The Host Plant as a Factor in the Synthesis and Secretion of Salivary Glucose Oxidase in Larval Helicoverpa zea

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We investigated the effect of the host plant on the synthesis and secretion of the elicitor glucose oxidase in the salivary glands of larval Helicoverpa zea. Glucose oxidase catalyzes the oxidation of D-glucose to produce D-gluconic acid and hydrogen peroxide. Previous studies have found that the product hydrogen peroxide is primarily responsible for suppressing the wound-inducible defenses of the host plant. Using an antibody specific for glucose oxidase, we determined the effect of the host plant on the rate of secretion of glucose oxidase. Larval H. zea secrete microgram amounts of the enzyme glucose oxidase from their principal salivary glands, the labial glands. Larvae reared on different host plants produce varying amounts of glucose oxidase in their labial glands. We used a tissue printing procedure with our antibody to determine if larvae secrete glucose oxidase directly at the feeding or wound sites. Significant amounts of the enzyme are deposited at the feeding site, although some is deposited outside the feeding margins. Arch. Insect Biochem. Physiol. 58:106–113, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

The saliva or oral secretions of herbivores may play important roles in the mediation of induced plant responses (Alborn et al., 1997, 2000; Eichenseer et al., 1999; Halitschke et al., 2001; Korth and Dixon, 1997; Musser et al., 2002a,b). The known oral elicitors from lepidopteran larvae fall into two principal categories: fatty acid-amino acid conjugates, e.g., volicitin (Alborn et al., 2002), and enzymes, e.g., β-glucosidase (Mattiacci et al., 1995) and glucose oxidase (Eichenseer et al., 1999).

In the case of the fatty acid-amino acid conjugates, there is debate as to their origin; it has been suggested that bacteria may contribute to their synthesis (Spiteller et al., 2000). However, they may be synthesized in the gut directly by the herbivore (Lait et al., 2003). Glucose oxidase (GOX) is produced primarily in the labial glands, but lesser amounts are found in other tissues such as the mandibular glands and hemolymph (Eichenseer et al., 1999). GOX mediates the oxidation of D-glucose with the concomitant production of D-gluconic acid and H₂O₂. The release of GOX from the spinneret during feeding is believed to inhibit the induced defenses of the tobacco plant; namely the alkaloid nicotine (Musser et al., 2002a). Applications of purified GOX (or the reaction product H₂O₂) to mechanical wounds decreased the amount of induced nicotine in wounded leaves (Musser et al., 2002a). Estimates of the amount of GOX to apply to mechanical wounds were based on recov-
erable GOX activity from nitrocellulose feeding disks. However, these estimates were subject to criticisms because of the artificial nature of the feeding and the lack of specific quantification of the GOX protein.

With few exceptions, investigators have largely ignored the amounts of any given elicitor that may be secreted during feeding (Gouinguene et al., 2003). Therefore, many of the early studies on elicitors have relied upon assumptions regarding the specific "dose" of an elicitor to be used to treat plants. Nevertheless, such quantitative information is critical to predict how oral secretions may function to trigger various plant responses. Without a clear understanding of dose-response, it is not possible to be certain about the function of oral secretions in situ. Recently, Truitt and Pare (2003) used a radiochemical method to elegantly demonstrate that third instar *Spodoptera exigua* secrete approximately 100 pmol of volicitin on a damaged site in corn seedlings. More detailed studies are needed to determine the rates of elicitor secretion for herbivores under a variety of host plant conditions.

In this study, we use an antibody specific for GOX from *Helicoverpa zea* to determine the amount of the enzyme secreted during feeding on various host plants and to determine if it is secreted primarily onto the larval feeding sites on the plant. We also assess the effect of the host plant on GOX activity in the labial salivary glands.

**MATERIALS AND METHODS**

**Plant and Insect Rearing**

*Helicoverpa zea* eggs were obtained from the insectary at North Carolina State University. Larvae were reared on a wheat germ and casein based artificial diet (Chippendale, 1970) with ingredients purchased from BIOSERV (Frenchtown, NJ) or Sigma (St. Louis, MO). Insects were kept at 27°C, with a 16-h photoperiod.

