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Diet Factors Responsible for the Change of the Glucose Oxidase Activity in Labial Salivary Glands of *Helicoverpa armigera*

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We investigated the change of the glucose oxidase (GOX) activity in labial salivary glands of *Helicoverpa armigera* larvae fed with the artificial diet or host plant tobacco and the major factors responsible for such a change. Throughout larval development, the labial salivary GOX activities in caterpillars reared on the artificial diet were remarkably higher than those fed with the plant. After fifth-instar plant-fed caterpillars were transferred to the artificial diet, their labial salivary GOX activity increased quickly, which was closely correlated with the time spent feeding on the artificial diet. The total sugar content of the artificial diet was 68 times higher than that of the tobacco leaves. We hypothesized that sugars and secondary metabolites are the possible causes of induction of GOX activity. When fifth-instar caterpillars were fed with tobacco leaves coated with glucose or sucrose, their labial salivary GOX activity was significantly higher than those fed with leaves without sugar coating. Following native PAGE, 1 single band of the labial salivary GOX was observed in all the caterpillars fed with different diets, implying that only the activity of the isoenzyme was changed in response to different diets. Furthermore, the labial salivary GOX activity was determined after caterpillars were fed with artificial diets containing chlorogenic acid, rutin, and quercetin. The results showed that all these phenolic compounds had no effect on the GOX activity. We conclude that sugar in diets was a major factor influencing the labial salivary GOX activity of the larvae. Arch. Insect Biochem. Physiol. 68:113–121, 2008.

KEYWORDS: glucose oxidase; *Helicoverpa armigera*; sugar; phenolics

INTRODUCTION

The oral secretions of herbivores play important roles in plant–insect interactions (Alborn et al., 1997, 2000; Halitschke et al., 2001; Musser et al., 2002a,b; Bede et al., 2006). One category of the oral secretory substances is fatty acid–amino acid conjugates, for example, volicitin (Alborn et al., 1997; Turlings et al., 2000; Mori et al., 2003), and the other is enzymes, for example, β-glucosidase (Mattiacci et al., 1995) and glucose oxidase (GOX) (Eichenseer et al., 1999).

By ablating caterpillar labial salivary glands, Musser et al. (2002a, 2006) demonstrated that the labial salivary GOX of *Helicoverpa zea* suppressed herbivore-induced nicotine production in *Nicotiana tabacum*. In this process, GOX converts D-glucose and molecular oxygen to D-gluconic acid and hydrogen peroxide (H_2O_2) (Eichenseer et al., 1999). The latter is a signaling molecule that can enhance ethylene levels in plants (Chamnongpol et al., 1998). Ethylene was found to suppress synthesis of nicotine in *N. sylvestris* (Kahl et al., 2000; Voelckel et al., 2001; Baldwin, 2001; Winz and Baldwin, 2001). Musser et al. (2005a) confirmed that GOX not only could suppress nicotine in tobacco, but could also affect the level of trypsin inhibitors in tomato. Besides its role in modulating

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induced plant defenses, GOX also acts as an antimicrobial agent against insect pathogens present on leaf surfaces (Eichenseer et al., 1999; Musser et al., 2005b).

GOX activity appears to be variable in labial glands among caterpillar species. It was detected in *H. zea* (Eichenseer et al., 1999), *H. assulta*, *H. armigera* (Zong and Wang, 2004), *Spodoptera exigua*, and *Mamestra configurata* (Merkx-Jacques and Bede, 2004), but not in all noctuid species. The GOX activity was not found in labial glands of *S. litura* (Zong and Wang, 2004), *Pseudaletia unipuncta*, or *Colias eurytheme* (Merkx-Jacques and Bede, 2004). Furthermore, it was discovered that larval salivary GOX activity of *S. exigua* reared on artificial diets was significantly higher than those reared on *Medicago truncatula* plants (Merkx-Jacques and Bede, 2004, 2005), and *H. zea* larvae reared on different host plants produced varying amounts of glucose oxidase in their labial glands (Peiffer and Felton, 2005), suggesting that diets were involved in the regulation of caterpillar salivary enzyme synthesis and secretion. However, they failed to identify factors in the diets responsible for mediating GOX activity changes. The chemical components in artificial diets and host plants are very different. Artificial diets often support insect growth better than natural host plants (Schoonhoven et al., 2005), probably because the natural host plants usually contain inadequate nutrients and a variety of secondary metabolites that need to be detoxified. We hypothesized that sugars and secondary metabolites might influence the level of GOX activity in salivary glands of insects.

