

GUT-BASED ANTIOXIDANT ENZYMES IN A POLYPHAGOUS AND A GRAMINIVOROUS GRASSHOPPER

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Abstract—Graminivorous species of grasshoppers develop lethal lesions in their midgut epithelia when they ingest tannic acid, whereas polyphagous grasshoppers are unaffected by ingested tannins. This study tests the hypothesis that polyphagous species are defended by higher activities of antioxidant enzymes (constitutive or inducible) in their guts than are graminivorous species. Comparisons were made between four antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), and glutathione transferase peroxidase (GSTPX). Enzyme activities were measured in the gut lumens and midgut tissues of *Melanoplus sanguinipes* (polyphagous) and *Aulocara ellioti* (graminivorous). The results of this study do not support the hypothesis that *M. sanguinipes* is better defended by antioxidant enzymes than is *A. ellioti*, nor are these enzymes more inducible in *M. sanguinipes* than in *A. ellioti* when insects consume food containing 15% dry weight tannic acid. Instead, tannic acid consumption reduced SOD, APOX, and GSTPX activities in both species. This study reports the first evidence that SOD is secreted into the midgut lumen in insects, with activities two- to fourfold higher than those found in midgut tissues. The spatial distribution of GSTPX and APOX activities observed in both species suggests that ingested plant antioxidant enzymes may function as acquired defenses in grasshoppers. In addition, the results of this study permit the first comparison between the antioxidant enzyme defenses of Orthoptera and Lepidoptera. Most notably, grasshoppers have higher SOD activities than caterpillars, but completely lack APOX in their midgut tissues.

Key Words—Grasshopper, Orthoptera, Acrididae, graminivorous, polyphagous, herbivore, *Melanoplus*, *Aulocara*, antioxidant enzyme, superoxide dismutase, catalase, ascorbate peroxidase, glutathione transferase peroxidase.

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INTRODUCTION

The ingestion of tannic acid by graminivorous grasshoppers (Orthoptera: Acrididae) greatly increases mortality, a result of the formation of ulcerlike lesions through the midgut epithelium (Bernays, 1978; Bernays et al., 1980). By comparison, the consumption of similar amounts of tannic acid by polyphagous grasshoppers produces neither lesions nor increased mortality (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980). The formation of midgut lesions has been attributed to the direct interaction of tannins with the midgut epithelia of grasshoppers (Bernays, 1978) and caterpillars (Steinly and Berenbaum, 1985). Another possible mode of action of ingested tannins is oxidative stress in epithelial tissues. For instance, in some caterpillars the consumption of low-molecular-weight phenols causes elevated levels of oxidative damage to proteins and lipids in midgut tissues (Summers and Felton, 1994; Bi and Felton, 1995), as well as midgut lesions (Lindroth and Peterson, 1988).

Ingested phenolic compounds can act as antioxidants or prooxidants, depending on the physicochemical environment of an insect's gut lumen. If ingested phenols do oxidize, they can produce oxidative stress by two primary mechanisms. The oxidation of phenols produces reactive oxygen species (ROS; Table 1) (Canada et al., 1990), including hydrogen peroxide in the gut lumens of caterpillars (Barbehenn et al., 2001). Hydrogen peroxide is relatively stable and permeates cell membranes (Grisham, 1992). However, its decomposition (via the Fenton reaction) produces highly reactive hydroxyl radicals. Cytotoxicity can result from hydroxyl radical damage to DNA, lipids, and proteins (Hanham et al., 1983; Imlay et al., 1988; Halliwell and Gutteridge, 1999). Secondly, quinones (oxidation products of phenols) may also be cytotoxic and can cause the formation of gut lesions (Gant et al., 1988; Zheng et al., 1997; Thiboldeaux et al., 1998). Ingested tannins appear to oxidize in the gut lumens of grasshoppers (Barbehenn et al., 1996), suggesting that, if antioxidant defenses were inadequate, oxidative stress could produce lesions in the midgut epithelium.

Little is known about the antioxidant enzyme defenses of grasshoppers. Previous work on antioxidant enzymes in insects has been done primarily on caterpillars

TABLE 1. ENZYME-CATALYZED REMOVAL OF REACTIVE OXYGEN SPECIES

Reactive oxygen species ^a	Antioxidant enzyme
$2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	Superoxide dismutase
$2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$	Catalase
$\text{AA} + \text{H}_2\text{O}_2 \rightarrow \text{DHA} + 2 \text{H}_2\text{O}$	Ascorbate peroxidase
$\text{ROOH} + 2 \text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG}$	Glutathione transferase peroxidase

^a AA = ascorbic acid, DHA = dehydroascorbic acid, GSH = glutathione, GSSG = glutathione disulfide, ROOH = hydroperoxide.

(e.g., Ahmad et al., 1987; Pritsos et al., 1988; Aucoin et al., 1991; Berenbaum, 1991; Felton and Duffey, 1992; Barbehenn et al., 2001). A variety of antioxidant enzymes protects caterpillar tissues and extracellular fluids from oxidative damage. Among the most widely studied enzymes for detoxifying ROS (including lipid peroxides) are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), and glutathione transferase peroxidase (GSTPX) (Table 1). Two types of evidence have supported the hypothesis that these enzymes serve a defensive role in insect herbivores that feed on plants containing high levels of potential prooxidants. First, antioxidant enzyme activities are high in the tissues of some insect species that feed on such host plants (Pritsos et al., 1988; Lee and Berenbaum, 1990; Aucoin et al., 1991; Felton and Duffey, 1992), and second, in some species, some antioxidant enzymes are induced by ingested prooxidants (Pritsos et al., 1988; Lee and Berenbaum, 1989, 1990; Ahmad and Pardini, 1990; Aucoin et al., 1991; Lee, 1991).

