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Insect-Bacteria interaction: identification of some of the bacterial proteins in the gut of Nezara viridula

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The existence of bacterial proteins was proved in the gut of animals. Many proteins with bacterial origin were reported from the alimentary canal in the invertebrate animals. Insects, as the largest group of animals, have known serious pests in agricultural ecosystems. Feeding and nutrition are the main ways that caused damages to economic crops. Identification of digestive proteins in the gut of insects is the first step in the clearance of biochemical degradation of food particles. Proteomics is a powerful tool to identify effective proteins in the physiological systems in animal and plants. Digestive proteins in the gut of insects have originated from innate cells and microorganisms like bacteria. In this research, for the first time, gut proteins of *Nezara viridula* were extracted. These proteins were visualized and identified with two-dimensional polyacrylamide gel electrophoresis and mass spectrometry, respectively. Eight proteins with bacteria origins were accumulated in the gut of adults. Identified proteins were phospho pyruvate dehydratase, amino acid –N-acetyl transferase, hypothetical protein, glycerol-3 phosphate dehydrogenases, ribulose-phosphate 3-epimerase, pyruvate kinase, chitinase, and catalase. Results suggest that the interaction of bacteria and lumen of Hemiptera has a high level of efficacy; hence, the nutrition process can be used as a new target for selecting proper inhibitors to use in the integrated pest management.

Introduction

Metagenomic analysis has been extensively used to study microorganism- insect interaction. The efficacy of this technique is not suitable for identifying the metabolically active microbiome in the gut of insects. Compared to genomics and transcriptomic, only proteomics can be considered a trusted approach to produce effective information based on the “function”. A variety of bacterial phyla are commonly present in insect guts, including Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Firmicutes including Lactobacillus and Bacillus species, Clostridia, Actinomycetes, Spirochetes, Verrucomicrobia, Actinobacteria,Candidatus, Pantoea, Burkholderia, Brevundimonas, Rhizobium, Pseudomonas, Tachikawaea, Rosenkranzia, Streptomyces, Buchera and other genus.

Nezara viridula is a known insect that can be considered a serious pest in agricultural systems. The common names of this pest are the southern green stink bug (USA), southern green shield bug (UK), or green vegetable bug (Australia and New Zealand). This pest is a cosmopolitan pentatomid, highly polyphagous, feeding on cultivated and

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uncultivated plants in the Middle East region, particularly in Iran. Using insecticides is one of the common approaches to control the hemipteran pests. On the other hand, overpressure of widespread pesticides like organophosphates and pyrethroids agents have disadvantaged effects on the non-target organisms, particularly their biological enemies in the dynamic ecosystems. Hence, finding new methods with no or minimal risks for insect control is essential. Disrupting sect pests' nutrition process is an ideal opportunity to find a proper inhibitors for using of direct or indirect in integrated pest management. Many protein inhibitors were reported against various pests' like protease inhibitors, α-amylase inhibitors, lectins, and chitinases (Jouanian et al., 1998).

The digestive system of insects is one of the main barriers against toxic xenobiotics that enter to the gut lumen. The basic structure of the digestive canal haschanged and adapted in relationto feeding approaches (Engel and Moran, 2013). In Hemiptera such as Pentatomidae and Scutelleridaethe alimentary canal is composed of foregut, midgut, and hindgut, among which foregut and hindgut are smaller than the midgut. Also, midgut divided into four parts may have differences, especially in the herbivorous species (Habibi et al., 2008; Saadati et al., 2008). Salivary glands are the second organs of the digestive system in animals, particularly hemipteran insects (Habibi et al., 2008, Saxena, 1963). Extra oral digestion in the Sap-feeding hemipterans is specialized in feeding on nectar or honeydew, which do not produce peritrophic matrix proteins (Engel and Moran, 2013). During extra-oral digestion, injection of saliva proteins to plant tissue occurred, and after liquefying of the food particles, they were pumped to the gut for the final digestive process (Boyd, 2003). The physiological functions of the gut as the main part of the alimentary canal are degradation, digestion, and absorption (Pauchet et al., 2008; Hou et al., 2010; Liu et al., 2009). On the other hand, the role of the gut in the defense system against plant toxins and other xenobiotics that can be taken while feeding should be considered (Yao et al., 2009). Hence, the gene expression and protein accumulation in the gut of insects are key factors in the study of the nutrition process.

