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Identification of salivary proteins in the adult of southern green stink bug, *Nezara viridula* using of gel-based proteomics

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ABSTRACT

The digestive system of hemipteran was adapted to extra-oral digestion. Salivary proteins were injected into the plant cells and after changing form to liquid were transferred into the midgut to complete digestion process. The salivary gland and its secretions have an important function in the feeding process in the insect world. In this work, protein patterns of salivary glands were determined using bottom-up proteomics. Using two-dimensional electrophoresis, gel analysis, gel digestion, liquid chromatography, mass spectrometry, and blast in the specific databases were performed, respectively. Finally, 49 spots were sequenced. Identified proteins classified their physiological roles into seven categories; carbohydrates metabolism, lipid metabolism, protein metabolism, nucleic acid metabolism, defense proteins, structure proteins, and the other groups. Identification of salivary proteins increases our knowledge about the physiology of salivary glands, but also, it can be used in protein engineering to produce new inhibitors that can disrupt insect-plant interaction that led to decreasing in nutrition in the insect pest. Also using new techniques like proteomics can be considered a great step in protein studies because it facilitates to study of a proteome instead of a protein.

Keywords: Inhibitor, Extra oral digestion, Pest management, Hemiptera, Mass spectrometry, Gel base approach

INTRODUCTION

Polyphagous insects are serious pests on garden and farm crops. One of the key pests is a southern green bug, *Nezara* viridula (Pentatomidae: Hemiptera) which is a cosmopolitan pest in many countries particularly Iran (Javaheri et al. 2009). The mouthparts of this insect is piercing-sucking that consist of tetramer beak which called the rostrum. In the first step, salivary proteins were interred to plant tissue and after primary degradation, fluid food was pumped up the midgut lumen. The most parts of plants are sufficient to feed, but some parts like growing shoots and developing fruit are more selected. It is possible that shoots destruct after damage and may die. The important sign of damage on fruit is reported as hard brownish or black spots. These damages affect marketing value and reduce farmers' income for a long time. In addition to the usual damage caused by

N.viridula feeding, it is a main vector for some of pathogens like the tomato bacterial spots, if well considered.

The biology cycle of southern green stink bug as multivoltine pest was studied and contained four generations every year in the Iran condition (Critchley 1998). If cotton is the main host for this pest, but more than 115 hosts were reported for this pest all over the world. Many nonchemical methods were used to control this pest, but there were not efficient to now. Chemical control is an interesting approach to pest management in almost of agricultural ecosystems. Overusing pesticides have caused serious problems for the non-target organism, particularly biological enemies like predators and parasitoids. Pesticides pressure is key agent in the outbreaking of non-important insects like trips and aphids in crop ecosystems (Saadati and Toorchi, 2021). Therefore, finding safer methods for pest management is necessary. One of the new and safe approaches in integrated pest management is using of protein inhibitors as biopesticides. Widespread proteins with insecticidal activity such as inhibitors against α-amylase, protease, lipase, lectins, and chitinases were known that can be proposed for the production of transformed plants (Jouanian et al. 1998). The study and dissection of the digestive canal like salivary glands and alimentary canals is the first step in finding new targets for toxic proteins (Saadati and Toorchi, 2021).

The digestive system of insects is consists of salivary glands and alimentary canals particularly heteroptera insects (Habibi et al 2008, Saxena 1963). The general form and structure of the salivary glands is similar in the species of heteroptera with somewhat modifications in adaptation to extra-oral digestion (Habibi et al 2008). Extraoral digestion as a unique approach to feeding has developed as the injection of salivary secreations into plant cells to liquefy food before passing to the alimentary canal (Boyd 2003). The function of saliva proteins is very important to begin insect-plant interaction as the first step of contact between herbivores and plants. Therefore, saliva proteins and up/down-regulation of them are key factors to improve defense reactions in plants. Some salivary proteins were identified in the sunn pest, Eurygaster integriceps and Aelia acuminate (Saadati et al., 2012a, c; Saadati and Nouripure, 2020). Proteomics is the study of the proteome investigating how different proteins interact with each other in specific organs. The proteomics term was first given by Wilkins in 1996 as new methods of study all expressed proteins in the specific organ instead of protein study separately in a particular time and situation (Saadati and Toorchi, 2017; Wilikins et al., 1996). Bottom-up proteomics as gel base method is a simple approach in comparison to Up-down proteomics as the gel-free method. Also, two main categories are been addressed in proteomics research: 1-Proteome Map considered as all expressed proteins in the special organ and 2-Proteome Analysis considered as changed proteins among different treatments. Determination of the proteome map of the salivary gland in the Eurygaster integriceps showed that 31 proteins were expressed (Saadati et al., 2012a). Proteome study of Salivary glands in the Aelia acuminate lead to identification of 29 proteins (Saadati and Nouripure, 2020).