Relatively little attention has been focused on the antioxidant defenses in the gut lumens of insects. Studies on the antioxidant enzymes of caterpillars commonly have examined enzyme activities in whole-body homogenates, providing no information on antioxidant defenses in the gut lumen. However, it is in the gut lumen that ingested phenolic compounds may become extensively oxidized (Barbehenn and Martin, 1994; Barbehenn et al., 1996). Previous work on antioxidant enzymes secreted into the midgut lumens of caterpillars has measured the activities of CAT, APOX, and dehydroascorbate reductase (Felton and Duffey, 1991; Mathews et al., 1997; Barbehenn et al., 2001). This study extends work on antioxidant enzymes to grasshoppers, and examines their activities in both the midgut tissues and gut lumen.

If antioxidant enzyme defenses are strengthened in species of insect herbivores that feed on plants that are rich in potential prooxidants, then polyphagous insects would be expected to have evolved higher antioxidant enzyme activities than have graminivorous insects. Insects that feed on mature grasses ingest far lower allelochemical concentrations, as well as a far lower diversity of these chemicals, compared to insects that feed on dicots (Bernays and Barbehenn, 1987). Secondly, the antioxidant enzymes of polyphagous insects might be more effectively induced by the consumption of potential prooxidants than are those of graminivorous species.

The purpose of this study was twofold: (1) to test the hypothesis that polyphagous grasshoppers are better protected from oxidative stress by antioxidant enzymes, either constitutive or induced, than are graminivorous grasshoppers; and (2) to compare the activities of a suite of antioxidant enzymes in grasshoppers and caterpillars. Two grasshopper species (*Melanoplus sanguinipes* and *Aulocara ellioti*) were compared to test the first hypothesis. *M. sanguinipes* is a polyphagous species in the subfamily Melanoplinae and is tannin tolerant (Bernays et al., 1980). *A. ellioti* is a member of the graminivorous subfamily Gomphocerinae and is

assumed to be tannin sensitive like other graminivorous species examined (Bernays et al., 1980). Four antioxidant enzymes were examined in the gut lumens and tissues of *M. sanguinipes* and *A. ellioti*: SOD, CAT, APOX, and GSTPX. To test the extent of antioxidant enzyme inducibility in the two grasshopper species, enzyme activities were compared after feeding each species control or tannic acid-treated wheat leaves. This study provides the first examination of grasshopper antioxidant enzyme activities, and these values are compared with the enzyme activities from previous studies on larval Lepidoptera.

METHODS AND MATERIALS

Insects

Eggs of *M. sanguinipes* were obtained from a nondiapause colony (USDA, Bozeman, Montana, USA). Insects were reared on romaine lettuce (*Lactuca sativa* var. *longifolia*) and wheat bran as previously described (Barbehenn et al., 1996). Third-instar nymphs of *A. ellioti* were collected in central Wyoming, USA, and reared to adulthood in Ann Arbor, Michigan, USA, in the same conditions as *M. sanguinipes*, with the exception that the host plant was seedling wheat (*Triticum aestivum*). Adult females were used for all experiments, unless otherwise noted. Each experimental insect was placed in a separate ventilated plastic container (470 ml) in an incubator (28°C; 16L:8D) for 2–2.5 days. Insects were assigned at random to feed on control or treated wheat leaves. Seedling wheat leaves were cut and treated with 13 μ l of 70% acetone (control) or 13 μ l of a 70% acetone solution of tannic acid (Sigma, lot 64F-0049) to give a final concentration of 15% dry weight. Fresh leaves were provided daily, and these were usually completely consumed. In some instances, *A. ellioti* exhibited reduced consumption rates on tannin-treated wheat, and in these cases the level of tannin was reduced to 10% dry wt.

Sample Collection

Each insect was chilled at -20°C for 10 min and dissected. The entire gut was removed from the body, rinsed in distilled water (2–3 sec), blotted dry, and the Malpighian tubules removed. Samples of foregut and midgut contents were weighed in tared microcentrifuge tubes flushed with N_2 , and extracted by shaking (10 sec) in N_2 -purged buffer containing 50 mg/ml hydrated insoluble polyvinylpolypyrrolidone (PVP). The midgut contents included the portion of the food bolus that extended into the anterior hindgut. Extracts of gut contents were incubated for a minimum of 5 min on ice with occasional mixing to remove tannic acid. Midgut tissues, including the caeca, were blotted dry on a paper towel, weighed, and homogenized in N_2 -purged buffer (without PVP) in a hand-held

glass tissue homogenizer (20 strokes). Sample extracts were centrifuged (8000g, 4° or 22°C, 3–5 min), and kept under a N₂ atmosphere on ice until analyzed. Samples were assayed within 1 hr from the time of collection, unless otherwise noted. Sample sizes are presented in Tables 2–5 below. All buffers were purged of O₂ by bubbling them with N₂ (1 min/ml), unless otherwise noted. Reagents for all assays were purchased from Sigma Chemical Co. or ICN Pharmaceuticals.

