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BORIC ACID-INDUCED EFFECTS ON PROTEIN PROFILES OF *GALLERIA MELLONELLA* HEMOLYMPH AND FAT BODY

P. HYRŠL,[1]** ENDER BÜYÜKGÜZEL[2]* and K. BÜYÜKGÜZEL[2]

[1] Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlarska 2, 611 37, Brno, Czech Republic
[2] Department of Biology, Faculty of Arts and Science, Karaelmas University, 67100, Zonguldak, Turkey

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The dietary effects of boric acid (BA) on the protein profiles of greater wax moth, *Galleria mellonella* (L.), were investigated in hemolymph and fat body of final instar (VIIth) and pupae. The insects were reared from first-instar larvae on an artificial diets containing 156, 620, 1250 or 2500 ppm of BA. We detected many undetermined protein fractions (6.5–260 kDa) in addition to well-defined protein fractions such as lipophorins and storage proteins in the tissues by using sodium dodecyl-sulphate polyacrylamide gradient gel electrophoresis. A marked quantitative change in the 45 kDa protein fraction of the hemolymph was observed in the VIIth instar larvae reared on 2500 ppm dietary BA.

Keywords: Galleria mellonella – boric acid – protein profiles – hemolymph – fat body

INTRODUCTION

Chemical pest management programs rely upon applications of broad-spectrum pesticides which pose a threat to nontarget organisms and the environment [6]. The problem have focused attention on less toxic natural insecticides and lesser-used compounds such as boric acid (BA). BA, a nonvolatile, slow-acting inorganic insecticide, has long been used in urban pest management, e.g. against cockroaches and other crawling household pests [7]. Boric acid is classified as preferred insecticides because they have relatively low toxicity to mammals and other vertebrates and a low environmental impact. Most BA toxicity studies have been directed toward biological fitness of adults of urban pest insects [9, 10, 33]. The oxidation of ingested alle-

* Contributed equally.
** Corresponding author; e-mail: hyrsl@mail.muni.cz

lochemicals is often associated with the production of oxidative radicals that damage insect tissues by oxidizing vital cell components in midgut of a lepidopterans [19, 20]. Boric acid act as stomach poisons, the exact mode of action has not been well established, although it has been proposed that BA produce free radicals leading to oxidative damage in some tissues and increasing mortality [12]. It follows that more knowledge of BA-induced toxicity is necessary.

Beyond these general responses, proteolysis with a concomitant increase in protease in hemolymph and fat body [26] and increased whole body protein content [17] are compensatory physiological mechanisms in response to insecticide toxicity. While effects of BA on life parameters of a few insects have been recorded, tissue-specific changes in protein profiles to BA challenge is an emerging field, thus we are investigating the mechanisms of BA-induced toxicity in insects. In the present paper, we report on outcomes of experiments our hypothesis that dietary BA affects protein profiles of the wax moth, *Galleria mellonella.*

MATERIAL AND METHODS

Insects

Last-instar larvae (upon reaching VIIth instars, 100–150 mg) and newly emerged pupae (90–100 mg) of the greater wax moth, *G. mellonella* (L.) were used in all experiments. The insects were reared in 1000 ml glass jars with an artificial diet [4], at 30 ± 1°C in constant darkness. The standard diet was composed of 420 g of bran, 150 ml of filtered honey, 150 ml of glycerol, 20 g of ground old dark honeycomb and 30 ml of distilled water. Fifteen newly emerged adult females were placed in the jars and provided a piece of old honeycomb on the diet for egg deposition and feeding of newly hatched larvae. The methods used to prepare and dispense diets into container, and the methods used to obtain eggs and larvae and their placement onto diets were described by İçen et al. [17].

BA (Crystal form, 99%, H_3BO_3, Eti Mine Works General Management, Ankara, Turkey) was directly incorporated into diets at concentrations of 156, 620, 1250 and 2500 ppm. First instar larvae were reared through VIIth instars on artificial diets amended with given concentrations of BA. The larvae were transferred into another jar lined with a filter paper for pupation. BA dietary treatments reduced survival to pupal and adult stages, thus no pupae at 2500 ppm of BA were obtained.