The function of salivary proteins in the insect-plant interaction was approved. In the current study, for the first time in the world, the proteins map of the salivary gland in the adults of N.viridula using bottom-up proteomics was determined. The known proteins were classified as their physiological roles in the biosystems. The identification of salivary proteins can increase our knowledge of the co-evolution of insects and plants. Any disruption and inhibition of salivary enzymes using plant compounds can be affected the feeding process as the first

barrier. Hence, finding new compounds against digestive enzymes is one of the practical results after the determination of the proteome map in the salivary glands. Using a new technique like proteomics is preferred to traditional methods because it can be considered all expressed proteins in one moment and it will be saving action in time and value. The practical data of this research will open a new contex for enzyme engineering to design new inhibitors for the production of transgenic plants containing fatal gens against *N.viridula*.

MATERIALS AND METHODS

Insects and dissection

Adults, *Nezara viridila* (Hemiptera, Pentatomidae) were collected from Torbat-e Jam, Iran, in September 2021 and were transferred to an insectarium for rearing at 27°C with a 16:8 L:D photoperiod. Forty adult insects were chosen to dissect of the alimentary system. Salivary glands were transferred to microtubes containing phosphate-buffered saline (pH: 6.9) and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany).

Protein extraction

Twenty salivary glands were powderd using a mortar and pestle. Salivary gland homogenates were mixed with 10 ml of 10% tricholoroacetic acid in acetone and 0.07% 2-mercaptoethanol in the 50 ml tube. The sample was ultrasonicate to 5 minutes and keep 45 min at -20°C. Centrifugation was performed at 11000 ×g for 30 min at 4° C. The pellets were washed using acetone containing and 2-mercaptoethanol. Drying of pellets and suspending in lysis buffer containing urea, thiourea, CHAPS, tributylphosphine were performed, respectively. The end step was centrifugation at 20000 ×g for 20 min at room temperature. The pellets should be kept at -80°C until used (Saadati Bezdi et al, 2012 a, b; Saadati and Nouripure, 2020).

Two-dimensional electrophoresis and image analysis

Three houndered microgram proteins of target organ were introduced to immobilized pH gradient strips (11 cm, pH 3-10 linear; Bio-Rad, Hercules, CA, USA). The first step was rehydration that done in the 50 V for 14 hours. The condition for Isoelectric focusing was 230 voltages for 15 minute and 8000 voltages for one hour on a linear ramp; and 8000 voltage for 4 hours on a rapid ramp (Saadati and Toorchi, 2021). The equilibration solution number one containing 20% glycerol, 130 mM dithiortheitol (DTT), 0.375 M Tris-HCl (pH 8.8), 2% SDS, 6 M urea, that used for floating of strips. This action was replicate with equilibration solution number 2 that was similar to solution 1 with DTT replaced with 135 mM iodoacetamide. The second stage of electrophoresis was performed with 13% SDS-polyacrylamide gels. The silver staining and Coomassie brilliant blue were used for staining of final gels, separately. Drying and scanning of gels were performed using densitometer (GS-800, Bio- Rad), and using PDQuest software (ver. 9.0.1, Bio-Rad) was used for data analysis (Saadati and Nouripure, 2020). Four important stages were occurred in the picture analysis containing image filtration, spot detection, background subtraction and spot matching (Saadati Bezdi et al., 2012a). The reference gel was selected as the best quality in the acquired images. The many pixels in the spots were defined as the amount of protein that measured with PDQuest software (Saadati Bezdi et al., 2012c; Saadati and Nouripure, 2020). Data normalization was used with local regression method that was very effective in reducing of experimental errors (Saadati and Toorchi, 2021).

