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Research Article

Identification of Bacterial Proteins in the Gut of Sunn pest, *Eurygaster integriceps*

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Abstract

Proteomics is a powerful tool to identify effective proteins in the biochemical reactions of the insect body. Many proteins were reported in the gut lumen and tissues which are essential to complete the physiological role of the alimentary canal of Sunn pest. The gut microbiome of insects has a key role in the digestive process. In this study, for the first time, gut proteins of adult Sunn pest were extracted. These proteins were visualized and identified with two-dimensional polyacrylamide gel electrophoresis and mass spectrometry, respectively. Newly identified proteins include pyruvate dehydrogenase, oxidoreductase FAD-binding protein, hypothetical proteins, glycerol-3 phosphate dehydrogenase, conserved hypothetical proteins, ABC excinucleases, ABC-type transport systems and molecular chaperones. The accumulation of these metabolism proteins in the gut of Sunn pest indicates the importance of symbiotic proteins in the improvement of digestive activities and insect-bacteria interactions. Results suggest that the identified bacterial proteins can be considered as effective proteins in the process of nutrition and provide more gut-derived targets for enzyme engineering and development of biopesticides.

***Keywords*:** Proteomics, Digestive system, Scutelleridae, Biochemistry, Bacteria, Two dimensional gel electrophoresis

Introduction

Many insects are serious pests of the grains, among them Sunn (Critchley, 1998). The active cycle begins in the spring with main devastating consequences on the farms. It includes feeding, reproduction, and appearance of new adults. Inactive phase occurs on the host in the winter shelters on hillsides (Critchley, 1998; Javaheri et al., 2009). The utilization of chemicals is one of the common approaches to control the Sunn pest in the farmers. Although, overuse of insecticides has affected the non-target organisms particularly their biological enemies in the current farms. This lead to the outbreak of secondary pests like wheat trips and some aphids in the grain farms of Iran. Therefore, the introduction of safer pest management methods is amenable. Protein inhibitors are a new class of biopesticides with the capacity for safe control of insect-plant interactions. Insecticidal proteins such as protease inhibitors, α-amylase inhibitors, lectins, and chitinases have been proposed as potential candidates for development of transgenic plants (Jouanian et al., 1998). The comprehensive study of the digestive system could be considered as the first step to find potent targets for toxic proteins.

The basic structure of the digestive canal is similar among various species of insects with some differences related to their adaptation for feeding process (Engel and Moran, 2013). In hemiptera and Sunn pest as special cases, the alimentary canal is composed of foregut, midgut and hindgut, among which foregut and hindgut are smaller than the midgut (Habibi et al., 2008; Saadati et al., 2008). Midgut as the key part of the digestive system contains four parts (Saadati bezdi et al., 2012a). Salivary glands and gut are main parts of the digestive system in the insects, particularly hemipterous insects (Habibi et al., 2008, Saxena, 1963). Sap-feeding hemipterans, and many beetle and ant species which are specialized on nectar or honey dew do not produce peritrophic matrix proteins (Engel and Moran, 2013). Extra oral digestion as a unique approach of feeding was reported from hemiptera. During extra oral digestion, they inject their salivary proteins to the

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target plant tissues to liquefy the food before entering to their gut for final digestive process (Boyd, 2003). The gut as the main part of the digestive system is responsible for converting food particles to small molecules and preparing them for absorption (Pauchet et al., 2008; Hou et al., 2010; Liu et al., 2009). Moreover, gut is the first natural barrier against plant toxins and other xenobiotics that can be taken while feeding (Yao et al., 2009). Hence, gut proteins and their gene expression modifications are key factors which improve efficiency of nutrition.

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

On the other hands, the functional integrity of bacterial proteins with digestive processes was approved in animals. It was proposed that gut microbiome secretions of insects may have different roles in their normal physiology. In the present study we were endeavored to identify gut bacterial proteins of Sunn pest for the first time. This will open up new opportunities to clarify the roles of bacterial proteins in digestive and immune systems of insects and will improve our knowledge about insect-bacteria interactions.

Materials and Methods

Animal sampling and insect dissection

Overwintering adults of Sunn pest (*Eurygaster integriceps Puton*, Hemiptera: Scutelleridae) were collected from Torbat-e Jam, Khorasan Razavi, Iran, in February 2018 and were transferred to insectarium for rearing on the wheat seeds (*Triticium aestivum* L.; Poales: Poaceae) of variety Roshan at 27°C with a 16:8 long-day (L:D) photoperiods. One-day-old new adult insects were selected for dissection of digestive system. Their midguts were then transferred to micro tubes containing a cocktail of protease inhibitors in phosphate buffered saline (PBS, pH: 6.9) (Roche Applied Science, Manneheim, Germany).

