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Bottom-up Proteomics: Identification of Salivary Gland Proteins in the Bishop's Mitre Shieldbug, *Aelia Acuminate*

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ABSTRACT

Proteomics is a powerful technique to identify proteins as important biomacromolecules. The determination of protein maps in the different organs using proteomics is the first step of molecular studies in the nutrition process. The salivary glands of Hemiptera are the main resource of secreting various proteins in the extra-oral digestion as a preliminary digestive process that occurs in the Hemiptera as specialty. The importance of salivary proteins is not only in the nutrition process but also they are key agents to elicit/suppress plant defense pathways in the insect-plant interaction. In this study, proteome map of salivary glands in the Aelia acuminata was visualized and some of the up-regulated proteins were identified using bottom-up proteomics. Final results lead to the identification of twenty-three proteins in the salivary gland tissues. Identified proteins belong to different categories contain digestive enzymes and their inhibitors, cell metabolism proteins, and cell defense proteins. Results indicate that the many known proteins in this research can be considered appropriate candidates for use in enzyme-engineering programs to produce and develop new protein inhibitors in the wheat structure that leads to disruption of the feeding process in the bishop's mitre shieldbug.

Keywords: Digestive system, Inhibitor, Hemiptera, Metabolism, Two-dimensional gel electrophoresis

INTRODUCTION

Global strategic crops are corn, wheat, rice and soybeans. In low-income countries like Iran, wheat is the dominant crop. Many insects and diseases can be affected yield production in agricultural ecosystems. Several important pests like *Eurgaster integriceps*, *Diuraphis noxia*, *Zabrus tenebrioids*, *Cephus pygmeaus*, *Haplothrips triticum* were reported from wheat farms in Iran. Bishop's mitre shieldbug, *Aelia acuminata* is a hemipterous pest that reported as potential pest that outbreaks after using widespread insecticides against sunn pest in recent decades. The biology cycle of this multivoltine pest was studied and it has not migratory- phase unlike sunn pest [1,2].

Salivary gland and gut are main parts of the digestive system in the insect, particularly hemiptera [3,4]. Extra oral digestion (EOD) is a common way of feeding in the predaceous land-dwelling arthropods and herbivorous hemiptera [5]. In the extra oral digestion, salivary proteins inject to the plant tissue to liquefy food into nutrient-rich slurry as the first step of digestion [6,7]. In this step, hydrolytic enzymes degrade some of the plant tissue and after liquefying, pumped them to gut for the final digestion [8]. Many proteins with structural and physiological roles were reported from the salivary gland of Insects. Alpha-amylase, serin protease, trypsin, chymotrypsin, glucose dehydrogenase and Phospholipase A2 from *E.integriceps* [9]; phosphatase and phosphorilase from *Pyrrhocoris apterus* [10]; serin protease from *Oncopeltus fasciatus* [11]; serin protease from *Miris dolabratus* [12]; glucose oxidase from *Helicoverpa zea* [13]; polygalacturonase from *L.hesperus* [14]; Protease, elastase, amylase, pectinase and phospholipase A2 from *L.hesperus* [15]; β-glucosidase from *Pieris brassicae* [16] and *Myzus persicae* [17], and α-amylase from *Lygus hesperus, Lygus lineolaris* and *Orius insidiosus* [18].

Any disruption in the normal feeding process can be considered potential factor in reducing losses. Overusing of pesticides as efficient and fast approach has adverse effects on the non-target organisms in agricultural ecosystems.

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Fig. 1. Adult insect of bishop's mitre shieldbug, *Aelia acuminate* (adapted from https://biodiversidade.eu/especie/aelia-acuminata-linnaeus-1758-/?lang=en).

Hence, finding environmental methods like using protein inhibitors such as protease inhibitors, α-amylase inhibitors, lectins and chitinases as biopesticides are necessary [16].

The identification of saliva proteins is essential action to study the insect-plant interactions because the first contact occurred between saliva and plant proteins. The aim of the present study is determination of proteome map of salivary glands in the *A.acuminata*. The results of current study will open new opportunity in the insect-plant interaction studies and improved our knowledge about physiological systems in the invertebrate animals.