Sample preparation

The larvae and pupae were chilled on ice for 5 min and surface sterilized in 95% ethanol. Larval hemolymph was collected into cold Eppendorf tubes by amputating the second pair of prolegs. Pupal hemolymph was collected into a cold tube by puncturing heads of pupa with a fine syringe needle [16]. For protein analysis, 5 mg of fat

body was homogenized in a diluted sample buffer using ultrasonic homogenizer (Elma D-78224, Germany) for 8 min. A few crystals of phenylthiourea (PTU) were added to each sample to prevent melanization. Fat body and hemolymph samples were centrifuged at 1100 × *g* for 10 min and denatured by heating at 90 °C for 2–5 min in a diluted sample buffer containing 7% β-mercaptoethanol and 0.37% of the anionic detergent SDS. After dilution, the samples applied into the gels contained finally 0.025 µl of hemolymph (containing 1.25–3 µg of proteins according to developmental stage) or 0.025 mg of fat body. Altogether 59 larvae and 34 pupae were used (at least 6 individuals per experimental group). The samples were frozen at –25 °C until use.

Electrophoresis

Sodium dodecyl-sulphate polyacrylamide gradient gel electrophoresis (SDS-PAGGE) was performed in a discontinuous buffer system according to Laemmli [21] modified according to Hyršl and Šimek [16]. Fourteen samples and one standard were applied to a 5% stacking gel (1 mm thickness) and 7.5–20.0% gradient separating gel (gels composition was essentially according to Bollag et al. [3] with the addition of saccharose) in electrophoresis buffer. All buffer systems were prepared according to Marshak et al. [22] and Bollag et al. [3] with the addition of EDTA. All chemicals used were obtained from Fluka (Germany), except acrylamide 30% solution (AppliChem, Czech Republic), amonium persulphate (Bio-Rad, USA) and glycine (Lachema, Czech Republic).

The electrophoresis (using Hoefer Scientific Instruments SE 600 device, San Francisco, CA, USA) with mini chiller (model 1000, Bio-Rad, Ramsey, Minnesota, USA) was carried out automatically in two steps, pre-electrophoresis for 1 h at 400 V and then at 610 V for 2500 Vh (volt-hours, values for two parallel gels, constant voltage power supply 1000/500, Bio-Rad) at 5 °C. The gels were fixed and stained with silver nitrate at room temperature essentially following the technique of Kirkeby et al. [18]. Broad range protein standards (161-0317, Bio-Rad) were used for calibration. The molecular weight of separated protein fractions was determined by densitometry using an imaging densitometer (GS-670, Bio-Rad) and Molecular Analyst software (Bio-Rad).

RESULTS

Approximately 15 and 13 protein fractions were detected after separation of larval and pupal hemolymph proteins, respectively (Fig. 1). Similarly, approximately 16–20 protein fractions were detected and some others were observed in a poor resolution (Fig. 2) following separation of fat body proteins. Larval and pupal hemolymph (Fig. 1) includes a dominant group of protein bands ranging in molecular mass approximately 72–84 kDa. These are generally recognized as storage pro-

