABR27868	5e-64	55	AB999699
Pe122	1	9.24	18.4	18–19	Td24, similar to salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50829	2e-68	74	AB999702
Pe124	1	9.07	20.1	19–20	salivary lipocalin (<i>Triatoma infestans</i>): ABR27831	1e-54	49	AB999704
Pe134	1	6.12	16.5	21–22	salivary protein MYS2 precursor (<i>Triatoma brasiliensis</i>): ABH09417	6e-95	87	AB999705
Pe156	1	4.72	21.4	28–19	Td33, similar to pallidipin-like salivary lipocalin (<i>Triatoma dimidiata</i>): BAI50837	9e-48	49	AB999707
Pe237	1	6.17	13.1	19–20	Td76, similar to heme-binding protein (<i>Triatoma dimidiata</i>): BAI53116	3e-49	71	AB999713
Pe266	1	4.86	3.5	28–29	salivary kazal-type proteinase inhibitor (<i>Triatoma infestans</i>): ABR27896	3e-05	38	AB999714
Pe294	1	10.51	8.6	24–25	antigen-5-like protein precursor (<i>Triatoma infestans</i>): ABR27897	1e-15	48	AB999718
Pe188	1	6.18	17.4	20–21	salivary lipocalin 1 (<i>Triatoma brasiliensis</i>): ABH09421	4e-26	35	AB999711

^aTruncated in the 5' region.