

Developmental changes in phenol-oxidizing activity in the greater wax moth *Galleria mellonella*

Jana BENEŠOVÁ, Pavel DOBEŠ, Pavel HYRŠL

Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

Abstract

Activity of enzymes oxidizing phenolic substrates commonly termed phenoloxidases (POs) was investigated during normal development of *Galleria mellonella* L. in haemolymph, intact cuticle and homogenized cuticle. PO activities were determined colorimetrically following chemical activation of prophenoloxidase with acetone or methanol using hydroquinone and pyrogallol as substrates. Hydroquinone with *para*-positioned hydroxyl group seems to be most suitable for spectrophotometrical detection of phenol-oxidizing activity in various insect tissues because its oxidation products interact only minimally with tissue material. Enzymatic activity changed markedly during development in larvae, pupae and adults. The highest levels of PO in haemolymph were detected using hydroquinone on the fifth day of the last larval instar and at the beginning and at the end of pupal stage. PO activity changed gradually during development except during larval-pupal and pupal-adult metamorphosis, where marked increases in activity occurred. Comparable changes with slight time shifts were determined in cuticle after its homogenization. Intact cuticles showed only minimal PO activity without distinct developmental changes. Experiments using pyrogallol as substrate confirmed the temporary decrease of overall PO activity during pupal stage of development and the subsequent return to high PO levels in adults. Developmental changes in phenol-oxidizing activity are independent of sex in both pupal and adult stage of *G. mellonella*.

Key words: development, *Galleria mellonella*, hydroquinone, laccase, phenol-oxidizing activity, phenoloxidase, pyrogallol.

Introduction

Insect immune responses against invading pathogens can be broadly categorized into two processes: cellular also called haemocytic and humoral. Both of these branches cooperate to protect animals against invaders that have breached their physical barriers such as integument, midgut epithelium and peritrophic membrane (Elrod-Erickson *et al.*, 2000). Cellular responses involving direct interaction of haemocytes with antigens include phagocytosis, microaggregation, nodulation and encapsulation. Bacteria that are entrapped in nodules (Stanley and Miller, 2006) as well as encapsulated larger objects such as parasites or parasitoid eggs (Gupta, 2002) can subsequently be melanized by action of phenoloxidases (POs). Humoral defense reactions involve the action of molecules constitutively present in tissues and haemolymph such as lectins or lysozyme, induced synthesis of antimicrobial peptides and the phenoloxidase cascade (Gupta, 2001). At the boundary of cellular and humoral immunity lies the coagulation cascade which includes action of both whole haemocytes and many coagulation factors released by them. The coagulation cascade closely cooperates with the PO cascade, which contributes to protein cross-linking, elimination of the pathogens and melanization in forming clot (Li *et al.*, 2002; Theopold *et al.*, 2004). In addition to its role in immune reactions POs in insects are essential to the fundamental physiological processes of pigmentation and sclerotization of new cuticle after each moult (Arakane *et al.*, 2005).

The PO cascade or prophenoloxidase-activating system comprises several serine proteinase zymogens and pattern-recognition proteins that are able to detect min-

ute amounts of antigens. Its function is to activate the enzyme PO which generally occurs as an inactive prophenoloxidase (proPO) (Ashida and Yamazaki, 1990; Jiravanichpaisal *et al.*, 2006; Cerenius *et al.*, 2008). POs *sensu lato* include enzymes with monophenoloxigenase (tyrosinase-type, EC 1.14.18.1), *o*-diphenoloxidase (tyrosinase-type, EC 1.10.3.1) and *p*-diphenoloxidase (laccase-type, EC 1.10.3.2) activity. In *Galleria mellonella* L. (Lepidoptera Pyralidae) as in other insects the presence of multiple proPO genes is assumed (Li *et al.*, 2002; Cerenius and Söderhäll, 2004) and some of the corresponding proteins were already purified (Kopáček *et al.*, 1995). Although laccase-type and tyrosinase-type POs prefer various substrates and differ in their primary function, both of them belong to the phenoloxidase group of copper-binding proteins (Hughes, 1999; Arakane *et al.*, 2005).