Wilkins proposed the term of proteomics in 1996 for the first time as a similar approach to genomics. It is defined as the large-scale investigation of proteins, emphasizing their specialized functions in particular times or situations. The proteome map of the Eurygaster integriceps and Aelia acuminata's midgut was determined previously by Saadati et al., (2012b, Saadati and Nouri, 2021). They identified more than 100 protein spots which were classified into 11 various functional categories. They demonstrated some changes in adult Sunn pests' gut and salivary gland proteins and their fifth instars in a comparative mode (Saadati et al., 2012c). Moreover, another study confirmed that some wheat proteins were accumulated in the gut of adult Sunn pests (Saadati and Toorchi, 2017). In the new finding, some bacteria proteins in the gut of Sunn pests were reported for the first time by Saadati and Toorchi (2021).

In the current study, bottom-up proteomics isused to identify some bacterial proteins in the gut of N.viridula. The study of bacteria-insect interaction is a new opportunity to use bacterial proteins as new targets in protein engineering to produce new inhibitors against the symbiotic proteins as a component of digestive proteins.

Mateial and Methods

Sampling and insect dissection

Adults of southern green stink bug (Nezaraviridula, Hemiptera: Pentatomidae) were collected from farms and gardens of Torbat-e Jam, Khorasan Razavi, Iran, in June 2016 and the digestive system were dissected. The midguts were then transferred to microtubes containing a cocktail of protease inhibitors in phosphate-buffered saline (PBS, pH: 6.9) (Roche Appli,Science, Mannheim, Germany).

Protein extraction

TCA/acetone that adapted to insect proteins was used for the extraction. Eight guts were selected to hemogynazied to a fine powder using a mortar and pestle. Then, gut tissues were transferred to a 50 ml tube containing 10 ml of 10% trichloroacetic acid in acetone and 0.07% of 2-mercaptoethanol. The tube was ultra-sonicated and kept at -20°C for one hour. Centrifugation was performed at 9000 ×*g* for 20 min at 4° C, and

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then the supernatant was removed. Washing with acetone containing 0.07% of 2-mercaptoethanol, air-dring, and resuspending in the lysis buffer (7 M Urea, 2 M Thiourea, 5% 3-[(3-cholamidopropyl) dimethylammonio]-1propanesulfonate (CHAPS), and 2 mM Tributylphosphine) were used for pellet recovery on thevigorous shake. The final step of centrifugation was performed at 20000 ×g for 20 min at room temperature for removing gross materials. The pellet was kept at -80°C until further analysis as it was described previously (Saadati Bezdi et al., 2012 a, b).

Two-dimensional polyacrylamide gel electrophoresis (2-DE) and image analysis

The amount of 350 µg of gut proteins was transferred to an immobilized pH gradient (IPG) strip (11 cm, pH 3-10 linear; Bio-Rad, Hercules, CA, USA). The rehydration was performed under the following condition: 50 V for 14 hours at 25 °C. Isoelectric focusing was performed under various conditions, including: 250 V for 15 min; and 8000 V for one h on a linear ramp; and 8000 V for four h on a rapid ramp. The equilibration solution (ES) 1 (6 M urea, 2% Sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 130 mM dithiothreitol (DTT)) was used to float the strips for 30 min. This process was repeated with equilibration solution (ES) 2. The DTT was substituted by 135 mM Iodoacetamide (Saadati and Toocchi, 2021) Using 13% SDS-polyacrylamide gel The second dimension electrophoresis was begun after the first dimension. Staining of gels using Coomassie Brilliant Blue and scanning with a calibrated densitometer (GS-800, Bio-Rad) were performed, respectively. PDQuest 2-D analysis software (ver. 8.0.1, Bio-Rad) is special proteomics software used for gel analysis. The analysis contains different stages such as image filtration, spot detection, background subtraction, and spot matching. As the principle of bottom-up proteomics gel with the highest quality was selected as the reference gel, and was used for the comparison with the other gels. According to the recommendation of PDQuest software, the amounts of protein per spot were expressed as the volume of the spot, which is defined as the sum of detected intensities of all pixels that make up that spot (Saadati bezdi et al. 2012c).

