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The saliva proteome of the blood-feeding insect *Triatoma infestans* is rich in platelet-aggregation inhibitors

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Abstract

The saliva of the bloodsucking bug *Triatoma infestans* vector of Chagas disease contains an anti-hemostatic molecular cocktail that prevents coagulation, vasoconstriction and platelet aggregation in a vertebrate prey. In order to characterize *T. infestans* saliva proteome, we separated the secreted saliva by two-dimensional gel electrophoresis (2-DE). More than 200 salivary proteins were detected on the 2-DE map, mainly in the alkaline region. By nanoLC–MS/MS analysis using a LTQ–Orbitrap equipment followed by a combination of conventional and sequence-similarity searches, we identified 58 main protein spots. Most of such proteins possess potential blood-feeding associated functions, particularly anti-platelet aggregation proteins belonging to lipocalin and apyrase families. The saliva protein composition indicates a highly specific molecular mechanism of early response to platelet aggregation. This first proteome analysis of the *T. infestans* secreted saliva provides a basis for a better understanding of this fluid protein composition highly directed to counterpart hemostasis of the prey, thus promoting the bug's blood-feeding.

Keywords: *Triatoma infestans*; Saliva proteomic; Chagas disease; Tandem mass spectrometry; Sequence-similarity search

1. Introduction

Chagas disease, one of the most devastating parasitic human infections, is wide spread in South America. It was estimated at 16–18 million people infected by the protozoan *Trypanosoma cruzi*, with an annual death rate of 50,000 cases [1,2]. The disease is transmitted during blood-feeding of triatomine bug (Hemiptera: Reduviidae; Triatominae) vectors, which release the parasite in the feces, infecting human through skin lesions.

The first line of vertebrate defense against blood loss is platelet aggregation that forms a hemostatic plug. Platelet aggregation is accomplished through a biochemical cascade triggered by collagen, thrombin, thromboxane A2 and ADP [3,4]. To preserve host blood flow during feeding, saliva of hematophagous insects contain platelet-aggregation inhibitors.

Triatoma infestans is considered a main vector in the epidemiology of Chagas disease in some ecosystems of South America, because it feeds more efficiently than its *Triatominae* competitors [5,6]. Several salivary proteins of triatomine bugs have been characterized so far, the majority consisting of platelet inhibitors. Two *T. infestans* salivary proteins, triplatin-1 and -2, inhibit platelet aggregation induced by collagen, but not by ADP or thrombin [7]. *Triatoma pallidipennis* saliva contains two inhibitors of platelet aggregation, pallidipin, which shares function and sequence similarities with triplatins [8] and triabin, a thrombin-induced platelet-aggregation inhibitor [9,10]. Another platelet-aggregation inhibitor from triatomine bugs is the *Rhodnius prolixus* ADP-binding protein *Rhodnius* Platelet Aggregation Inhibitor 1 (RPAI1) [11] that inhibits ADP-induced platelet aggregation [12]. Triplatin, pallidipin, triabin and RPAI, all belong to the family of lipocalins that are extracellular

Abbreviations: IEF, isoelectric focusing; IPG, immobilized pH gradient; 2-DE, two-dimensional gel electrophoresis; MS BLAST, mass spectrometry driven BLAST; TiAPY, *Triatoma infestans* apyrase; DTT, dithiothreitol

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transport proteins [13]. In *R. prolixus* saliva, the strongest anti-platelet aggregation activity triggered by ADP is attributed to apyrase, a nucleoside triphosphate-diphosphohydrolase that hydrolyses ATP or ADP to AMP [14,15]. This *R. prolixus* enzyme has not been isolated yet and, like other apyrases, does not belong to the lipocalin family.

T. infestans saliva also manifests salivary apyrase activity [16]. It appears that *T. infestans* salivary apyrases play an important role in the insect feeding habit, and half of its storage is used during single blood meal [17]. It has been shown that the apyrase activity of *T. infestans* saliva should be related to five glycosylated proteins with apparent molecular masses of 88, 82, 79, 68 and 67 kDa, that can not be distinguished by non-denaturing one-dimensional gel electrophoresis due to homo-oligomerization [18]. Only the full sequence of the *Tiapy79* gene encoding the 79 kDa apyrase was reported, confirming that it belongs to the 5′-nucleotidase family [16].

