Türk. entomol. derg., 2012, 36 (2): 215-223
ISSN 1010-6960

Orijinal araştırma (Original article)

Protein patterns in the salivary gland of the sunn pest, *Eurygaster integriceps* (Put.) (Hemiptera: Scutelleridae)[1]

Süne [*Eurygaster integriceps* (Put.) (Hemiptera: Scutelleridae)]'nin salya bezindeki protein desenleri

Mohammad SAADATI BEZDI[2] Reza FARSHBAF POURABAD[2]*
Mahmoud TOORCHI[2] Nosratollah ZARGHAMI[3] Setsuko KOMATSU[4]

Summary

Salivary glands of the members of the Order Hemiptera contain proteins which are involved in extra oral digestion and insect-plant interactions. Identification of proteins and determination of their physiological roles in the salivary gland is an essential step to elucidate sunn pest-wheat interactions. In this study, proteomic techniques were used to compare protein patterns in the salivary gland of fifth-instar nymphs and adults of the sunn pest, *Eurygaster integriceps*. Among 139 detected spots, 31 spots were selected for protein identification. Thirteen spots were differentially expressed between the two life stages. Trypsin, arginine kinase and phospholipase A2 levels were higher in the adult stage whereas chymotrypsin, arginine methyltransferase and salivary endonuclease levels were greater in fifth-instar nymphs. The remaining eighteen spots were selected for protein mapping of the salivary gland. Serine protease ssp3, alpha-amylase, glucose dehydrogenase, vacuolar H+ATPase and thrombostasin-3 were expressed at similar levels in fifth-instar nymphs and adults

Key words: Salivary gland, proteomics, sunn pest, enzyme, digestion

Özet

Hemipteradaki salya bezleri, ağız dışındaki sindirimde etkili olan, böcek-bitki ilişkileri açısından önemli proteinleri içerir. Bu çalışmada Süne'nin beşinci dönem nimfleri ve ergin bireylerinde salya bezlerinin protein dokularının karşılaştırılmasında protomiks tekniklerinden yararlanılmıştır. Belirlenen 139 leke şeklindeki desenlerden 31 tanesi, proteinlerin tanılanması için seçilmiştir. 13 protein lekesi iki yaşam dönemi arasında farklı olarak belirlenmiştir. Ergin bireylerde tripsin, arginin kinaz ve fofolipaz A2 artış gösterirken, beşinci dönem nimflerde ise kimotripsin, arjinin metiltransferaz ve endonukleaz salyası daha yüksek miktarda gözlenmiştir. Diğer 18 leke salya bezinin protein haritasını oluşturmak için seçilmiştir. Beşinci dönem nimfler ile ergin bireylerde; ssp3 serin proteazları, alfa-amilaz, glukoz dehidrojenaz, vakuolar H+ATPase ve trombostasin-3'ün aynı oranlarda olduğu belirlenmiştir. Proteinlerin tanılanması ve salya bezlerindeki fizyolojik rollerinin belirlenmesi Süne-buğday etkileşimlerini aydınlatmak için önemli bir adımdır.

Anahtar sözcükler: Salya bezi, proteomikler, pis kokulu böcek, enzim, sindirim

[1] This study is a part of Ph.D thesis of Mohammad Saadati Bezdi in field of Insect Physiology
[2] Faculty of Agriculture, University of Tabriz, Tabriz, Iran
[3] Faculty of Medicine, Tabriz University of Medical sciences, Tabriz, Iran
[4] National Institute of Crop Science, Tsukuba 305-8518, Japan
* Sorumlu yazar (Corresponding author) e-mail: rfpourabad@yahoo.com
Alınış (Received):21.07.2011 Kabul ediliş (Accepted): 13.12.2011

Introduction

Sunn pest is a serious insect pest of wheat and barley in the Middle East, particularly in Iran (Javaheri et al., 2009). Currently, chemical control is an effective method for suppressing its damage. However, over-using chemicals carries concerns about environmental risks against non-target organisms, especially for humans and natural enemies that exist in ecosystems (Jouanian et al., 1998). Finding safe alternative methods in pest management is necessary in the near future. Protein inhibitors which act against digestive proteins and defense proteins are suitable candidates for use in pest management in the form of transgenic plants.

