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Short commmunication

Effect of different host substrates on hemipteran salivary protein profiles*

Javad Habibi[1], Elaine A. Backus[1], Thomas A. Coudron[2] & Sandra L. Brandt[2]
[1]*Department of Entomology, University of Missouri, Columbia, MO 65211, USA (Phone: (573) 882-8243; Fax: (573) 882-1469; E-mail: habibij@missouri.edu);* [2]*USDA-Agricultural Research Service, Biological Control of Insects Research Laboratory, 1503 South Providence Rd., Research Park, Columbia, MO 65203, USA*

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Abbreviations: kDa – kilodalton; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

The chemical composition of the watery saliva of hemipteran (both homopteran and heteropteran) insects is crucial for effective feeding, because these insects rely heavily on saliva for extra-oral digestion (Cohen, 1995, 1996, 1998) or detoxification of defensive chemicals (Miles & Oertli, 1993). For phytophagous hemipterans, saliva can also be involved in plant injury. Yet, salivary composition has been studied in relatively few species, primarily aphids (Miles, 1999) and predaceous heteropterans (Cohen, 1995, 1998), and infrequently in direct relation to plant injury. Also, most studies have used homogenates or extracts of salivary glands coupled with colorimetric assays. Only a few studies have used electrophoretic methods to examine secreted saliva of hemipterans, e.g., aphids (Baumann & Baumann, 1995; Madhusudhan & Miles, 1998), a phytophagous heteropteran (Taylor & Miles, 1994), and some predaceous heteropterans (Cohen, 1990).

Our goal was to determine whether changes in host substrates caused modification in the protein content within the secreted saliva of several hemipteran species. In particular, we were interested in comparing related species that cause different plant injuries, and to compare phytophagous and entomophagous species. Thus, our approaches were: (1) to recover secreted saliva without causing damage to insect tissues, (2) to use electrophoretic separation methods to detect trace quantities of all proteins, i.e., enzymatic and non-enzymatic proteins, and (3) to determine whether different host substrates cause variation in the protein profiles. We studied three representative species of phytophage and, for comparison, one species of entomophage. Our work provides, for the first time for our test species of Hemiptera, total protein profiles of secreted saliva via electrophoresis.

Materials and methods

Insect rearing. *Empoasca fabae* (Harris), the potato leafhopper, was reared in growth chambers on broad bean, *Vicia faba* L., as in Habibi et al. (1993). *Empoasca abrupta* De Long, the western potato leafhopper, was reared similarly in growth chambers on pinto bean, *Phaseolus vulgaris* L. Eggs of *Lygus hesperus* Knight, the western tarnished plant bug, were obtained from BioTactics Co. (Riverside, California) and reared on an artificial diet, described below. *Podisus maculiventris* (Say), the spined soldier bug, was reared on *Trichoplusia ni* (Hübner), the cabbage looper, and larvae of *T. ni* and *Spodoptera frugiperda* (J. E. Smith), the fall armyworm, were reared on a modified wheat germ diet (Coudron et al., 2000). Larvae of *Leptinotarsa decemlineata* (Say), the Colorado potato beetle, were reared on leaves of red potato, *Solanum tuberosum* (L).

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Table 1. Summary of experimental design: test insect, rearing host, and treatment hosts for each experiment

Species	Rearing host	Treatment host
Empoasca fabae	Broad bean	Broad bean
		Pinto bean
		Simple diet
Empoasca abrupta	Pinto bean	Pinto bean
		Simple diet
Lygus hesperus	*Lygus* artificial diet	*Lygus* diet
		Lygus diet (starved insects)
		Cotton
		Cotton (starved insects)
		Pinto bean
		Pinto bean (starved insects)
Podisus maculiventris	*Trichoplusia ni*	*T. ni*
		T. ni (starved insects)
		S. frugiperda
		S. frugiperda (starved insects)
		L. decemlineata
		L. decemlineata (starved)
		Podisus diet
		Podisus diet (starved insects)

Diet composition and sachet construction. The composition of the artificial diet used to rear *L. hesperus* (BioTactics Co., Riverside, CA) was similar to that of DeBolt (1982), and will hereafter be referred to as '*Lygus* diet'. The artificial diet for rearing *P. maculiventris* was similar to the formulation of a diet developed for *Diapetimorpha introita* (Carpenter & Greany, 1990), and will hereafter be referred to as '*Podisus* diet'. An agarose/sucrose medium, hereafter referred to as 'collection diet', was composed of 3% (in the case of *Lygus* and *Podisus*) or 4% (in the case of the *Empoasca* spp.) agarose (SeaPlaque agarose, FMC, Rockland, Maine) plus 5% sucrose (Sigma Chemical Co., St. Louis, Missouri), except in the case of *P. maculiventris*, whose diet lacked sucrose.