Protein identification

In the first step, gel 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). Using of trypsin in comparison to the other proteases lead to more identification of spots. Incubation with acetonitrile, washing with 50 mM NH4HCO3 for 15 min, reduction 10 mM DTT in 50 mM NH4HCO3 for 20 min and alkylation with 40 mM iodacetamide in 50 mM NH4HCO3 for 15 min were four stages of spot preparing (Saadati and Toorchi, 2021). Finally, samples were digested using trypsin at 37°C for 16 h. Desalting stage as necessary action for removing impurities was performed using NuTip C-18 pipet tips. Auto-sampler was used for injection of peptides 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 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 and sprayed at voltage of 1.8 kV (Saadati and Toorchi, 2021). The MS operated in positive ion mode using Xcalibur software (ver. 1.4, Thermo Fisher) and data acquisition was 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 National Centre for Biotechnology Information 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 two missed trypsin cleavage was allowed and carbamidomethylation of cysteins and oxidation of methionines were selected as fixed and variable modification, respectively (Saadati Bezdi et al, 2012 b, c). Proteins with more than five matched peptides with at least eigh percent sequence coverage were considered. The proteins with ion scores greater than 42 were significant for NCBI database.

RESULTS AND DISCUSSION

Salivary glands are the key components of the digestive system for the extra-oral digestion process (Denecke et al., 2020). The salivary proteins are key factors in insect-plant interaction. The role of salivary proteins was known in the inducing or inhibiting of plants' defense reactions against many pests (Fransichetii et al., 2007; Pauchet et al., 2008). The Proteomics technique is significant progress in protein studies. Although, many approaches were used in proteomics, “Proteome Map” is the basic study that should be done in the first step for every organ (Saadati and Nouripure, 2022). In the current studies for the first time, proteome maps of salivary glands in the adults of N.viridula were visualized using bottom-up proteomics (Figure 1).

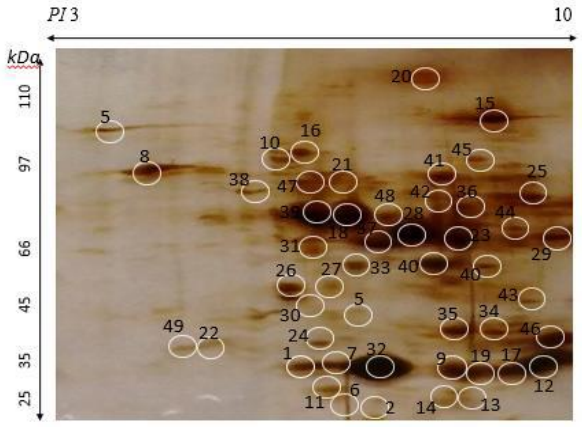

Figure1. Protein expression patterns in gut of adult insects of Nezara viridula. Adult insects were dissected and proteins were extracted from salivary glands, separated by 2-DE and visualized CBB staining. Circles indicate position of accumulated proteins in salivary glands.

Overall, 49 spots were sequenced which led to the identification of 40 proteins (Table 1). The Proteome map of the salivary gland in the adults of Eurygaster integriceps, was determined (Saadati et al., 2012a). Their results showed that 31 protein spots in the final pattern were identifiable. In another study, on the sunn pest, proteome maps of guts in the adult insect were studied (Saadati et al., 2012b). 100 protein spots were sequenced which led to the identification of 63 proteins in their results. The expressed proteins in the salivary gland of A.acuminata using proteomics were identified (Saadati and Nouripure, 2020). In their results, 29 spots were sequenced but 23 proteins were identified, finally (Table 1). The identified proteins in the current study were classified into seven groups contains as effective proteins in carbohydrates metabolism, lipid metabolism, protein metabolism, nucleic acid metabolism, structure proteins, defense proteins, and the other groups (**Figure 2**).