Protein extraction

At first, ten dissociated guts were grounded to a fine powder using a mortar and pestle. Then, gut tissues were transferred to a 50 ml tube containing 10 ml of 10% tricholoroacetic acid in acetone and 0.07% of 2-mercaptoethanol. The tube was ultra-sonicated and kept at -20°C for one hour. After one round of centrifugation at 9000 ×*g* for 20 min at 4° C, the supernatant was removed. The pellet was washed with acetone containing 0.07% of 2-mercaptoethanol, air-dried, and resuspended in the lysis buffer (7 M Urea, 2 M Thiourea, 5% 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS), and 2 mM Tributylphosphine) while vigorously shake. The final step of centrifugation was performed at 20000 ×g for 20 min at room temperature to remove 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

A total of 400 µg of gut proteins were 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 1 h on a linear ramp; in addition to 8000 V for 4 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 dithiortheitol (DTT)) was used to float the strips for 30 min. This process was repeated with equilibration solution (ES) 2 in which the DTT was substituted by 135 mM Iodoacetamide. The second dimension electrophoresis with 13% SDS-polyacrylamide gel was begun after isoelectric focusing. The gels were finally stained with Coomassie Brilliant Blue and were scanned with a calibrated densitometer (GS-800, Bio-Rad). Results were analyzed using PDQuest 2-D analysis software (ver. 8.0.1, Bio-Rad). This includes different stages such as image filtration, spot detection, background subtraction and spot matching. The gel with the highest quality was selected as the reference; and was used for spot analysis of other gels. 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). To reduce errors during image analysis, spots volumes were normalized using local regression method.

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 automated protein digestion system (Digest Pro 96; Intavis, Koeln, Germany). To prepare spots these steps were performed sequentially: Incubation (acetonitrile), washing (50 mM NH_4HCO_3, 15 min), reduction (10 mM DTT in 50 mM NH_4HCO_3, 20 min), and alkylation (40 mM Iodacetamide in 50 mM NH_4HCO_3,15 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) and data acquisition was set to cover a scan range of 100- 2000 *m/z* that resulted by three MS/MS scans after considering of 60 min as 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 which are 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 cysteins and oxidation of methionines were selected as fixed and variable modifications, respectively (Saadati Bezdi et al., 2012b, c). The three match peptides and at least five percent coverage in the peptides sequences were selected to beginning of evaluations of probably proteins. Finally, the proteins with ion scores greater than 36 which were significant in the NCBI database ($P<0.01$) selected as target proteins.

Results

One-day-old adult sunn pests were selected for dissecting. 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 288 protein spots were detected. The most spots were also identified in previous studies (Saadati et al., 2012a; Saadati et al., 2012b; Saadati et al., 2012c., Saadati and Toorchi, 2017). Fifteen protein spots which were not introduced previously were selected for further analysis via sequencing (Figure 1).

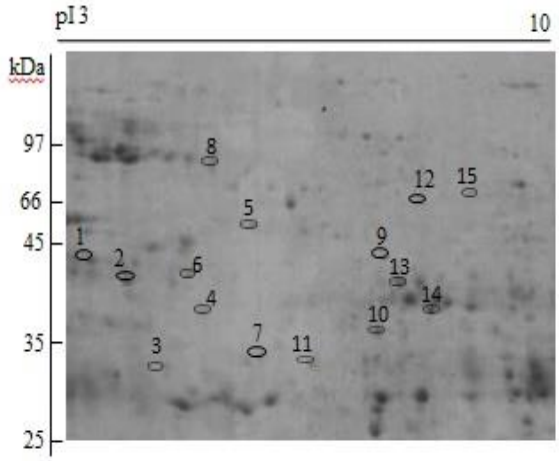

Figure 1. Gut protein expression patterns of Sunn pest adult insects (*Eurygaster integriceps*). One-day-old insects rearing on the wheat seeds were dissected and their gut proteins were extracted, separated by two-dimensional gel electrophoresis, and visualized by CBB staining. Circles indicate positions of accumulated proteins in the guts.

The resulted sequences that prepared with mass spectrometry searched in the different database. In the first step animal database and plant database were used to identify new proteins. Finally, all organism databases used to search new sequence for covering prokaryotes and eukaryotes proteins. Eight proteins were identified for the first time in the present study. Although, they were extracted from sunn pest gut, they had a bacterial origin (Table 1). Newly identified proteins includes: Pyruvate dehydrogenases (spot 1a), oxidoreductase FAD-binding proteins (spot 1b), unidentified proteins (spot 1c), glycerol-3 phosphate dehydrogenases (spot 2a), conserved unidentified proteins (spot 2b), excinuclease ABC (spot 3), ABC-type transport systems (spot 4), and molecular chaperons (spot 5). In the proteomics researches, targets proteins were selected from current database. Hence, there is more chance to this point that unknown proteins (Table 1) be new proteins that not addressed with identified proteins. Some of spots in the gel patterns contain