Members of the Order Hemiptera have extra oral digestion which involves the injection of salivary proteins into plant tissue, liquefying and then pumping out to the gut for final digestion (Zeng & Cohen, 2000). Salivary glands in Hemiptera consist of the principle gland (anterior lobe and posterior lobe) and accessory gland. The principle gland in the sunn pest consists of a number of lobules which discharge at hilus (Habibi et al., 2008). Proteins in salivary gland of hemipterans have a critical role in the initiation of feeding. Digestive proteins such a alpha-amylase, protease, lipase, alpha-carbohydrase and beta-carbohydrase were reported from the salivary glands of different insects (Applebaum, 1985; Strobl et al., 1998; Barbosa Pereira et al., 1999; D'Amico et al., 2000; Lulek et al., 2000; Carlini & Grossi-de-Sa, 2002; Franco et al., 2002; Oliveira- Neto et al., 2003;). On the other hand, some salivary proteins, such as glucose oxidase from *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) (Eichenseer et al., 1999) and *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphidiidae) (Harmel et al., 2008), volicitin from *Spodoptera exigua* (Hubner, 1808) (Alborn et al., 1997) (Lepidoptera: Noctuidae), caeliferin from *Schistocerca americana* (Drury, 1773) (Orthoptera: Acrididae) (Alborn et al., 2007), have a role in plant-insect interactions as elicitors on the plant defense reactions.

Proteomics is a new approach for studying the proteome in different tissues of insects. In this technique, usually two-dimensional electrophoresis patterns are determined and then some selected proteins are identified by LC-MS/MS or protein sequencing. In this study, proteomic techniques were employed for the identification of proteins which were differentially expressed between fifth-instar nymphs and adults of sunn pest. Differentially expressed proteins were detected in both stages on the basis of molecular weight and isoelectric points of each spot. This knowledge of the proteins in sunn pest salivary glands will be helpful in understanding sunn pest-wheat interactions and raises the possibility of their use as targets for inhibitors. The 2-DE patterns of salivary glands in sunn pest may also be useful as a model for the other hemipteran studies.

Material and Methods

Materials

Overwintering adults of *Eurygaster integriceps* were collected from Tabriz, Iran, in February 2010 and reared in an insectary room on wheat grains at 27°C and with a 16 h light/ 8 h dark photoperiod. One day old adults and fifth-instar nymphs were selected for dissection of their salivary glands. The chemicals used were a combination of protease inhibitors, tricholoroacetic acid, acetone, thiourea, tributylphosphine, urea, 2-mercaptoethanol, phosphate buffer, CHAPS, SDS, glycerol, trypsin, dithiortheitol, iodoacetamide, acetonitrile and NH_4HCO_3.

Methods

Sample Preparation

The salivary glands of adults and fifth-instars were dissected, rinsed in ice-cold phosphate buffer and the 10 pairs were transferred to microtubes containing phosphate-buffered saline and the mixture of

protease inhibitors (Roche Applied Science, Manneheim, Germany). The extracted proteins from three independent biological replicates were dried and kept at -80°C until use.

Protein extraction

Proteins were extracted with the acetone/trichloroacetic acid method. Thirty salivary glands were ground with mortar and pestle, homogenized with 10 mL of a solution containing 10% tricholoroacetic acid in acetone and 0.07% 2-mercaptoethanol, and followed by ultrasonication for 10 min. Total proteins were precipitated for 1 h at -20°C followed by centrifugation at 9000 ×g for 20 min at 4°C. The pellet was washed three times with acetone containing 0.07% 2-mercaptoethanol and dried under a vacuum for 15 min. The pellet was resuspended in 400 µL of lysis buffer containing 7 M urea, 2 M thiourea, 5% CHAPS, 2 mM tributylphosphine and shaken vigorously for 1 h at room temperature. Insoluble materials were removed by centrifugation at 20000 ×g for 20 min at room temperature.