Enzyme Assays

Superoxide Dismutase. Dissected gut contents were extracted in 350 μ l of sodium phosphate buffer (pH 7.0, 50 mM). Midgut tissues were homogenized in 200 μ l of phosphate buffer and centrifuged. Centrifuged extracts were stored at –75°C as they were collected. Thawed supernatant solutions were ultrafiltered to remove low-molecular-weight antioxidants. Sample supernatant solutions (150 μ l from gut contents; 100 μ l from tissues) were placed in 10,000 molecular-weight cutoff ultrafilters (Millipore Centrifree 0.5). The extracts were reduced to approximately 10 μ l by centrifugation of the ultrafilters (8000g, 4°C). The retentate (containing SOD) was resuspended with 250 μ l of N₂-purged buffer (pH 7.0) and filtered again. The washing process was repeated a second time, after which the retentate was reconstituted to a final volume of 200 μ l with phosphate buffer. Sample retentates and SOD standards were stored in microcentrifuge tubes under N₂ at –75°C without loss of activity. Frozen solutions of purified SOD (Sigma) were stable for at least one month. Thawed samples were kept on ice until analyzed. Boiled samples were analyzed to control for nonenzymatic activity. No SOD activity was observed in boiled samples.

The SOD reaction mixture was composed of 145 μ l of carbonate buffer (pH 10.2; 62.5 mM with 0.125 mM EDTA), 20 μ l of catalase (prepared as 1.3 enzyme units (EU) ml of 50 mM phosphate buffer, pH 7.0), 15 μ l of sample extract, and 20 μ l of epinephrine (13.6 mM in 100 mM HCl) to start the reaction. The rate of change absorbance at 490 nm was measured with a Biorad Benchmark microplate reader. Kinetic measurements were made using three different dilutions of each sample extract with pH 7.0 phosphate buffer (range = 5–100% of original sample extract concentration). Dilutions were chosen to fall within the linear region in regressions of percent inhibition of the browning reaction versus milligrams of protein in the reaction mixture. One unit of SOD activity was defined as the mass of protein (milligrams) corresponding to a 50% inhibition of the rate of epinephrine oxidation (Misra and Fridovich, 1972; Munkres, 1990). SOD activity was expressed as enzyme units/per milligram of protein. Protein concentrations in the samples were measured with the modified Bradford assay (Stoscheck, 1990), using Bradford reagent and bovine serum albumin as a standard.

Catalase. Samples of foregut and midgut contents from male and female *M. sanguinipes* were extracted in 400 μ l of phosphate buffer (pH 7.0, 66 mM).

Tissues were homogenized in 200 μl of buffer. The reaction mixture was composed of 665 μl of potassium phosphate buffer (pH 7.0, 66 mM), 25 μl of sample extract, and 10 μl of 3% hydrogen peroxide. Change in absorbance was measured at 240 nm over a 30-sec period using a Zeiss spectrophotometer. The protein concentration in each extract was measured as described above. CAT activity was expressed as micromoles of hydrogen peroxide reduced per minute per milligram of protein, using an extinction coefficient of $39.4 \text{ M}^{-1} \text{ cm}^{-1}$ (Aebi, 1984). CAT activity in *A. ellioti* was measured as described above, with the exception that females were fed wheat treated with 10% tannic acid (dry wt), 50 mM phosphate buffer was used for extraction and the reaction mix, and 400 μl of buffer was used to homogenize tissues. Rapid bubble formation was observed in cuvettes containing high levels of CAT (e.g., gut tissues), consistent with the formation of O_2 by this enzyme. Negligible CAT activity was observed in boiled samples.

Ascorbate Peroxidase. Samples from the foregut and midgut lumen were extracted in 400 μl of potassium phosphate buffer (pH 7.0, 50 mM) containing 0.5 mM ascorbic acid. Midgut tissues were homogenized in 400 μl of buffer. Samples of seedling wheat (16–21 mg fresh wt) from plants fed to the insects were homogenized in 500 μl of buffer (50 strokes). The reaction mixture was composed of 75 μl of supernatant solution, 646 μl of potassium phosphate buffer (containing 0.5 mM ascorbic acid), and 18 μl of hydrogen peroxide (0.3% in double-distilled water). The rate of change in absorbance at 290 nm was measured with a Zeiss spectrophotometer. Reaction rates were measured in boiled samples (10 min) to correct for nonenzymatic ascorbate oxidation. Protein levels in the samples were measured as described above, and enzyme activity was expressed as micromolar ascorbate oxidized per minute per milligram of protein, using a molar extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ (Asada, 1984). No enzyme activity was measurable using cumene hydroperoxide as the substrate.

Glutathione Transferase Peroxidase. Samples were extracted or homogenized in potassium phosphate buffer (pH 7.0, 50 mM), containing 1 mM EDTA. Foregut and midgut contents were extracted in 150 μl of buffer, and tissues were homogenized in 150 μl of buffer. Wheat leaf samples were collected and homogenized as described above. The reaction mixture consisted of 20 μl of supernatant solution, 20 μl of glutathione reductase (1.67 EU/ml), 100 μl of buffer (pH 7.0, containing 1.0 mM GSH and 0.2 mM NADPH), and 50 μl of cumene hydroperoxide (1.4 mM in buffer) (Ahmad and Pardini, 1990; Weinhold et al., 1990). The rate of decrease in the absorbance at 340 nm was measured for 4 min using a Biorad microplate reader. Boiled samples (10 min) and reaction mixtures lacking certain reagents were assayed as controls. The average reaction rate in boiled samples ($N = 5\text{--}6/\text{type}$) was subtracted from the rates in active samples to correct for the nonenzymatic oxidation of NADPH. The reaction rate was negligible in the absence of added glutathione or cumene hydroperoxide, and autoxidation of NADPH was negligible in the absence of added samples. Protein levels were

measured as described above, and GSTPX activity was expressed as nmol NADPH oxidized per minute per milligram of protein, using a molar extinction coefficient of $6220 \text{ M}^{-1} \text{ cm}^{-1}$. Although GSTPX activity was measured promptly in fresh extracts, full activity was recovered after storage at -75°C (two weeks).