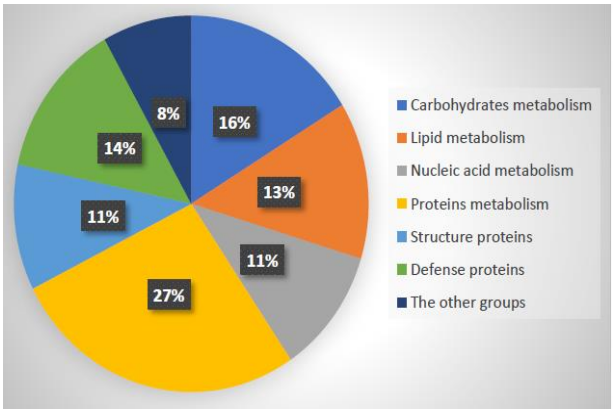

Figure 2. The percentage of identified proteins in the salivary gland of adult insects of *Nezara* viridula based on the physiological roles.

The glycerol-3-phosphate dehydrogenase (spot 1a), alpha-amylase (spot 5), maltase 2-like protein (spot 21), Endo-beta-1,4-glucanase (spot 32c), and glucokinase-like (spot 42) were reported as salivary proteins in the carbohydrates metabolism (Babitt et al., 1996).The glycerol-3-phosphate dehydrogenase as membrane-connected protein is one of the oxidoreductase enzymes in the respiratory electron transport chain and is considered an main component in aerobic growth according to glycerol (Hou et al., 2010). This enzyme is responsible for converting of glycerol-3-phosphate to dihydroxyacetone phosphate in glycolysis, with the production of FADH2, and facilitates passing electrons on to membrane protein and oxygen, finally (Kunieda et al., 2006). Alpha-amylase is a starch hydrolase with several amino acid sequences that produced very isoforms that are highly conserved in their family. This enzyme is considered mainly responsible for the hydrolysis of internal α-1, 4-glycosidic linkages in polysaccharides like starches into glucose, maltose, and maltotriose units (Saadati bezdi et al., 2012b). Maltase 2-like protein catalyzes the hydrolysis of disaccharide maltose into two simple sugars of glucose. Endo-beta-1,4-glucanase from glycosyl hydrolase family 9 (GH9) digests cellulose like β-1,4-glucans in various animals. The main role of glucokinase enzyme is production of glucose-6-phosphate after adding phosphor to glucose. This enzyme is vital in the glycolysis reactions in animals (Serteyn and Francis, 2019). The specific proteins to lipid metabolism in the salivary glands of N.viridula were annexin-like protein (spot 17), alkaline phosphatase (36), phosphotransferase (spot 37), and lipase (spot 45). Annexin-like protein is phospholipid-binding proteins that is effective for forming an evolutionary conserved proteins that expressed throughout animal and plant kingdoms (Pauchet et al., 2008). Annexin is also known as lipocortin. Lipocortins can be used as a suppressor of phospholipase A2 (Tunaz and Stanley, 2004). One of the vital enzyme in the phospholipid degradation is alkaline phosphatase that catalyzes the hydrolysis of phosphate monoesters at basic pH values. This protein is an important enzyme in the fighting of