Two-dimensional polyacrylamide gel electrophoresis

A total of 400 µg of proteins from 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 35000 Vh 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 electrophoresis after transferring onto a 13% SDS-polyacrylamide gels. The gels were stained with Coomassie brilliant blue (CBB).

Image analysis

Gels from the three replicates were scanned with a calibrated densitometer (GS-800, Bio-Rad), and analyzed with PDQuest software (ver. 8.0.1, Bio-Rad). Image analysis included the steps of image filtration, spot detection and measurement, background subtraction and spot matching. The best gel was selected as the reference gel to compare all other gels accordingly. 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 the pixels that made up that spot. To accurately compare spot quantities between gels, spot volumes were normalized using the local regression method. Significantly changed spots between adults and fifth-instar nymphs were determined according to the *t*-test analysis ($p < 0.05$).

Protein identification

Protein spots excised from CBB-stained 2-DE gels were subjected to in-gel trypsin digestion (Wako, Osaka, Japan) using an automated protein digester (Digest Pro 96; Intavis, Koeln, Germany). Spots placed in 96 well plates 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 a NuTip C-18 pipet tips (Glygen, Columbia, MD, USA). Peptides were injected with 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 C_{18} PepMap trap column with a 25 µL/ min flow rate. The peptides were eluted and separated from the trap column using 0.1% formic acid in acetonitrile in a 75 µm id × 12 cm C_{18} column (Nikkyo Technos, Tokyo, Japan) at a flow rate of 200 nL/ min and sprayed at a voltage of 1.8 kV. The MS operated in positive ion mode using Xcalibur software (ver. 1.4, Thermo Fisher)

and data acquisition covered a scan range of 100- 2000 *m/z* followed by three MS/MS scans in exclusion dynamic mode during the 60 min retention period. Tandem mass spectrum DTA files were converted to MGF files using Bioworks software (ver. 3.3.1, Thermo Fisher). Peptide masses were searched for in National Center for Biotechnology Information (NCBI) database using the Mascot search engine (ver. 2.3.02, Matrix Science, London, UK). The proteins with ion scores greater than 36 were significant for NCBI database (significant score was calculated by Mascot engine) ($p < 0.01$).

N-terminal amino acid sequencing

After 2-DE, gels were transferred by electroblotting onto polyvinylidene difluoride membrane (Pall, Port Washington, NY, USA) and detected by CBB staining. Stained protein spots were excised from the membrane and directly subjected to Edman degradation in a gas-phase protein sequencer (Procise cLC, Applied Biosystems, Foster City, CA, USA). The amino acid sequences were compared with those of known proteins in the Uniprot database by using the web-accessible search program of FASTA.

Protein identification by molecular weight and isoelectic point

After the molecular weight and isoelectric point of all protein spots were determined, these parameters were used to search in Swiss-Prot for those of known proteins. Furthermore, we ran a blast search in the NCBI database for the identified proteins and maximum percent identity in insect world was employed for identification of the target protein.

Results

One-day-old adult insects and fifth-instar nymphs were dissected and crude proteins were extracted from salivary glands. Proteins were separated by 2-DE and visualized with CBB. Protein expression patterns were analyzed using image analysis and a total of 139 protein spots were detected. Thirteen protein spots were significantly changed between fifth-instars and adults, whereas 18 spots were only accumulated in either of the stages (Figure 1). Out of the differentially regulated proteins, six and seven protein spots were up-regulated in adults and fifth-instars, respectively (Figure 2). Using mass spectrometry, two protein spots containing arginine kinase (spot 3, gi|116235665) and hypothetical protein (spot 5, gi|124008418) were identified. For the remaining 11 differentially expressed spots, the Edman degradation system was used; spots 9 and 10 were identified as phospholipase A2 and the remaining spots were N- terminally blocked.

