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EFFECT OF DIMBOA, A HYDROXAMIC ACID FROM CEREALS, ON PEROXISOMAL AND MITOCHONDRIAL ENZYMES FROM APHIDS: EVIDENCE FOR THE PRESENCE OF PEROXISOMES IN APHIDS

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Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid involved in the resistance of cereals to aphids, was administered to adult individuals of the aphid *Sitobion avenae* in artificial diets. Effects on the cellular metabolism were inferred from the evaluation of several organelle marker enzymes. Catalase from peroxisomes and cytochrome *c* oxidase from mitochondria increased their activities about twofold when aphids were fed with 2 mM DIMBOA. The role of these enzymes in the metabolizing of xenobiotics by aphids is discussed. Biochemical and cytochemical evidences for the presence of peroxisomes in aphids are reported here for the first time.

Key Words—*Sitobion avenae*, Aphididae, aphids, hydroxamic acids, DIMBOA, catalase, cytochrome *c* oxidase, peroxisomes, mitochondria, xenobiotics.

INTRODUCTION

Aphids (Homoptera: Aphididae) are important crop pests that can cause serious agronomical losses by consuming nutrients from the host plant, injecting toxins,

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providing a medium for fungal development, and transmitting plant viruses. The presence of secondary metabolites in plants is of importance in their protection against herbivores such as aphids. Thus, phenolic compounds (Leszczynski et al., 1989), indole alkaloids (Zúñiga and Corcuera, 1986), and particularly hydroxamic acids (Hx) (Niemeyer, 1988; Argandoña et al., 1980; Bohidar et al., 1986; Thackray et al., 1990; Nicol et al., 1992; Niemeyer et al., 1993) have been described as defensive compounds in cereals. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is the main Hx present in wheat extracts. This allelochemical produces several effects on aphids, including feeding deterrence, antibiosis, decreased reproduction, and decreased performance (Argandoña et al., 1983; Bohidar et al., 1986; Niemeyer et al., 1989; Givovich and Niemeyer, 1994, 1995). Little is known about the detoxification systems of plant allelochemicals, such as Hx, by aphids. A decrease in the activity of UDP-glucose transferases, involved in the detoxification of phenolic aglucones, has been reported in aphids fed with Hx (Leszczynski et al., 1992); an induction of glutathione-*S*-transferase activity has been shown in aphids from cereals with high concentrations of allelochemicals (Leszczynski et al., 1994); and an *in vitro* inactivation of aphid acetylcholinesterase by DIMBOA has also been reported (Cuevas and Niemeyer, 1993). In this study we evaluate the effect of DIMBOA on the activity of some subcellular organelle marker enzymes to determine their possible participation in Hx detoxification. We also report new evidence for the presence and function of peroxisomes in insects. These ubiquitous subcellular organelles participate in a variety of metabolic pathways, including detoxification of xenobiotics (Mannaerts and Van Veldhoven, 1993).

METHODS AND MATERIALS

Aphid Culture. One aphid clone collected and developed on wheat (*Triticum aestivum* cv. Paleta) under controlled conditions (20°C and 16L : 8D), and determined in our laboratory as *Sitobion avenae* with molecular markers (Figueroa et al., 1999), was used in this study. Previous to the experiments, the aphids were kept for at least four generations on oat (*Avena sativa* L. cv. Nahuén), an Hx-lacking cereal, to avoid the influence of Hx through maternal effects.

Treatment of Aphids with DIMBOA. Artificial diets containing 30% sucrose, amino acids, vitamins, and mineral salts, pH 5.5, were prepared as previously reported (Auclair, 1965; Argandoña et al., 1980). Fifty adult aphids were placed on a Parafilm sachet containing an artificial diet and encompassing a 60-mm-diam. Petri dish. The diets contained 0–8 mM DIMBOA, a concentration range comparable to that found in whole leaf extracts (Niemeyer et al., 1992) and in different plant compartments (Argandoña et al., 1987). DIMBOA was

prepared as solutions in dimethyl sulfoxide (DMSO), which were added to the artificial diets. The final concentration of DMSO was 0.001% v/v in all cases, including the control diets. Adult aphids were counted after 6–72 hr, and mortality was determined.