Tomato seeds (cv. Betterboy), tobacco seeds (cv. K316), and cotton seeds (cv. Acala maxxa, CPCSD, Bakersfield, CA) were grown in Metromix 400 potting mix (Griffin Greenhouse & Nursery Supplies, Tewksbury, MA) in a greenhouse at Penn State University, University Park, PA. The greenhouse was maintained on a 16-h photoperiod. Plants were watered every other day and fertilized 1 time per week with 20-20-20 Scotts Peter Professional fertilizer (Griffin Greenhouse & Nursery Supplies).

**Ablation of Spinneret**

We have previously used a method of cauterizing the spinneret with a hot needle in order to prevent larvae from salivating (Musser et al., 2002a). We have since modified this technique as follows. To ablate the spinneret, larvae were placed on ice and chilled until flaccid. Larvae were then laid ventral side up on a dish of ice and the spinneret was cauterized by touching briefly with a heat pen (Electron Microscopy Sciences, Fort Washington, PA). Following ablation, larva were returned to artificial diet for recovery.

**Rotofor Purification of GOX**

Labial glands were dissected from day-two fifth-instar larvae and stored at −80°C. To purify GOX, 200 pairs of glands were homogenized in phosphate buffered saline (PBS), then separated by isoelectric point using a Rotofor (Biorad, Hercules, CA) containing biolyte ampholytes pH 3–10. Rotofor fractions with GOX activity, as determined by a dianisidine assay (Eichenseer et al., 1999), were combined and concentrated with a Nanosep 10,000 MWCO centrifugal device (Pall Sciences, Ann Arbor, MI). Protein was quantified by the Bradford assay (Vincent and Nadeau, 1983) and stored at −20°C until use.

**Recovery of GOX From Leaves**

Day-one fifth-instar larvae were starved for 24 h, then caged on leaves and allowed to feed. We also tested larvae with ablated spinnerets to verify that glucose oxidase is secreted solely or primarily from the labial glands. After 4 h, larvae were removed and 0.05 to 0.1 g of leaf tissue was taken from around the feeding site. The leaf tissue was
placed in a Nanosep 10,000 MWCO centrifugal device with 0.5 ml of 0.065 M Tris-HCl, pH 6.8, with 0.7% SDS. Tubes were vortexed for 20 sec, then the leaf tissue was removed with forceps, and the remaining wash fluid centrifuged at 14,000g for 40 min. Concentrated proteins were then recovered by adding 25 µl of SDS sample buffer to the tubes. Proteins were separated on a 12% Tris/glycine gel (Gradipore LTD, Salt Lake City, UT), then transferred to 0.1 µm nitrocellulose (Schleicher & Schuell, Keene, NH). Western blots were blocked with Superblock blocking buffer in PBS (Pierce, Rockford, IL) probed with Anti-GOX (produced by a synthetic peptide based on H. zea GOX sequence derived from the cDNA; unpublished data) diluted 1:5,000, and detected with the Vector ABC kit (Vector Laboratories, Burlingame, CA), and Vector DAB kit. After detection, the blots were scanned at 720 dpi, and then analyzed using Sigmascan Pro 5.0 (SPSS Science, Chicago, IL). For each gel, the contrast was adjusted to standardize the background pixel intensity, and then average band intensity was measured. Known amounts of purified GOX were resolved and blotted at the same time and the average band density plotted against the concentration to create a standard curve for determining µg GOX recovered from the leaves.

**Tissue Printing**

To visualize secreted GOX on leaves, sixth-instar H. zea were allowed to feed overnight on detached tomato leaves. Leaves were then placed against nitrocellulose and subjected to electrotransfer for 5 h at 15 V, 0°C. After transfer, the nitrocellulose was allowed to air dry at room temperature and then blocked 1 h with Superblock. Membranes were incubated in Anti-GOX, diluted 1:5,000 overnight at room temperature, then detected with Vector ABC Elite and Vector DAB kit. Control leaves were not fed on by H. zea.