invasive bacteria in the midgut of insects (Saadati bezdi et al., 2012a). Phosphotransferase has a key role in phosphorylation reactions. The acceptor group can be alcohol, carboxyl, nitrogenous, phosphate, or a pair of groups. Lipase has key roles in insect lipid metabolism and is also fundamental to many physiological processes in energy production (Prado et al., 2006). The ability of insects to energy production is related to lipids as important component of diets. These materials are hydrolyzed in the midgut lumen, absorbed, and used for the synthesis of complex lipids in the membranous and mucus. Some of the key proteins in the nucleic acid metabolism were observed in the protein pattern of the salivary gland in the adult insects like salivary secreted ribonuclease (spot 11), DNA polymerase (spot 13), thymidylate synthase-like protein (spot 14) and exonuclease ABC (spot 17). Salivary-secreted ribonuclease is an extracellular enzyme that is characterized by a high specific activity in the single and double stranded RNA (Liu et al., 2009). The important function of DNA polymerases is related to improve to accurate and efficient replication in the genome and its faithful transmission through generations. Thymidylate synthase-like protein is responsible for the methylation of dUMP by 5,10-methylenetetrahydrofolate to create thymidylate and dihydrofolate. On the other hand, this reaction provides is used as an intracellular new source of thymidylate which is necessary for DNA biosynthesis. In addition to its role in enzyme catalysis, there is a report that shows Thymidylate synthase also acts as an RNA-connecting enzyme. Excinnuclease ABC is a multi-subunit enzyme composed of three proteins, UvrA, UvrB, and UvrC that has a key role in the repairing of nucleotide sequences (Saadati and Noirpure, 2020). The protein metabolism process in the salivary glands of adult insects in the N.viridula is related to some proteins such as nitric oxide synthase (Spot 1b), cysteine protease (Spot 7, 27), serine protease (spot 9), salivary nitrophorin (spot 19), calmodulin (spot 24), nitrophorin 3 (spot 28a), peptidase inhibitor (38a), trypsin protein inhibitor (spot 38b), glutamate dehydrogenase (39), serpin (spot 40), trypsin (spot 43) and polygalacturonase (spot 46) (Figure 1). Nitric oxide synthases (EC 1.14.13.39) used from l-arginine and nicotinamide-adenine-dinucleotide phosphate as the substrate and co enzyme, respectively. Flavin mononucleotide, 5,6,7,8-tetrahydro-l-biopterin and Flavin adenine dinucleotide were considered as cofactors for these enzymes (Tunaz and Stanley, 2004). Nitric oxide synthases are homodimers. The main function of this enzyme is transferring of electrons from nicotinamide-adenine-dinucleotide phosphate, using Flavin mononucleotide and Flavin adenine dinucleotide in the carboxy-terminal to the amino-terminal in the oxygenase domain. This domain can be bound to the Tetrahydrobiopterin as one of the cofactors, molecular oxygen, and l-arginine. At the haem site, the role of the electrons is reduced and activate oxygen molecule to nitric oxide, citrulline and arginine. Cysteine protease is present in all living organisms, particularly insects. Besides their fundamental functions in the catabolism of proteins, they can perform diverse functions in the biochemical cycles according to organisms. Cysteine proteases as one of the famous proteases in the insect world are using from S− anion of a cysteine side chain as the nucleophile in protein degradation (Kunieda et al., 2006). The new role of cysteine proteases as proteolytic enzymes is chitin degradation of the exoskeleton and peritrophic matrix in the midgut of insects (Habibi et al., 2008). Proteolytic activity is necessary for survival in organisms, so it is not unusual which all insects have different proteases, especially serine, cysteine and metallo proteases. Cysteine protease has various isozymes in the salivary

gland and gut of insects. Many functions were reported for this enzyme in the molecular level such a forming of secreted molecules, surface proteins, and venom in specialized glands, plant latex and digestive enzymes. Calmodulin acts as a regulatory protein which change amount of calcium ions that related to activity of ion channels and some of pumps in the membrane of nervous cells (Chandra et al., 2006). The form of calmodulin is dumbbell-shape with two circular domains. The head and tail of the polypeptide bind to two calcium ions. Peptidase inhibitor (spot 31a), Trypsin protein inhibitor and serpin are regulated proteins that affected protease activity according to organ requirements. Glutamate dehydrogenase catalyzes the reversible inter-conversion of glutamate to α-ketoglutarate. This reaction is very important in the Krebs cycle. Trypsin is the most famous protease in the insect world. This enzyme is an endopeptidase that cleaves peptide bonds than contains arginine and lysine. As the eldest proteinase, trypsin was reported from gut and salivary glands of many hemipterans in Iran and world (Liu et al., 2009; Saadati Bezdi et al., 2012a). Polygalacturonases have a critical role in catalyzing the degradation of pectin in the cell wall. The main role of this enzyme in the pathogen-plant interaction as catalyzing breakdown of the cell wall was proved in the pathogenesis (Dillon and Dillon, 2004). Plants also can produce polygalacturonases as a defense reaction or as a part of normal physiological systems. Structural proteins like salivary sheath protein (spot 1c), salivary glue protein (spot 2b), and actin (spot 19) were identified in this research. Salivary sheath protein is essential for forming sheath in the extra-oral digestion process in the hemipteran. Salivary glue protein is an important protein with a key role in the metamorphosis and forming of the salivary sheath (Baumen, 2005). Actin is known as the most abundant protein in the animal body. This protein is highly conserved and is very important in the protein-protein interactions in comparison to the other proteins (Hou et al., 2010). The result of this led to the point that actin protein have many physiological roles, ranging from expansion and contraction of muscles to the regulation of transcription. HSP90 (spot 24) is a member of the heat shock proteins family which has important function against environmental stress in the different cells. This protein was classified as the defense protein (Mahroof et al., 2005). Also, some proteins were identified that do not place in the mentioned category; hence they were placed in the other groups.

