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ANTIOXIDANT ENZYMES OF HONEYBEE LARVAE EXPOSED TO OXAMYL

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ABSTRACT

Oxamyl is a carbamate insecticide used to control a broad spectrum of insects. It can also affect non-targeted organisms when applied incorrectly. The world food production depends partially on honeybee pollination abilities and therefore it is directly linked to the health of bees. The success of the colony development depends, among other factors, on the health of the larvae. The first 6 days are crucial for their development. In this stage, the worker larvae grow exponentially and may be exposed to xenobiotics via their diet. In this study, we investigated the effect of oxamyl on honeybee larvae (*Apis mellifera*) by monitoring the changes in their antioxidant enzyme system. The activities of superoxide dismutase, catalase and glutathione-S-transferase were determined in the homogenates of *in vitro* reared honeybee larvae after their single dietary exposure to oxamyl at doses of 1.25, 2.5, 5, 10 and 20 µg a.i./larva (a. i.—active ingredient). The doses of oxamyl did not cause statistically significant changes in the activities of the enzymes. Even a slight activation of these enzymes protected the larvae from the adverse effects of the reactive oxygen species (ROS). Marked changes in both the enzyme activity and the content of lipid peroxidation products were observed at the oxamyl dose of 10 µg a. i./larva. This fact may indicate a potential oxidative damage to the larvae. These results allowed us to assume that the toxic effects of oxamyl involves not only the inhibition of acetylcholine esterase but is also associated with ROS production.

Key words: antioxidant enzyme system; honeybee; larvae; oxamyl

INTRODUCTION

The honeybee (*Apis mellifera*) is undoubtedly the economically most important insect. Up to 35 % of the world agricultural production depends upon the pollination of crop plants by bees [9]. The pollination capabilities of bees are directly conditional on the health of honeybee colony. This is the reason why the health of bees is currently a subject of considerable attention. The decreasing tendency of

bee keeping and survival of bee colonies is associated with environmental abuse [1]. As a sensitive biosensor of environmental changes, the bee colony reacts even to small deviations caused by pollutants. Sublethal effects of plant protection products can be manifested by the changed behaviour of bees when: searching for food, memory disorders, ability to learn, rejection of food, loss of orientation and similar situations [16, 24]. The flying worker bees directly affected by insecticide may not have enough energy to return to the bee hive [21]. Those which survived the contact with such pollutants carry them back to the hive. There, the xenobiotics and their residues may be stored for considerable periods and may accumulate in the wax and honey. These compounds which accumulated in the hive, can induce in a short time, negative long-lasting effects on the colony behaviour [12].

By means of contaminated food and wax, all developmental stages of the honeybee can be exposed to a broad spectrum of xenobiotics. The presence of multiple residues can result in mutual interactions even at sublethal concentrations and cause a permanent "pesticide" stress manifested by delayed development of the larvae or shortened life of the bees [19].

Reactive oxygen species (ROS) are the side product of aerobic metabolism. The antioxidant system and antioxidant enzymes as its part constitute one of the protective mechanisms against oxidation damage. Antioxidant enzymes are involved in important physiological processes and can affect the health and survival of bees, their cognitive abilities, immune response to pathogens and longevity [5, 15]. If the free radicals are capable of overcoming the protective antioxidant system of the insect, an oxidation stress arises. The potential oxidation damage increases the demands of the insects on oxygen due to their way of life and the food rich in pro-oxidants. Carbamate insecticides cause reversible inhibition of acetylcholine esterase. According to a number of authors the primary toxic effect of carbamates may increase as a result of their ability to generate reactive oxygen species [17, 23]. The action and effects of such insecticides may be elucidated also on the basis of changes in the enzyme system of the exposed individuals.

The aim of this study was to evaluate the effects of oxamyl on the activity of antioxidant enzymes in honeybee larvae under *in vitro* conditions, 72 hours after their single exposure to this insecticide [18].