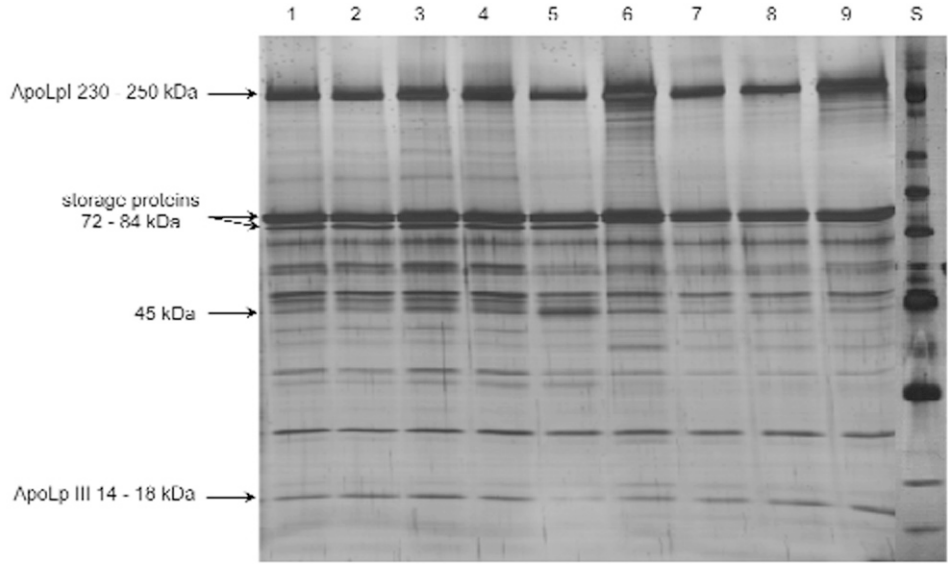

Fig. 1. Acrylamide gradient gel of proteins extracted from last instar larvae (samples 1–5) and pupae (samples 6–9) hemolymph of *G. mellonella.* Samples with increasing concentration of boric acid: 0.00 ppm (1, 6); 156 ppm (2, 7); 620 ppm (3, 8); 1250 ppm (4, 9) and 2500 ppm (5). Main protein groups of proteins are described on the left margin as well as 45 kDa protein fraction according to protein standards (S)

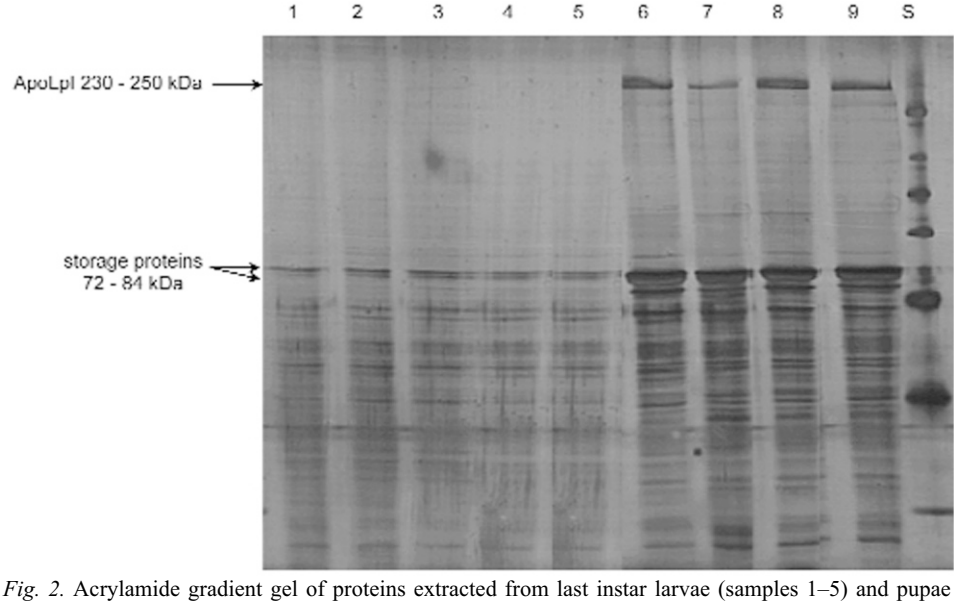

Fig. 2. Acrylamide gradient gel of proteins extracted from last instar larvae (samples 1–5) and pupae (samples 6–9) fat body of *G. mellonella.* Samples with increasing concentration of boric acid: 0.00 ppm (1, 6); 156 ppm (2, 7); 620 ppm (3, 8); 1250 ppm (4, 9) and 2500 ppm (5). Main protein groups of proteins are described on the left margin according to protein standards (S)

teins. Other well-determined fractions in larval hemolymph are lipophorins including apoLp I (230–250 kDa) and apoLp III (14–18 kDa). ApoLpI was present in tested tissues of larvae or pupae, whereas apoLp III was detected in only hemolymph of larvae and pupae. In addition to well-defined protein fractions, some undetermined protein fractions varying from 6.5 to 260 kDa were also detected in larval as well as pupal hemolymph. Clearly visible differences between these two developmental stages are demonstrated.