Statistical Analyses

All analyses were performed using SAS (version 8e; PROC MIXED) (SAS Institute, 2000). Comparisons between means were made using differences of least square means (SAS Institute, 2000).

RESULTS

Antioxidant Enzyme Activities

Superoxide dismutase. Overall, SOD activities were similar in *A. ellioti* and *M. sanguinipes* (Table 2 and Table 6 below). Tannin consumption decreased SOD activity in both species ($P = 0.018$). However, activity levels in the midgut tissues of *M. sanguinipes* were unaffected by tannic acid consumption (94.8% of control levels). By comparison, SOD activities in the midgut tissues of *A. ellioti* were reduced to 73.4% of control levels. SOD activity varied across sites in the gut in a similar fashion within each species: maximal activity was found in the midgut lumen, followed by the foregut lumen, and similar lower levels were found in

TABLE 2. SUPEROXIDE DISMUTASE (SOD) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID^a

Species and site	Tannic acid in diet (%)	SOD activity (units/mg protein)	N
<i>A. ellioti</i>			
Foregut contents	0	$102.7 \pm 16.2^{\text{cd}}$	9
Foregut contents	15	$56.6 \pm 12.2^{\text{ab}}$	5
Midgut contents	0	$151.2 \pm 24.0^{\text{ef}}$	9
Midgut contents	15	$121.0 \pm 23.8^{\text{cde}}$	5
Midgut tissue	0	$68.9 \pm 4.9^{\text{bc}}$	9
Midgut tissue	15	$50.6 \pm 4.7^{\text{a}}$	12
<i>M. sanguinipes</i>			
Foregut contents	0	$88.5 \pm 15.1^{\text{bcd}}$	7
Foregut contents	15	$62.8 \pm 20.2^{\text{abc}}$	6
Midgut contents	0	$218.2 \pm 37.0^{\text{f}}$	7
Midgut contents	15	$137.6 \pm 18.4^{\text{def}}$	8
Midgut tissue	0	$57.8 \pm 5.2^{\text{ab}}$	8
Midgut tissue	15	$54.8 \pm 7.2^{\text{ab}}$	8

^a Data are presented as mean \pm SE. Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

TABLE 3. CATALASE (CAT) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID^a

Species and site	Tannic acid in diet (%)	CAT activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	N
<i>A. ellioti</i>			
Foregut contents	0	26.9 \pm 5.5 ^a	9
Foregut contents	15	41.3 \pm 7.9 ^{ab}	10
Midgut contents	0	184.9 \pm 32.2 ^{efg}	9
Midgut contents	15	188.9 \pm 15.9 ^{fg}	9
Midgut tissue	0	182.5 \pm 25.3 ^{defg}	10
Midgut tissue	15	148.0 \pm 11.6 ^{defg}	10
<i>M. sanguinipes</i>			
Foregut contents	0	52.0 \pm 9.4 ^{bc}	7
Foregut contents	15	54.5 \pm 7.4 ^{bc}	8
Midgut contents	0	102.5 \pm 28.0 ^{cd}	9
Midgut contents	15	124.4 \pm 27.8 ^{def}	8
Midgut tissue	0	181.8 \pm 42.5 ^{defg}	10
Midgut tissue	15	235.9 \pm 59.7 ^g	9

^a Data are presented as mean \pm SE. Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

the midgut tissues (Table 2). SOD activities were three- to fourfold higher in the midgut lumens of grasshoppers than in their midgut tissues (Table 2).

Catalase. Overall, CAT activities were similar in *A. ellioti* and *M. sanguinipes* (Tables 3 and 6). CAT activity was unaffected by tannin consumption in both species at each site in the gut (Table 3 and Table 6 below). Within different sites in the gut, however, CAT activity varied between the species: CAT activity in the foregut of *M. sanguinipes* was higher than that in *A. ellioti* ($P = 0.020$), but the reverse was true for CAT activity in the midgut lumen ($P = 0.016$) (Table 3). The greatest CAT activity was present in midgut tissues and midgut lumen contents in *A. ellioti*. The lowest levels of CAT activity were present in the foregut contents, which varied from as little as 14% of midgut lumen levels (*A. ellioti* controls) to 51% of midgut lumen levels (*M. sanguinipes* controls).

Ascorbate Peroxidase. APOX activities were similar in *A. ellioti* and *M. sanguinipes* (Table 4 and Table 6 below). Tannic acid consumption reduced APOX activity ($P = 0.030$) (Tables 4 and 6). This effect was most pronounced in the midgut lumen of *M. sanguinipes*, in which APOX activity in insects fed tannic acid was 21% of control levels (Table 4). A similar treatment effect was observed in *A. ellioti*. APOX activities were substantially higher in the foreguts of both species than in their midgut lumens. No APOX activity was found in the midgut tissues of either species. APOX activity in the seedling wheat leaves consumed by *M. sanguinipes* and *A. ellioti* was 1007.0 ± 128.8 ($N = 3$) and $1017.9 \pm 103.3 \mu\text{M}/\text{min}/\text{mg}$ ($N = 5$), respectively.