Homogenization and Subcellular Fractionation. At the end of the testing period, the living and effectively feeding aphids were transferred to 1.5-ml Eppendorf tubes containing ice-cold homogenization buffer [0.25 M sucrose, 3 mM imidazole, 1 mM EDTA, and 0.1% (v/v) ethanol, pH 7.4]. The aphids were homogenized with a plastic pellet pestle (Kontes) in ice. The homogenates were filtered through a single layer of cheesecloth, recovered in a fresh tube, and then centrifuged at 800*g* for 10 min to obtain a nuclear pellet and a postnuclear supernatant (PNS), according to conditions established previously (Santos et al., 1988). The PNS fraction was centrifuged at 105,000*g* for 35 min to obtain a pellet (P fraction), equivalent to the classic De Duve's MLP (M: heavy mitochondria; L: light mitochondria; P: microsomes) fraction, containing most of membranous organelles, and a supernatant (S fraction), containing mainly the cytosol. All these procedures were performed in the presence of a cocktail of protease inhibitors (0.5 mM each of chymostatin, leupetin, antipain, and pepstatin).

Density Gradient Fractionation. PNS fractions were subfractionated in a 56% Nycodenz continuous density gradient (density ranging from 1.00 to 1.30 g/ml) and used to separate and characterize animal peroxisomes and mitochondria. The PNS fraction was loaded over the gradient and subsequently centrifuged at 40,000 rpm for 49 min at 4°C in a Vti65 rotor. After centrifugation, fractions of 12 drops were collected from the bottom (heavier) to top (lighter) of the gradient solution, in preweighed tubes. The density was determined by measuring the refractive index of each fraction (Santos et al., 1994).

Enzyme Assays. Established procedures were used for the determination of activities of marker enzymes of subcellular organelles in homogenates, subcellular fractions, and density gradient fractions [catalase for peroxisomes, cytochrome *c* oxidase for mitochondria, *N*-acetyl-β-glucosaminidase (Naβgase) for lysosomes, and NADPH cytochrome *c* reductase for microsomes] (Santos et al., 1988). Proteins were determined by the Lowry method, with bovine serum albumin as standard (Lowry et al., 1951). The distribution of enzyme markers in fractionation experiments was calculated and represented as previously described (Bowers and de Duve 1967; De Duve and Baudhuin, 1966). Catalase latency was determined by measuring the activity of the enzyme in the presence/absence of detergent (Triton X-100, Sigma Co.).

Transmission Electron Microscopy and Catalase Cytochemistry. Aphids were fixed for 3 hr as a pellet with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PIPES buffer, pH 7.2, containing 0.2 M sucrose. The samples were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon.

Ultrathin sections were further stained with uranyl acetate and lead citrate, and examined in a Phillips electron microscope (Figueroa et al., 1997).

Cytochemistry for catalase was performed by a modification of the alkaline diaminobenzidine method of Roels and Goldfischer (1979), exactly as described previously (Santos et al., 1994). Incubations in the absence of H_2O_2 and in the presence of the catalase inhibitor, 3-amino-1,2,4-triazole, served as controls.

Statistical Analysis. Comparison of means among different treatments were performed with the Mann-Whitney U test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

The presence of four subcellular organelle marker enzymes in nontreated aphids was established (Table 1). To evaluate the effect of DIMBOA over the activity of these enzymes, aphids were fed with artificial diets containing different concentrations (0–8 mM) of the allelochemical during 48 hr. The most pronounced effect was observed with 2 mM DIMBOA (data not shown). At this concentration, cytochrome *c* oxidase and catalase increased their specific activities about twofold while Naβgase and NADPH cytochrome *c* reductase were not significantly altered (Figure 1). At higher concentrations of DIMBOA (>2 mM), a strong antifeeding effect and increased mortality were observed (data not shown), making these data less reliable. A range of feeding times (6–72 hr) with 2 mM DIMBOA was also tested. The effects on enzymes were highest at 48 hr of treatment (data not shown).