Laccase (spot 25a) is a copper-containing oxidase enzyme exist in many animals. The important role of this enzyme is the degradation of lignin and its similar materials.on the other hand, it can hydrolyze lignocellulosic compounds for removing of agricultural residues, nowadays (Chandra et al., 2006). Hypothetical protein (spot 4, 25b) is a specific protein whose genes contains a numerous not-usual fractions of the unspecific parts. The hypothetical genes were considered conserved hypothetical because they have wide phyletic distribution (Engel and Moran, 2013). Although species small fraction of these gens not known, conserved hypothetical proteins create a big question not just to basic genome, but also for all aspect of general biology. At now, very isoforms from this protein were reported from different tissue and organs in various insects. Salivary protein Bt56 (spot 8) is a key salivary effector that promotes whitefly performance as an elicitor of the salicylic acid-signaling pathway in the insect-plant interaction (Barbehenn, 2002). Salivary nitrophorin (spot 13) is a hemoprotein found in the saliva of Hemiptera. Nitrophorins are containing nitric oxides (NO) ligated to the ferric (FeIII) haem iron. Histamine is another nitrophorin ligand, this carbohydrate is released by the host in response to tissue damage. The main role of

nitrophorins is the transportation of NO to the feeding site. Salivary gland secreted protein (spot 15) is a known protein with an unknown role in the physiological systems. Glucose oxidase (spot 21b) as an elicitor is known in some insect-plant interactions (Dilon and Dilon, 2004). This enzyme is a subset of oxidoreductase enzymes that catalyzes the transfer of electrons from an oxidant to a reductant.

CONCLUSION

The determination of the different organ's proteome is the first step in to study of protein interactions. In his research, existing proteins in the salivary glands of southern green stink bug were identified using the proteomics technique in Iran. The reported proteins in this research can be used as potential candidates to use in protein engineering for the production of new inhibitors as digestive disruptors. Many inhibitors as biopesticides are used in integrated pest management, nowadays. These results open a new opportunity for researchers to improve non-chemical approaches in pest control and then it will increase health indexes on food and fruit consumption in the sociality. Finding safe methods in agricultural entomology is the ideal goal for specialists hence, it is necessary to increase our knowledge of the physiological systems using the new and better technique to understand the pros and cons of the target organs in the insect body. However, further experiments are essential to find out which proteins in the salivary glands will be selected as the target for doing decrease/suppressing the nutrition process.

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Table 1. 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 molecular weight and p*I*; d Exp., experimental molecular weight; e M.P., number of query matched peptides; the proteins with more than 5 matched peptides were included; f Score, ion score 42 of identified protein using NCBI (all entries) database; g Cov., Sequence coverage, the proteins with more than 8% sequence coverage were included.

Spotno.[a]	Description	Acc. no.b	Organism	Theoc.	Expd.	M.P.e	Scoref	Cov g. %
Mr (kDa)/pI				Mr (kDa)				
1 1b 1c	glycerol-3-phosphate dehydrogenase Nitric oxide synthase salivary sheath protein	gi|62526114 gi|607352984 gi|723001886	*A.mellifera* *O.biroi* *N.lugens*	40.1 12.9 79.1	6.67 7.1 9.8	33 33 33	11 12 8	51 48 48	11 11 9
2 2b	nitric oxide synthase salivary glue protein	gi|2252275310 gi|1841821854	*D.ponderase* *T.palmi*	12.6 27.1	7.1 4.9	25 25	13 10	52 55	12 9
3					Not identified				
4					Not identified				
5	Alpha-amylase	gI:25992514	*A.grandis*	52.5	5.1	44	15	66	14
6	Hypothetical protein	gI:133916468	*T.domestica*	10.9	5.5	25	13	58	11