TABLE 4. ASCORBATE PEROXIDASE (APOX) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID^a

Species and site	Tannic acid in diet (%)	APOX activity ($\mu\text{M}/\text{min}/\text{mg}$ protein)	N
<i>A. ellioti</i>			
Foregut contents	0	282.3 \pm 49.7 ^c	7
Foregut contents	15	196.7 \pm 39.8 ^{bc}	9
Midgut contents	0	96.0 \pm 12.7 ^{ab}	8
Midgut contents	15	33.6 \pm 9.7 ^a	9
Midgut tissue	0	0.0 \pm 0.0	8
Midgut tissue	15	0.0 \pm 0.0	9
<i>M. sanguinipes</i>			
Foregut contents	0	237.8 \pm 31.2 ^c	13
Foregut contents	15	215.8 \pm 87.2 ^{bc}	9
Midgut contents	0	178.4 \pm 39.8 ^{bc}	11
Midgut contents	15	42.3 \pm 14.6 ^a	8
Midgut tissue	0	0.0 \pm 0.0	8
Midgut tissue	15	0.0 \pm 0.0	9

^a Data are presented as mean \pm SE. APOX activity in wheat leaves consumed was 1007.0 \pm 128.8 ($N = 3$) and 1017.9 \pm 103.3 ($N = 5$) in experiments on *M. sanguinipes* and *A. ellioti*, respectively. Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

Glutathione Transferase Peroxidase. *M. sanguinipes* maintained higher GSTPX activities in its foregut and midgut lumens than did *A. ellioti* ($P < 0.001$) (Tables 5 and 6). By comparison, GSTPX activities in the midgut tissues were remarkably similar across species and treatments (Table 5). GSTPX activity in control insects decreased along the length of the gut, dropping over 50% from the foregut to the midgut lumen in both species. Tannin consumption reduced GSTPX activity in both species, most strikingly in the gut lumens. GSTPX activity in the foregut lumen was reduced to 7.2% and 10.4% of control levels in *A. ellioti* and *M. sanguinipes*, respectively. In the midgut lumen, tannic acid consumption reduced GSTPX activities to 39.6 and 78.8% of control levels in *A. ellioti* and *M. sanguinipes*, respectively. GSTPX activity in the seedling wheat leaves consumed by *A. ellioti* was 46.0 \pm 11.1 nmol/min/mg ($N = 3$).

Comparison of Orthoptera and Lepidoptera

Table 7 summarizes examples of SOD, CAT, APOX, and GSTPX activities previously measured in caterpillars. Among the quantitative differences between Orthoptera and Lepidoptera, the most striking is the higher activity of SOD in grasshoppers; activities in grasshopper midgut tissues (Table 2) range from 10- to 50-fold greater than in the midgut tissues and whole body homogenates

TABLE 5. GLUTATHIONE TRANSFERASE PEROXIDASE (GSTPX) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID^a

Species and site	Tannic acid in diet (%)	GSTPX activity (nmol/min/mg)	N
<i>A. ellioti</i>			
Foregut contents	0	11.1 ± 2.8 ^{cd}	10
Foregut contents	15	0.8 ± 0.5 ^a	9
Midgut contents	0	5.3 ± 1.5 ^{abc}	10
Midgut contents	15	2.1 ± 1.1 ^{ab}	10
Midgut tissue	0	15.2 ± 1.0 ^d	10
Midgut tissue	15	10.2 ± 0.8 ^c	10
<i>M. sanguinipes</i>			
Foregut contents	0	28.8 ± 6.9 ^e	9
Foregut contents	15	3.0 ± 1.1 ^{abc}	10
Midgut contents	0	12.3 ± 3.4 ^{cd}	9
Midgut contents	15	9.7 ± 2.3 ^{bc}	9
Midgut tissue	0	15.5 ± 1.6 ^d	10
Midgut tissue	15	10.5 ± 0.6 ^c	10

^a Data are presented as mean ± SE. GSTPX activity in wheat leaves consumed by *A. ellioti* was 46.0 ± 11.1 nmol/min/mg ($N = 3$). Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

of caterpillars. Among the qualitative differences that are evident, the most notable is the absence of APOX from the midgut tissues of grasshoppers (Table 4), but its presence in the midgut tissues and whole body homogenates of caterpillars. CAT activities in grasshopper gut tissues and gut fluids (Table 3) are in the range of those found in the midgut tissues and whole-body homogenates of caterpillars (Table 7). APOX activities present in the gut lumens of the grasshoppers in this study also lie within the broad range measured in caterpillars (Tables 4 and 7). GSTPX activities in grasshopper midgut tissues fall at the low end of the range of GSTPX activities found in caterpillar whole-body homogenates (Tables 5 and 7).