MATERIALS AND METHODS

Honeybee (*Apis mellifera carnica*) larvae were obtained from three healthy queen-right colonies reared in an experimental apiary of the University of Veterinary Medicine and Pharmacy in Košice, SR, during the summer of 2017. Synchronised first instar larvae were transferred to a laboratory where they were reared under *in vitro* conditions according to the method of A u p i n e l et al. [2] and OECD 237 [18]. Each experimental group comprised 12 larvae from the transferred colonies (three parallel groups, 12 larvae in each). The larvae were maintained in a 48-well plate in commercial grafting cells of 9 mm internal diameter (ref CNE/3, Nicotplast, France). Throughout the acute oral test (D1–D7), the larvae were kept in an enclosed box at 34.5 °C and a relative humidity of 90 ± 5 % (Sicco, Germany). With the exception of D2, they were fed a diet adjusted to the needs of the larvae, depending on the developmental stage [18]. The diet was prepared using royal jelly (Institute of Apiculture, Liptovský Hrádok, Slovakia), D-glucose, D-fructose, yeast extract and distilled water:

Diet A (D1): 50 % fresh royal jelly + 50 % water solution containing
2 % yeast extract, 12 % glucose and 12 % fructose;

Diet B (D3): 50 % fresh royal jelly + 50 % water solution containing
3 % yeast extract, 15 % glucose and 15 % fructose;

Diet C (D4—D6): 50 % fresh royal jelly + 50 % water solution containing
4 % yeast extract, 18 % glucose and 18 % fructose.

The stock solution of oxamyl in a glucose-fructose solution (6.6 mg a .i./500 µl Glu-Fru solution) was prepared on the day of administration (D4). By serial dilution of the stock solution with the fresh diet we prepared five dilutions with the final contents of oxamyl in the feed rations of: 1.25; 2.5; 5; 10; and 20 µg a. i./larva (a. i.—active ingredient). Dimethoate (organophosphate) at the concentration of 8.8 µg a. i./larva was used as a positive control. For the validity of the test, the following was required: cumulative mortality between days 4 and 7 of larvae on the control plate ≤ 15 %; mortality of larvae on Day 7 in the positive control ≥ 50 % [18].

On the first day of the test (D1), the larvae were transferred with a special grafting tool to the surface of the grafting cell with Diet A (20 µl). On D4, the larvae from the experimental groups were fed diet C with the respective

doses of oxamyl. The control group received a diet with the addition of distilled water while the positive control group received a diet with dimethoate. On D5 and D6, larvae of all groups received diet C. The test was terminated on day 7 (D7). The mortality of the larvae was recorded on D5, D6 and D7. The larvae that did not move nor reacted to the touch were considered dead. On the basis of mortality, the 72h LD50 was calculated using ToxRat software.

The D7-survived larvae were frozen (–50 °C) and later homogenized in 5 mmol·l^{-1} TRIS-HCl buffer solution (pH 7.8) containing 0.15 mol·l^{-1} KCl, 1 mmol·l^{-1} Na2EDTA and 2 mmol·l^{-1} glutathione using a homogeniser Ultra-Turrax T25 (Germany). The homogenates (25 % w/v) were centrifuged at 105 00 g and °C, for 1 hour (Beckman L8-60, USA). They were stored at –50 °C (LTF 325 Arctiko, Denmark) until the enzyme analysis.

The total proteins were determined by the method of B r a d f o r d [4]. The determination of the activity of superoxide dismutase (SOD) was based on the measurement of the inhibition rate of cytochrome c reduction at 550 nm. The xantin/xantinoxidase system was responsible for the formation of the superoxide radical in the reaction mixture [8]. The activity of catalase (CAT) was based on the determination of the decrease in hydrogen peroxide in the reaction mixture at 240 nm [22]. The activity of glutathione-S-transferase (GST) was determined by the measurement of the increment conjugate of reduced glutathione at 340 nm with 1-chloro-2,4-dinitrobenzene as a substrate [11]. Thiobarbituric acid reactive substances (TBARS), the products of lipid peroxidation, gave a colour reaction with thiobarbituric acid at the development of compounds absorbing light at 535 nm [10]. The specific activity of enzymes was expressed in U·mg^{-1} protein. The chemicals used were of the highest analytical purity and were purchased from the following companies: Sigma, Merck and Boehringer.

The results of the enzyme analysis are presented as means ± SD (n = 3). The statistical evaluation was carried out by the Student t-test ($P < 0.05$ was considered significant).

RESULTS

The aim of this study was to observe the effect of the carbamate insecticide oxamyl on the antioxidant system of honeybee larvae *in vitro*. The oxamyl doses were selected on the basis of preliminary testing. The LD50 calculated on the basis of larvae mortality was 7.15 µg a. i./larva.