During normal development laccase-type POs commonly termed laccases participate especially in sclerotization of cuticle (Sugumaran *et al.*, 2000; Sugumaran, 2002). In this non-immune process laccases in conjunction with other enzymes such as quinone isomerase catalyze the production of highly reactive quinones and quinone methides. Products of these cuticular reactions are responsible for cross-linking of cuticular proteins and their incorporation into the cuticle leads to its hardening and coloration. In contrast to tyrosinase-type POs the role of laccases during immune responses is still unclear.

Tyrosinase-type POs sometimes referred to simply as phenoloxidases or tyrosinases mediate melanin synthesis, protection against potential pathogens and are also involved in immune reaction such as nodulation and encapsulation (Mandato *et al.*, 1997; Zhao *et al.*, 2007).

Reminiscent of laccases the tyrosinases act in conjunction with other enzymes especially with dopa decarboxylase (Sideri *et al.*, 2007). During immune response tyrosinases respond on the presence of antigen, proPO is activated and the production of melanin leads to the sequestration of invaders. Furthermore, intermediates with potential cytotoxic activity are produced during proPO activation which contribute to the elimination of pathogens (Nappi and Ottaviani, 2000). Similarly, tyrosinases are important in wound healing and clot formation, where they contribute to protein cross-linking and microbe killing (Theopold *et al.*, 2002).

The greater wax moth *G. mellonella* is frequently used as a model for investigating insect immunity and physiology, but little is known about the development-dependence of immune reactions, including PO activity, both in this and other insect species. The aim of the present work was to describe developmental changes in oxidation activity for phenolic substances in *G. mellonella* with accent on cuticular laccases.

The PO activity was determined in several tissues of *G. mellonella* including haemolymph, intact cuticle (IC) and homogenized cuticle (HC). Inactive proPO is generally produced by subsets of insect haemocytes and released into haemolymph. It was proven in *Bombyx mori* L. that some haemocyte-derived proPO can be post-translationally modified and then transported through the cuticle (Asano and Ashida, 2001). Moreover, laccase genes are highly expressed in epidermal cells participating in cuticle formation (Dittmer *et al.*, 2004) and the presence of inactive form of laccase-type PO in cuticle of *B. mori* was proven in recent study (Yatsu and Asano, 2009). Here POs are involved, apart from other functions, in sclerotization and pigmentation thus developmental changes in PO activities linked to growth and molting are likely.

Materials and methods

Insects

Larvae of greater wax moth were reared on an artificial diet (Haydak, 1936) at 30 ± 1 °C in constant darkness. For the experiments larvae from the same day of development were used. We established several experimental groups covering each day of the 7th instar of larval stage, including the last two days also called prepupa. In some experiments selected days of pupae or adults were used. To discover possible differences between sexes we divided pupae into two subgroups. There were at least ten larvae in each experimental group.

Reagents

In all experiments sodium phosphate buffer (pH 5.8) consisting of 0.1 M NaH₂PO₄ and 0.1 M Na₂HPO₄ was used. DOPA [3-(3,4-Dihydroxyphenyl)-DL-alanine]; hydroquinone (1,4-Dihydroxybenzene); pyrocatechol (1,2-Dihydroxybenzene); pyrogallol (1,2,3-Trihydroxybenzene) and tyrosine [3-(4-Hydroxyphenyl)-DL-alanine] were purchased from Sigma Aldrich Chemical Co.

Determination of phenol-oxidizing activities in haemolymph

Haemolymph was collected by amputation of a proleg and pooled into cold tubes without using any anticoagulant. 50 µl of haemolymph was mixed with 3 ml of ice-cold acetone and incubated 30 min at -4 °C. After centrifugation (5 min, 5000 g, -4 °C) the supernatant was removed and the sediment dried at room temperature. The acetone-treated dry material was then mixed with 3 ml of phosphate buffer (pH 5.8) and preincubated 10 min at 30 °C before 0.6 ml of 0.1 M hydroquinone (or 0.1 M pyrogallol) were added. The reaction was allowed to proceed for 25 min at 30 °C and then stopped by adding a few drops of inhibitor (2.5% phenylthiourea in distilled water). Controls with no PO activities used for determination of background absorbance values were prepared in the same way apart from phenylthiourea solution which was added before the substrate. After centrifugation (5 min, 5000 g, RT) the absorbance was measured spectrophotometrically at 470 nm (410 nm for pyrogallol) using a cuvette spectrophotometer (Spekol 210, Germany). The PO activities determined as the difference between absorbance values of controls and experimental samples was expressed in absorbance units per µl of haemolymph.

