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Proteomic profiling of the midgut contents of *Haemaphysalis flava*

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ABSTRACT

Scant information is available regarding the proteins involved in blood meal processing in ticks. Here, we aimed to highlight the midgut proteins involved in preventing blood meal coagulation, and in facilitating intracellular digestion in the tick *Haemaphysalis flava*. Proteins were extracted from the midgut contents of fully engorged and partially engorged ticks. We used liquid chromatography tandem-mass spectrometry (LC–MS/MS) analysis to identify 131 unique peptides, and 102 proteins. Of these, 15 proteins, each with at least two unique peptides, were recognized with high confidence. We also retrieved 18 unigenes from our previous published transcriptomic libraries of the midguts and salivary glands of *H. flava,* and inferred the primary structures of nine proteins and fragments of five proteins. There were 23 and 21 unique proteins in the midgut contents of fully engorged and partially engorged ticks, respectively. We detected 58 shared proteins in the midgut contents of both fully engorged and partially engorged ticks. Of these, seven were significantly differentially expressed between fully engorged and partially engorged ticks: actin, calmodulin, elongation factor-1α, hsp90, multifunctional chaperone, tubulin α, and tubulin β. Our results demonstrated that the proteome of the midgut contents, combined with the transcriptome of the midgut, was a viable method for the reinforcement of protein identification. This method will facilitate further study of blood meal processing by ticks, as well as the identification of clues for tick infestation control. The existence of numerous proteins detected in the midgut contents also highlight the complexity of blood digestion in ticks; this area is in need of further investigation.

1. Introduction

Haemaphysalis flava, a tick belonging to the family Ixodidae, has a broad geographic distribution in Asia. This species parasitizes human, cattle, wildlife, and a variety of birds. *H. flava* sheds both viral and bacterial pathogens, including thrombocytopenia syndrome, tick-borne encephalitis (Yun et al., 2016), *Borrelia* spp. (Ishiguro et al., 2000), *Cercopithifilaria* spp. (Nematoda: Onchocercidae) (Uni et al., 2013), *Anaplasma,* and *Bartonella* spp. (Kang et al., 2016). Previously, we characterized microbial community diversity in *H. flava* using NGS, and showed that eight bacteria were highly abundant throughout the life cycle of the tick (*Rickettsia* spp., *Coxiella* spp., *Pseudomonas* spp., *Ehrlichia* spp., *Escherichia* spp., *Acinetobacter* spp., *Citrobacter* spp., and *Cupriavidus* spp.; Duan and Cheng, 2017). As *H. flava* has a wide geographic distribution, and harbors dozens of pathogens through numerous hosts, this species is especially significant for public health.

TickGARD and the Gavac vaccine, both based on the Bm86 antigen, are effective, economic, and environmentally friendly strategies for tick control (Rodríguez-Mallon, 2016). The identification of additional antigen candidates for further vaccine development is critical. High-throughput omics technologies, including proteomics and transcriptomics, have greatly facilitated this. To date, proteomic analyses of the saliva and feces of *H. flava,* as well as transcriptomic analyses of the midgut and salivary glands in this species, have identified several antigen candidates, including subolesin, hsc70, and enolase.

As the host-tick-pathogen interface, the tick midgut is recognized as a reservoir of antigen candidates (Oleaga et al., 2017); Bm86 is expressed there. Midguts are also major sites for the storage and digestion of blood meals in ticks. Although the molecular physiology of blood meal processing in the midgut epithelia has received intense study (Sojka et al., 2013), the molecules and mechanisms in the midgut that prevent blood meal coagulation and that mediate intracellular digestion remain largely unknown. As blood-proteins provide ticks with essential nutrients, gut contents, which may include food residues, molecules released by upper tissues, and microorganisms, are directly associated with digestion and the efficient utilization of blood meals; gut contents are thus closely related to tick vitality and vector capability (Connat, 1991). However, integrated analyses of tick midgut contents are

Abbreviations: ACN, acetonitrile; BCA, bicinchoninic acid; DTT, DL Dithiothreitol; FASP, filter aided sample preparation; FDR, false discovery rate; HCD, higher-energy collisional dissociation; KO, knocked out; LC–MS/MS, liquid chromatography tandem-mass spectrometry; LFQ, label free quantification; NGS, next generation sequencing; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UA buffer, urea acid buffer; Vitellogenin, Vg

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extremely rare. We therefore aim to identify the proteins in the midgut of *H. flava* with LC–MS/MS, and to combine these identifications with our previous published transcriptome data from the midgut and salivary glands of this species. Using these two datasets, we aim to determine the primary structures of related proteins. These structures will provide a basis for further study of the mechanisms of blood meal anticoagulation and digestion, as well as a framework for the identification of feasible antigen candidates for future vaccine development.

2. Materials and methods

2.1. Tick specimens

Live *H. flava* specimens were collected in Xinyang, Henan Province, China (32°13′N,114°08′E). Identification was confirmed using morphological and molecular analysis (Yan and Cheng, 2015). Ticks were fed on hedgehogs in our laboratory at Hunan Agricultural University (HUNAU), Hunan Province, China. All experimental procedures were approved and overseen by Institutional Animal Care and Use Committee at HUNAU (No.43321503). No animals were subject to unnecessary suffering in the present study.

2.2. Collection and pretreatment of midgut contents

We dissected 12 adult female ticks, six fully engorged and six partially engorged, under a stereo microscope. Midgut contents, about 30 μL from each tick, were carefully squeezed into a clean tube containing 90 μL sodium citrate-physiological saline. Tubes were vortexed and centrifuged at 5 000 rpm for 15 min. We removed 4 μL aliquots of each supernatant, and pooled these in two groups based on the feeding state of the source tick: fully engorged or partially engorged. We added an equal volume of SDT buffer (4% SDS, 100 mM DTT, and 150 mM Tris-HCl pH 8.0) to each supernatant pool. We vortexed the supernauts, and placed the tubes in a water bath at 100 °C for 5 min. We preliminarily analyzed the proteins in the two pools with the conventional BCA method and with SDS-PAGE (Song et al., 2016).