Protein identification

To identify proteins, gels excised from Coomassie brilliant blue CBB -stained 2-DE gels were subjected to in-gel trypsin digestion (Wako, Osaka, Japan) using an automated protein digestion system (Digest Pro 96; Intavis, Koeln, Germany). To prepare spots these steps were performed sequentially: Incubation (acetonitrile), washing (50 mM NH4HCO3, 15 min), reduction (10 mM DTT in 50 mM NH4HCO3, 20 min), and alkylation (40 mM Iodacetamide in 50 mM NH4HCO3, 15 min). Finally, samples were digested using trypsin at 37°C for 16 h. desalting was performed using NuTip C-18 pipet tips (Glygen Inc., Columbia, *NY*). Peptides were then injected with an autosampler into an Ultimate 3000 nanoLC (Dionex, Germering, Gemany) coupled with a nanospray LTQ Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA, USA). Peptides (1 µl) were reconstituted in 0.1% formic acid (5 µl) and loaded onto the trap column (PepMap, C18, 300 µm ID × 5 mm, pump at 25 µl/ min flow rate). Then, peptides were eluted and separated from the trap column using 0.1% formic acid in acetonitrile on a 75 µm ID × 12 cm C18 column (Nikkyo Technos, Tokyo, Japan) at a flow rate of 200 nl/ min and sprayed at a voltage of 1.8 kV. The mass spectrometry (MS) was applied in the positive-ion mode using Xcalibur software (ver. 1.4, Thermo Fisher). Data acquisition was set to cover a scan range of 100-2000 m/z by three MS/MS scans after considering of 60 min as the retention time. Tandem mass spectrum data files were converted to MGF files using Bioworks software (ver. 3.3.1, Thermo Fisher). Peptide masses were searched against protein sequences available from the National Centre for Biotechnology Information using the Mascot software (ver. 2.3.02, Matrix Science, London, UK). Search parameters were set equal to 0.5 Da for product mass tolerance and 10 ppm for peptide mass accuracy. Only one missed trypsin cleavage was allowed during analysis, and carbamidomethylation of cysteines and oxidation of methionines were selected as fixed and variable modifications, respectively (Saadati Bezdi et al., 2012b, c). The four-match peptides and at least six percent coverage in the peptides sequences were selected at the beginning of evaluations of probable proteins. Finally, the proteins with ion scores greater than 40which were significant in

the NCBI database (P<0.01) selected as target proteins.

Results

Adults southern green stink bug, *N.viridula* were selected for dissecting in the insect-bacteria interaction study. In the first step, crude proteins which were extracted from their guts were separated by 2-DE and visualized by CBB. Protein expression patterns were analyzed using image analysis software, and a total of eight protein spots were selected to identify. Many spots were observed in the gels that were not related to the bacteria and hence should be discussed in the separated manuscripts. (Figure 1).

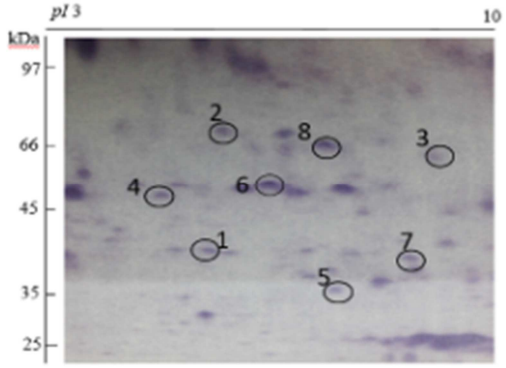


Figure 1. Gut protein expression patterns of southern green stink bug (*Nezara viridula*). Adults were dissected, and their gut proteins were extracted, separated by two-dimensional gel electrophoresis, and visualized by CBB staining. Circles indicate positions of accumulated proteins in the guts.