These 9 co-expressed proteins, along with the 18 spots detected in the adults or fifth-instars, were identified using molecular weight and isoelectric point. For these spots, the molecular weight and isoelectric point were used in searching in Swiss-Prot, salivary gland database of *Oncupeltus fasciatus* and Aphid EST Database for tagging the target proteins (Table 1). The digestive enzymes trypsin (spot 1), chymotrypsin (spots 8, 11), serin protease ssp3 (spots 19, 20, 21, 31) and alpha-amylase (spots 17, 18) were identified. The other identified proteins were hypothetical proteins (spots 2, 4, 14), thrombostatin-3 (spots 15, 16), glucose dehydrogenase (spots 22, 23, 24, 25, 26), vacuolar H+ ATPase (spots 27, 28, 29, 30), arginine methyltransferase (spots 6, 7) and salivary endonuclease (12, 13).

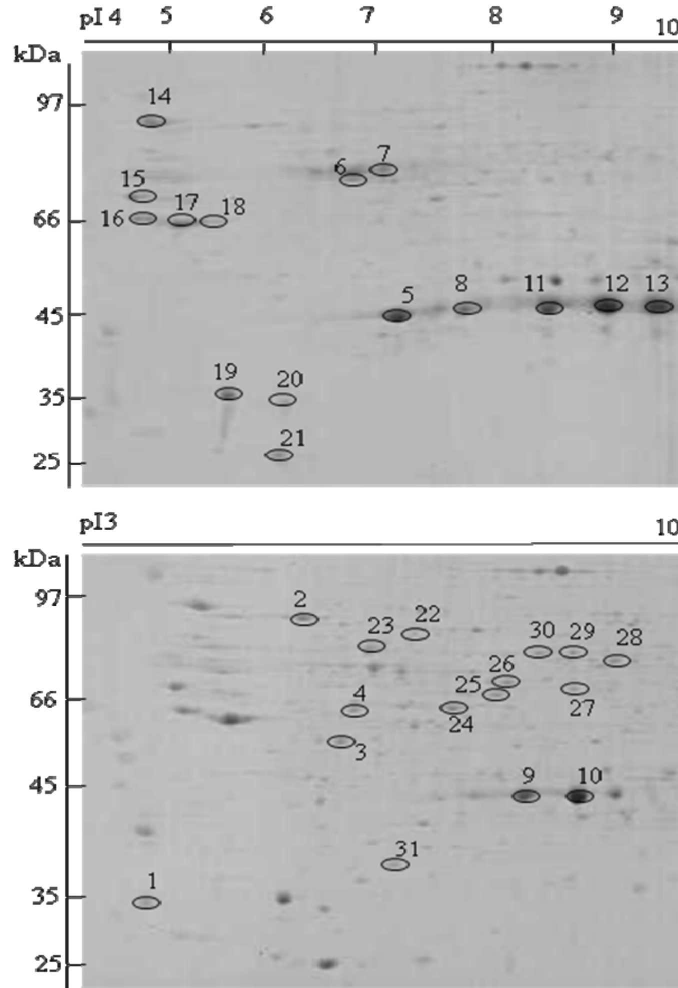

Figure 1. Protein expression patterns in salivary gland of fifth-instar nymphs and adults of sunn pest. One-day-old insects were dissected and proteins were extracted from salivary glands, separated by 2-DE and visualized by CBB staining in (A) fifth-instar nymphs and (B) adults. Circles indicate position of accumulated proteins in salivary glands.