The effect of tannic acid consumption on APOX activity in grasshoppers and caterpillars was similar: activity is reduced in the midgut fluids of both Orthoptera and Lepidoptera (*O. leucostigma* and *M. disstria*) (Tables 4 and 7). GSTPX activity is also reduced in grasshoppers and some caterpillars by ingested phenols (tannic acid and quercetin, respectively) (Tables 5 and 7). By comparison, SOD activity is increased in caterpillars by ingested quercetin (Table 7), but is reduced or unaffected by tannin consumption in the examined grasshoppers. CAT activity is unaffected by ingested tannic acid in species of Lepidoptera and Orthoptera (Tables 3 and 7). The specific effects of ingested tannic acid on SOD and GSTPX activities have not been measured in caterpillars.

TABLE 6. PROBABILITY VALUES FROM ANALYSES OF ANTIOXIDANT ENZYME ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes*^a

Effect	Superoxide dismutase		Catalase		Ascorbate peroxidase		Glutathione transferase peroxidase	
	F	P	F	P	F	P	F	P
Species	0.26	1, 34	0.00	1, 35	0.26	1, 34	13.37	1, 36
Site	33.26	2, 34	38.92	2, 35	27.76	1, 34	11.64	2, 36
Treatment	6.18	1, 34	0.55	1, 35	5.15	1, 34	26.94	1, 36
Species × site	2.35	2, 34	8.31	2, 35	1.17	1, 34	5.82	2, 36
Species × treatment	0.01	1, 34	0.95	1, 35	0.00	1, 34	2.00	1, 36
Site × treatment	2.28	2, 34	0.20	2, 35	0.63	1, 34	7.72	2, 36
Species × site × treatment	0.90	2, 34	0.87	2, 35	1.48	1, 34	2.21	2, 36

^a SAS PROC MIXED analysis. Bold highlights significant cases.

TABLE 7. ANTIOXIDANT ENZYME ACTIVITIES IN LARVAL LEPIDOPTERA^a

Enzyme and species	Enzyme		Effect of ingested phenol on enzyme activity (phenol)	Reference
	Source	Activity		
Superoxide dismutase				
<i>Trichoplusia ni</i>	whole body	0.9, 2.3 units/mg	Increased (quercetin)	Lee and Berenbaum, 1989; Ahmad and Pardini, 1990
<i>Papilio polyxenes</i>	whole body	2.0, 2.9 units/mg	Increased (quercetin)	Pritsos et al., 1988; Berenbaum, 1991
<i>Papilio glaucus</i>	whole body	1.9 units/mg	ND	Berenbaum, 1991
<i>Depressaria pastinacella</i>	whole body	2.6 units/mg	ND	Lee and Berenbaum, 1990
<i>Anaitis plagiata</i>	whole body	5.5 units/mg	ND	Aucoin et al., 1991
<i>Ostrinia nubilalis</i>	whole body	1.4 units/mg	ND	Aucoin et al., 1991
<i>Manduca sexta</i>	whole body	1.5 units/mg	ND	Aucoin et al., 1991
<i>Lycantria dispar</i>	midgut tissues	5.8 units/mg	Increased (locust compounds)	Peric-Mataruga et al., 1997
Catalase ^b				
<i>Trichoplusia ni</i>	whole body	153, 302 $\mu\text{mol/mg/min}$	Decreased (quercetin)	Ahmad and Pardini, 1990, Lee and Berenbaum, 1989
<i>Papilio polyxenes</i>	whole body	128, 289 $\mu\text{mol/min/mg}^c$	Decreased (quercetin)	Pritsos et al., 1988; Berenbaum, 1991
<i>Papilio glaucus</i>	whole body	73 $\mu\text{mol/min/mg}^c$	ND	Berenbaum, 1991
<i>Depressaria pastinacella</i>	whole body	585 $\mu\text{mol/min/mg}$	ND	Lee and Berenbaum, 1990
<i>Anaitis plagiata</i>	whole body	171 $\mu\text{mol/min/mg}$	ND	Aucoin et al., 1991
<i>Ostrinia nubilalis</i>	whole body	34 $\mu\text{mol/min/mg}$	ND	Aucoin et al., 1991
<i>Manduca sexta</i>	whole body	154 $\mu\text{mol/min/mg}$	ND	Aucoin et al., 1991
<i>Orgyia leucostigma</i>	midgut tissues	611 $\mu\text{mol/min/mg}$	NS (tannic acid)	Barbehenn, unpublished data
<i>Malacosoma dissrta</i>	midgut tissues	802 $\mu\text{mol/min/mg}$	NS (tannic acid)	Barbehenn, unpublished data
<i>Lycantria dispar</i>	midgut tissues	251 nmol/min/mg	Decreased (locust compounds)	Peric-Mataruga et al., 1997
<i>Orgyia leucostigma</i>	midgut fluid	103 $\mu\text{mol/min/mg}$	NS (tannic acid)	Barbehenn et al., 2001
<i>Malacosoma dissrta</i>	midgut fluid	192 $\mu\text{mol/min/mg}$	NS (tannic acid)	Barbehenn et al., 2001
<i>Helicoverpa zea</i>	regurgitate	3 $\mu\text{mol/min/mg}$	ND	Felton and Duffey, 1991