Determination of phenol-oxidizing activities in intact cuticle (IC)

Freshly prepared cuticles from the abdomen were divided lengthwise into halves, which facilitated processing of samples and controls simultaneously. To adjust for weight differences between six and eight halves of cuticle were mixed with 3 ml of ice-cold acetone and incubated 30 min at -4 °C. Concentration of cuticular material in all samples was adjusted to 5 mg/ml in order to ensure identical conditions for all groups. After centrifugation (5 min, 5000 g, -4 °C) the supernatant was removed and cuticles were dried at room temperature. Cuticles were then mixed with 3 ml of phosphate buffer (pH 5.8); preincubated 10 min at 30 °C and 0.6 ml of 0.1 M hydroquinone (or 0.1 M pyrogallol) was added as substrate. After 25 min at 30 °C the reaction was stopped by adding 2.5% phenylthiourea solution. Controls were prepared simultaneously from the same cuticles as the samples and treated with phenylthiourea to block PO activities before substrate addition. Finally, cuticles were removed carefully from both samples and controls and the absorbance of supernatants was measured at 470 nm or 410 nm for hydroquinone and pyrogallol, respectively. The PO activity was expressed as absorbance per mg of cuticle.

Determination of phenol-oxidizing activities in homogenized cuticle (HC)

Homogenized cuticle was prepared by mixing ten to fifteen cuticles from the abdomen (fixed concentration as above) with 6 ml of phosphate buffer (pH 5.8) and subsequent homogenization on ice. For each HC assay 30 mg of cuticular material was used. Compared to IC assay double amount of cuticle was used due to ensure optimal absorbance values. The homogenized mixture was centrifuged (5 min, 5000 g, -4 °C) and the super-

nantant was used for determination of PO activity. 2 ml of supernatant was mixed with 0.2 ml of methanol and 0.5 ml of 0.1 M hydroquinone. The reaction was allowed to proceed for 25 min at 30 °C and then stopped by adding 2.5% phenylthiourea. In controls the phenylthiourea solution was added at the same time as methanol. Debris was removed by centrifugation (5 min, 5000 g, RT) and absorbance was measured spectrophotometrically at 470 nm. The enzymatic activities were determined as the difference between absorbance of samples and controls and expressed in absorbance units per mg of homogenized cuticle.

Statistical analysis

To determine significant differences between means Kruskal-Wallis analysis of variance (Statistica 8.0 software) followed by Tukey's test was used. The results were expressed as mean \pm standard deviation and considered significantly different at $p < 0.05$.

Results

In preliminary tests the dependence of PO activity on time was determined. Samples from both haemolymph and cuticle were prepared as described above and absorbance was measured spectrophotometrically in 5min intervals during 30 min after addition of substrate. Both 0.1 M hydroquinone and 0.1 M pyrogallol were used as substrates measured at 470 nm and 410 nm, respectively. In all samples the increase in absorbance was nearly linear during this time period both with hydroquinone (figure 1) and pyrogallol (data not shown) which confirms earlier observations (Goldsworthy *et al.*, 2002; Adamo, 2004). Because PO activity can be considered constant under the used conditions during the first 30 min of the reaction and for simplicity, we decided to express PO activity as the absorbance units (au) reached after 25 min of incubation with substrate per μ l of haemolymph or mg of cuticle.

Significant differences in laccase-type PO activity during development were detected using hydroquinone as substrate in haemolymph samples (figure 2). Enzymatic activity increased gradually from the first to the fifth day of 7th instar larvae where it reaches the maximum (0.00634 ± 0.00058 au₄₇₀/μl). During subsequent days of the larval stage the laccase activity decreased until the end of this stage. Rapid growth in activity appeared during the first day of the pupal stage and then in the last day of the same stage. Enzymatic activity on these days was as high as in five day old 7th instar larvae. There was no significant difference in laccase activity between male and female pupae.

When pyrogallol was used as substrate we weren't able to detect any developmental changes of PO activity in haemolymph except a small non-significant decrease connected with change of prepupae to pupae (figure 3). This decrease appeared in both males and females. During other days of the 7th larval instar overall PO activity changed only minimally with no significant differences.