more than one protein like spot 1 and 2 (Figure 1, Table 1). Cutting of large spot from fresh gels may be reducing quality of target spots. The replicable experiment is necessary action against this challenge. Distribution of appearing spots in the final gels showed that bacteria can be secreted various proteins in the wide range of *pH* in the gut of sunn pest (Figure 1). The most of identified proteins belongs to energy and metabolism proteins such pyruvate dehydrogenases, oxidoreductase FAD-binding proteins, glycerol-3 phosphate dehydrogenases and ABC-type transport systems. The other proteins from this research are effective in the protein and nucleic acid synthesis like unidentified proteins (spot 1c), conserved unidentified proteins (spot 2b), excinuclease ABC (spot 3), and molecular chaperons (spot 5).

(Saadati et al., 2012a). Our results showed that some of bacterial proteins were accumulated in the alimentary canal of adult sunn pest. The function of microbial proteins in the digestive system of insects is improvement of digestive and immune process (Demandel et al., 2020). Alkaline condition in the gut of some insects like Lepidoptera and termites were not proper to symbiotic bacteria, generally. There are new reports from resistant bacteria to alkalinity phase like a *Firmicutes*, *Clostridium*, and *Planctomycetes* in the digestive system of insects (Engel and Moran, 2013). Aerobic and anaerobic conditions are very effective to microbiome variability in the gut of insects. It is reported that anaerobic phase was more occurred in the larger insects in comparison to smaller insects.

The presence of many protists, fungi, archaea, and bacteria was reported in the guts of various insects

Table 1. Expressed proteins with bacterial origins in the gut of adult sunn pest, Eurygaster integriceps, using all entries (organisms) in NCBI database.

Spot no.[a]	Description	Acc. no.[b]	Organism	Theo[c].		Exp[d].	M.P.[e]	Score[f]	Cov[g].
				M_r (kDa)/p*I*		Mr (kDa)			%
1	Pyruvate dehydrogenase	gi| 97898	*E.faecalis*	26.2	4.36	44	15	59	19
1b	Oidoreductase FAD-binding protein	gi| 291333815	*Marine bacterium*	37.8	3.62	44	11	48	17
1c	Hypothetical protein	gi| 145543418	*P.tetraurelia*	35.2	3.88	44	8	45	13
2	Glycerol-3-phosphate dehydrogenase	gi| 28377286	*L.plantarum*	38.2	4.72	41.3	12	44	8
2a	Conserved Hypothetical protein	gi| 303247419	*D.fructosovoranus*	36.40	4.92	41.3	9	38	7
3	Not identified								
4	Not identified								
5	Not identified								
6	Not identified								
7	ABC-type transport system	gi| 237745572	*O.formigenes*	26.17	6.18	34.7	16	64	15
8	Not identified								
9	Molecular Chaperons	gi| 120436429	*G.forsetti*	46.3	7.63	46.2	20	85	11
10	Not identified								
11	Not identified								
12	Excinnuclease ABC	gi| 15892189	*R.conorii*	17.71	6.9	70.2	11	48	8
13		Not identified							
14		Not identified							
15		Not identified							

a) Spot no., the spot numbers as given in the Figure 1.
b) Acc. no., accession numbers 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 3 matched peptides were included.
f) Score, ion score of identified protein using NCBI database.
g) Cov., Sequence coverage, the proteins with more than 5% sequence coverage were included.

Discussion

Microbial colonization of the gut lumen depends on the variations of both pH and oxygen levels (Engel and Moran, 2013; Demandel et al., 2020). Our previous data showed that the *pH* along the gut axis of sunn pest is about 5.5 to 6.5 for the midgut

(Dillon and Dillon, 2004). Termits as the main host of protists, xylophage insects as vectors of fungi, and methanogenic archaea and bacteria as common microorganisms in the animal world, are well-known examples of the symbiotic insect-bacteria interactions (Engel and Moran, 2013; Dillon and Dillon, 2004). There are few or no detectable

bacteria in the gut of hemiptera insects. On the other hands, detritivores and wood-feeders are famous groups which have the highest ratios of total gut microbial biomass to the host mass.

Dillon and Dillon (2004) suggested that there is a direct relationship between communities and the compartmentalizations of the gut. They proposed that small communities of microorganisms such as Drosophila, Mosquitoes, and Aphids have a correlation with the narrow gut. 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). In a previous study, Baumann showed that heritable symbionts can be divided into two intergrading categories as follows: obligate and facultative endosymbionts (Baumann, 2005).

The presence of the *Ishikawaella capsulate*, as an obligate bacterium, was reported from the midgut of Stink bugs. This bacterium has a key role in the coevolution of the gut-microorganism interactions. During this process some genes which are related to the cell wall synthesis or lipids metabolism were drifted, which indicates the specialization of the microorganisms to the conditions provided by their hosts (Engel and Moran, 2013).