Diets were dispensed as liquid into hemispheres constructed of stretched Parafilm®. Hemispheres were covered with Mylar® that was heat-sealed to the Parafilm®. Sachets containing diets, 40 μl, were stored at 4 °C and used within 3 d of preparation.

Experimental design. Table 1 has a summary of the experimental design. For each species studied, insects were removed from their rearing host (either a plant or diet), then placed on one of the treatment hosts for a designated period of time. After feeding on treatment hosts, the insects were immediately moved to a collection diet for recovery of saliva. For the purpose of this study, we defined all proteins introduced into the collection diet to be from saliva. Starvation of *L. hesperus* and *P. maculiventris* was done to test the possibility of regurgitation into the collection diet. We were unable to starve the *Empoasca* spp. for a sufficient duration without causing mortality.

For *Empoasca* spp., about 200, 1–5 d old adults were transferred from a rearing host onto a treatment host, fed for 72 h, then 100 were transferred onto 4 sachets (160 μl) of collection diet for 48 h. For *L. hesperus*, 30 adult, 1–3 d old females (10 adults per each of 3 treatment hosts, diet, cotton and pinto bean), were transferred from a rearing host onto a treatment host for 72 h. Five insects from each treatment were then starved for 24 h while the other 5 insects remained on the treatment host. Starved and non-starved insects were then transferred onto 6 sachets (240 μl) of collection diet for 24 h. For *P. maculiventris*, 15 third instar nymphs per treatment were transferred onto the treatment host until 1–2 d past adult eclosion. Thereafter, insects were treated the same as for *L. hesperus* (see Table 1 for more detail).

Saliva analysis. Fed-upon collection diet sachets were stored at minus 70 °C. For analysis, the diet was removed from the sachets, melted by heating to 65 °C in loading buffer, then loaded (160 μl for *Empoasca* spp., 240 μl for *L. hesperus* and *P. maculiventris*) directly into the wells of a mini or large gel. Protein markers were included for molecular weight estimations, proteins separated under denaturing conditions, and then visualized with a silver stain, following the methods of Coudron et al. (1998). Separation was performed using 8% and 10% gels in order to improve the resolution of both high and low molecular weight proteins, respectively. Protease K digestion was used to confirm that the bands from fed-upon collection diet were proteinaceous. For *Empoasca* spp., 25 μl of protease K (1.8 mg ml^{-1})was added to 160 μl of fed-upon collection diet; for *L. hesperus* and *P. maculiventris*, 50 μl was added to 240 μl of diet. All diets were incubated for 20 min at 70 °C.

Results

In most cases, sufficient amounts of proteins were recovered in the collection diet for comparative purposes. Every effort was made to minimize dilution of these proteins, to aid in their visualization on gels.