Enzymes are generally considered a sensitive parameter indicating exposure of an organism to xenobiotics. The most important biomarkers are enzymes that participate in the development of oxidative stress or catalyse detoxication processes in the exposed organism.

Superoxide dismutase catalyses dismutation of the superoxide radical to hydrogen peroxide and oxygen. The results of our study showed that the specific activity of SOD in bee larvae from the experimental groups did not change significantly in comparison with the control. A slightly decreased activity of SOD in comparison with the control was recorded at the oxamyl dose reaching 2.5 µg a. i./larva. With the increasing dose of oxamyl, the activity of this enzyme gradually increased. The highest specific activity was determined at the dose of 10 µg a. i./larva. Exposure to the dose of 20 µg a. i./larva caused a decrease in the activity of SOD (Tab. 1). Catalase is the most effective eliminator of hydrogen peroxide. The catalase activities failed to show significant changes (Tab. 1). The changes observed in individual experimental groups copied the changes in the activities of SOD.

Glutathione-S-transferase is a primary detoxifying enzyme which participates in biotransformation of xenobiotics by means of conjugation reactions with glutathione. With an increasing dose of oxamyl, the specific activity of

Table 1. Specific activity of superoxide dismutase and catalase in homogenates of honeybee larvae following single exposure to the insecticide oxamyl

Oxamyl dose [µg a. i./larva]	**SOD** [U·mg^{-1}]	**CAT** [U·mg^{-1}]
0	52 ± 11	475 ± 43
1.25	71 ± 46	439 ± 100
2.5	41 ± 6	537 ± 200
5	55 ± 20	452 ± 85
10	100 ± 40	680 ± 180
20	70 ± 0,0	541 ± 0,0
Dimethoate (positive control)		
8.8	**67 ± 37**	**439 ± 100**

SOD—superoxide dismutase; CAT—catalase; a. i.—active ingredient. The values are presented as the arithmetic means ± SD (n = 3)

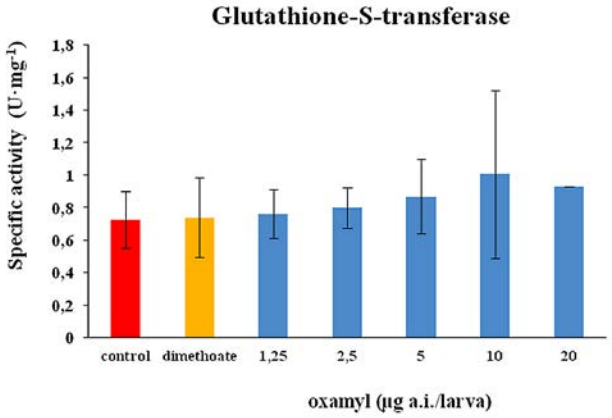

Fig. 1. Specific activity of GST in the homogenates of honeybee larvae following a single exposure to the insecticide oxamyl

GST—glutathione-S-transferase; dimethoate—positive control; a. i.—active ingredient; the values are presented as the arithmetic means ± SD (n = 3)

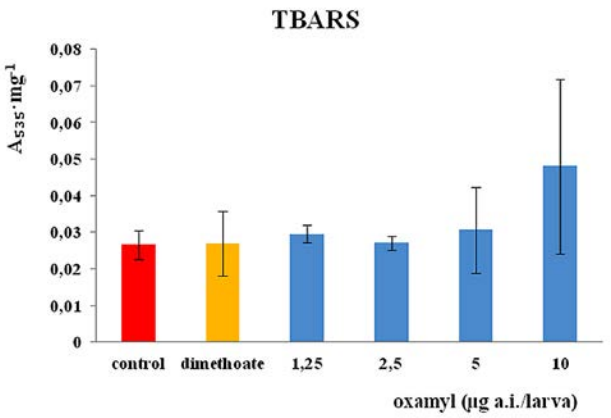

Fig. 2. Content of TBARS in the homogenates of honeybee larvae following a single exposure to the insecticide oxamyl

TBARS—thiobarbituric acid reactive substances; dimethoate—positive control; a.i.—active ingredient. A535.mg^{-1}—absorbance.mg^{-1} protein. The values are presented as the arithmetic means ± SD (n = 3)

GST increased. The highest activity (1.006 U·mg^{-1}) was determined in larvae exposed to the dose of 10 µg a.i/larva (Fig. 1).