Cytochrome *c* oxidase is a protein of the inner membrane of mitochondria, which participates in the terminal complex of the mitochondrial respiratory chain (Van Kuilenburg et al., 1991). This enzyme has been the subject of several evolutive and physiological studies in insects such as flies, beetles, honeybees, cockroaches (De Bruijn, 1983; Hall and Smith, 1991; Azeredo-Espin et al., 1991; Martínez-González and Hegardt, 1994) and also aphids (Sunnucks and Hales, 1996).

TABLE 1. SPECIFIC ACTIVITIES OF SUBCELLULAR ORGANELLE MARKER ENZYMES IN HOMOGENATES OF APHID *Sitobion avenae*

Enzyme	Specific activity (mU/mg of protein)[a]
Catalase (peroxisomes)	0.64 ± 0.064 (14)
Cytochrome *c* oxidase (mitochondria)	9.2 ± 1.1 (7)
Naβgase (lysosomes)	11.7 ± 0.69 (8)
NADPH cytochrome *c* reductase (microsomes)	976 ± 84 (4)

[a]Mean ± standard error. Parentheses indicate the number of replicates.

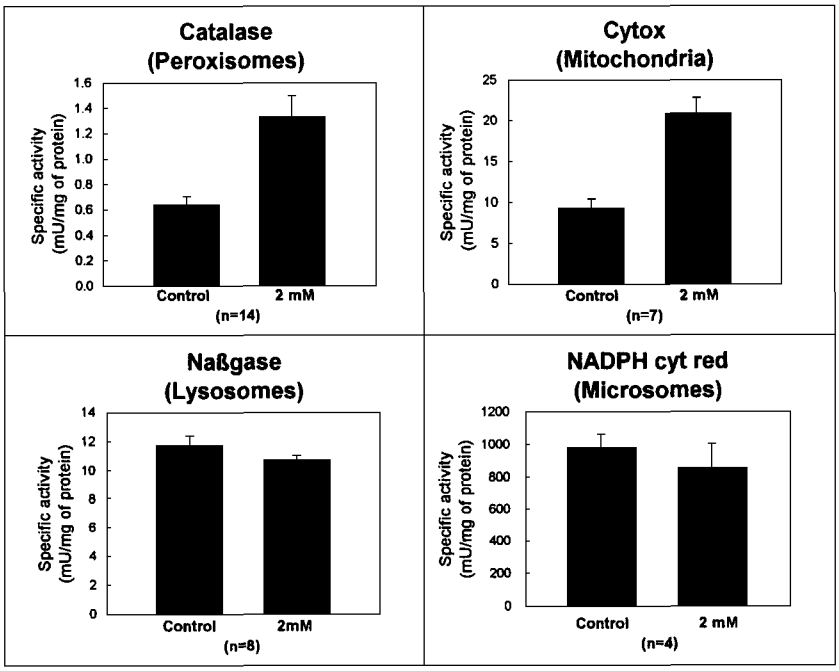

FIG. 1. Effect of administration of 2 mM DIMBOA in the diet of aphids during 48 hr, on organelle marker enzymes. The specific activities of four enzymes (cytox: cytochrome *c* oxidase; Naβgase: *N*-acetyl-β-glucosaminidase; NADPH cyt red: NADPH cytochrome c reductase) were measured in controls and treated (2 mM) aphid homogenates. Only the activities of catalase and cytochrome *c* oxidase were statistically different ($P < 0.05$) between treated and control aphids.

