Potential role of hydrogen peroxide and melanin in the cold hardiness of *Ostrinia nubilalis* (Lepidoptera: Pyralidae)

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Abstract. The aim of this study was to investigate the relationship between antioxidant enzymes and reactive oxygen species production in diapausing larvae of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae) kept at 5°C, –3°C and –16°C for two weeks. The amount of hydrogen peroxide (H_2O_2), activity of antioxidant enzymes, copper zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutases (MnSOD) and catalase (CAT) in whole body homogenates, as well as the electron paramagnetic resonance (EPR) spectroscopy of this insect's whole body were analysed. A higher level of melanin radical and lower CuZnSOD and CAT activities were found in larvae kept at –3°C than at 5°C and –16°C. At the same temperature (–3°C) an elevated H_2O_2 concentration was recorded. A possible regulatory role of H_2O_2 at –3°C, which is the temperature that triggers freezing tolerance, is suggested.

INTRODUCTION

Studies on the mechanisms of cold-hardiness in insects include the regulation of physiological (Ramlov, 2000) and biochemical (Storey, 1997; Duman, 2001) processes that are involved in insect survival at subzero temperatures. A significant number of results indicate a relationship between the biochemical mechanisms of cold hardiness and processes regulated by free radicals and the antioxidant system (Rojas & Leopold, 1996; Grubor-Lajsic et al., 1997; Joanisse & Storey, 1998; Jovanovic-Galovic et al., 2004, 2007).

One of the most common responses of many species of insect when exposed to low environmental temperatures is the accumulation of low molecular weight organic solutes that act as cryoprotectants (Asahina, 1966; Lee et al., 1987; Storey & Storey, 1988). Our previous studies (Stanic et al., 2004) show that the biosynthesis of glycerol as a cryoprotectant is closely associated with antioxidant enzymes in larvae of the European corn borer, *Ostrinia nubilalis*. The elevated activity of enzymes in the pentose phosphate pathway in *Ostrinia* larvae exposed to cold accord with data on the glycerol synthesis pathway (Nordin et al., 1984). Flux through the pentose phosphate pathway is critically important not only for polyol synthesis but also for generating the reducing equivalents (NADPH) needed for the antioxidant defense system (ADS). Investigations of the role of the ADS in the adaptation of insects to low temperatures (Blagojevic, 2007) show that ADS reduces the risk of oxidative damage and, also, optimizes concentration of reactive oxygen species (ROS). This is particularly important because ROS is involved in cellular signaling as part of the complex redox based physiological regulation (Schafer & Buettner, 2001), including adaptation. At low/moderate concentrations hydrogen peroxide and other ROSs are involved in redox signalling and are key regulators of many intracellular pathways (Valko, 2007).

The aim of this work was to determine whether the response to cold is modulated by ROS and, also, whether the ROS homeostasis is adapted to the regulatory processes resulting in the cold resistance of larvae of *Ostrinia nubilalis*. We examined (1) the concentration of H_2O_2, and (2) the activities of some antioxidant enzymes (CuZnSOD, MnSOD and CAT), which may regulate the concentration of H_2O_2 in larvae of the European corn borer, *Ostrinia nubilalis*. In adittion, entire *O. nubilalis* larvae were analyzed by Electron Paramagnetic Resonance (EPR) spectroscopy.

MATERIAL AND METHODS

Insects

Diapausing fifth instars larvae of *Ostrinia nubilalis* were collected from maize plants growing in Vojvodina Province, Serbia. Insects were kept in a laboratory in humid conditions at 5°C for a week and then divided into the two groups, i.e. the first was kept at 5°C while the second was gradually exposed to –3°C and –16°C (temperature decreased over 8 days at –3°C per day). They were kept at these temperatures for the next 2 weeks, when the samples were collected.