**Comparison of Saliva, Labial Glands, and Regurgitant**

To compare the electrophoretic profile of saliva, regurgitant, and labial glands, 48-h-old sixth-instar H. zea were chilled on ice and 2–5 µl of homogenized leaf was pipetted onto the mandibles. Saliva secreted from the spinneret was then collected in a gel-loading pipette tip containing 1 µl glycerol. Saliva from 3–5 larvae was collected into one tip, then the contents expelled into a microcentrifuge tube where 10 µl of SDS sample buffer was added.

Regurgitant collected from 10 larva was combined with 100 µl PBS and centrifuged at 5,000g for 5 min, the supernatant was recovered and protein quantified via the Bradford assay. Dissected salivary glands were homogenized in PBS and Bradford assayed as well. The proteins were then separated on a 12% tris-glycine gel and silver stained.

**Effect of Jasmonic Acid on GOX**

To investigate the effect of the plant-signaling compound on GOX in H. zea, jasmonic acid was dissolved in ethanol and added to freshly made, cooled artificial diet. H. zea that had slipped the head capsule in preparation for molting to 6th instar were placed on the diet. Larvae were dissected and the labial glands removed 48 h after they molted. Glands from 5 larvae were combined, homogenized in 150 µl PBS, and assayed for GOX activity as previously described (Eichenseer et al., 1999).

**Effect of Different Host Plants on GOX**

To determine if GOX levels changed in response to feeding on different plants, 6th-instar H. zea were allowed to feed on detached leaves of tobacco, cotton, or tomato. After 48 h, larvae were dissected and labial glands were removed and assayed for GOX as described above.

**RESULTS**

**Recovery of GOX From Leaves**

To quantify the amount of GOX recovered from leaves fed upon by H. zea, a standard curve was created from 11 different immunoblots of known amounts of GOX. We were able to measure band
Effect of the Host Plant on *H. zea* intensity when a minimum of 0.25 µg of purified GOX was resolved on the gel. The relationship between average band intensity and µg GOX loaded on the gel was linear for the amounts used in this study, 0.25 to 1 µg (Fig. 1). To determine the percent of GOX recovered from the leaf, known amounts of purified GOX were pipetted onto the leaf; the leaf was then washed and GOX quantified as described. We were able to recover 25% of the applied GOX from tomato leaves and 30% from cotton leaves. Percent recovery for tobacco was assumed to be the same as tomato. We were unable to detect less than 1 µg pipetted onto the leaf. Therefore, assuming recovery is similar for GOX secreted onto the leaf by *H. zea*, in order to be measurable the larvae would have to secrete a minimum of 1 µg in the allotted time period. From 39 tomato leaves, the average GOX secreted onto the leaf in 4 h was 1.56 µg (Table 1). In 19 of 39 tomato leaves, we were unable to detect GOX. Since only leaves that insects actually fed on were used, we can conclude using our method that less than 1 µg of GOX was secreted onto these leaves in the 4-h time period. Figure 2 shows a typical immunoblot from this experiment.

From 26 tobacco leaves, the average GOX secreted onto the leaf in 4 h was determined to be 2.47 µg (Table 1), with 8 of 26 leaves containing undetectable amounts of GOX. From 30 cotton leaves, the average GOX secreted onto the leaf in 4 h was determined to be 2.61 µg with 8 of 30 larvae secreting undetectable amounts.

We were not able to detect GOX in any of 10 tomato leaves fed on by ablated *H. zea*; previous studies show that ablation stops GOX excretion (Musser et al., 2002a). We believe that this current method of ablation provides more satisfactory results in that salivation appears to be completely impaired by the procedure.