To determine changing patterns of GOX activity in the labial salivary glands of the insects reared on different diets and factors involved in the regulation of the GOX activity, we compared the difference of GOX activity in the labial salivary glands of *H. armigera* reared on *N. tabacum* and artificial diets during larval development. We also investigated the effect on the GOX activity of 2 sugars, glucose and sucrose, and 3 common phenolic compounds found in plants (Harborne, 1979; Parejo et al., 2004), chlorogenic acid, rutin, and quercetin.

MATERIALS AND METHODS

Plant and Insect

Seeds of tobacco (*Nicotiana tabacum* L.) cultivar "Putongyan" were provided by Institute of Crop Germplasm Resources of the Chinese Academy of Agricultural Science (CAAS). They were germinated in 16-cm (diameter) × 15-cm (deep) flowerpots in a growth chamber at 24 ± 1°C with a 16-h light/8-h dark photoperiod. The fertilized soil was obtained from the Institute of Vegetables and Flowers, CAAS. Three months later, the tobacco plants were used for caterpillar feeding studies.

H. armigera were collected in the field as larvae from Zhengzhou, Henan province of China. The larvae were reared on an artificial diet (Wu and Gong, 1997) for many generations in the laboratory at 26 ± 1°C with a 16-h light/8-h dark photoperiod.

Changing Patterns of GOX Activity of Larvae

We fed *H. armigera* from first instar with artificial diet or tobacco leaves. The labial salivary glands of larvae in each treatment were collected at age 24 h in the third-, fourth-, and fifth-instar larvae. The whole experiment was repeated separately 3 times, and in each replication 15–30 pairs of glands at each time point of each treatment were pooled together for the GOX activity determination. Meanwhile, the total sugar contents of the artificial diet and tobacco leaves were measured (as described later).

To investigate the effect of diets on GOX activity further, caterpillars were reared on tobacco leaves until the beginning of fifth instar, and then transferred to the artificial diet. At 0, 6, 12, 18, and 24 h after transfer, labial salivary glands were collected and analyzed for GOX activity. The control caterpillars were fed with tobacco leaves or the artificial diet all the time. Three replications were run, and in each replication 6 pairs of glands were used at each time point of each treatment.

Effect of Sugars on GOX Activity of Larvae

Tobacco leaves covered with sucrose or glucose were used as test diets for insect rearing, and those

without any sugar coating were used as control. The tobacco leaves were dipped into 1% of sucrose or glucose solutions so that the surface of the leaves was covered with an appropriate sugar solution. After water evaporated from the leaf surface, the leaves were used as the test diets, and the total sugar contents of all the leaves were determined (as described later).

H. armigera were reared on the artificial diet from neonate, and then moved to the test diets from the second instar onward. Caterpillars fed with normal tobacco leaves dipped in water were used as control. Labial salivary glands were removed from the 24-h-old fifth-instar caterpillars for analysis of GOX activity. The whole experiment was repeated separately 5 times, and in each repeat experiment 6 pairs of labial salivary glands were used at each time point of each treatment.

To determine whether different diets induce different GOX isozymes, cell-free extracts of labial salivary glands of caterpillars from different diet treatments were analyzed following electrophoresis on 10% polyacrylamide gels under native or nondenaturing conditions. The native gels were specially stained for GOX activity using 6 mM D-glucose, 0.3 mM o-dianisidine, and 60 U/ml horseradish peroxidase (HRP). GOX from *Aspergillus niger* was used as control. The HRP was purchased from Roche, Germany; all other chemicals were obtained from Sigma (St. Louis, MO).