Levels of laccase-type PO activity determined in IC were very low if hydroquinone was used as substrate

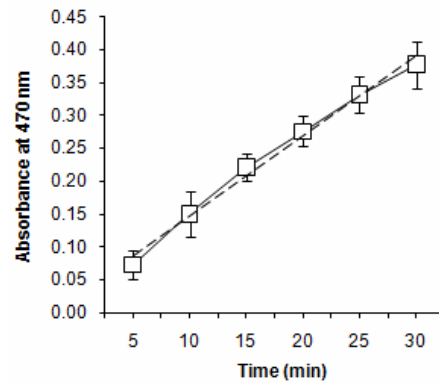


Figure 1. Time course of hydroquinone conversion mediated by laccase-type PO from haemolymph (means \pm SD, $n = 4$). Dashed line demonstrates linearity for comparison with observed increase in absorbance (solid line).

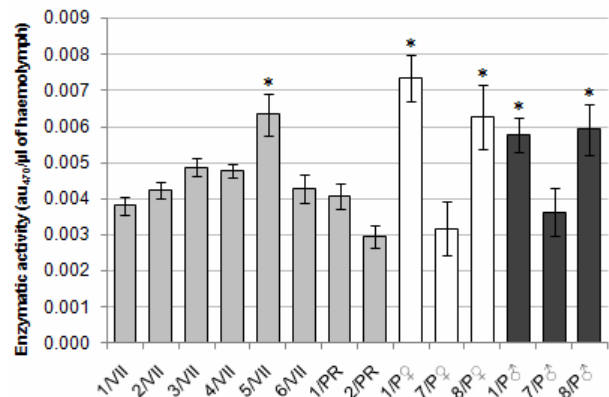


Figure 2. Enzymatic activity in *G. mellonella* haemolymph using hydroquinone as substrate [means \pm SD, $n = 3$, significance level is <0.05 (*)] and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR) and pupae (P). Sex not determined (grey); females (white); males (black).

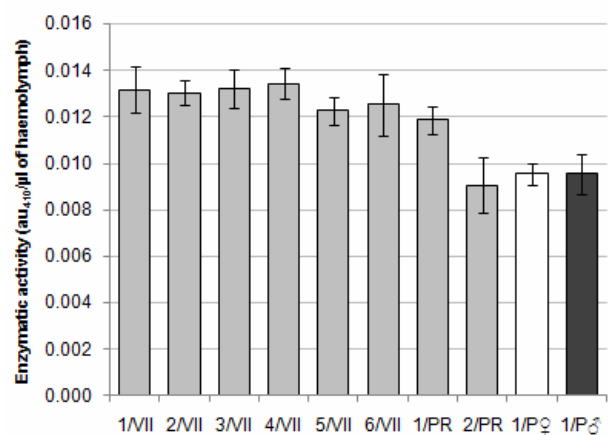


Figure 3. Enzymatic activity in *G. mellonella* haemolymph using pyrogallol as substrate (means \pm SD, $n = 3$) and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR) and pupae (P). Sex not determined (grey); females (white); males (black).

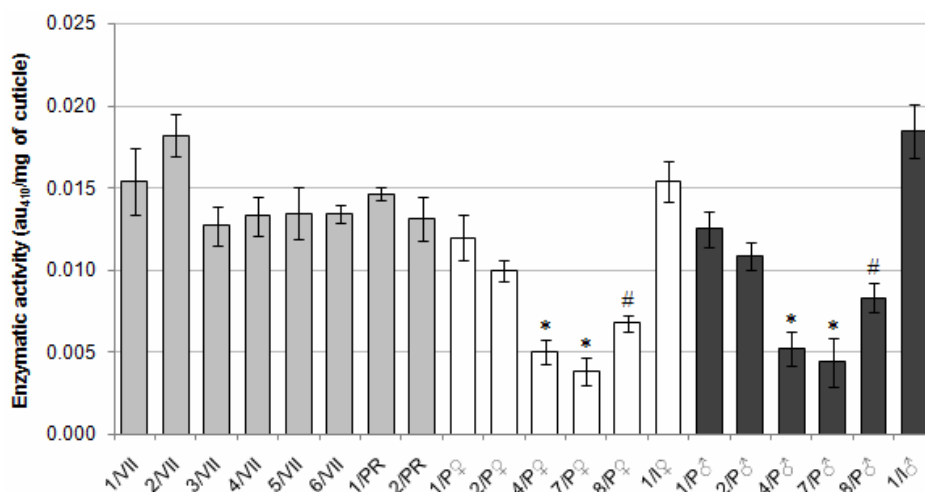


Figure 4. Enzymatic activity in *G. mellonella* intact cuticle using pyrogallol as substrate [means \pm SD, $n = 3$, significance level is <0.05 (*), marked values (#) indicate no significant difference to all other groups] and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR); pupae (P) and imagoes (I). Sex not determined (grey); females (white); males (black).