**Tissue Printing**

Initial attempts to label GOX directly on the leaf using fluorescent or HRP conjugated secondary antibodies were unsuccessful. Experiments quantifying the recovery of GOX from the leaves illustrate that GOX is not firmly attached to the leaf and can easily be removed by washing. Immunodetection requires washing away excess antibody to ensure specificity and is not compatible with labeling directly on the leaf. For this reason, we transferred the proteins on the leaf surface to nitrocellulose. We were then able to visualize the location of GOX as in an immunoblot. As seen in Figure 3, most of the GOX is detected along the edge of the feeding site. On leaves exposed to larvae, GOX was also detected on undamaged areas of the leaf as well. GOX was not detected in any leaves not exposed to larva (Fig. 3).

**Comparison of Saliva, Labial Glands, and Regurgitant**

Previous studies of salivary gland proteins have focused on salivary gland homogenate or regur-
gitant, both of which are easy to collect. We sought to compare these to what is secreted onto the leaf during the course of normal feeding. Initial attempts to concentrate the saliva washed from fed-upon leaves or glass fiber filters were unsuccessful. When resolved on gels, the proteins from these samples were badly degraded despite the use of protease inhibitors at all steps. We observed that during feeding, the larva is also excreting feces, which contaminate the samples and degrade the proteins. Attempts were also made to stimulate salivation by interhemocoelic injection of dopamine and serotonin. While these did stimulate minimal salivation, the simplest and most reliable method was to pipette 2–5 µl of homogenized leaf onto the mandibles. This resulted in a small drop of saliva emerging from the spinneret, which, when resolved by gel electrophoresis, showed that saliva contains multiple proteins (see Fig. 4). A 50% glucose solution applied to the mandibles also induced salivation. Glucose oxidase and other high molecular weight proteins were not detectable in the regurgitant (Fig. 4). No obvious differences were detected in the saliva induced by either glucose or extracts from tomato, tobacco, or cotton (data not shown).

**Effect of Jasmonic Acid on GOX**

Jasmonic acid was added to the diet at rates of 0.29, 2.9, or 290 µg/ml; in control treatments, 0.1% ethanol was added to the diet. Total protein in the glands increased with jasmonic acid, but no statistically significant differences were detected. GOX activity per gland was similar in all treatments (Table 2; \( P > 0.05 \)). Specific activity for GOX was processed in parallel with samples of GOX secreted onto the leaf during larva feeding.

Fig. 2. Immunoblot blot of purified GOX and GOX recovered from leaves fed on by larval *H. zea*. Known amounts of purified GOX were loaded onto the gel and processed in parallel with samples of GOX secreted onto the leaf during larva feeding.

Fig. 3. A: Tissue print of tomato leaves fed on by *H. zea*. B: Control tomato leaf. After being fed on by *H. zea*, secreted proteins on the leaf surface were transferred to nitrocellulose and GOX was detected by immunoblotting.
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Effect of Different Host Plants on GOX

When fifth instars were allowed to feed on detached leaves of tomato, tobacco, or cotton, then assayed for GOX activity in labial glands, glands from larvae that fed on tobacco contained more protein per gland than those that fed on tomato or cotton (Table 3; $P < 0.05$). In addition, larvae that fed on tobacco contained more GOX activity per gland pair than larvae feeding on cotton ($P < 0.05$).

**DISCUSSION**

The host plant affects the protein composition of the labial salivary glands in both a qualitative and quantitative manner. Larvae feeding on the comparatively poor host tobacco had the highest amounts of salivary protein and the highest amount of GOX activity. Surprisingly, the amount of protein in the salivary glands was inversely related to the quality of the host. Larvae grow considerably better on cotton, followed by tomato and tobacco (Eichenseer et al., 2002; unpublished data). It should be noted that not only is there a quantitative effect of host plant on salivary proteins, but also qualitative effects. Salivary lysozyme, for instance, follows a very different expression pattern in larvae feeding on these hosts, with the lowest expression found when they ingest tobacco (unpublished data). Other findings in our laboratory indicate that the host plant has a profound effect on non-protein components (e.g., carotenoids) of the labial and mandibular salivary glands (Eichenseer et al., 2002).