Catalase is a known peroxisomal marker enzyme, present in the matrix of peroxisomes from animals and plants (glyoxisomes) and also in the cytosol, that decomposes H_2O_2 into water and oxygen (De Duve and Baudhuin, 1966; Mannaerts and Van Veldhoven, 1993). Since no evidence was available on the presence of catalase or peroxisomes in aphids, the subcellular distribution of this enzyme was determined in *S. avenae* by cell fractionation in nontreated aphids. The sedimentable subcellular fraction (P fraction, containing most of the membranous organelles) was separated from the cytosolic fraction (S fraction, containing the cell cytosol) by high-speed centrifugation of a postnuclear supernatant extract. Figure 2a shows the distribution of catalase from nontreated aphids in the P and S fractions. Mitochondrial cytochrome *c* oxidase was used as an internal control for the fractionation experiments (Figure 2b). As expected, cytochrome *c* oxidase was found only in the P fraction, i.e., mitochondria. The

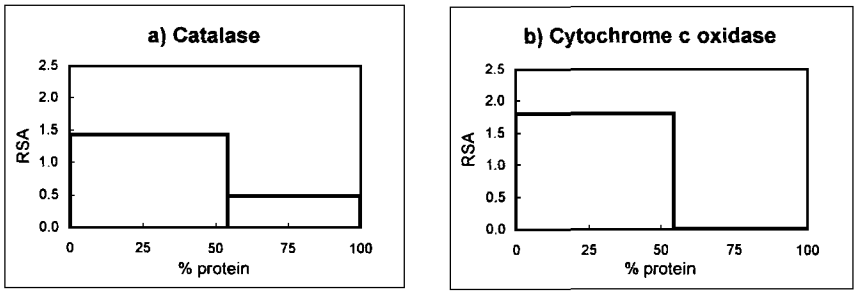

FIG. 2. Subcellular distribution of catalase (a) and cytochrome *c* oxidase (b) in a differential fractionation. A postnuclear supernatant was fractionated into an organellar pellet (P fraction, bar at left) and a supernatant (S fraction, bar at right). In the graphs the abscissa represents the cumulative protein content for each fraction as a percentage of the total protein of the homogenate. The ordinate represents the relative specific activity (RSA), i.e., percentage of activity of each enzyme in a given fraction of the homogenate relative to total activity over the percentage of homogenate protein in that fraction relative to total protein.

subcellular fractionation experiments showed the presence of catalase activity largely recovered in the organellar pellet (threefold more than in the S fraction), suggesting that this protein is contained in a sedimentable structure. Catalase latency in the P fraction was $73 \pm 1\%$, indicating that the majority of catalase in P is contained in a membrane organelle. These data are concordant with the proportion of relative specific activity found in the P fraction versus the S fraction. To isolate particles containing catalase, we used a Nycodenz density gradient subfractionation of aphid extracts. The particles containing catalase activity equilibrated in Nycodenz at 1.19 g/ml (Figure 3a), a density described for mammalian peroxisomes (Santos et al., 1994), and were clearly separated from mitochondria (1.14 g/ml) (Figure 3b). A morphological characterization of catalase-containing structures was undertaken by using the cytochemical diaminobenzidine method (Roels and Goldfischer, 1979) at the electron microscopy level (Figure 4). The diaminobenzidine reaction product in membrane-bound structures (Figure 4A) was not observed when the aphid sections were incubated in the presence of 3-amino-1,2,4-triazole, an inhibitor of catalase (Figure 4B and C). The presence of catalase in sedimentable particles showing an equilibrium density known for peroxisomes, and the structure of catalase-active particles observed under the electron microscope, indicate that aphids do contain peroxisomes and that catalase is a valid marker enzyme for peroxisomes in aphids. This organelle is known to participate in a variety of metabolic pathways such as lipid, purine, eicosanoid, phospholipid, and also xenobiotic metabolism (Mannearts and Van Vendhoven, 1993) and is an essential component in the cellular economy of several organ-