Despite of the medical importance of *T. infestans* vectoring, relatively little is known about its genome. Currently, sequences of 35 *T. infestans* proteins are available in NCBI database. Therefore, proteomics efforts utilizing conventional database searching approaches that rely on exact correlations of masses of intact peptides (in peptide mass fingerprinting) or masses of precursors and fragments (in tandem mass spectrometry) to corresponding database entries were expected to deliver limited identification efficiency (reviewed in [19]). The identification could rely on cross-species matching of spectra from *Triatoma* peptides to identical peptide sequences in known homologous proteins. Alternatively, unmatched peptide spectra could be completely or partially interpreted de novo (reviewed in [20]) and obtained sequence candidates used in error-tolerant searches (reviewed in [21]). Both stringent and error-tolerant approaches can be combined in a layered fashion such that, first, the known and highly homologous proteins are identified in stringent cross-species searches, and then the rest of spectra is interpreted de novo and complete or partial peptide sequences are applied for similarity driven identifications [22,23]. The former approach is considerably faster since it utilizes uninterpreted tandem mass spectra, however it typically produces hits with only a few matched peptides and therefore their statistical confidence could be compromised. The sequence similarity approach is more challenging technically, however, by tolerating multiple mismatches in aligned peptide sequences, it almost doubles the number of identified proteins [23–26].

Mass spectrometry-based strategy has been used for characterizing the proteome of salivary gland from *Anopheles gambiae*, the major malaria vector. That study provided validation for the existing genome annotation, and also discovered novel proteins, which had been represented only as predicted transcripts in the databases and not previously identified as cDNAs [27]. This illustrates the complementary nature of different strategies for identification of gene products.

Herein, we present the first proteomic study of the *T. infestans* saliva. 2-DE maps revealed more than 200 salivary protein spots, most of them in the alkaline pH range. The majority of proteins identified by mass spectrometry is potentially engaged in platelet-aggregation inhibition and belongs to triabin subfamily and apyrase family. The results also revealed that several proteins were present in multiple isoforms having partial sequence redundancy.

2. Materials and methods

2.1. Harvest of triatomine saliva

T. infestans colony was reared in the insectarium of the Chagas Disease Multidisciplinary Research Laboratory, University of Brasília, maintained at 28 °C, 70% relative humidity, with photoperiods of 12 h. Secreted *T. infestans* saliva was collected by using pipette tips placed in contact with the insect mouthparts at 7–9 days following the blood meal. Protease Inhibitor Mix without EDTA (GE Healthcare, Uppsala, Sweden) was added to the saliva and samples stored at −80 °C. For tissue localization experiments, saliva samples were extracted from the salivary glands D1, D2 and D3. Glands in 0.9% saline solution were carefully punctured with a needle, and extracted intra-luminal saliva was harvested by centrifugation.

2.2. Quantification of sample

Samples of pooled saliva obtained from several adult insects, were quantified using the Plus One 2D Quant Kit (GE Healthcare) and, in parallel, by amino acid analysis on a Hitachi L8500 amino acid analyzer using ninhydrin post-column derivatization.

2.3. Two-dimensional gel electrophoresis

2.3.1. Wide pH range

T. infestans saliva samples containing 80 or 300 μg protein were diluted and incubated for 1 h at room temperature in 350 μL final volume of denaturing sample buffer containing 7 M urea, 2 M thiourea, 66 mM DTT, 2% Triton X-100, 0.5% Pharmalyte 3–10, and 10% isopropanol. Samples were centrifuged (12,000 × *g*, 10 min) prior to IEF.

The supernatants were applied to 18 cm IPG gel-strips with linear separation pH range of 3–10 (GE Healthcare) by in-gel rehydration [28] for 6 h without current followed by 6 h at 30 V. IEF was carried out at 20 °C using an Ettan IPGphor3 unit (GE Healthcare) with the following conditions, 500 V for 1 h, 1000 V for 1 h and 8000 V for 4 h with a maximum current of 50 μA per strip. Prior to SDS-PAGE, the IPG strips were subjected to reduction with equilibration buffer (50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 4% SDS) supplemented with 125 mM DTT for 40 min and alkylation with 300 mM acrylamide in equilibration buffer for additional 40 min. SDS-PAGE was performed on 12% T polyacrylamide gels run on a Protean II system (Bio-Rad, Richmond, CA, USA) at 20 °C. Proteins were visualized by silver staining [29] and the gels were stored in 1% acetic acid before protein digestion. Alternatively, the 2-DE gels were submitted to Western blotting.

2.3.2. Basic pH range

IPG gel-strips of 11 cm with a linear separation alkaline pH window of 6–11 were rehydrated in 200 μL of denaturing sam-