<i>Spodoptera exigua</i>	regurgitate	98 $\mu\text{mol}/\text{min}/\text{mg}$	ND	Felton and Duffey, 1991
<i>Heliothis virescens</i>	regurgitate	16 $\mu\text{mol}/\text{min}/\text{mg}$	ND	Felton and Duffey, 1991
Ascorbate peroxidase				
<i>Helicoverpa zea</i>	whole body	16, 21 $\mu\text{M}/\text{min}/\text{mg}$	ND	Mathews et al., 1997
<i>Orygia leucostigma</i>	midgut tissue	600 $\mu\text{M}/\text{min}/\text{mg}$	Decreased (tannic acid)	Barbehenn unpublished data
<i>Malacosoma dissiria</i>	midgut tissue	890 $\mu\text{M}/\text{min}/\text{mg}$	NS (tannic acid)	Barbehenn unpublished data
<i>Orygia leucostigma</i>	midgut fluid	1100 $\mu\text{M}/\text{min}/\text{mg}^d$	Decreased (tannic acid)	Barbehenn et al., 2001
<i>Malacosoma dissiria</i>	midgut fluid	1700 $\mu\text{M}/\text{min}/\text{mg}^d$	Decreased (tannic acid)	Barbehenn et al., 2001
<i>Helicoverpa zea</i>	regurgitate	11 $\mu\text{M}/\text{min}/\text{mg}$	ND	Mathews et al., 1997
Glutathione transferase peroxidase				
<i>Trichoplusia ni</i>	whole body	50, 200 nmol/min/mg	Decreased (quercetin)	Ahmad and Pardini, 1990; Weinhold et al., 1990
<i>Papilio polyxenes</i>	whole body	253 nmol/min/mg	ND	Weinhold et al., 1990
<i>Spodoptera eridania</i>	whole body	106 nmol/min/mg	ND	Weinhold et al., 1990
<i>Lycantria dispar</i>	midgut tissues	6 nmol/min/mg ^e	NS (locust compounds)	Peric-Mataruga et al., 1997

^a Data for mid- and final-instar larvae are reported when available. ND = not determined. NS = not statistically significant.

^b CAT activities are proportional to substrate concentration and may not be completely comparable between studies.

^c Published in Berenbaum (1991) as mol/mg/min, but presumably is $\mu\text{mol}/\text{min}/\text{mg}$.

^d Note corrected values here.

^e GSTPX assays used cumene hydroperoxide, with the exception of Peric-Mataruga et al. (1997), which used *t*-butyl hydroperoxide.

DISCUSSION

The results of this study demonstrate that the superior defenses of polyphagous grasshoppers against the effects of ingested tannic acid are not the result of consistently elevated activities of four of the major antioxidant enzymes. Contrary to the main hypothesis, few cases were observed in which *M. sanguinipes* was found to have greater enzyme activities than *A. ellioti*. Among the antioxidant enzymes measured in *A. ellioti* and *M. sanguinipes*, only GSTPX in the foregut and midgut lumen was consistently higher in the tannin-tolerant species. In addition, SOD activities in *M. sanguinipes* gut tissues were unaffected by tannic acid, whereas tannic acid reduced SOD activity in *A. ellioti* tissues. CAT activity was also higher in the foregut lumen of *M. sanguinipes* than in this region of *A. ellioti*. However, the opposite pattern exists for CAT activity in the midgut lumen, where its activity was higher in *A. ellioti* than in *M. sanguinipes*. Enzyme activities in the polyphagous and graminivorous grasshopper species are remarkably similar in many cases, most notably in the tissues of the midgut.

The results of this study also show that antioxidant enzymes are not induced to a greater extent in a polyphagous grasshopper than in a graminivorous species. None of the enzymes examined in either species was significantly induced by tannic acid during a two-day period. On the contrary, lower enzyme activity was associated with tannin consumption, where a significant treatment effect was observed (SOD, APOX, and GSTPX). Ingested tannic acid had a strong inhibitory effect on APOX activity in the midgut fluids of both species. GSTPX activity was also strongly inhibited in the foreguts of both species. Among the enzymes studied, only CAT activity was unaffected by tannic acid in all cases.

In this study it is assumed that the pattern of tannic acid sensitivity observed in examined graminivorous grasshoppers is shared by *A. ellioti* (Bernays et al., 1980). Although this assumption was not tested, an examination of the morphological features found in strictly graminivorous grasshoppers confirmed that *A. ellioti* has both the flattened mandibles and shortened posterior midgut caeca that are typical of graminivorous species (Isely, 1944; Chapman, 1988; Barbehenn, personal observation). Care must also be taken in generalizing from results on the nondiapauses strain of *M. sanguinipes*; elevated levels of some detoxification enzymes have been found to arise as artifacts of laboratory rearing in this variety (Isman et al., 1996). The midgut tissues of grasshoppers, particularly those of the midgut caeca, contain the highest detoxification enzyme activities in the body (Feng and Isman, 1994). In this study, it is assumed that these tissues also represent a key site at which the capabilities of antioxidant enzyme defenses can be compared among grasshopper species.

Two distinct sources of antioxidant enzymes in the gut lumens of grasshoppers are apparent: secretion by the midgut and ingestion. The results of this study suggest that luminal SOD and CAT activities are due to enzymes secreted by the

midgut, whereas luminal APOX and GSTPX activities are due to ingested plant enzymes. The spatial pattern of SOD and CAT activity (i.e., higher activity in the midgut lumen than the foregut lumen, and activity in midgut tissues) is consistent with their secretion into the midgut lumen by midgut tissues. The foregut is lined with an impermeable cuticle (Maddrell and Gardiner, 1980), precluding enzyme secretion. However, if antioxidant enzymes were secreted into the midgut lumen, regurgitation into the foregut would provide a source of these enzymes in the foregut. For example, regurgitated gut fluid from the midgut provides digestive enzymes to the foregut (e.g., Evans and Payne, 1964; Ferreira et al., 1990). Secretion of antioxidant enzymes in the saliva cannot be ruled out as an additional enzyme source.