MATERIALS AND METHODS

Insects and Dissection

Adults, *Aelia acuminata* (Hemiptera, pentatomidae) were collected from Torbat-e Jam, Iran, in February 2016 and reared in an insectary room on wheat, *Triticium aestivum* L. (Poales: Poaceae) variety Roshan at 27 ℃ with a 16:8 L:D photoperiod (Fig. 1). Starved adults; 24 h after feeding; were selected for the dissection of salivary glands. The glands (10 pairs) were transferred to microtubes containing one milliliter phosphate buffered saline (pH: 6.9) and a cocktail of protease inhibitors (Roche Applied Science, Manneheim, Germany).

Protein Extraction

The acetone/trichloroacetic acid approach with tiny modification was used for protein extraction [17,18]. The twenty glands were ground and homogenized with 10 ml of a solution containing 10% tricholoroacetic acid in acetone and 0.07% 2-mercaptoethanol, followed by ultrasonication for 10 min [7] Total proteins were precipitated and then centrifuged at 9000 × g for 20 min at 4 °C. The product pellet was washed with acetone (three times) and then was dried for 20 min under vacuum conditions. The final pellet was resuspended using lysis buffer containing 7 M urea, 2 M thiourea, 5% CHAPS, 2 mM tributylphosphine. The final stage was centrifugation at 20000 × g for 20 min to remove insoluble materials.

Two-dimensional Polyacrylamide Gel Electrophoresis

A total of 500 µg proteins of salivary glands were applied to immobilized pH gradient (IPG) strips (11 cm, pH 3-10 linear; Bio-Rad, Hercules, CA, USA) with rehydration at 50 V for 14 h followed by isoelectric focusing at 250 V for 15 min on a linear ramp, 8000 V for 1 h on a linear ramp and 8000 V for 4 h on a rapid ramp. After isoelectric focusing separation, the strips were equilibrated for 30 min in a solution containing 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 130 mM dithiortheitol (DTT), followed by 30 min in the same equilibration solution with DTT substituted by 135 mM iodoacetamide. The IPG strips were subjected to the second dimension of electrophoresis after transferring onto a 13% SDS-polyacrylamide gel. The

gels were stained with Coomassie brilliant blue (CBB) [17,18].

Image Analysis

The PDQuest software (ver. 8.0.1, Bio-Rad) was used for the analysis of gel images. In the process of image analysis, image filtration, spot detection and measurement, background subtraction and spot matching was done, respectively, after selecting one gel as reference. The amount of protein in a spot was expressed as the volume of the spot, which was defined as the sum of the intensities of all pixels that make up that spot [17]. In the final step, the local regression method was used to normalize spot quantities.

Protein Identification

Protein spots excised from CBB-stained 2-DE gels were subjected to in-gel trypsin digestion (Wako, Osaka, Japan) using automated protein digestion (Digest Pro 96; Intavis, Koeln, Germany). Spots placed in 96-well plate were incubated in 50% acetonitrile and then washed in 50 mM NH_4HCO_3 for 15 min. Proteins were reduced with 10 mM DTT in 50 mM NH_4HCO_3 for 20 min and alkylated with 40 mM iodacetamide in 50 mM NH_4HCO_3 for 15 min, then digested with 1 pM trypsin at 37 °C for 16 h. The resulting peptides were concentrated and desalted using NuTip C-18 pipet tips (Glygen, Columbia, MD, USA). Peptides were injected using an autosampler into an Ultimate 3000 nanoLC (Dionex, Germering, Gemany) coupled to a nanospray LTQ XL Orbitrap MS (Thermo Fisher, San Jose, CA, USA). Peptides (1 µl) were loaded in 0.1% formic acid onto a 300 µm id × 5 mm C18 PepMap trap column at a 25 µl min^{-1} flow rate. The 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^{-1} and sprayed at voltage of 1.8 kV. The MS operated in the positive ion mode using Xcalibur software (ver. 1.4, Thermo Fisher) and data acquisition were set to cover a scan range of *m/z* 100-2000 followed by three MS/MS scans in exclusion dynamic mode in 60 min retention time.