The gastric caeca are special part of digestive system to colonize of bacterial symbionts in the other hemiptera insects. Some of them are transmitted vertically through smearing of eggs by the mother as in *I. capsul*ata, and in the other species environmental acquisition is entrance main way. In the last way, 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, *Nezara viridula* (Heteroptera: Pentatomidae), contains symbiotic bacteria in the alimentary canal as both way innate and acquiring shelter in every generation (Prado et al., 2006).

Previous data demonstrated the proteome map of the gut and salivary glands of the adult Sunn pests using tube-based 2-dimensional gel electrophoresis (Saadati Bezdi et al., 2012a and 2012b). The previously identified proteins were classified into six functional groups based on their physiological roles including metabolism proteins, musculature proteins, immune related proteins, transport proteins, nutrition storage, and other proteins (Saadati Bezdi et al., 2012b). Various proteins were reported from the gut of Sunn pest: *i)* Proteins associated with the gut muscles such as myosin heavy chain, arginine kinase, actin 3, and tropomyosin; *ii)* effective proteins of the carbohydrate metabolism like glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase, α-amylase, β-galactosidase, glycoside hydrolase, enolase, and aldose reductase; *iii)* protein related to protein metabolism such as aspartate aminotransferase, glyoxylate reductase, glutamate dehydrogenase, and trypsin (Saadati Bezdi et al., 2012b and 2012c). Our data indicated that glyceraldehyde 3-phosphate dehydrogenase, and pyruvate dehydrogenase, two key enzymes in the carbohydrate metabolism, are expressed in the Sunn pest gut with an origin of the *Lactobacillus plantarum* and *Enterococcus faecalis*, 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. Through the combination of this data with our previous ones, we can suggest that both innate cells of the host and some symbiotic bacteria have the amenable capacity for the synthesis of key proteins of the carbohydrates metabolism. Furthermore, the supplementary roles of some microorganisms in the nutrition process were approved. The pyruvate dehydrogenase-mediated reaction of pyruvate conversion to the acetyl co enzyme A occurs in the inner membranes of mitochondria (Chandra et al., 2006). The 1c and 2b spots consist of some hypothetical proteins which their existence has been predicted. These proteins are interesting candidates for further biochemical investigations as our current knowledge is not sufficient to identify their properties. Although, in the near future their complete sequences, structures and functional features will be provided based on the molecular dynamic studies in the context of the progressive proteomics. Molecular chaperons (spot 5) are among the critical proteins during the protein synthesis. In fact, most of the times, chaperons are heat shock proteins which are expressed in response to the cellular stress or the elevation of the temperature. Upon activation they can act as foldases, holdases or protein disaggregates (Mahroof et al., 2009). Our results propose that the existence of chaperons with bacterial origins can be considered as an effective folding agent in the conformational determination of the bacterial proteins. Some chaperons of the Sunn pest gut were reported by Saadati bezdi et al., (2012 a, c).

Oxidoreductase FAD-binding protein (spot 1b) is the main protein in the electron transport chain that acts in the redox reaction with flavin adenine

dinucleotide (FADH). This protein is necessary to complete ATP production after the reduction of nicotinamide adenine dinucleotide (NADH) and FADH (Chen et al., 2019). Similar to our previous results, it was demonstrated that this protein is also accumulated in the guts of adult Sunn pests (Saadati Bezdi et al., 2012c). The presence of similar proteins from different origins confirmed the importance of some host reactions in the gut of the Sunn pest. It is interesting that some coevolutionary pathways select proper symbiotic bacteria of the digestive system in the Sunn pests.

ABC-type transporter system (ATP-binding cassette transporter) (Spot 4) is one the largest gene families of animals and prokaryotes. This protein composed of a transmembrane and a membrane-associated ATPase subunit (Chen et al., 2019).
Excinuclease or excision endonuclease (spot 3) is a key enzyme of the DNA repair. The existence of this enzyme with the bacterial origin suggests that the DNA reproductions of symbiotic bacteria is a common mechanism. So far, the digestive system of the Sunn pest was one of the interesting targets for proteome analysis by our research team. We studied the proteome map of the gut and the salivary glands (Saadati bezdi et al., 2012a, 2012b). We compared the proteome contents of adult Sunn pets with their fifth instars (Saadati bezdi et al., 2012c). Also, we demonstrated the accumulation of some plant proteins in the guts of the Sunn pests (Saadati and Toorchi, 2017). These results will complete our knowledge about the gut proteins of Sunn pest in Iran. However, further experiments are essential to find more appropriate approaches to apply these proteins as potential candidates during protein engineering and design of biopesticides against sunn pest populations.

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