(data not shown) and almost all led to OD values of $0.00100 \text{ au}_{470}/\text{mg}$ independently on age of larvae or pupae. Distinctively different laccase activity was determined only on the first day of 7th instar larvae where the activity was nearly three times lower ($0.00038 \pm 0.00002 \text{ au}_{470}/\text{mg}$). In IC as in haemolymph there was no difference between sexes.

Much more data from IC were gained using pyrogallol as substrate (figure 4). Also here PO activity changed minimally during larval stages, but there was a substantial decrease in enzymatic activity during the pupal stage. In cuticles from one day old female imagoes the PO activity was again as high as in larvae; in males it was even slightly higher. Except for this difference the changes in PO activity during pupal stage were the same both in males and females.

In HC using hydroquinone as substrate the changes of phenol-oxidizing activity were much more pronounced (figure 5). During the first two days of the 7th instar the laccase-type PO activity was only minimal, but then gradually increased with the age of the larvae similar to haemolymph. This time the maximum of enzymatic activity was reached on about the sixth day of larval stage ($0.00779 \pm 0.00052 \text{ au}_{470}/\text{mg}$) and first day of prepupae ($0.00799 \pm 0.00053 \text{ au}_{470}/\text{mg}$). Then the activity started to decrease again and at the beginning of the pupal stage it was more than three times lower both in males and females.

Discussion and conclusions

In this work we show that the activity of phenol-oxidizing enzymes is strongly influenced by development in *G. mellonella* and that the developmental changes appear both in haemolymph and in the cuticle. However, we detect a difference of about two days in the maxima of activities between haemolymph and cuticle in larval stage. This may confirm the transport of proPO

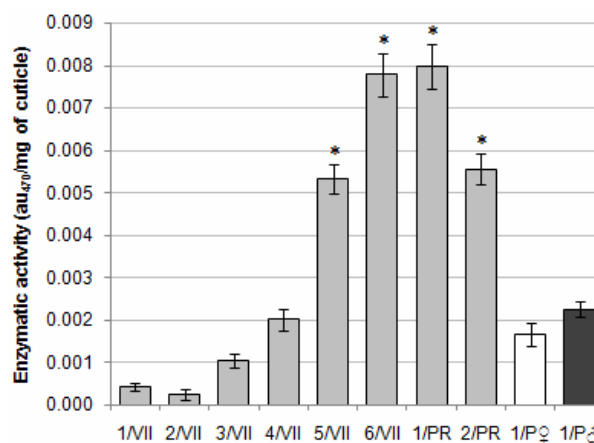


Figure 5. Enzymatic activity in *G. mellonella* homogenized cuticle using hydroquinone as substrate [means \pm SD, $n = 3$, significance level is <0.05 (*)] and its dependence on the day of development in seventh instar larvae (VII); prepupae (PR) and pupae (P). Sex not determined (grey); females (white); males (black).

from haemolymph, where it is synthesized by haemocytes, to the cuticle as suggested before (Ashida and Brey, 1995; Asano and Ashida, 2001). On the other hand, laccase genes are expressed mainly in epidermal cells, fat body and other tissues, but their expression in circulating haemocytes is low as observed in *Manduca sexta* L. (Dittmer *et al.*, 2004). Therefore, regarding laccases it is conceivable that there exist two different enzymes with similar functions, one responsible for *p*-diphenoloxidase activity in cuticle and a second functioning in haemolymph.