Preliminary evidence indicates that GOX has the strongest effect of mitigating induced resistance in tobacco (Musser et al., 2002a), which could suggest that this serves an adaptive response to the inducible defenses of tobacco. In tomato, GOX may not be as effective in mitigating induced resistance. In fact, it has been shown that purified fungal GOX fed through cut stems of tomato plants elicits the production of serine protease inhibitors (Orozco-Cardenas et al., 2001). Thus, as Farmer (2000) states: "Well adapted attackers must therefore minimize the display of elicitors and also avoid injuring their host since both types of input will

![Silver-stained 12% gel of homogenized salivary gland (Gl), regurgitant (Rg) and saliva (Sa) from H. zea.](image)

**TABLE 2. Effect of Jasmonic Acid on Labial Glucose Oxidase Activity***

<table>
<thead>
<tr>
<th>Jasmonic acid level ($\mu$g/gm diet)</th>
<th>Protein concentration ($\mu$g protein/pair of glands)</th>
<th>GOX specific activity (mols/min/mg protein)</th>
<th>GOX activity (mols/min per pair of glands)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.4 (62.2) a</td>
<td>5.18 (4.34) a</td>
<td>15.51 (7.11) a</td>
</tr>
<tr>
<td>0.29</td>
<td>133.4 (66.9) a</td>
<td>3.61 (2.21) ab</td>
<td>16.55 (7.55) a</td>
</tr>
<tr>
<td>2.9</td>
<td>120.7 (59.2) a</td>
<td>3.67 (2.76) ab</td>
<td>15.38 (5.02) a</td>
</tr>
<tr>
<td>290</td>
<td>134.7 (85.6) a</td>
<td>3.29 (2.49) b</td>
<td>13.43 (6.48) a</td>
</tr>
</tbody>
</table>

*GOX activity and protein concentration were measured in labial glands after larva fed 48 h on artificial diet containing jasmonic acid. Means followed by the same letter are not statistically different at $P < 0.05$ by Tukey’s pairwise comparison. Numbers in parentheses are standard deviations.
surely lead to better detection and stronger defense." This could be an example of where an herbivore is able to manipulate its display of elicitors to be most effective depending upon its host. These findings warrant further study on the ability of herbivores to adaptively respond to hosts by altering their composition and production of elicitors.

Although we did not find statistically different amounts of GOX secreted on the varied host plants, differences could be greater if larvae were first reared on the respective hosts rather than ingesting artificial diet prior to the experiment. Nevertheless, we found that larvae secrete microgram quantities of GOX onto the leaf as they feed. In the case of larvae feeding on tobacco, it would be estimated that they may secrete nearly 15 µg of protein during a 24-h period. This is considerably more than we estimated in our initial studies on the effect of GOX on induced responses in tobacco (Musser et al., 2002a) and suggests we have underestimated the impact of GOX in this example. It should be recognized that a good portion of the secreted GOX is probably ingested during feeding, so that the total amounts of GOX secreted may be considerably larger than what we can recover on the leaf. The tissue print of the leaf indicates that there are substantial amounts of GOX found at the feeding sites, although some of the enzyme is distributed on surfaces outside the feeding margins. Thus, there is ample opportunity for GOX to react with plant glucose at the feeding sites to produce \( \text{H}_2\text{O}_2 \) bursts. However, we have found no evidence that GOX is transported through the plant vascular system. Preliminary evidence using our antibody indicates that GOX does not move significantly from the wound site (unpublished data). However, because the reaction product \( \text{H}_2\text{O}_2 \) is readily diffusible, it likely moves considerable distances from the initial feeding site.

Future studies are directed at determining if the host plant affects GOX at the transcriptional and/or translational level. The impact of differential synthesis and secretion of GOX on induced responses in various host plants is the goal of our ongoing investigations.

**LITERATURE CITED**


vore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. Plant Physiol 125:711–717.