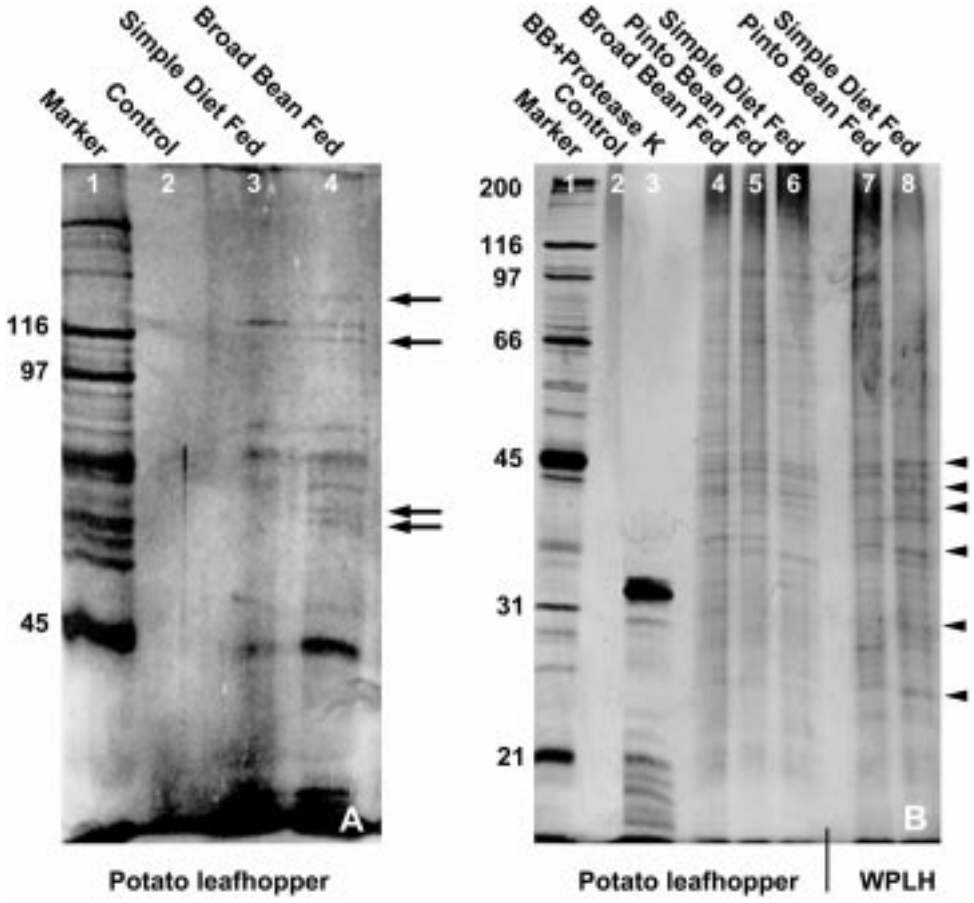

Figure 1. (A) SDS-PAGE separation of the salivary proteins of the potato leafhopper in a mini-gel (10 × 7.5 cm, 4 and 8% acrylamide for stacking and resolving gels respectively, 1.5 mm). Lane 1: marker. Lane 2: control (non-fed simple diet) showing no bands. Lane 3: bands from insects fed treatment simple diet. Lane 4: bands from insects fed treatment broad bean. (B) SDS-PAGE separation of the salivary proteins of the potato leafhopper (lane 1–6) and western potato leafhopper (WPLH) (lane 7 and 8) in a large gel (20 × 20 cm, 4 and 10% acrylamide for stacking and resolving gels respectively, 1.5 mm), Lane 1: marker. Lane 2: control (non-fed simple diet). Lane 3: digestive activity of protease K combined with proteins from insects fed treatment broad bean. Lane 4, 5, 6: bands from potato leafhopper fed treatments broad bean, pinto bean and simple diet, respectively. Lane 7, 8: bands from western potato leafhopper (WPLH) fed treatments pinto bean and simple diet, respectively. Molecular markers are Sigma broad range SDS-PAGE molecular weight marker, representing the molecular weights of 200, 116, 97, 66, 45, 31 and 21 kDa, respectively.

The electrophoretic separation of the denatured proteins from saliva of *E. fabae* is shown in lanes 2–4, Figure 1A and lanes 2–6, Figure 1B. A greater number of bands are visible in the separation of the saliva proteins from insects fed on treatment broad bean, lane 4, Figure 1A, versus when insects were fed treatment simple diet, lane 3 Figure 1A. There are four distinct bands ranging in size from about 50–130 kDa in lane 4, Figure 1A, that are not visible in lane 3 (see arrows in Figure 1A). In addition, one band about 45 kDa is present at much greater percentage composition after insects fed on treatment broad bean than the treatment simple diet (lane 4 vs. 3, respectively, Figure 1A). In an effect opposite that observed for *E. fabae*, *E. abrupta* demonstrated a greater number of bands after being fed treatment simple diet (lane 8, arrow heads, Figure 1B) than on treatment pinto bean (lane 7, Figure 1B).