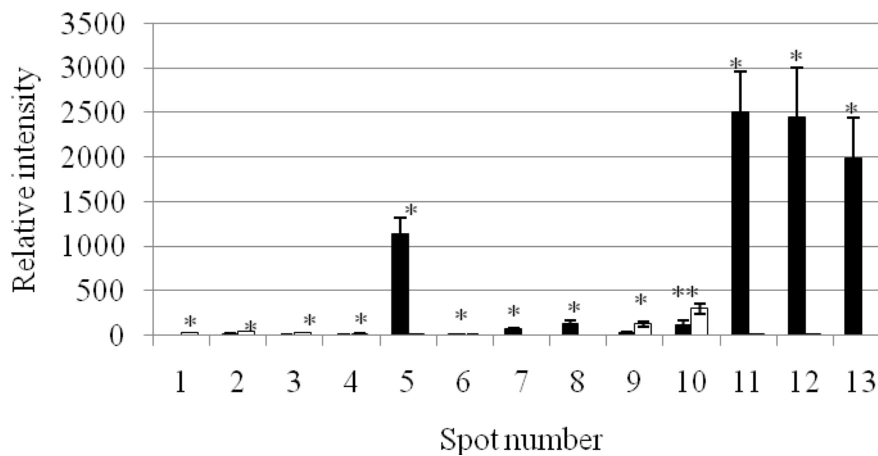

Figure 2. Relative intensities of protein expression in salivary glands of sunn pest. Relative intensities were analyzed using PDQuest software. Values are the average relative intensities ± SE from three independent experiments. Spot numbers are as given in Figure 1. Asterisks indicate significant difference between fifth-instar nymphs (black column) and adults (white column) (*: $p < 0.05$ and **: $p < 0.01$).

Table 1. Proteins identified from salivary glands of sunn pest

Spot	Mr	PI	Protein	Method	Database
1	34	4	Trypsin	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
2	90	5.4	Hypothetical protein	Experimental M*r* and PI	Swiss-Prot
3	55	5.8	Arginine kinase	nanoLC-MS/MS(Mascot)	NCBI
4	64	6	Hypothetical protein	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
5	45	6.9	Hypothetical protein	nanoLC-MS/MS(Mascot)	NCBI
6	75	6.8	Arginine metyltransferase	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
7	77	7	Arginine metyltransferase	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
8	47	7.5	Chymotrypsin	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
9	43	7.6	Phospholipase A2	Edmen degradation	Uniprot database
10	43	8	Phospholipase A2	Edmen degradation	Uniprot database
11	49	8.4	Chymotrypsin	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
12	49	8.9	Salivary endonuclease	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
13	49	9.4	Salivary endonuclease	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
14	93	4.9	Hypothetical protein	Experimental M*r* and PI	Swiss-Prot
15	72	4.6	Thrombostatin 3 Putative 4.7 KDa secreted	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
16	66	4.6	a) Thrombostatin 3 b) Putative 4.7 KDa secreted	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
17	66	5.1	Alpha-amylase	Experimental M*r* and PI	Aphid EST Database
18	66	5.3	Alpha-amylase	Experimental M*r* and PI	Aphid EST Database
19	37	5.6	Serin protease ssp3	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
20	35	6.3	Serin protease ssp3	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
21	25	6.4	Serin protease ssp3	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*
22	85	7	Glucose dehydrogenase	Experimental M*r* and PI	Aphid EST Database
23	74	6.3	Glucose dehydrogenase	Experimental M*r* and PI	Aphid EST Database
24	66	7	Glucose dehydrogenase	Experimental M*r* and PI	Aphid EST Database
25	74	7.3	Glucose dehydrogenase	Experimental M*r* and PI	Aphid EST Database
26	77	7.4	Glucose dehydrogenase	Experimental M*r* and PI	Aphid EST Database
27	75	8	Vacuolar H+ ATP ase	Experimental M*r* and PI	Swiss-Prot
28	78	8.3	Vacuolar H+ ATP ase	Experimental M*r* and PI	Swiss-Prot
29	80	7.9	Vacuolar H+ ATP ase	Experimental M*r* and PI	Swiss-Prot
30	80	7.6	Vacuolar H+ ATP ase	Experimental M*r* and PI	Swiss-Prot
31	39	6.5	Serin protease ssp3	Experimental M*r* and PI	Salivary gland database of *Oncopeltus fasciatus*

Discussion

The salivary glands in the Order Hemiptera have a critical role in extra oral digestion (Boyd, 2003). Salivary proteins are mainly responsible for the starting of feeding; after injection into plant tissue, liquefying it in the external environment, and pumping out to the gut for final digestion (Habibi et al., 2008). The salivary gland in wheat-bugs inject proteinases that destroy the gluten structure in seed, hence we were interested in the proteinases usually express in this organ. Our results showed that serine protease ssp3, trypsin and chymotrypsin were expressed in both the adult and fifth-instar stages. Trypsin cleaves peptide chains on the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. Chymotrypsin preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond is a tyrosine, tryptophan, or phenylalanine. Serine protease ssp3 is a novel protease from the S1 family of serine proteases which is involved in immunity responses to bacterial pathogens (Hamilton et al., 2002). These proteases are suitable candidates for use as targets for protease inhibitors in transgenic plants. Protease inhibitors have been used as defense proteins in crops such as potato, tomato, soybean and corn against some insect pests (Jouanian et al., 1998). Identification of suitable protease inhibitors from biological sources e.g. plant protease inhibitors (PPIs) or the design of new inhibitors by adapting biotechnology techniques or protein engineering can be considered as a new opportunity for sunn pest management in near future.