Effect of Phenolic Compounds on GOX Activity of Larvae

Chlorogenic acid, rutin, and quercetin (Beijing Chemical Reagent Co., China) were first dissolved in 1 ml 70% (v/v) ethanol. They were then incorporated into the artificial diet singly, at a final concentration of 0.2% dry weight of the artificial diet. *H. armigera* were reared on the artificial diet with phenolics from fifth-instar caterpillars for 1 day, and those fed with the artificial diet treated with 70% ethanol in the same way were used as control. We collected the labial salivary glands for analysis of GOX activity. The whole experiment was repeated separately 5 times, and in each replication 6 pairs of labial salivary glands were used at each time point of each treatment.

Preparation of Extracts From Labial Salivary Glands

Labial salivary glands were removed with the aid of an anatomical lens from anesthetized *H. armigera* kept on ice and immediately homogenized in chilled potassium phosphate buffer (0.1 M, pH 7.0). The homogenates were centrifuged at 4°C, 12,000*g* for 15 min, and the supernatants were collected as the labial gland extracts.

Glucose Oxidase Assay

Glucose oxidase activity was determined by measuring the change in absorbance at 460 nm of the reaction mixture on a Beckman DU 800 spectrophotometer. The reaction mixture in a total volume of 3.1 ml contained 0.17 mM o-dianisidine-HCl (Sigma) in 0.1 M potassium phosphate buffer (pH 7.0), 95 mM D-glucose (Sigma), 60 U/ml HRP (Roche, Germany), and 0.1 ml of the labial salivary gland extract (Kelley and Reddy, 1988). For the control, 0.1 ml potassium phosphate buffer (0.1 M, pH 7.0) was added instead of the labial salivary gland extract. The extinction coefficient was 8.3 cm^{-1} μM^{-1}. Before salivary gland extracts were added, the reaction mixtures were incubated at 35°C and saturated with oxygen. Over 5 min, the change in absorbance at 460 nm/min was calculated to obtain the slope of the linear portion. Protein concentrations were determined following the method of Bradford (1976) using bovine serum albumin (BSA) (Amresco) as a standard.

Determination of the Glucose and Total Sugar Contents

Glucose content of the diets was determined with glucose oxidase and peroxidase (Bergmeyer and Bernt, 1974; Frost, 2004). o-Dianisidine-HCl (5 mg/ml, Sigma) was dissolved in buffer–enzyme

mixture consisting of 0.12 M phosphate buffer (pH 7.0), 40 μg/ml HRP (Roche), and 250 μg/ml glucose oxidase from *Aspergillus niger* (Sigma). To 5 ml of this, 0.2 ml of an extract containing glucose was added. The change in absorbance of the reaction mixture was measured at 436 nm using a Beckman DU 800 spectrophotometer. D-Glucose standard was purchased from Sigma.

The total sugar content of the diets was determined as described by Frost (2004). One gram of a diet, either the artificial diet or tobacco leaves, was dissolved in 6 M HCl and boiled for 30 min. After this was hydrolyzed, 1% (w/v) 3,5-dinitrosalicylic acid (DNS, dissolved in 10%, w/v, NaOH), 10% (w/v) phenol 22% (w/v) sodium potassium tartrate, and 10% (w/v) $NaHSO_4$ were added. All these reagents were bought from Beijing Chemical Reagents Co. (Beijing, China). The absorbance of the reaction mixture was measured at 540 nm using a Beckman DU 800 spectrophotometer.

STATISTICAL ANALYSIS

The data were analyzed with one-way analysis of variance (ANOVA). Differences among the means were compared with least significant difference (LSD) at the $P = 0.05$ level of significance. All the above analyses were carried out with SPSS 12.01 (2001) software package.

RESULTS

GOX Activity of Larvae Fed on Artificial and Plant Diets

The GOX activity of labial salivary glands of *H. armigera* larvae increased to the highest levels in fifth instar, whether they were reared on an artificial diet or tobacco leaves (Fig. 1). However, the GOX activity of labial salivary glands from the third-, fourth-, and fifth-instar larvae fed with the artificial diet were all significantly higher than that of the corresponding larvae reared on tobacco leaves. The salivary GOX activity of the fifth-instar caterpillars reared on the artificial diet was 1.02 ± 0.05 μmol/min/mg protein, which was 4 times higher than that of the caterpillars fed on tobacco leaves.