Enzyme assays

The enzyme activities were assayed in the supernatant of larval homogenates (20% w/v 0.05 M phosphate buffer, pH 7.0, with 0.05% phenylthiourea). The total SOD activity was measured using the method described by McCord & Fridovich (1968)
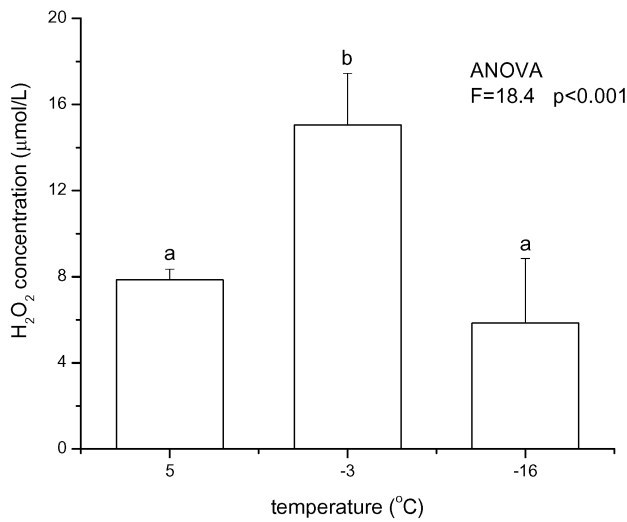
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Fig. 1. Concentration of hydrogen peroxide in *Ostrinia nubilalis* larvae kept at 5, –3 or –16°C. Results are presented as mean ± SD (n = 3). Statistical significance of the differences was tested using one-way ANOVA and post hoc Tukey's HSD test. Different letters above bars indicate significant differences.

and the MnSOD activity by the same method after the sample was incubated with 2 mM KCN for 30 min. The CuZnSOD activity was calculated as the total minus MnSOD activity. The activity of CAT was measured using the method of Aebi (1984). The protein content was determined using the method of Lowry (Lowry et al., 1951), with bovine serum albumin as the protein standard.

H_2O_2 assay

The H_2O_2 in body homogenates was detected using the Amplex Red reagent (Invitrogen, USA). Individual 100 μl reactions included a 50 μl sample of the homogenate (5% whole body homogenate in 50 mM sodium phosphate buffer, pH 7.4), 50 μM Amplex Red reagent and 0.1 U/ml horseradish peroxidase (HRP). After incubation for 30 min at room temperature, fluorescence was measured using a fluorescence microplate reader with excitation at 544 nm and detection at 590 nm.
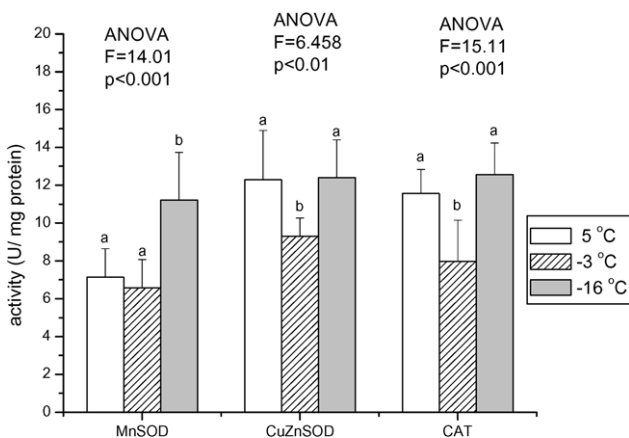

Fig. 2. Antioxidant enzymes in *Ostrinia nubilalis* larvae kept at 5, –3 or –16°C. Results are presented as mean ± SD (n = 8). Statistical significance of differences was tested using one-way ANOVA and post hoc Tukey's HSD test. Different letters above bars indicate significant differences.
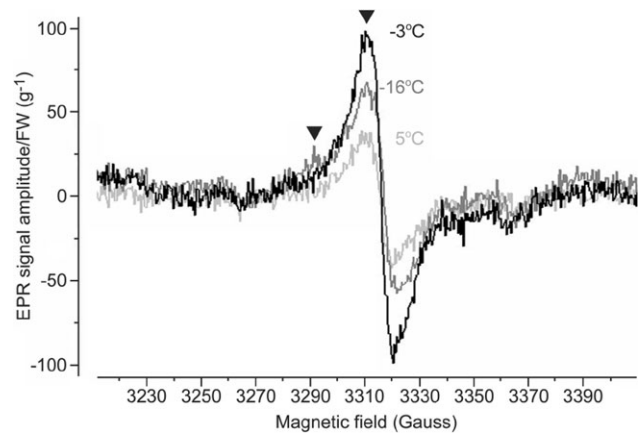

Fig. 3. EPR spectra of larvae kept at –3°C (black trace), –16°C (gray trace) or 5°C (light gray trace). Downward triangle (▼) marks two characteristic melanin-related EPR peaks. Measurements were performed at –150°C. Measurements are for 8 larvae from each experimental group. Results are means ± S.D. values of relative peak intensity/larval body mass.