Alpha-amylase is an important digestive enzyme that breaks down macromolecules. It hydrolyzes starch to maltose and then it is converted to glucose by an α-glucosidase (Applebaum,1985; Strobel et al., 1998). It therefore plays a major role in carbohydrate metabolism. Insects with a carbohydrate-rich diet depend on the efficiency of their amylases for growth and development (Applebaum, 1985; Strobl et al., 1998; Barbosa Pereira et al., 1999; D'Amico et al., 2000; Iulek et al., 2000; Carlini & Grossi-de-Sa, 2002; Franco et al., 2002; Oliveira-Neto et al., 2003). Alpha-amylase inhibitors are effective defense proteins which exist in various organisms. These inhibitors can be used as suppressing agents of feeding by sunn pest, if they are expressed in grains of wheat at a high enough level. We believe that the use of bifunctional inhibitors (alpha-amylase and protease inhibitors) is more effective than the use of single inhibitors against insect targets because bifunctional inhibitors not only increase the capacity for inhibition of digestive proteins but also reduce the probability of the occurrence of resistance to these inhibitors.

Thrombostatin-3 has anti-hemostatic activity which plays an important role in maintaining the ectoparasitic lifestyle of insects (Zhang et al., 2002). Thrombostatin -3 was reported only from the salivary glands of blood-sucking insects and here we report it for first time from sunn pest. The putative 4.7 KDa secreted is a new protein whose physiological role is not clear, but in the other organisms it is prerequisite for secretion of P80 proteins.

Arginine kinase is a type of phosphagen kinase which catalyzes the transfer of a phosphoryl group from a guanidino phosphagen. This enzyme has a critical role in production of ATP in muscle contraction processes which occurs in cytoskeleton muscles (Yao et al., 2009). Arginine methyl transferase plays a key role in the posttranslational modification commonly found in RNA-binding proteins. This enzyme catalyzes the methylation process that modulates protein-protein interactions (Yu et al., 2004).

Phospholipases A2 (PLA2s) EC 3.1.1.4 are enzymes which hydrolyze glycerol to fatty acids at its second carbon group. These enzymes exists as two forms in which extracellular forms have been isolated from different venoms (bee and wasp), mammalian tissue and bacteria. On the other hand, the intracellular phospholipases A2 have completely different 3D structures and they are significantly larger than secreted phospholipases A2. Extracellular and intracellular forms of phospholipaseA2 are Ca-dependent (Rodrigues et al, 2008). This is the first report of this enzyme in the salivary gland proteome of

sunn pest. Up-regulation of this protein in adults suggests that their ability to use lipids as a substrate is higher than that of fifth-instar nymphs.

Salivary endonucleases are enzymes capable of hydrolyzing nucleic acids. These enzymes belong to the phosphodiesterase family and are capable of cleaving phosphodiester internal bonds within double-stranded or single-stranded DNA and RNA substrates. Eukaryotic endonucleases (EC 3.1.30.-) require a divalent ion such as magnesium for their activity in neutral or alkaline conditions. These enzymes have various roles such a salvage, repair, recombination, transposition, degradation and cell division, cell defense and degradation of foreign nucleic acids (Calvo & Rebirieo, 2006). The role of this enzyme in salivary gland of insects is yet to emerge.

Glucose dehydrogenase belongs to the family of oxidoreductases which generally display chemical reactivity on the CH-OH group of the donor, with NAD+ or NADP+ as acceptor. This enzyme was reported from the salivary gland of *Myzus persicae* (Harmel et al., 2008). It has a key role in immunity reactions, particularly in cellular immune defense against foreign pathogens. It is also active in the encapsulation some of external invaders.