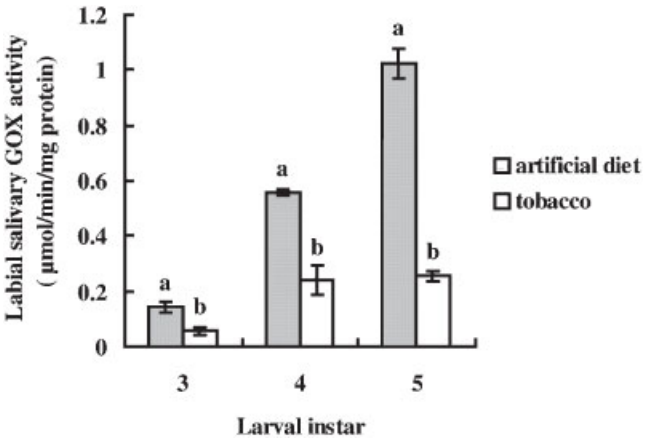

Fig. 1. Glucose oxidase (GOX) activity in labial salivary glands of *Helicoverpa armigera* fed with an artificial diet or tobacco leaves during larval development (third to fifth instars). Different letters represent statistically significant differences (LSD) between treatments at $P < 0.05$. Bars indicate mean ± SE.

When tobacco-fed caterpillars were transferred to artificial diet from the beginning of the fifth instar, GOX activity in labial glands was gradually increased with the time caterpillars spent on artificial diet (Fig. 2). In the diet-transferring treatment, GOX activity of labial salivary glands from those caterpillars transferred rose 10 times from 0.09 ± 0.002 μmol/min/mg protein at the beginning to 0.98 ± 0.002 μmol/min/mg protein in 24 h, while that of plant-fed caterpillars only rose 2.5 times from 0.09 ± 0.003 μmol/min/mg protein to 0.25 ± 0.004 μmol/min/mg protein in the same period of time. At 18 h, GOX activity of labial salivary glands in the transferred caterpillars had nearly reached that of caterpillars reared on the artificial diet.

Since diets had great effects on labial salivary GOX activity of larvae, we studied the level of the substrate for GOX, glucose, as well as total sugar content, in the artificial diet and tobacco leaves. The results showed that the artificial diet contained much more glucose (2.2 ± 0.32 mg/g fresh diet) and sugar contents (85.8 ± 6.14 mg/g fresh diet), which are about 9 and 68 times higher than those in the control tobacco leaves, respectively.

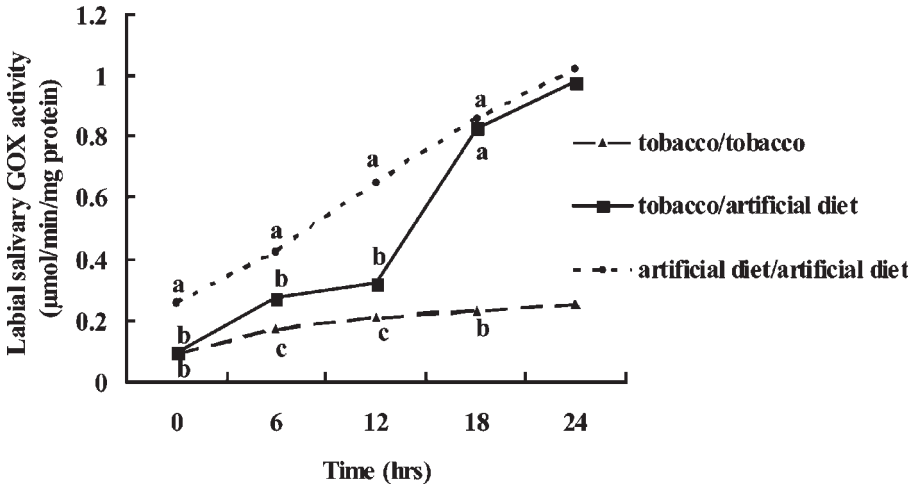

Fig. 2. Labial salivary gland glucose oxidase (GOX) activity after caterpillars transferred from tobacco leaf diet to an artificial diet. Fifth-instar caterpillars were transferred from the tobacco leaf diet to an artificial diet. At 0, 6, 12, 18, or 24 h, GOX activity was measured. Different letters represent statistically significant differences (LSD) between treatments at $P < 0.05$. Bars indicate mean ± SE.

