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Effects of multigenerational cadmium exposure of insects (*Spodoptera exigua* larvae) on anti-oxidant response in haemolymph and developmental parameters

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ABSTRACT

Biochemical and organismal indices of metal tolerance were studied in *Spodoptera exigua* exposed to a cadmium-contaminated diet for one or many (33 or 61) generations. Reduced and oxidised glutathione, protein thiols, total anti-oxidant capacity level, glutathione transferase activity, and Cd accumulation were assayed in the haemolymph of the last instar larvae. The cadmium concentration in the whole larval body as well as larval survival, larval duration time and last instar body weight were also measured. Elevated cadmium concentration in the whole body, higher mortality and longer duration of the larval stage in one-generation exposed insects in comparison with those exposed for many generations suggest that metal tolerance builds over time. For the larvae from multigeneration metal treatment, the higher cadmium concentration in larval haemolymph positively correlated with glutathione oxidation and total anti-oxidant capacity. One-generation exposed insects had lower metal concentration in haemolymph than did 33-generation exposed insects.

1. Introduction

Cadmium pollution has increased for decades due to industrial, agricultural and municipal wastes (Ursinova and Hladikova, 2000). Because cadmium is a non-degradable metal, it may be accumulated in animal tissues and may disturb their physiological functions (Augustyniak and Migula, 2000). A contaminated ecosystem may exert strong selection on populations, resulting in their higher metal tolerance. This phenomenon was found by Van Ooik and Rantala (2010) for individual autumnal moths (*Epirrita autumnata*) that inhabited an area locally polluted with heavy metals. Some herbivorous insects are strong accumulators of cadmium, e.g., *Locusta migratoria*, *Oxya chinensis*, *Acrida chinensis* (Orthoptera), *Eligma narcissus* or *Lymantria dispar* (Lepidoptera) larvae (Gintenreiter et al., 1993; Li et al., 2006; Zhang et al., 2009). Cadmium is a persistent environmental pro-oxidant, which causes a wide variety of detrimental effects in organisms that eventually lead to higher mortality (e.g., Augustyniak and Migula, 2000; Zhang et al., 2009). Thus, the evolved tolerance to metals may be due to an increasingly effective anti-oxidant defence (Limon-Pacheco and Gonsebatt, 2008). Pro-oxidants, which accelerate the generation of reactive oxygen substances (ROS) instead of their degradation, increase oxidative stress. ROS interact with vast number of biomolecules and degrade them. These detrimental effects can be indicated by the elevation of carbonyl groups of proteins (Headlam and Davies, 2004; Halliwell and Gutteridge, 2005; Lushchak and Bagnyukova, 2006).

Sulphydryl substances, like protein thiols (tSH) or the tripeptide glutathione, are important anti-oxidant agents. The latter seems to be the main thiol that is responsible for redox homeostasis in most organisms. The ratio of the reduced (GSH) to the oxidised (GSSG) form of glutathione serves as an important index of oxidative stress (Halliwell and Gutteridge, 2005; Oktyabrsky and Smirnova, 2007; Kemp et al., 2008). Acting as a universal radical scavenger and cofactor for glutathione transferases (GST), GSH diminishes the detrimental effects of a variety of toxins (Oktyabrsky and Smirnova, 2007; Kemp et al., 2008). The activity of GST, which catalyses the conjugation of glutathione, may be up-regulated in response to contamination stressors, such as excessive amounts of heavy metals (Korashy and El-Kadi, 2006). This up-regulation may provide enhanced GSH utilisation and higher levels of GSSG, which may react with protein thiols (Garcia-Fernandez et al., 2002; Krężel and Bal, 2004). Cadmium causes higher GSH consumption and an increase in GSSG concentration, decreasing the ratio of GSH/GSSG (Wang and Wang, 2008). Different anti-oxidants seem to be useful as sensitive warning signals, however, interactions among different anti-oxidants may complicate the prediction of responses to given toxicant (Regoli et al., 2006). Individual anti-oxidants, such an

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enhanced action seems to be useful as sensitive warning signals. The biochemical defence may facilitate the successful adaptation of the organism in metal contaminated environment (Augustyniak and Migula, 2000). Thus, in addition to the assessment of well-known anti-oxidants, it is important to investigate total anti-oxidant capacity (TAC), which reflects a crude measurement of all anti-oxidant processes. One radical that is suitable for such measurements is 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), ABTS (Re et al., 1999).