Tandem mass spectrum DTA 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 Center for Biotechnology Information (NCBI; 2019-11-24) using the Mascot search engine (ver. 2.3.02, Matrix Science, London, UK). Search parameters were 0.5 Da for mass tolerance and 10 ppm for peptide mass accuracy. Only one missed trypsin cleavage was allowed and carbamidomethylation of cysteine and oxidation of methionines were selected as the fixed and variable modification, respectively. Proteins with more than five matched peptides with at least eight percent sequence coverage were considered. Proteins with ion scores greater than 52 were significant for NCBI database ($p < 0.01$).

RESULTS

The previously starved adults; 24 h; were selected for dissecting of salivary glands. According to bottom-up technique, salivary gland proteins were extracted, separated by 2DE and visualized by CBB, respectively [17,18]. Gel patterns were analyzed using PDQeuest software and 121 protein spots were detected in the first step. Twenty-nine spots; 24% of spots; were selected to identify as possibility to cut (frequency of intensity) (Fig. 2).

Twenty-three new proteins, 79% from selected spots, were identified and reported for the first time from salivary glands of *A.acuminata* (Table 1). Seven spots, 21% from selected spots, contain number 5, 9, 13, 24, 25, 28 and 29, were not addressed in the current databases. New proteins were macrophage erytroblast attacher (spot 1), nitric oxide synthase (spot 2), Sucarase (spot 3), ecdyson-induced protein (spot 4), beta glucosidase (spot 6), nitrophorin 1 (spot 7), maltase A1 (spot 8), putativae phosphoglycerate mutase (spot 10a), glutathione-S-transferase (spot 10b), alpha amylase (spot 11), chymotrypsinogen A (spot 12), protein tyrosinase kinase (spot 14), thymidine kinase (spot 15), chymotrypsin (spot 16), trypsin-7 (spot 17), phenoloxidase-activating enzyme (spot 18), trypsin inhibitor (spot 19), kunitz protease inhibitor (spot 20), trypsin precursor (spot 21), putative secreted protein (spot 22), hypothetical protein (spot 23), salivary secreted protein (spot 26), 60 s ribosomal protein (spot 27).

DISCUSSION

Salivary glands in hemiptera are important resources to digestive enzyme production in the extra oral digestion [8].

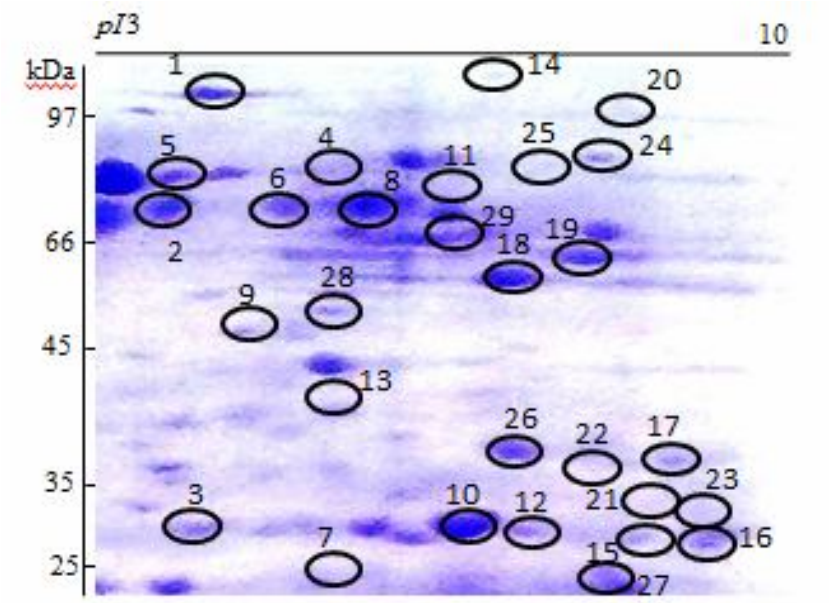

Fig. 2. Protein expression patterns in the salivary glands of adult insects of *Aelia acuminata*. Starved adults were dissected and proteins were extracted from salivary glands, separated by 2-DE and visualized by CBB staining. Circles indicate position of accumulated proteins in salivary glands.