The results of this work also suggest that ingested plant antioxidant enzymes are functional in grasshopper gut fluids. Unlike SOD and CAT, the spatial distribution of GSTPX and APOX activities in the guts of grasshoppers is characterized by maximal activity in the foregut lumen. In the case of APOX, the absence of activity in midgut tissues suggests that it is either inactive at this site or is not synthesized in grasshoppers. On the other hand, the activities of APOX and GSTPX in seedling wheat are sufficiently high to account for their activities in the foreguts of *M. sanguinipes* and *A. ellioti*. APOX activity in wheat is approximately fourfold higher than that found in grasshopper foregut lumens. Similarly, GSTPX activity in wheat leaves ranges from fourfold higher than the maximal (foregut) activity in *A. ellioti* to roughly twofold higher than the maximal (foregut) activity in *M. sanguinipes* (assuming similar enzyme levels across seedling wheat used in separate experiments).

Previous work on plant enzymes ingested by insects has focused on defensive enzymes, such as polyphenol oxidase and guaiacol peroxidase (Duffey and Felton, 1989; Felton et al., 1989; Felton and Duffey, 1991). A considerable body of evidence demonstrates that ingested enzymes also play beneficial roles in the gut lumens of insects. For instance, previous work on ingested fungal enzymes demonstrates that they play an essential role in cellulose digestion in a variety of saprophagous plant-feeding insects (Martin, 1987). The physicochemical conditions in grasshopper gut fluid are conducive to the continued activity of ingested plant enzymes, which function in an acidic pH (ca. 4–6) in the plant (Schultz and Lechowicz, 1986). The pH of grasshopper gut fluid (ca. 6–7) (Barbehenn et al., 1996; Appel and Joern, 1998) is far more favorable for continued plant enzyme activity than the high pH conditions commonly found in caterpillars (ca. 8–12) (Dow, 1984; Appel and Martin, 1990). Do the more extreme conditions in Lepidoptera preclude the acquisition of plant antioxidant enzymes, selecting for the secretion of endogenous APOX in caterpillars? Cofactors necessary for the function of APOX (ascorbate) and GSTPX (glutathione) are present in grasshopper gut fluid in adequate concentrations to permit enzyme activity. Ascorbate is present in the midgut and foregut lumens of both grasshopper species examined at levels

of 300–5000 μM (Barbehenn, in preparation), compared to 500 μM in the APOX assay reaction mixture. Glutathione is present in the midgut and foregut lumens of both species at levels of 200–800 μM (Barbehenn, in preparation), compared to 1000 μM in the GSTPX assay reaction mixture. The potential importance of acquired antioxidant enzymes in insect herbivores needs further work.

If APOX is not present in grasshopper midgut tissues, then what controls hydrogen peroxide levels in these insect tissues? Mathews et al. (1997) propose that CAT is inefficient at reducing hydrogen peroxide to low levels because of its high K_m and that APOX better serves this role. It is possible that CAT could effectively reduce hydrogen peroxide to low levels, despite its high K_m , if high activity levels of this enzyme were produced. However, CAT activities in the grasshoppers examined in this study are not higher than those found in Lepidoptera (Table 7). It is also possible that other, unmeasured enzymes function in grasshoppers and that these are measured in assays intended to measure the degradation of hydrogen peroxide by CAT. This limitation of the assay performed cannot be addressed in this study, although the rapid production of gas bubbles in the “CAT” reaction mixtures is consistent with the activity of CAT.

Neither SOD nor GSTPX has been examined in the gut lumens of Lepidoptera, to my knowledge. SOD would likely be even more useful for catalyzing the dismutation of superoxide in the high pH midguts of larval Lepidoptera; at the high pH of caterpillar gut fluids, the stability of superoxide anion radical is on the order of 100,000-fold greater than in the mildly acidic pH of grasshopper gut fluids (Rosen et al., 1999).

In conclusion, the relative susceptibilities of graminivorous and polyphagous grasshoppers to fatal midgut lesions from ingested tannic acid do not appear to be determined by variation in the activities of the antioxidant enzymes examined in this study. Several alternative hypotheses may be proposed: (1) antioxidant enzymes that were not measured in this study differ between the two species, (2) low-molecular-weight antioxidants are more important than antioxidant enzyme activities, and (3) antioxidant defenses do not explain the relative susceptibility of grasshoppers to develop gut lesions (i.e., a mechanism other than oxidative stress is at work). Additional enzymes that might be examined in grasshopper tissues include ascorbate free radical reductase, quinone reductase, glutathione reductase, and in the lumen and tissues, thioredoxin reductase, dehydroascorbate reductase, and glutathione peroxidase. Low-molecular-weight antioxidants play a critical role in protecting tissues and extracellular fluids from oxidative stress, and compounds such as ascorbate, glutathione, and α -tocopherol could vary among grasshopper species independent of the activities of antioxidant enzymes, such as those measured in this study. In addition, the basis for proposing an antioxidant defense hypothesis to explain the taxonomic variation in grasshopper midgut lesions would be strengthened by an examination of levels of ROS and oxidative stress (biomarkers) in tannin-tolerant and -sensitive grasshoppers.

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