EPR spectroscopy

The EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) and a temperature of –150°C. The following settings were used: 2 G modulation amplitude, 100 kHz modulation frequency, 10 mW microwave power, 200G field width and 3310 G field centre. The spectra were recorded using EW software (Scientific Software Inc, Bloomington, IL, USA). Results are presented as mean ± S.D. values of relative peak intensity / unit larval body mass.

Data analysis

All the values are expressed as mean ± SD. The data were analyzed using one-way analyses of variance (ANOVA) followed by Tukey's HSD test (Hinkle, 1994). The test criterion for statistical significance was p < 0.01.

RESULTS

The concentration of H_2O_2 was higher in larvae exposed to –3°C than in those kept at 5°C and –16°C (Fig. 1). Lower CuZnSOD and CAT activities were found at –3°C than at the other two temperatures (Fig. 2). MnSOD activity was higher in larvae exposed to –16°C than in those kept at 5°C and –3°C. (Fig. 2).

EPR spectra (Fig. 3) show the characteristic signal of melanin. Melanin-related peaks represent the stable semiquinone radicals from eumelanin (central peak) and pheomelanin (inflexion on the left side of the eumelanin signal), as reported earlier (Buszman et al., 2006; Meredith et al., 2006). Melanin-related signals were obtained at both –150°C (higher S/H ratio) and at room temperature (data not shown). The eumelanin signal was significantly stronger in larvae exposed to –3°C (198.1 ± 25.9 g^{-1}) than those kept at –16°C (125.1 ± 19.9 g^{-1}) or 5°C (82.0 ± 33.2 g^{-1})

DISCUSSION

In this study the concentration of H_2O_2 and the activities of SOD and CAT, which regulate the concentration of H_2O_2, and the presence of melanin (polymeric pigments capable of mediating oxydative stress) in the larvae of the European corn borer, *Ostrinia nubilalis*, reared at 5°C and subsequently exposed to –3°C and –16°C, were determined. The higher level of H_2O_2 at –3°C than at either 5°C or –16°C (Fig. 1) indicate that this reac-

tive molecule may be involved in the metabolic adjustments characteristic of cold-hardy insects. It is known that H_2O_2 induces the intracellular accumulation of trehalose (Benaroudj et al., 2001), which is an important anhydroprotectant found in many freeze tolerant organisms.

The increased level of H_2O_2 could be a consequence of reduced CAT activity, which is in agreement with the significant decrease in CAT activity in larvae exposed to $-3°C$ (compared to those exposed to $5°C$ or $-16°C$) (Fig. 2). Moreover, the increased level of H_2O_2 at $-3°C$ correlates with the decrease in CuZnSOD activity, possibly because H_2O_2 provokes a slow, irreversible inactivation of CuZnSOD (Bray, 1974; Salo et al., 1990) without inactivating MnSOD (Asada et al., 1975; Fridovich, 1986). This could explain why the larvae exposed to $-16°C$ exhibited higher levels of MnSOD activity than those exposed to either $5°C$ or $-3°C$ (Fig. 2). The biological significance of MnSOD as a mitochondrial antioxidant enzyme and its contribution to cell signalling is under intensive investigation (Murphy, 2009).

In addition to dismutation of superoxide radicals, H_2O_2 can be generated in cells via polymerization reactions of quinonic melanogenic intermediates during melanin synthesis (Jimenez-Cervantes et al., 2001). This may explain the increased levels of two characteristic melanin-related EPR signals (eumelanin and pheomelanin) coincident with the elevated H_2O_2 concentration ($-3°C$) we recorded (Fig. 3). Moreover, as these two semiquinone radicals result from the reaction of melanin with free radicals (Meredith et al., 2006), the observed increase in the eumelanin signal could indicate the scavenging of radicals generated in response to exposure to cold stress.

On the basis of our results it may be concluded that the H_2O_2 level, tuned via antioxidant enzymes optimizing the concentration of ROS and radical properties of melanin, could be a part of the alterations in redox involved in processes that account for the cold-hardiness of *Ostrinia nubilalis*.

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