Many proteins existed in the lumen and tissues of salivary glands. The role of these proteins in the insect-plant interaction is unclear and it is necessary to improve our knowledge in this background.

In the current study, identified proteins were divided into four categories contain digestive enzymes and their inhibitors that conclude sucarase, beta-glucosidase, alpha-amylase, chymotrypsinogen-A, chymotrypsin, trypsin-7, trypsin precursor, kunitz protease inhibitor and trypsin inhibitor; metabolism proteins contain protein tyrosinase kinase, thymidine kinase, 60s ribosomal protein and putative phosphoglycerate mutase; defense and immune proteins contain macrophage erythroblast attacher, nitric oxide synthase, nitrophorin 1, glutathione S-transferase and phenoloxidase-activiting enzyme; and uncategorized proteins contain Ecdyson-induced protein, salivary secreted protein, hypothetical protein and putative secreted protein (Fig. 2, Table 1).

Sucarase (spot 3), is responsible for the hydrolysis of sucrose into glucose and fructose, which is essential in the feeding process of insects. Insect sucarase activity is thought to depend mainly on alpha-glucosidase [19]. Also, sucarase was reported as potential elicitor in the insect-plant interaction. Our results showed that salivary sucarase as important protein can be considered as elicitor and digestive enzyme in the salivary gland of *A.acuminata*. Sucarase were reported from salivary gland of blattidae, gryllidae, acridaidae *etc.* (Pauchet *et al.,* 2008). Beta-glucosidae (spot 6), as hydrolyzing enzyme breakdown β(1-4) linkages between two glucose or glucose-substituted molecules like cellobiose. Also, it can act as elicitor in the insect-plant interaction in which triggering plant defense mechanisms [20]. Beta-glucosidae was reported from salivary glands of *E.integriceps* using proteomics technique [17,18]. MaltaseA1 (spot 8), as an isoform of the maltase family, cleaves the α(1-4) linkages of maltose and convert it to the simple sugar glucose. The activity of maltose A1 on the sucrose is more important compared to maltose [21]. Maltase A1 as a vital carbohydras in the internal metabolism of digestive system was reported from salivary glands of hemiptera [18]. Chymotrypsin (spot 16) as serine protease was originating from Chymotrypsinogen A (spot 12) after activating stage. The main function of chymotrypsin is hydrolyzing amide bonds in the protein

Table1. Expressed Proteins in the Salivary Glands of *Aelia acuminata* Using Metazoa in NCBI Database

Spotno[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	Macrophage erytroblast attacher	gi| 109043138	*Bemisia tabaci*	106.6	4.58	110	5	54	9
2	Nitric oxode synthase	gi| 1370627585	*Blattella germanica*	67.5	5.14	70	6	68	11
3	Sucarase	gi| 388556076	*Aedes aegypti*	31.8	5.47	34	6	55	8
4	Ecdyson-induced protein	gi| 386771349	*Drosophila melanogaster*	88.5	6.06	92	8	69	14
5	Not identified								
6	Beta glucosidase	gi| 636630776	*Perplaneta americana*	57.3	4.98	72	12	71	8
7	Nitrophorin 1	gi| 3219825	*Rhodnius prolixus*	22.7	6.81	25	9	56	11
8	Maltase A1	gi| 1152526134	*Nilaparvata lugens*	71.6	6.91	74	9	63	9
9	Not identified								
10[a]	Putative phosphoclycerate mutase	gi| 925207831	*Rhodnius negelectus*	29.1	6.51	30	11	71	11
10[b]	Glutathion S-transferase	gi| 7302612	*D.melanogaster*	23.4	6.64	30	8	65	9

a) Spot no., the spot number as given in figure. 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 5 matched peptides were included. f) Score, ion score of identified protein using NCBI database. g) Cov., Sequence coverage, the proteins with more than 8% sequence coverage were included.