Many spots were observed in the final gels, but only eight spots were related to the bacteria. Raw data from mass spectrometry that conclude sequence of amino acids were blast in the animal database and plant database to identify proteins. For the 8 sots that were not addressed and reported as unknown, all organism databases were used. For the first time in the present study, eight proteins were identified as bacterial proteins using proteomics (Table 1). Newly identified proteins include: Phospho pyruvate dehydratase (spot 1), Amino acid –N-acetyl transferase (spot 2), Hypothetical protein (spot 3), glycerol-3 phosphate dehydrogenases (spot 4), Ribulose-phosphat 3-epimerase (spot 5), Ptruvat kinase (spot 6), Chitinase (spot 7), and Catalase (spot 8) (Table 1).

Dillon, 2004). The bacterial activity in the gut of insects was reported in both Aerobic and anaerobic conditions, as that aerobic phase was more occurred in the smaller insects compared to larger insects.

Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, and Firmicutes such as Lactobacillus and other Bacillus species, Clostridia, Actinomycetes, Spirochaetes, Verrucomicrobia, and Actinobacteria are commonly present in the gut of insects (Engel and Moran, 2013). The heritable symbionts can be divided into two intergrading categories obligate and facultative endosymbionts (Baumann, 2005).

Engel and Moran (2013) showed that Ishikawaella capsulate, as an obligate bacterium in the midgut of Stink bugs,had a key role in the gut-microorganism interactions. The gastric caeca is a special part of the digestive system to colonize bacterial symbionts in the other Hemiptera. Some of them are transmitted vertically through smearing of eggs by the mother as in *I. capsul*ate, and in the other species, the environmental acquisition is entrance way. Lastly, specific symbiont strain may be selected every generation after suitable ingested organisms (Engel and Moran, 2013; Demandel et al., 2020). The southern green stink bug, N.viridula (Heteroptera: Pentatomidae), contains symbiotic bacteria in the alimentary canal as innate and acquiring shelter in every generation (Prado et al., 2006).

Our data indicated that glyceraldehyde 3-phosphate dehydrogenase (Spot 4), and pyruvate kinase (Spot 6), two key enzymes in the carbohydrate metabolism, are expressed in the southern green stink bug gut with an origin of the P.capeferrum and K.piersonii,respectively. Glyceraldehyde 3-phosphate dehydrogenase breaks down the glucose to produce cellular energy (Chandra et al., 2006; Kunieda et al., 2004). This enzyme is necessary to convert glyceraldehyde 3-phosphate to the 1, 3 bisphosphoglycerate during the glycolysis cycle. Our results showed that both innate cells and symbiotic bacteria effectively synthesize and secret key proteins in the carbohydrates metabolism. The pyruvate kinase is the vital enzyme in the glycolysis process in which glucose is broken down into two pyruvates. The role of pyruvate kinase was occurred in the final step, in

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which a phosphate group in phosphoenolpyruvate is transferred to ADP(Chandra et al., 2006).Pyruvate kinase has four different tissue-specific isozymes in animals, PKL, PKR, PKM1, and PKM2, but there are no evidence of its isoforms in the gut of insects.

Table 1. Expressed proteins with bacterial origins in the gut of adult, *Nezera viridula* using all entries (organisms) in NCBI database.

Spotno.[a]	Description	Acc. no.[b]	Organism	Theo[c]. I_r (kDa)/p*I*		Exp[d]. Mr (kDa)	M.P.[e]	Score[f]	Cov[g]. %
1	Phospho pyruvate	gi| 751600640	*I.capsulata*	47.5	5.74	38	9	51	16
2	hydratase		*P.vagans*	49.4	5.58	59	14	63	19
3	Aminoacid-N-acetyl transferase	gi| 1694344347	*T.gelatinosa*	51.9	9.16	62	13	57	11
	Hypothetical protein	gi| 686207646							
4	Glycerol-3-phosphate	gi| 666446524	*P.capeferrum*	52.3	6.90	58	8	52	15
5	dehydrogenase		*I.capsulata*	24.5	6.37	38	14	55	9
	Ribulose-phosphate 3-epimerase	gi| 751601753							
6	Pyruvat kinase	gi| 218762995	*K.piersonii*	51.49	5.80	64	19	76	21
7	Chitinase	gi| 1826397527	*Steroptomyces sp*	26.8	9.28	42	9	48	7
8	Catalase	gi| 354528412	*R.conorii*	17.71	6.9	70.2	11	48	8