The products of lipid peroxidation react with thiobarbituric acid to provide coloured products. Their quantity manifests the degree of damage to the lipids due to undesirable oxidative changes. A marked increase in the content of TBARS was observed in the group exposed to the action of oxamyl at a dose of 10 µg a. i./larva (Fig. 2).

DISCUSSION

Oxamyl acts as a reversible inhibitor of acetylcholine esterase which results in the accumulation of acetylcholine. The primary toxic effect of oxamyl may be supported by the development of reactive oxygen species as was described also in association with the action of other carbamates [23]. The increased production of ROS in insects is associated with the detoxication processes [7]. Antioxidant enzymes play an important role in the regulation of the level of ROS and are produced by cells as a response to stress. The SOD and CAT constitute the primary protection system of an organisms against oxidation stress. GST is an early marker of the induction of the detoxication system and contributes also to the protection of cells against oxidative damage [3]. The superoxide that serves as a substrate of SOD is responsible for the direct damage to macromolecules but also as a source of additional ROS. The role of SOD is to keep the level of superoxide in the cell low. Inhibition of its activity may result in increased production of ROS depending on the degree of oxidation stress. A slight decrease in the activity of SOD was recorded at the dose of oxamyl reaching 2.5 µg a. i./larva. However, in general the changes in the activity of SOD in homogenates of honeybee larvae showed no significant changes (Tab. 1). The presence of CAT was confirmed in the honey. Its probable role consists in the maintenance of H2O2 in honey under the toxic level. High activity of CAT was observed also in bees and can be affected by the presence of environmental contaminants [5]. The slight increase in the activity of CAT observed in our experiment (Tab. 1) indicated that the larvae were exposed to higher concentration of hydrogen peroxide originating from the diet or produced following the exposure to xenobiotics, i. e. oxamyl.

The important function of GST involves the detoxification of insecticides and secondary metabolites and protection against oxidative stress [3]. Some GST isoenzymes exhibit peroxidase activity [6]. This activity is especially important for invertebrates because they lack selenium-dependent glutathione peroxidase [25]. The GST of the delta class participate in the resistance of insects to insecticides.

The mechanism of this resistance at the metabolic level involves the induction of detoxification enzymes capable of transforming xenobiotics to less toxic and more soluble compounds that can then be eliminated [14]. Our study showed a gradual increase in the activity of GST (from 0.739 to 1.006 $U \cdot mg^{-1}$) with increasing doses of oxamyl in comparison with the control (0.724 $U \cdot mg^{-1}$). An increased specific activity of GST was observed in honeybees exposed to permetrin, deltametrin and flumetrin [20]. The GST of the class sigma showed high affinity to products of lipid peroxidation. Because they are located in the metabolically active tissues of insects, one may assume that these enzymes play an important role in the protection of insects against oxidative stress [7].

In our study, the activity of enzymes was determined on day D7, 72 hours following the single exposure to oxamyl in homogenates of the larvae *in vitro*. The characteristic feature of this larval stage is the highest content of lipids [13]. Lipids are considered as molecules most susceptible to oxidative damage. An increased level of TBARS was determined already at the dose of oxamyl reaching 10 μg a. i./larva. Our results allowed us to conclude that the toxic action of oxamyl involves not only the inhibition of acetylcholine esterase but also the production of ROS. However, the doses of oxamyl caused no damage to the larval tissues. The adverse effects of pesticides on *in vitro* larvae was frequently manifested by a decreased survival rate and weight loss [2]. Y a n g et al. [26] observed that sublethal doses of imidacloprid had no effect on the larvae but interfered with memory and the ability to learn in adult honeybees developed from these larvae.

CONCLUSIONS

The first 6 days of development of worker bee larvae are of key importance because of their exponential growth during this stage. Exposure to residues of pesticides in the diet and wax can have undesirable effects. The determination of enzyme activities carried out in our study allowed us to conclude that the antioxidant enzyme system of larvae reacted to the doses of oxamyl in their diet. Already moderate induction of these enzymes ensured the protection of the larva against the harmful action of ROS. Only the highest tested dose of oxamyl induced an increased content of products of lipid peroxidation which can indicate potential oxidative damage to larval tissues. As the success of the bee colony depends on the health of developing larvae, the potential adverse effects of pesticides on larval stages should become a part of any overall analysis. The results of our study are a small contribution to this effort.

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