2.3. Protein analysis with LC–MS/MS

A filter aided sample preparation (FASP) was used to purify and digest proteins before instrumental analysis (Wiśniewski et al., 2009). Briefly, 200 μL UA buffer (150 mM Tris-HCl and 8 M urea pH 8.0) was added to 10 μL each midgut extract (not pooled), and well vortexed. Samples were then transferred to ultrafiltration tubes fitted with 10 kDa membranes, and centrifuged at 14,000 × *g* for 15 min. Proteins remained on the membrane, and the filtrate was discarded. We performed this filtration twice to maximize the removal of impurities. Residues on the membrane were reconstituted in 200 μL UA buffer containing 50 mM iodoacetamide, shocked at 600 rpm for 1 min, and allowed to stand for 30 min at room temperature in the dark. Samples were centrifuged at 14 000*g* for 10 min. Filtrate was discarded, and residue retained on the membrane was washed twice with 200 μL UA buffer, and then with 200 μL dissolution buffer (25 mM NH_4HCO_3).

Protein resides were digested with 40 μL trypsin buffer (3 μg trypsin (Promega, WI, USA) in 40 μL 25 mM NH_4HCO_3) in a 37 °C water bath for 16–18 h. This digest was transferred to clean ultrafiltration tubes fitted with 10 kDa membranes, and centrifuged at 14,000 × *g* for 10 min. We collected 5 μL filtrate from each sample, and measured the peptide levels at OD280.

We diluted each filtrate sample to 1 μg/μL peptide, and 5 μL of each diluted sample was loaded onto an EASY-nLC1000 system (Thermo Scientific, MA, USA). Mobile phase A was 0.1% formic acid, and mobile phase B was 84% acetonitrile in 0.1% formic acid. Chromatographic columns were balanced with 95% mobile phase A before sample loading. Samples were injected onto a trap column (Thermo EASY column SC001 traps 150 μm × 20 mm (RP-C18)), and then onto an analytical column (EASY column SC200 150 μm × 100 mm (RP-C18)). The flow rate was set to 250 nL/min. Mobile phase B was used as the eluent. After HPLC separation, both samples were analyzed with a Q-Exactive mass spectrometer (Thermo Scientific, MA, USA). Nano spray ionization was used as the ion source, and argon was used as the collision gas. We set the analysis time to 120 min.

MS data was obtained using a data-dependent top10 method, where we dynamically chose the most abundant precursor ions from the survey scan (300–1 800 *m/z*) for higher-energy collisional dissociation (HCD) fragmentation. Determination of the target value was based on predictive automatic gain control. Dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70 000 at *m/z* 200, and the resolution for the HCD spectra was set to 17 500 at *m/z* 200. Normalized collision energy was 30 eV, and the underfill ratio was defined as 0.1%. The mass spectrometer was run with peptide recognition mode enabled. We performed three biological and three technical replicates of the LC–MS/MS analysis.

2.4. MS data analysis

MS data were processed with MaxQuant (version 1.3.0.5). We identified proteins by searching against the Uniprot database uniprot_Ixodidae_80119_20151102. We constructed fasta and putative peptide libraries based on our previously published transcriptomes of the midgut and salivary glands of *H. flava.* Carbamidomethylation of cysteine was used as a fixed modification, while oxidation of methionine was defined as a variable modification. Searches were carried out with tryptic specificity, allowing two missed cleavage sites at most, and a mass measurement tolerance of 20 ppm in MS mode and 0.5 Da for MS/MS ions. The global false discovery rate (FDR) cutoff for peptide and protein identification was set to 0.01. Hits with at least two unique peptides were considered proteins with high confidence. Label-free quantification (LFQ) was carried out in MaxQuant as previously described by Luber et al. (2010). Protein abundance was calculated on the basis of the normalized spectral protein (LFQ) intensity. For each protein common to the two engorgement states, we calculated the LFQ intensity ratio between fully engorged and partially engorged. We also compared LFQ intensities using the Student's *t* test. The difference between LFQ intensities was considered significant if the LFQ ratio was ≥ 1.5 or ≤ 0.67, and p was < 0.05.

The raw data files used for these analyses are available from the integrated proteome resource (http://iprox.org/page/SSV024.html;url=1511751279689Dh3L with the key: zORj; project ID: IPX0001041003).

2.5. Bioinformatics analysis of identified proteins

We used our protein identifications and annotations to retrieve unigenes from the previously published *H. flava* transcriptome libraries of the midgut (NCBI Gene Expression Omnibus ID: GSE69721; https://www.ncbi.nlm.nih.gov/gds/?term=GSE69721) and the salivary glands (NCBI Gene Expression Omnibus ID: GSE67247; https://www.ncbi.nlm.nih.gov/gds/?term=GSE67247). The primary structures of the proteins were predicted based on gene sequences.

3. Results

3.1. Protein analysis with SDS-PAGE

Our BCA indicated that there was 9.8 μg/μL protein in the midguts of partially engorged ticks, and 22.4 μg/μL protein in the midguts of fully engorged ticks. SDS-PAGE protein bands were clear, and ranged from 10 to 170 kDa (Fig. 1). Protein band patterns were generally similar between engorgement states: both states had very intense bands at about 70 kDa (Fig. 1). Our analyses therefore indicated that our midgut protein extraction was effective.