Although the changes in larval protein profiles at low BA concentrations were initially not detected a clear influence of the highest concentration of BA (2500 ppm) was observed. One 45 kDa protein fraction (Fig. 1) was significantly more abundant in insects reared on BA-supplemented diets. The quantity of the 45 kDa fraction increased in connection with increasing of BA concentrations, about 10% increase was present by 620 and 1250 ppm and at highest BA concentration (2500 ppm) was 23% increase of this fraction in comparison to controls. This observation was confirmed by all samples used in the experiment. The other main proteins were not altered in insects reared on diets amended with BA. No differences were observed in pupal hemolymph proteins. In the analysis of protein spectrum of larval and pupal fat body, no changes between control and BA-treated groups were noted.

DISCUSSION

In our previous study we suggested that BA concentrations till 2500 ppm are sufficient for developmental as well as biochemical changes [15]. The data reported in this paper support our hypothesis that dietary BA treatments influence the protein composition of *G. mellonella* hemolymph. Larval and pupal hemolymph protein profile highlights a dominant group of protein bands ranging in molecular mass from 72 to 84 kDa. These are thought to be storage proteins (also known as 81/82, 76 and 74 K) according to Miller and Silhacek [23, 24] and Godlewski et al. [8]. Storage proteins are used as an amino acid reserve for the production of adult proteins. They are synthesized in the fat body and later released into the hemolymph. Other well-determined fractions in larval hemolymph are lipophorins: ApoLp I (230–250 kDa) and apoLp III (14–18 kDa), they were determined in this study according to Wiesner et al. [32] and Halwani et al. [14].

One 45 kDa protein fraction in larval hemolymph (Fig. 1) was significantly more abundant in larvae reared on diets supplemented with the increasing BA concentrations. The molecular weight of this protein was similar in size to cytochrome P450 enzymes reported for other insects [30]. The cytochrome P450 monooxygenases are an important metabolic system included in the detoxification of xenobiotics and insecticide resistance whose level of activity can be influenced by several dietary constituents [2, 5]. We suggest that appearance of 45 kDa protein band may be a result of metabolic resistance reaction to BA or its oxidatively activated products, because substrates for P450 include insecticides, allelochemicals, hydrocarbons and drugs [13]. We hypothesize that accumulation of 45 kDa protein was also necessary

to serve as a compensatory pool to restore antioxidant enzymes lost in *G. mellonella* larvae to cope with BA-induced oxidative stress on the anti-oxidant systems [15]. Miota et al. [25] further supported our hypothesis when they showed the induction of a 45 kDa protein in atrazine-treated midges, *Chironomus tentans* larvae. The intensity of this atrazine-induced protein was associated with a P450 membrane protein.

Proteins are the main molecules that are used as an compensatory energy source for insect activities [31]. Appearance of this protein band may also be a result of energy depletion as a compensative response to BA toxicity. The results of Park and Keeley [28] provide support for this hypothesis because certain insecticides leads to a depletion of energy reserves of the cockroach, *Blaberus discoidalis* (Serville), resulting in increased mortality. Furthermore, involvement of protein reserve in adaptation of insecticide intoxication has also been reported by Nath et al. [26] and Ahmed et al. [1]. Increased whole body protein content is also compensatory physiological mechanism in response to some insecticides [17, 27]. Results of more recent studies show that insecticide-resistant species have more proteins than susceptible insects and the increase in protein content has also been correlated with development of resistance towards toxic effects of insecticides [11, 29]. In conclusion, we suggest that physiological response to BA in *G. mellonella* is associated with the changes in the expression of stress-related proteins.

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