After exposure to Cd, increased metal concentration was found in the haemolymph (Martin and Rainbow, 1998). There is also the evidence that ROS production in haemolymph is promoted by Cd (Jo et al., 2008). This is important because Cd may be transferred to other internal tissues via the haemolymph. Additionally, the haemolymph helps to protect the other organismal systems, through immunological and anti-oxidant defences (Franciosa and Berge, 1995; An and Choi, 2010).

The aim of our study was to compare the differences in anti-oxidant defence and developmental parameters between herbivorous insects derived from multigenerational rearing on a diet contaminated with cadmium to those reared on a non-contaminated diet. As a model animal, we have chosen to use the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), a well-known polyphagous pest of many vegetable crops, widely distributed across Mediterranean Europe, Asia, America, Africa and Australia (Smits et al., 1987; Goh et al., 1991). There are several examples of this species resistance developed under the pressure of different toxicants, mainly pesticides (e.g., Moulton et al., 2000; Ahmad and Arif, 2010). We measured the following parameters: glutathione, protein thiols, total anti-oxidant capacity, and glutathione transferase activity in the haemolymph of larvae (last larval stage). The hypothesis was that insects exposed to cadmium stress over multiple generations will have greater metal resistance than control animals.

2. Materials and methods

2.1. Experimental insects and chemical treatment

The insects underwent continuous multigenerational rearing (over four years) at 25 °C and a photoperiod of 16L:8D in two strains exposed constantly to control and contaminated with metal larval diet (44 mg Cd per kg of dry weight of food). The used concentration of cadmium may be available in plants from highly contaminated or metalliferous environments (e.g., Wang et al., 2009) and was chosen as a result of our earlier examinations to obtain mild mortality of larvae, but significantly higher than existing among larvae kept in control conditions. It resulted in a survival rate of 75%, during the period from hatching to the early phase of pupation, in the Cd1 group as compared to the survival rate of the control group (Kafel, unpublished data).

Larvae were kept in 90 mm Petri dishes, with ten individuals per each plate. Larvae were offered a semi-synthetic diet of wheat germ prepared according to the recipe described by Poitout and Bues (1970). Pupae were collected from all Petri dishes before imagos hatched and transferred to 2.5 L containers. Adults were offered a 10% aqueous solution of honey. Eggs were collected on blotting-paper and sterilised in formaldehyde, 5% (v/v) aqueous solution. The first instar larvae were allowed to hatch and then transferred to Petri dishes.

The insects were divided into four experimental groups, differing in number of generations exposed to cadmium:

- Control (animals fed a control diet through 61 generations),
- Cd1 (animals fed a control diet through 60 generations and transferred to a diet contaminated with Cd in the last generation),
- Cd33 (animals fed a control diet through 28 generations and transferred to a diet contaminated with Cd for 33 generations),
- Cd61 (animals fed a diet contaminated with Cd for 61 generations).

2.2. Insect development

In our study, the survival and the duration of larvae were calculated for 100 larvae from each experimental group, over a period from the first instar stage until pupation. The body weight of the three-day-old last (fifth) instar larvae was measured.

2.3. Haemolymph collection and preparation

For haemolymph collection, the larvae were randomly chosen among ca. 400 individuals reared in each experimental groups, gathering larvae of the same age. The haemolymph was collected from three-day-old last instar larvae anaesthetised at low temperature (five replications were used for measurements within each experimental group, and for each replicate 25 μl of haemolymph was collected from three individuals). The collection was done shortly after a cut of the last proleg. The haemolymph was mixed immediately with cold anticoagulant (41 mM citric acid, 17 mM ethylenediaminetetraacetic acid (Na_2-EDTA), 98 mM NaOH and 186 mM NaCl) in a 1:4 (v/v) proportion and then centrifuged at 8000 *g* for 10 min at 4 °C. The supernatant, depending on its intended use, was mixed either 1:1 (v/v) with cold 20% trichloroacetic acid (TCA), for glutathione and thiol measurements, or 1:4 (v/v) with 0.1 M phosphate buffered saline (PBS), pH 7.4, for analysis of glutathione transferase activity. In a separate analysis, the haemolymph (50 μl from six individuals) was collected and mixed with HNO_3 ultrapure acid immediately after collection and used for metal concentration measurement. For this analysis, five replications were also used.