It is known that POs are able to process various substrates (Hall *et al.*, 1995; Ashida and Yamazaki, 1990; Dittmer *et al.*, 2004; Munoz *et al.*, 2006). Therefore the PO assays used in this article were also tested with DOPA, hydroquinone, pyrogallol, pyrocatechol and tyrosine as

substrates and their suitability for our experiments was compared. While DOPA is traditionally used as substrate for determining of insect POs (Li *et al.*, 1994; Kopáček *et al.*, 1995; Mandato *et al.*, 1997; Goldsworthy *et al.*, 2002; Hattori *et al.*, 2005), we found it unsuitable for spectrophotometrical detection of PO activity in samples containing whole tissues or tissue debris. Reaction products generated by enzymatic conversion of DOPA and pyrocatechol were kept on tissue surfaces causing its darkening and only a small fraction was released into solution where it could be measured spectrophotometrically. This was a problem mainly in HC assay during which substrates and cuticular material are both present in the reaction mixture. Tyrosine-based reactions were very slow and produced weak coloration. Therefore, we used diphenol hydroquinone and triphenol pyrogallol as substrates, which both appear to be efficient in detecting PO activity.

Hydroquinone was chosen as the most suitable substrate for our experiments, because only minimal interaction of its oxidation products with tissue debris and cuticular surfaces was observed. This enables the use of hydroquinone for colorimetric assays of PO activity both in haemolymph and cuticle. The disadvantage of hydroquinone is its *para*-positioned hydroxyl groups which makes it suitable substrate for laccase-type PO but not for tyrosinases with *o*-diphenoloxidase activity (Chase *et al.*, 2000). That is why we used also pyrogallol as substrate wherever possible. This triphenolic substance can be processed by both laccase-type and tyrosinase-type PO, but causes the darkening of tissues containing POs, which complicate its use in HC assay. Discrepancies between chemical structures of used substrates can also lead to differences in PO levels; using hydroquinone we found greater fluctuations in PO activity than with pyrogallol as substrate. The fact that different activity profiles were observed when pyrogallol and hydroquinone were used as substrates suggests that hydroquinone was detecting a different enzyme(s) than pyrogallol.

Because the level of PO activity is very low in haemolymph and tissues of *G. mellonella* that are not immune challenged with pathogens or their components (unpublished observation), we decided to measure and compare PO activity obtained after *in vitro* conversion of proPO to active enzyme by organic solvents (Goldsworthy *et al.*, 2002; Adamo, 2004). Samples were treated with acetone or methanol that interact with proPO and are able to change it into active PO. Although methanol is a more effective activator of proPO in *G. mellonella* than acetone we observed the developmental changes in total PO activity are not affected by the type of used organic solvent (unpublished observation). The exact molecular mechanism of this chemical activation is still not fully understood and remains to be elucidated.

During the larval stage the laccase activity in haemolymph determined using hydroquinone gradually increases, followed by a partial decrease prior the pupal stage. The same trend was observed before in larval haemolymph proteins of *G. mellonella* using SDS-PAGE (Godlewski *et al.*, 2001). The largest increase in laccase activity was detected in haemolymph at the beginning and at the end of the pupal stage. This makes sense since the animal undergoes large developmental changes in

this period connected with pupating and adult eclosure (Krämer *et al.*, 2002; Hyršl and Šimek, 2005). At these two important milestones of development laccases participate to a large extent in sclerotization and pigmentation of the new cuticle which forces the organism into the larger synthesis of PO precursors that is subsequently demonstrated in observed increase of enzymatic activity. These results agree with former observations in other insect species, which showed increased expression of laccase-like gene in epidermal cells of the pharate pupae of *M. sexta* (Dittmer *et al.*, 2004) and prepupae of *B. mori* (Yatsu and Asano, 2009). Insect development is controlled hormonally and the direct correlation was shown before between hormones and proPO gene expression. In *Anopheles gambiae* Giles 20-hydroxyecdysone (Ahmed *et al.*, 1999; Müller *et al.*, 1999) and in *M. sexta* the juvenile hormone (Hiruma and Riddiford, 1988) were shown to modulate proPO gene expression and thus are able to cause the observed changes in phenol-oxidizing activity during moulting.

In IC laccase activity changed only slightly during development of the larvae. This is probably caused by the small amount of enzyme bound to the inner and outer surface of the cuticle, which is able to interact directly with substrate present in reaction mixture. On the other hand, considerable age-dependent fluctuations in PO levels were determined in HC. Homogenization combined with methanol treatment supposedly led to the release of proPO from cells and its conversion to the active enzyme.