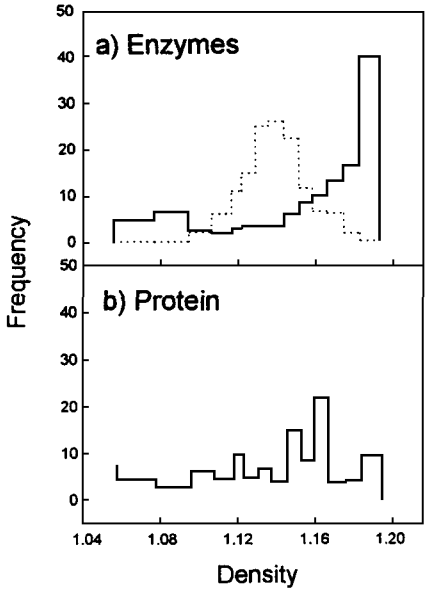

FIG. 3. Equilibrium density centrifugation of aphid postnuclear supernatants: (a) catalase (——), and cytochrome *c* oxidase (-----); and (b) proteins. The distribution pattern of catalase and cytochrome *c* oxidase corresponds to a representative fractionation experiment. For each distribution pattern, the ordinate represents the average frequency of the enzymes for each fraction. Frequency is plotted against density in a histogram form.

isms from yeasts to mammals (Lazarow and Fujiki, 1985). Although peroxisome functions in insect metabolism are still poorly understood, an organelle-bound catalase activity equilibrated in sucrose gradients at densities ranging between 1.19 and 1.22 g/ml and in Metrizamide at 1.15 and 1.21 g/ml has been reported recently in the honeybee midgut (Jiménez and Gilliam, 1996). This fact can also support the use of catalase as a peroxisomal marker enzyme in insects. In this case, the presence of peroxisomal catalase was associated with the metabolism of deleterious prooxidants from aerobic metabolism.

To investigate if the inducible catalase was peroxisomal and/or cytosolic, aphids treated with 2 mM DIMBOA for 48 hr were homogenized and fractionated by differential centrifugation. Figure 5a shows a significant twofold increment of sedimentable peroxisomal catalase activity in aphids treated with DIMBOA, while the effect on the cytosolic catalase activity was not significant (Figure 5b). The nonsignificant increment in the cytosolic catalase activity could be a consequence of the rupture of peroxisomes during the manipulations. The internal control of this fractionation experiment, the mitochondrial cytochrome

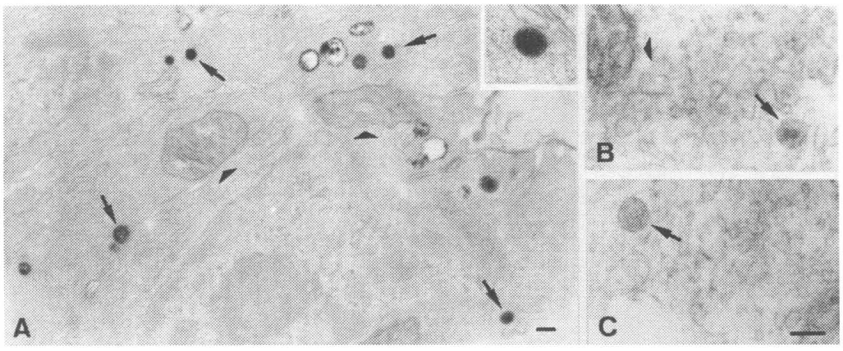

FIG. 4. Electron microscopy and catalase cytochemistry of epithelial tegumental cells of aphids. The aphids were fixed and subjected to conventional EM after cytochemistry for catalase in the absence (A) and in the presence of aminotriazole (B and C). The cytochemical reaction is clearly shown in thin sections, in which lead citrate staining has been omitted (A–C). A positive diaminobenzidine staining is concentrated inside of rather spherical granules (arrows in A–C), surrounded by a single membrane. A faint staining is also observed in the mitochondrial inner membrane (arrowheads). In the presence of aminotriazole, the reaction in the spherical granules is almost absent, showing that this reaction corresponds to catalase (B and C). Bar = 0.1 μm (A–C). Inset corresponds to a peroxisome at higher magnification.

c oxidase, was altered only in the pellet as expected (Figure 5c and d). Microsomal NADPH cytochrome *c* reductase and lysosomal Naβgase did not show any change.