Table1. Continued

11	Alpha-amylase	gi| 205638829	*A.aegypti*	80.8	7.17	84	21	77	9
12	Chymotrypsinogen A	gi| 560905860	*Camelus ferus*	27.2	7.44	29	12	58	14
13	Not identified								
14	Protein tyrosinase kinase	gi| 560905860	*Triatoma didmidiata*	101.6	6.62	110	8	62	12
15	Thymidin kinase	gi| 1101358166	*B.tabaci*	28.8	9.14	23	7	68	14
16	Chymotrypsin	gi| 32395297	*Stomoxys calcitrans*	28.4	8.52	27	24	82	16
17	Trypsin -7	gi| 170058933	*Culex quinuefasciatus*	30.6	8.99	36	18	79	15
18	Phenoloxidase-activating enzyme	gi| 1533734057	*Bombyx mandarina*	48.2	8.43	60	8	58	9
19	Trypsin inhibitor	gi| 1829478868	*D.mauritiana*	58.2	8.33	62	12	71	13
20	Kunitz protease inhibitor	gi| 197260710	*Similium vittatum*	88.8	8.95	99	14	76	12
21	Trypsin precursor	gi| 18034141	*Lygus lineolaris*	31.3	9.04	33	14	68	10
22	Putative secreted protein	gi| 18568284	*A.aejypti*	28.8	9.14	37	8	57	10
23	Hypothetical protein	gi| 124704698	*Heliothis virescens*	24.2	9.27	30			
24	Not identified								
25	Not identified								
26	Salivary secreted protein	gi| 37048801	*Mayetiola destructor*	12.2	9.97	41	18	82	11
27	60 s ribosomal protein	gi| 907681951	*S.calcitrans*	13.5	11.27	23	25	91	36
28	Not identified								
29	Not identified								

chains at the carboxyl side of aromatic amino acids. Chymotrypsin activity and its optimum condition such as instability at acidic *pH* and inhibition patterns are different between insect and vertebrate [22]. This enzyme is an abundant protein in the digestive system of insects. Hence, it can be selected as proper target for using of new enzyme inhibitors in the transgenic plants researches. Trypsin-7 (spot 17) and trypsin precursor (spot 21), as proteolytic enzymes, breakdowns amide bonds in the interior protein chains. These proteins belong to the serine protease family, which is found in the digestive system of vertebrate and invertebrate animals [23]. This enzyme was known protease in the salivary glands of hemiptera such as *E.itegriceps* [17,18]. Trypsin inhibitor (spot 19) and kunitz protease inhibitor (spot 20) belong to serine protease inhibitor family. If protease activities exceed tissue requirement, a subsequent cascade of events using serine protease inhibitors leads to reduction or suppression of proteolytic activities [23]. Many reports from accumulation of these inhibitors in the gut of herbivorous insects were recorded [8,17,24]. Alpha amylase (spot 11) split the α(1-4) glycosidic linkages in starch, glycogen and dextrins. The products of this process are oligosaccharide, maltose and glucose [25]. This enzyme has key role in the external and internal digestion in the cereal pest such a *E.integriceps* [8].

Digestive enzymes in the salivary glands of hemiptera are considered as effective proteins in the starting of extra oral digestion. Some of these enzymes were reported from salivary glands of *E.integriceps*, *Lygus hesprus*, *M.persicae*, *Miris dolabratus* and similar species [26,17,9]. Only for the limit species, protein map was determined using proteomics such as *E.integriceps*, *M.perscice*, *Rhodnius prolixus* and *Acyrthsiphon pisum* [27,28,18]. Results of this study confirm the potential importance of protease, amylase and the other carbohydrases in the insect-plant interaction.