The proteinaceous composition of these bands was confirmed by proteolytic digestion with protease K (lane 3, Figure 1B). All bands of molecular weight >31 kDa (molecular weight of protease K) were digested.

The electrophoretic separations of the denatured proteins from saliva of *L. hesperus* are shown in Figure 2. Again, different banding patterns were observed

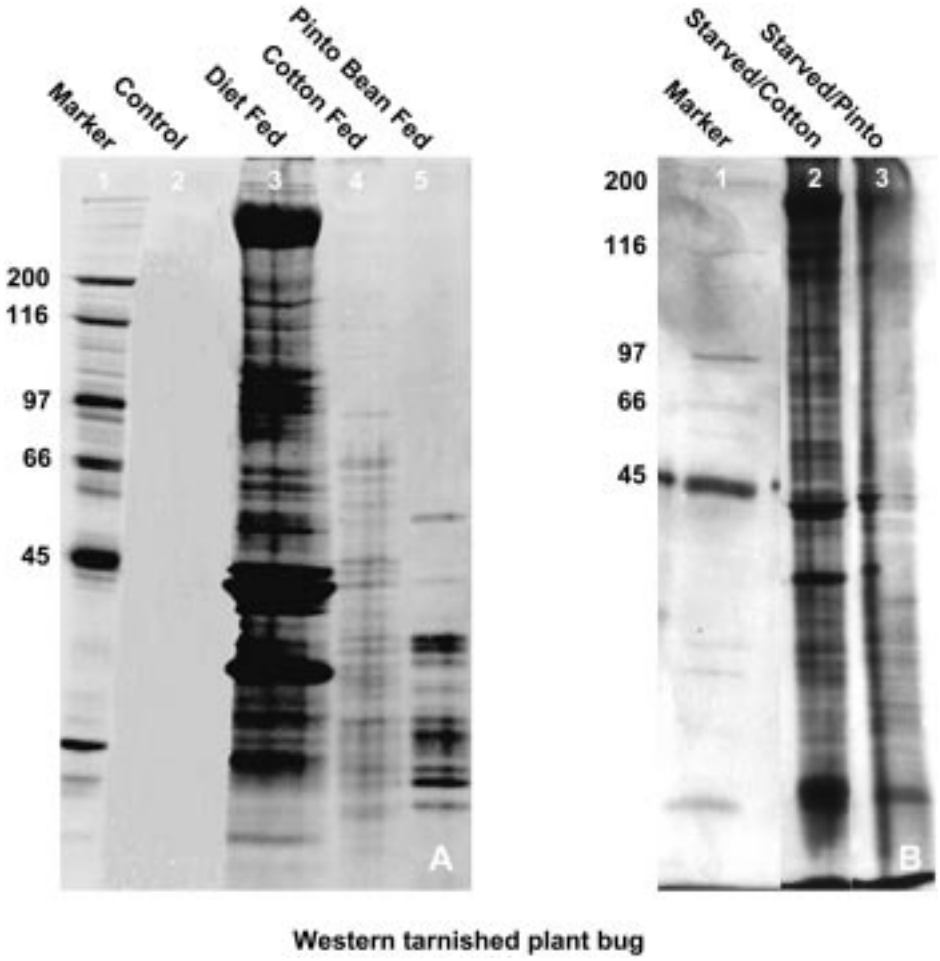

Figure 2. (A) SDS-PAGE separation of salivary proteins of the western tarnished plant bug in a large gel (20 × 20 cm, 4 and 10% acrylamide for stacking and resolving gels, respectively, 1.5 mm). Lane 1: marker. Lane 2: control (non-fed simple diet) showing no bands. Lane 3, 4, 5: bands from insects fed treatments *Lygus* diet, cotton and pinto bean, respectively. (B) SDS-PAGE separation of saliva proteins of the western tarnished plant bug in a large gel (same as 2A). Lane 1: marker. Lane 2: bands from insects starved for 24 h after being fed on treatment cotton. Lane 3: bands from insects starved for 24 h after being fed on treatment pinto bean. Marker molecular weights same as Figure 1.