Effect of Sugars on GOX Activity of Larvae

Tobacco leaves coated with glucose, sucrose, or water (control) were used to determine the role of sugars in induction of labial salivary gland GOX activity of the caterpillars. Among them, the control tobacco leaves had the lowest sugar content, which was 1.25 ± 0.44 mg/g fresh diet. There was no significant difference in the total sugar contents between the glucose-coated and the sucrose-coated tobacco leaves, but both had significantly higher sugar contents than the control tobacco (df = 2,6; F = 6.59; $P = 0.03$; Fig. 3).

The caterpillars reared on glucose- or sucrose-coated tobacco leaves had a higher level of the labial salivary GOX activity, which was about 2 times higher than those only fed with the control tobacco leaves (df = 2,12; F = 36.07; $P < 0.0001$). However, the GOX activity of the caterpillars fed with the glucose- and sucrose-coated tobacco leaves was

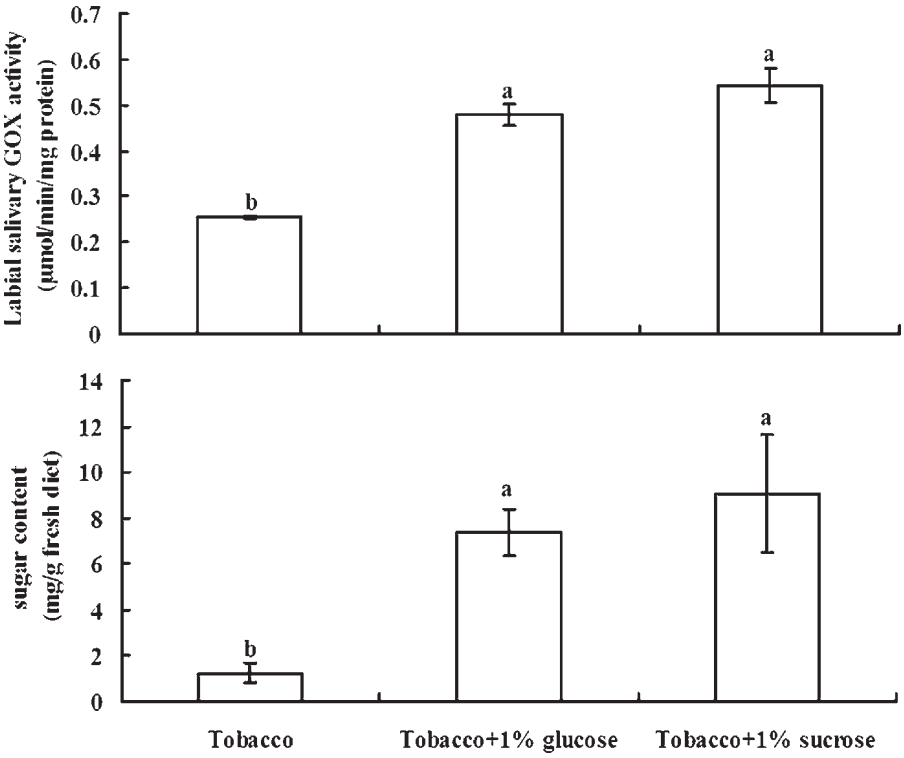

Fig. 3. Total sugar contents of tobacco leaves without any sugar coating, dipped in 1% glucose or sucrose and GOX activity in labial salivary glands of fifth-instar caterpillars feeding on these tobacco leaves. Different letters represent statistically significant differences (LSD) between treatments at $P < 0.05$. Bars indicate mean ± SE.