7	cysteine protease	GI:1061156822	*L.lineolaris*	38.9	4.8	36	17	54	11
8					Not identified				
9	serine protease	GI:1061156810	*L.lineolaris*	28.9	6.8	36	10	51	9
10					Not identified				
11	salivary secreted ribonuclease	gi|114050819	*B.mori*	11.2	5.5	22	12	61	13
12	salivary protein Bt56	gi|1321103906	*B.tabaci*	12.3	9.8	25	12	51	17
13	DNA polymerase	gi|262090487	*M.domestica*	21.2	8.9	27	21	88	22
14	thymidylate synthase-like protein	gi|984290650	*G.pallides*	10.4	8.6	22	15	62	13
15					Not identified				
16					Not identified				

17	annexin-like protein	gi|50510056	*N.lugens*	34.9	9.3	33	13	59	9
18	Excinnuclease ABC	gi|15892189	*R.conorii*	83.8	5.3	75	14	79	23
19	salivary nitrophorin	gi|46411093	*R.prolixus*	33.5	8.5	35	22	67	14
20					Not identified				
21	maltase 2-like protein	gi|1035439611	*N.lugens*	75.4	5.6	64	18	62	11
22	salivary gland secreted protein	gi|613447216	*H.armigera*	13.4	4.1	21	12	49	8
23					Not identified				
24	Calmodulin	gi|1035439601	*N.lugens*	16.8	4.1	21	9	48	9

25					Not identified				
26	Actin	gi|1035439589	*N.lugens*	41.8	5.3	45	13	55	19
27	cysteine protease	gi|1061156822	*L.lineolaris*	38.8	4.7	45	17	62	11
28a	nitrophorin-3	gi|4204974	*L.lineolaris*	22.1	6.1	25	9	51	13
28b	glucose oxidase	gi|215982092	*R.prolixus*	66.9	5.2	25	14	53	9
29					Not identified				
30	Ferritin	gi|1402400458	*H.armigera*	44.9	5.2	47	19	71	22
31	HSP90	gi|984290654	*B.tabaci*	37.6	4.6	63	11	52	9
32a	Laccase	gi|295292761	*G.pallidipes*	8.1	6.1	33	15	49	12
32b	hypothetical protein	gi|723001894	*N.cincticeps*	91.3	3.7	65	11	48	9
32c	endo-beta-1,4-	gi|538774655	*N.lugens*	24.3	8.5	33	19	51	12

	glucanase								
33	ribonuclease	gi|140763147	*E.okinawaensis*	41.3	5.8	58	13	62	21
34	Not identified								
35	Serin protease	gi|1061156810	*L.lineolaris*	28.9	6.8	41	9	48	8
36	alkaline phosphatase	gi|1004169304	*S.furcifera*	85	7.9	83	21	68	19
37	phosphotransferase	gi|1845937245	*D.melanofaster*	41.9	5.1	59	14	61	10
38a	peptidase inhibitor	gi|1407634100	*L.lineolaris*	40.6	4.8	71	13	52	17
38b	Trypsin protein inhibitor	gi|1407937996	*L.lineolaris*	24.3	4.9	65	11	51	14
39	glutamate dehydrogenase	gi|518545891	*L.lineolaris*	46.6	6.2	61	15	71	29
40	Serpin	gi|24647008	*B.mori*	48.4	5.3	51	15	56	11
41	Not identified								
42	glucokinase-like	gi|1035439601	*D.melanogaster*	38.5	5.5	45	11	51	15
43	Trypsin	gi|410329	*L.linolaris*	12.6	6.1	21	14	63	13
44	Not identified								
45	Lipase	gi|1035439601	*A.mellifera*	46.7	8.8	68	12	52	11
46	polygalacturonase	gi|1061156756	*N.lugens*	38.9	9.3	33	17	69	14
47	Not identified								
48	Not identified								

49	Not identified

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; Mr, 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.