a) Spot no., the spot number as given in figure 1.
b) Acc. no., accession number according to the NCBI (all entries) database.
c) Theo., theoretical; M_r, molecular weight; *pI*, isoelectric point.
d) Exp., experimental.
e) M.P., number of query matched peptides; the proteins with more than 4 matched peptides were included.
f) Score, ion score of identified protein using NCBI database.
g) Cov., Sequence coverage, the proteins with more than 6% sequence coverage were included.

Ribulose-phosphate 3-epimerase (Spot 5) catalyzes the reversible epimerization of D-ribulose 5-phosphate to D-xylulose 5-phosphate. This enzyme is classified as the enzyme effective for carbohydrates metabolism. The main function of this protein in the gut of insects was related to the non-oxidative phase of the pentose phosphate pathway and the implication of additional pentose and glucuronate inter conversions. Hypothetical proteins (Spot 3) as predicted proteins in the different databases were reported in many proteomic researches. These proteins are interesting candidates for further biochemical investigations as our current knowledge is insufficient to identify their properties. Aminoacid-N-acetyl transferase (Spot 2) is a key enzyme during protein synthesis. This enzyme catalyzes the transfer of acetyl groups from acetyl-CoA to arylamines, arylhydroxylamines, and arylhydrazines. The arginine production pathway is this enzyme's target in the proteins' metabolism reactions.

Phospho pyruvate hydratase (Spot 1), also known as enolase,is a metalloenzyme which catalyses the reversible dehydration of D-2-phos-phoglycerate to phosphoenolpyruvate. Enolase belongs to the family of lyases that cleave carbon-oxygen bonds. The main function of this enzyme is the stability of glycolysis reactions in the prokaryote and eukaryotes. Chitinase (Spot 7) is the main protein

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in the degradation of chitin polysaccharides. This protein belongs to the carbohydrate metabolism group. Although there is no chitinase gene in most insects, bacterial origins in the southern green stink bug proved the symbiotic role of some bacteria in the gut of some Hemiptera.

Catalase (Spot 8), as antioxidant protein, acts in the redox reaction and improves the immune system's efficacy against oxidative stress. This enzyme was reported in similar insects but was reported from symbiotic bacteria for the first time in Iran (Chen et al., 2019). In the previous studies, accumulation of this protein in the guts of similar insects like adult sunn pests was reported (Saadati Bezdi et al., 2012c). The presence of similar proteins from different origins in the gut of Hemiptera confirmed the interaction of microorganisms with gut tissue as the main part of the feeding process (Chen et al., 2019).

So far, the digestive system of some of the hemipteran as important pests in the agricultural ecosystems was studied by Saadati et al (2012a, b,c, 2020, 2021). Proteome map of salivary glands and gut in the adult and fifth instar nymphs of sunn pest, proteome map in the *Aelia acuminate*, tracing of plant proteins in the gut of sunn pest and bacteria proteins in the gut of *E.integriceps* were reported in the previous data (Saadati et al., 2012a, b,c; Saadati and Nouri, 2020; Saadati and Toorchi, 2021). Combination of these results with previous data about existing proteins in the gut of herbivorous insects increase our knowledge about how to digest the food particle in the lumen.

In this study, a new technique like proteomics can complete the mechanism of degradation at the molecular level. However, further experiments are essential to improve appropriate approaches to study bacteria-insect interaction to find new targets as accumulated proteins in the alimentary canal of insects as bio pesticides in the near future.

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