with insects that had fed on various diets. A greater number of bands was observed for insects fed treatment *Lygus* diet (lane 3, Figure 2A) than when fed treatments cotton or pinto bean (lanes 4 and 5, respectively, Figure 2A). Similarly, a greater number of bands were observed after insects were fed treatment cotton versus treatment pinto bean (compare lanes 4 and 5, respectively, Figure 2A). Interestingly, it is apparent from the greater staining intensity of all bands in lane 2, Figure 2B, versus lane 4, Figure 2A, as well as lane 3, Figure 2B, versus lane 5, Figure 2A, that starved insects fed (and therefore salivated) more than the non-starved insects. Yet, the protein components of the saliva were similar. Digestion with protease K again confirmed the proteinaceous nature in Figure 2 (data not shown).

The electrophoretic separations of the denatured proteins from saliva of *P. maculiventris* are shown in Figure 3. In contrast to the phytophagous hemipterans, *P. maculiventris* manifested minimal differences in banding patterns of the saliva proteins after feeding on treatment hosts of *T. ni* or *Podisus* diet (lanes 2 and 4, respectively, Figure 3). Banding patterns from *P. maculiventris* that were fed treatment *L. decemlineata* and *S. frugiperda* were similar to those fed treatment *T. ni* (data not shown). Again, starved insects appeared to feed (and salivate) more, resulting in a higher total composition of certain proteins (compare lanes 2 with 3, 4 with 5, in Figure 3). The proteolytic nature of

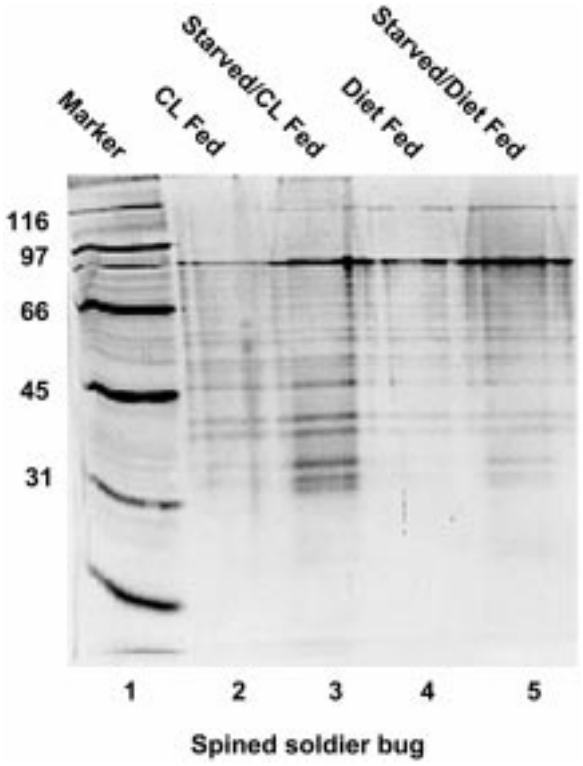

Figure 3. SDS-PAGE separation of salivary proteins of the spined soldier bug in a mini-gel (10 × 7.5 cm, 4 and 10% acrylamide for stacking and resolving gels, respectively, 1.5 mm). Lane 1: marker. Lane 2, 3: bands from insects not starved or starved, respectively, after being fed treatment cabbage looper (CL). Lane 4, 5: bands from insects not starved or starved, respectively, after treatment *Podisus* diet.

the salivary material was again confirmed by digestion with protease K (data not shown).

Additionally, results of our starvation tests show that the proteins in the collection diet are most likely to be saliva, not regurgitant. After 24 h of starvation, both *P. maculiventris* and *L. hesperus* guts had presumably absorbed or excreted all material ingested from the treatment hosts.