Putative phosphoglycerate mutase (spot 10a), is zymogen phosphoglycerate mutase, which is vital enzyme in the conversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis and gluconeogenesis. Protein tyrosinase kinase (spot 14), was a single-pass transmembrane protein that transfers a phosphate group from ATP to the tyrosine residues on the intracellular and extracellular proteins [29]. Thymidin kinase (spot 15), was fundamental enzyme in DNA production process and cell division in way using introducing thymidine into the DNA formation. This enzyme transfers a phosphate group from ATP to the thymidine [30]. 60 s ribosomal protein (spot 27) is innate protein that play a main role in protein formation. In similar to our results, many of metabolism proteins were reported in the protein map of salivary glands in the *E.integriceps* [17]. Macrophage erytroblast attacher (spot 1), is the main protein at the cell surface of mature macrophages that mediates the attachment of erythroid cell to mature macrophages [31,32]. This process is necessary to inhibit apoptosis during terminal maturation and nucleation of erythroblasts. Existence of this protein can be related to contrast between insect and plant interaction. Nitric oxide synthase (spot 2) (EC 1.14.13.39) is a key enzyme in the nitric oxide (NO) production through enzymatic conversion of L-arginine to citrulline. Nitric oxide (NO) is a biological effector molecule that acts as a multifunctional macromolecule in the physiological systems such as in the imaginal disc development, synaptogenesis, formation of retinal projection patterns, behavioral responses and modulate various cellular responses to stressors such as radiation, bacterial infection and hypoxic environment [33]. In similar to our results, up-regulation of this protein was reported in the salivary proteome of hemipterous pest [17,18]. Nitrophorin 1 (spot 7), is one isoform from nitrophorin family that is found in the saliva of blood-feeding insects [34,35]. Nitrophorins contain nitric oxide ligated to the ferric hem iron. The main function of nitrophorins is transporting nitric oxide to the feeding site. Results suggest that accumulation of twin proteins, nitrophorins and nitric oxide synthase, can be improved defense ability of salivary proteome against various stressors. Glutathione-S-transferase (spot 10b), is a famous enzyme to detoxify xenobiotics and noxious compounds and oxidative stress. This enzyme used from reduced glutathione to conjugate with target molecules [7,36]. Phenoloxidase-activating enzyme (spot 18), is endopeptidase with selective post-Arg cleavage site using calcium ions activate phenoloxidase system as a part of the innate immune system [37]. Several proteins that were related to the defense system were identified in the salivary proteome of sunn

pest [8,17,18]. The accumulation of many defense proteins in the salivary gland of *A.acuminata* indicated that co-evolution in the *A.acuminta*-wheat interaction is in the sensitive stage in which defense proteins of adults overcome to plant barriers in the first line of feeding.

Putative secreted protein (spot 22) and salivary secreted protein (spot 26) are proteins that there is no evidence to discuss about their physiological roles. Digestive, elicit, metabolism effects may be occurred after theirs secretion [38,39]. Hypothetical protein (spot 23) is protein with unknown function and there is no experimental evidence to discuss about its gene expression *invivo* [19,40]. More than 20 percent of proteins encoded in each newly sequenced genome, nominated as hypothetical protein [41,42]. Ecdyson-induced protein (spot 4), contain both a canonical DNA binding domain and a ligand-binding domain that define members of the nuclear receptor superfamily, although these proteins are referred to as orphan nuclear receptors because no corresponding hormonal ligand has yet been identified [43,44]. Many proteins with unknown function are reported from different insects that are needed to extract separately and after sequencing, find a biological role for them in the physiological systems.

CONCLUSIONS

Proteomic technique is very helpful to identify protein map in the different organs of insects. In this work, proteome map of salivary glands of aduts *A.acuminata* were detrmined and many proteins were reported for the first time in Iran. Identification of salivary proteins in the herbivorous hemiptera is the critical stage in the plant-insect interactions. Several proteins from salivary glands were documented that are proper targets to use in the production of transgenic plants to reduce application of widespread insecticides in the near future.

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