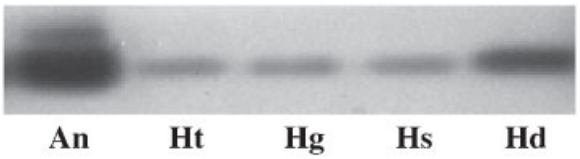

Fig. 4. Native PAGE of 5 μg protein from *Aspergillus niger* GOX (An); Ht, Hg, Hs, Hd denote extracts from 3 pairs of labial glands of *Helicoverpa armigera* that were fed with tobacco leaves without any sugar coating, dipped in 1% glucose or sucrose, and those reared on the artificial diet, respectively.

about the same, 0.48 ± 0.02 μmol/min/mg protein and 0.54 ± 0.04 μmol/min/mg protein, respectively (Fig. 3).

The possibility that the differences in the GOX activity observed above were due to a change in different GOX isozymes was investigated in the native PAGE experiment (Fig. 4). No new GOX isozymes were formed in response to different diets. However, the activity of the single GOX isoform appeared to be higher in the extracts from the salivary glands of the caterpillars fed with artificial diet compared to that of those reared on the tobacco leaves coated without a sugar, or with glucose or sucrose.

Effect of Phenolic Compounds on GOX Activity of Larvae

Chlorogenic acid, rutin, and quercetin (0.2%, dry weight) in the diets had no effect on the relative larval growth rate and GOX activity of labial salivary glands after *H. armigera* were fed with these diets for 24 h (Table 1).

TABLE 1. Glucose Oxidase Activity: μmol/min/mg Protein, Means ± SE, and Relative Growth Rate*

Diet types	Relative growth rate	GOX activity of labial salivary glands
Normal artificial diet	1.06 ± 0.017	1.00 ± 0.046
Control artificial diet	1.08 ± 0.018	0.97 ± 0.048
Artificial diet with chlorogenic acid	0.98 ± 0.040	1.10 ± 0.207
Artificial diet with rutin	1.07 ± 0.035	0.93 ± 0.030
Artificial diet with quercetin	1.07 ± 0.038	0.86 ± 0.090

*Means ± SE of fifth instar of *Helicoverpa armigera* reared on diets supplemented with chlorogenic acid, rutin, or quercetin. All secondary compounds, each at a concentration of 0.2% dry weight of the artificial diet, were dissolved in 1 ml of 70% (v/v) ethanol. Twenty caterpillars were used for each treatment; insects fed with artificial diet containing 1 ml of 70% ethanol were used as control. Numerical values were analyzed statistically (LSD at $P < 0.05$).

DISCUSSION

GOX was first discovered from *Aspergillus niger* (Muller, 1928) and was commonly regarded a fungal enzyme (Eichenseer et al., 1999). Some of the biochemical properties of insect GOX differ from fungal GOX (Eichenseer et al., 1999), but the basic oxidation process is the same, glucose + O_2 → gluconic acid + H_2O_2. In insects, GOX plays important roles in the plant-insect interactions (Alborn et al., 1997, 2000; Musser et al., 2002a). GOX can suppress synthesis of nicotine through interference in signal transduction (Musser 2002a, 2005a; Zong and Wang, 2004) and also have an antimicrobial characteristic (Eichenseer et al., 1999; Musser et al., 2005b).

In *H. zea* reared on an artificial diet, the labial salivary GOX activity increased from fourth to sixth instar and the highest GOX activity in sixth instar (Eichenseer et al., 1999). The labial salivary GOX activity of *H. armigera* fed with an artificial diet also had the similar trend. The results implied that GOX activity increased with the development of larvae and the advance of ingestion, and the older caterpillars were more adaptable to plant defense. However, *Spodoptera exigua* labial salivary GOX activity had a different trend of change, and the GOX activity of fourth-instar caterpillars was the highest (Merkx-Jacques and Bede, 2005). Although the level of the labial salivary GOX activity of caterpillars fed with their host plants was variable in different studies, the host plant appears to be an important factor in the synthesis and secretion of the enzyme. Labial glands from larvae of *H. zea* that fed on tobacco contained more GOX activity per gland pair than larvae feeding on cotton (Peiffer and Felton, 2005). A common result was that caterpillars reared on an artificial diet had higher labial salivary GOX activity than those fed with plants (Eichenseer et al., 1999; Merkx-Jacques and Bede, 2004, 2005). To determine the effect of diets on GOX activity further, fifth-instar *H. armigera* caterpillars were transferred from feeding on plants to the artificial diet. It was discovered that GOX activity of labial salivary glands increased with time spent on the new diet. It is clear that