It has been shown that DIMBOA is effectively ingested by aphids when they feed on artificial diets (Niemeyer et al., 1989; Givovich et al., 1992). The present results show an alteration of the oxidative cellular metabolism when aphids are treated with this plant secondary metabolite. Considering the lipophilic characteristics of DIMBOA, its incorporation into the cellular machinery through the cell membrane is likely. Within the cell, it would be metabolized by enzymes involved in defense against xenobiotics, such as glutathione-*S*-transferase, which has been shown to be inducible by the presence of high levels of cereal allelochemicals (Leszczynski et al., 1994). Their metabolism would produce oxidizable intermediates, which could induce the increase in the activity of enzymes involved in oxidative metabolism such as catalase and cytochrome *c* oxidase.

The increment in the activity of catalase and cytochrome *c* oxidase in aphids may represent another mechanism of insect resistance against plant secondary metabolites or insecticides, similar to the case of other xenobiotic metabolizing enzymes, i.e., cytochrome P-450s, glutathione-*S*-transferase, etc. (Mullin and

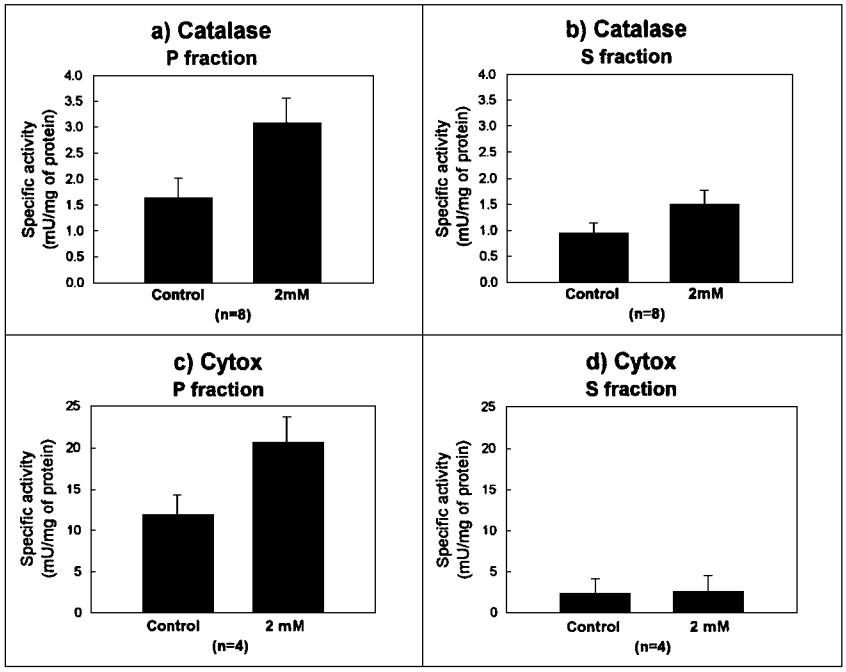

FIG. 5. Differential effect of DIMBOA on P and S subcellular fractions in aphids. (a) and (c) show the significant effects of 2 mM DIMBOA during 48 hr on specific activities of catalase ($P = 0.012$) and cytochrome *c* oxidase (cytox) ($P = 0.043$), respectively in the P fractions; (b) and (d) show lack of significant effects on both enzymatic specific activities in the S fractions ($P = 0.17$ and 0.773, respectively).

Scott, 1992). The evaluation of other enzymatic systems as well as the characterization of the metabolization route of DIMBOA will be essential to the understanding of the molecular mechanisms of action of cereal allelochemicals on aphids.

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