Discussion

Salivary protein profiles in relation to plant injury. Different salivary protein profiles were observed for *E. fabae* and *E. abrupta* when fed on the same treatment. This is interesting because, although both species rely on saliva during laceration feeding, they cause strikingly different plant injuries. *E. fabae* causes a chlorosis and stunting condition, called hopperburn, by feeding in several types of phloem cells in vascular tissues (Ecale & Backus, 1995a, b), while *E. abrupta* produces white leaf spots, known as 'stipples', by emptying mesophyll cells (Smith & Poos, 1931). Because the burner and the stippler both perform similar stylet penetration behaviors (i.e., similar tactics of the lacerate-and-flush feeding strategy; E.A.B., personal observation), we hypothesized that their saliva was similar, and different plant injuries were related more to the differential plant responses by chosen tissues. This hypothesis is supported by Miles (1999), who suggests that members of the same hemipteran family may possess similar salivary enzymes. Also, Berlin & Hibbs (1963) have shown that homogenized salivary glands of the potato leafhopper, *E. fabae* and *E. flavescens* (both phloem- feeders) contain invertase, amylase and protease. Yet, the different electrophretic patterns of our test species show that their salivary proteins are not identical. Because *E. abrupta* (a mesophyll-feeder) ingests whole cell contents more than *E. fabae*, it may require more diverse salivary proteins. However, further analysis of their enzymatic properties is required. Indeed Madhusudhan & Miles (1998) showed that salivary proteins with similar enzymatic activity may have different electrophoretic motilities, especially under denaturing conditions. Further testing of our hypothesis is clearly warranted.

We also found a larger number of proteins in *L. hesperus* secreted saliva, in all of our treatments, than has been reported in the literature for other *Lygus* species (Hori, 1975; Laurema, et al., 1985). Strong & Kruitwagen (1968) showed that homogenized salivary glands of *L. hesperus* contain a polygalacturonase, and proposed that the enzyme contributes to the burning-like plant injury caused by the plant bug. Recently, Cohen (1996, 1998) and Agusti & Cohen (2000) found that *L. hesperus* saliva (presumably from macerated glands) contains protease (especially trypsin-like enzymes), elastase, phospholipase A_2, amylase and pectinase. Given the large number of bands in our salivary protein profiles, it seems likely that other proteins are present that may be involved in plant injury or digestion.

Salivary protein profiles in relation to past dietary history. The past dietary history of the phytophagous hemipterans tested clearly influenced their salivary protein composition. For example, in the saliva of *E. fabae* fed treatment broad bean, four extra bands became visible compared with treatment simple diet; different banding patterns were also observed when

broad bean was compared with pinto bean. Drastically different salivary protein profiles were found when *L. hesperus* was fed 3 treatments consisting of *Lygus* diet, cotton or pinto bean; the greatest number of bands was observed on the *Lygus* diet.

Miles (1987) states that 'changes in diet are reflected in salivary composition of various Heteroptera and Homoptera'. Thus, our findings confirm and expand on those of previous authors. However, understanding of the mechanism and utility of these changes in salivary composition is very limited. Differences in responses among our primarily-phytophagous test insects may reflect that these insects ingest different tissues of the host plant (i.e., mesophyll versus vascular tissues). Thus, variable composition may be in part a passive process revealing only metabolic 'leftovers' from past food consumption. However, Miles (1999) says this is the case for amino acids and amides, not proteins. Alternatively, we speculate that a more active process may be involved. An insect's variable salivary protein profiles might indicate induction of new, unique proteins, in preparation for continued feeding on a substrate different from a previous one. Host plants can be induced *de novo* to produce compounds in response to feeding by herbivorous insects (as reviewed in Karban & Baldwin, 1997); such compounds can often change the taste of the plant. We hypothesize that highly specialized hemipteran herbivores may be able to counter plant compounds with inducible salivary proteins. Further, an awareness of the ability of hemipteran to alter their saliva proteins in response to changes in diet composition will aid understanding of diverse aspects of the insect's biology. Examples may include interaction with host plants (possibly chemical defenses) and other organisms (i.e., symbiotic microorganisms or transmission of pathogens), as well as the insect's ability to homeostatically balance its own biochemical milieu.

Unlike the phytophagous hemipterans, the entomophagous generalist species, *P. maculiventris*, showed no differences in salivary protein profiles dependent on host dietary treatment. This was even the case for *P. maculiventris* fed a plant-reared host, *L. decemlineata* (all other insect hosts were diet-reared). If phytochemicals were sequestered by *L. decemlineata*, no salivary proteins were induced by *P. maculiventris* in response. Thus, the capacity to distinguish and respond to dietary stimuli appears to differ between tested phytophagous hemiterans and *P. maculiventris*, a generalist entomophgous species.

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