The vacuolar (H^+)-ATPases (or V-ATPases) function as proton pumps is acidifies (with entrance of $H^{+)}$ of intracellular compartments in eukaryotic cells. The V-ATPases consist of multisubunit complexes with two functional domains. The cytoplasmic V_1 domain, a 500-kDa complex, is responsible for ATP hydrolysis, and contains at least 8 different subunits. The integral V_0 domain, a 250-kDa complex, transports protons across membranes and contains at least 5 subunits (Syntichaki et al., 2005).

Hypothetical proteins are predicted from nucleic acid sequences but their physiological role is unknown. These proteins are mostly found in organisms which have not been sequenced. The genome of sunn pest, like that of many other organisms, has not been sequenced, but the identification of such proteins is anticipated.

This study is the first to report proteome analysis of the salivary gland of sunn pest. We could not identify all the proteins using mass spectrometry because of the lack of genome sequencing in data banks. Therefore, most of the proteins were identified using molecular weight and isoelectric point. Some proteins which were differentially expressed between fifth-instars and adults could be identified by LC-MS/MS and protein sequencing. The challenge in the near future will be the completion of the genome sequencing of sunn pest for use as a genomic platform for other Hemiptera.

Acknowledgments

We thank Dr. Y. Nanjo and Dr. K. Nishizawa affiliations for their critical comments and kind collaboration. We also appreciate the assistance of M. Hashemi in insect collection. The authors also thank the University of Tabriz, Iran and the National Institute of Crop Science, Japan.

References

Alborn, H. T., T. V. Hansen, T. H. Jones, D. C. Bennett, J. H. Tumlinson, E. A. Schmelz & E. A. Peter, 2007. Disulfooxy fatty acids from american bird grasshopper *Schistocerca americana*, elicitors of plant volatiles. Proceeding of the National Academy of Sciences of the USA, 104: 12976-12981.

Alborn, H. T., T. C. J. Tulings, T. H. Jones, G. Stenhagen, J. H. Loughri & J. H. Tumlison. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. Science, 276: 945-949.

Applebaum, S. W. 1985. "Biochemistry of Digestion. pp. 279-307". In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol.4. (Eds: G. A. Kerkut and L. L. Gilbert). Pergamon Press, London, 743 pp.

Barbosa Pereira, P. J., V. Lozanov, A. Patthy, R. Huber, W. Bode, S. Pongor & S. Strobl, S. 1999. Specific inhibition of insect α-amylases: yellow meal worm α-amylase in complex with the Amaranth α- amylase inhibitor at 2.0 Å resolution. Structure, 7: 1079-1088.

Boyd, D. W. 2003. Digestive enzymes and stylet morphology of *Deraecoris nigritulus* (Uhler) (Hemiptera: Miridae) reflect adaptions for predatory habits. Annals of the Entomological Society of America, 96: 667- 671.

Calvo, E & C. M. Ribeiro, 2006. A novel secreted endonuclease from *Culex quinquefasciatus* salivary glands. The Journal of Experimental Biology, 209: 2651-2659.

Carlini, C. R. & M. F. Grossi-de-Sa, 2002. Plant toxic proteins with insecticidal properties: A review on their potentialities as bioinsecticides. Toxicon, 40: 1515-1539.

D'Amico, S., C.Gerday, & G. Feller, 2000. Structural similarities and evolutionary relationships in chloride-dependent α-amylases. Gene, 253: 95-105.

Eichenseer, H., M. C. Mathews, J. L. Bi, J. B. Murphy & G. W. Felton, 1999. Salivary glucose oxidase: multifunctional roles for *Helicoverpa zea*?. Archives Insect Biochemistry and Physiology, 42: 99-109.

Franco, O. L., F. R. RigdenMelo, & M. F. Grossi-de-Sa, 2002. Plant α-amylase inhibitors and their interaction with insect α-amylases, structure, function and potential for crop protection. European Journal of Biochemistry, 269: 397-412.