some factors in artificial diets influence the GOX activity of the labial salivary glands in caterpillars.

The factors inducing the production of enzymes in insects could be related to the amount of the appropriate substrates. For example, α-amylase induction in *Zabrotes subfasciatus* is related to the different starch granules from seeds of cowpea and the common bean (Silva et al., 2001). Sugars are important nutrients for caterpillars and other insects, and also universal phagostimulants (Schoonhoven and van Loon, 2002). In plants, sucrose as a major intermediate product of photosynthesis is transported from the leaves to other parts of plants via the phloem (Lalonde et al., 1999). In certain insects, sucrose is hydrolyzed to its constituent monosaccharides, glucose and fructose, and these are then metabolized (Wilkinson, 1997; Al-Waili, 2004). Furthermore, glucose is the major substrate of GOX in *Helicoverpa* species (Eichenseer et al., 1999; Zong and Wang, 2004).

We found that the glucose and total sugar contents of tobacco were significantly lower than those of the artificial diet, suggesting that GOX activity induction in the labial salivary glands of *H. armigera* probably is related to the high sugar contents in the artificial diet. This is supported by the finding that the labial salivary GOX activity of caterpillars feeding on tobacco leaves coated with glucose or sucrose was significantly higher than those feeding on control tobacco leaves (without sugar coating).

The natural food of phytophagous insects usually contains not only dilute nutrients, but also a variety of secondary metabolites that often affect physiology and behavior of insects (Bernays and Chapman, 2000). It is possible that secondary metabolites in tobacco may also be involved in suppression of the salivary GOX activity. Chlorogenic acid, rutin, and quercetin are widely distributed secondary metabolites in the plant kingdom including tobacco (Harborne, 1979; Parejo et al., 2004). From a purely ecological perspective, we speculated that polyphagous insects such as *H. armigera* would be able to detoxify or avoid toxicity from such ubiquitous secondary metabolites in some manner. When we incorporated them into the artificial diet at a moderate concentration for feeding the caterpillars, we found that all 3 chemicals had no effect on growth of the caterpillars, and also had no effect on their labial salivary GOX activity. However, we could not yet preclude that the artificial diet masked the effects of the phenolics, and other chemical factors in plants may have functions on suppression of the salivary GOX activity of the caterpillars.

Recent findings and our results have indicated that sugars may play some new roles besides their nutritional effect in plant-insect interactions. *Helicoverpa* species prefer host-plant buds and fruits, which usually contain more sugars than leaves. Based on our results, we speculate that the higher sugar content in these organs not only can provide enough carbohydrate nutrients for insect growth, but can also induce production of more salivary GOX. Furthermore, this elevation of salivary GOX activity would suppress nicotine synthesis in the tobacco host plant resulting benefits to the insects. On the other hand, Schwachtje et al. (2006) found that *N. attenuate* can increase the allocation of sugars to roots after herbivore attack and use their enhanced root reserves for plant tolerance. It is also possible that by allocating sugars to roots, tobacco shoots can maintain a high nicotine level for plant defense as far as minimizing sugar content to restrain the labial salivary GOX activity is concerned.

Based on native PAGE analysis, we confirmed that change of the GOX activity was due to a change in quantity, but not quality, of the enzyme, which migrated similarly as the fungal GOX. However, Eichenseer et al (1999) found that *H. zea* GOX migrated slower than fungal GOX. Further characterization of the labial salivary GOX of *H. armigera* is presently under investigation.

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