2.4. Assessments of oxidative stress indices and anti-oxidant responses

2.4.1. Glutathione and protein thiols concentration

Concentrations of total and oxidised glutathione were measured according to Griffith (1980) in the samples of haemolymph deproteinised with cold 10% TCA (in proportion 1:1, v/v). They were centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatants were used for further glutathione measurements and the pellets were used for thiols measurements. The supernatants were then neutralised with 0.125 M phosphate buffer (pH 7.4) containing 1 mM EDTA (in proportion 1:5, v/v) and mixed with 0.2 mM NADPH, glutathione reductase (0.01 U/ml) and 6 mM 5,5′-dithiobis(2-nitrobenzoic) acid for a colorimetric assay. The linear rate of change of absorbance at 412 nm was recorded for 5 min. The oxidised form of glutathione was quantified after incubation with vinylpyridine (96% solution, Aldrich), which was added to the homogenate at 1:100 (v/v). The quantity of tripeptide was calculated based on a calibration curve prepared from a series of solutions with known concentrations of oxidised glutathione. The quantity of glutathione reduced was calculated after abstracting the amounts of glutathione oxidised from the total amounts of glutathione. The concentration of glutathione (GSH or GSSG) was expressed in μg/mg protein.

Thiols (tSH) concentration was determined in the pellets obtained from haemolymph solution deproteinised with TCA. The pellet was solubilised in 0.1 M buffer phosphate (pH 7.6) and used for measurements at 324 nm, using 4,4′-dithiopyridine, according to the method of Riener et al. (2002) and expressed as nmol SH groups/mg protein. The reaction mixture consisted of 263 μl PBS buffer pH 7.4 with 1 mM EDTA, 50 μl sample and 12.5 μl 4,4′-dithiopyridine. The extinction coefficient was equal to 21,400 M^{-1} cm^{-1}.

2.4.2. Glutathione transferase activity

Glutathione transferase activity was measured as described by Yu (1982) using 15 mM ethanol solution of 1-chloro-2,4-dinitrobenzene (CDNB). The reaction mixture consisted of 100 μl 0.1 mM Tris–HCl buffer (pH 7.5), 10 μl 15 mM GSH in buffer, 5 μl CDNB and 5 μl of sample. Blank values (in the absence of sample) were subtracted to yield the final absorbance values. The enzyme assay was carried during first three minutes, within the linear range of reaction rate, at 340 nm, using a microplate spectrophotometer (Infinite M200, Tecan). Enzyme activity was expressed as nmol of GSH conjugates/min/mg protein. The coefficient extinction was equal to 9,6 mM^{-1} l^{-1} cm^{-1}.

2.4.3. Total anti-oxidant capacity assay

The total anti-oxidant capacity was determined according to Re et al. (1999). The haemolymph was homogenised in 0.1 M PBS, pH 7.4. Then, the samples were centrifuged at 20,000 *g* for 10 min at 4 °C and the precipitates were re-homogenised in PBS. The extract was filtered and used to determine the total anti-oxidant capacity. The radical of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), or $ABTS^-$, was prepared by reacting 19.5 mg $ABTS^-$ with 3.3 mg potassium persulphate in 7 ml sodium phosphate buffer pH 7.4 for 12–16 h in the dark. The blue-green solution was kept at −20 °C prior to use. Decolourisation of ABTS−.was measured at 734 nm. Results were expressed in terms of equivalent anti-oxidant capacity to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and expressed in μg Trolox/mg protein.

2.4.4. Protein assays

The protein concentrations were assessed as described by Bradford (1976), using bovine serum albumin (BSA) as a standard.