Habibi, J., T. A. Courdon, E. A. Backus, S. L. Brandt, R. M. Wagner, M. K. Wright & J. E. Huesing, 2008. Morphology and histology of the alimentary canal of *Lygus hesperus* (Heteroptera: Cimicomorpha: Miridae). Annals of the Entomiological Society of America, 101: 159-171.

Hamilton, J. V., R. J. L. Munks, S. M. Lehane, & M. J. Lehane, 2002. Association of midgut defensin with a novel serine protease in the blood- sucking fly *Stomoxys calcitrans*. Insect Molecular Biology, 11: 195-207.

Harmel, N., E. Letocart, A. Cherqui, P. Giordanengo, G. Mazzucchelii, F. Guillonneau, E. Depauw, E. Haubruge & F. Francis, 2008. Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. Insect Molecular Biology, 17: 165-174.

Iulek, J., O. L. Franco, M. Silva, C. T. Slivinski, C. Bloch Jr., D. J. Rigden & M. F. Grossi-de- Sa, 2000. Purification, biochemical characterization and partial primary structure of a new α-amylase inhibitor from secale cereal (rye). International Journal of Biochemistry and Cell Biology, 32: 1195-1204.

Javaheri, M., C. W. Scheafer & J. D. Lattin, 2009. "Shield bugs (Scutelleridae). pp 457-503". In: Heteroptera of economic Importance, (Eds: C. W. Scheafer & A. R. Panizi). CRC Press, Washington, USA, 856 pp.

Jouanian, L., M. Bonade-Bottino, C. Giard, G. Morrot & M. Giband, 1998. Transgenic plants for insect resistance. Plant Science, 131:1-11.

Oliveira-Neto, O. B., J. A. N. Batista, D. J. Rigden, O. L. Franco, R. Falcao, R. R. Fragoso, L. V. Mello, R. C. dos Santos & M. F. Grossi-de-Sa, 2003. Molecular cloning of α- amylases from cotton boll weevil, *Anthonomus grandis* and structural relations to plant inhibitors: an approach to insect resistance. Journal of Protein Chemistry, 22: 77-87.

Rodrigues, F. G., M. N.,Santos,T. X., de Carvalho, B. C., Rocha, M. A.,Riehle, P. F., Pimenta, E. G., Abraham, M., Jacobs-Lorena,C. F., Alves de Brito & L.A.Moreira, 2008. Expression of a mutated phospholipase A2 in transgenic *Aedes fluviatilis* mosquitoes impacts *Plasmodium gallinaceum* development. Insect Molecular Biology, 17: 175-183.

Strobl, S., K. Maskos, G. Wiegand, R. Huber, F. X. Gomis-Ruth, & R. Glockshuber, 1998. A novel strategy for inhibition of α-amylases: yellow meal worm α-amylase in complex with the ragi bifunctional inhibitor at 2.5 Å resolution. Structure, 6: 911-921.

Syntichaki, P., C. C. Samara, & N. Tavernarakis, 2005. The vacuolar H+- ATPas mediates intracellular acidification required for neurodegeneration in *C. elegans*. Current Biology, 15: 1249–1254.

Yao, H., X. Xiang., L. Chen., A. Guo., F. He., L. Lan., X. Lu. & X. Wu, 2009. Identification of the proteomeof the midgutof silkworm, *Bombyx mori* L. by multidimension liquid chromatographt LTQ-Orbitrap mass spectrometry. Bioscience reports, 29: 363-373.

Yu, M. C., F. Bachand & A. E. McBride, 2004. Arginine methyltransferase affects interactions and recruitment of mRNA processing and export factors. Gene, 18: 2024-2035.

Zeng, F. & C. A. Cohen, 2000. Partial characterization of α-amylase in the salivary glands of *Lygus hesperus* and *L. linealaris*. Comparative Biochemistry and Physiology, 126: 9-16.

Zhang, D., M. S. Cupp & E. W. Cupp, 2002. Thrombostatin purification, molecular cloning and expression of novel anti-thrombin protein from horn fly saliva. Insect Biochemistry and Molecular Biology, 32: 321–330.