Unlike laccase activity, the phenol-oxidizing activity determined using pyrogallol in haemolymph and intact cuticles was more uniform during development. As mentioned above this is caused by different chemical structure of pyrogallol, which makes it suitable for enzymes with both *o*-diphenoloxidase and *p*-diphenoloxidase activity. With this in mind we propose that not only the activity of one enzyme, but common phenol-oxidizing ability including activity from laccases, tyrosinases and other enzymes with potential phenoloxidase activity was measured using pyrogallol. Our results suggest that even if the activity of individual enzymes such as laccases changes markedly during development, the overall phenol-oxidizing activity shows less fluctuation during development.

It seems that developmental changes of PO activities are not affected by sex in *G. mellonella*. Similarly, no gender-specific differences in cecropin-like antibacterial activity were detected in *G. mellonella* haemolymph (Meylaers *et al.*, 2007).

To conclude, our study provides new data on phenol-oxidizing activities in the haemolymph and cuticle of *G. mellonella*. We show strong effect of development especially on laccase-type POs in haemolymph and homogenized cuticle.

Acknowledgements

We are grateful to Ulrich Theopold (Stockholm University, Sweden) for providing useful comments on draft of this article and three anonymous referees for their suggestions. All authors of the manuscript contributed equally.

References

- ADAMO S. A., 2004.- Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*.- *Journal of Insect Physiology*, 50: 209-216.
- AHMED A., MARTÍN D., MANETTI A. G., HAN S. J., LEE W. J., MATHIOPOULOS K. D., MÜLLER H. M., KAFATOS F. C., RAIKHEL A., BREY P. T., 1999.- Genomic structure and ecdysone regulation of the prophenoloxidase 1 gene in the malaria vector *Anopheles gambiae*.- *Proceedings of the National Academy of Sciences of the USA*, 96 (26): 14795-14800.
- ARAKANE Y., MUTHUKRISHNAN S., BEEMAN R. W., KANOST M. R., KRAMER K. J., 2005.- Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning.- *Proceedings of the National Academy of Sciences of the USA*, 102 (32): 11337-11342.
- ASANO T., ASHIDA M., 2001.- Cuticular Pro-phenoloxidase of the Silkworm, *Bombyx mori*.- *The Journal of Biological Chemistry*, 276 (14): 11100-11112.
- ASHIDA M., BREY P. T., 1995.- Role of the integument in insect defense: Pro-phenol oxidase cascade in the cuticular matrix.- *Proceedings of the National Academy of Sciences of the USA*, 92: 10698-10702.
- ASHIDA M., YAMAZAKI H. I., 1990.- Biochemistry of the phenoloxidase system in insects: with special reference to its activation, pp. 239-265. In: *Molting and Metamorphosis* (OHNISHI E., ISHIZAKI H., Ed.).- Japan Scientific Societies Press, Tokyo, Japan/Springer-Verlag, Berlin, Germany.
- CERENIUS L., SÖDERHÄLL K., 2004.- The prophenoloxidase-activating system in invertebrates.- *Immunological Reviews*, 198: 116-126.
- CERENIUS L., LEE B. L., SÖDERHÄLL K., 2008.- The proPO-system: pros and cons for its role in invertebrate immunity.- *Trends in Immunology*, 29 (6): 263-271.
- CHASE M. R., RAINA K., BRUNO J., SUGUMARAN M., 2000.- Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*.- *Insect Biochemistry and Molecular Biology*, 30: 953-967.
- DITTMER N. T., SUDERMAN R. J., JIANG H., ZHU Y., GORMAN M. J., KRAMER K. J., KANOST M. R., 2004.- Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*.- *Insect Biochemistry and Molecular Biology*, 34: 29-41.
- ELROD-ERICKSON M., MISHRA S., SCHNEIDER D., 2000.- Interactions between the cellular and humoral immune responses in *Drosophila*.- *Current Biology*, 10 (13): 781-784.
- GODLEWSKI J., KLUDKIEWICZ B., GRZELAK K., CYMBOROWSKI B., 2001.- Expression of larval hemolymph proteins (Lhp) genes and protein synthesis in the fat body of greater wax moth (*Galleria mellonella*) larvae during diapauses.- *Journal of Insect Physiology*, 47: 759-766.
- GOLDSWORTHY G., IPOKU-WARE K., MULLEN L., 2002.- Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*.- *Journal of Insect Physiology*, 48: 601-608.
- GUPTA A. P., 2001.- *Immunology of invertebrates: humoral*.- Encyclopedia of life sciences, John Wiley and Sons Ltd.
- GUPTA A. P., 2002.- *Immunology of invertebrates: cellular*.- Encyclopedia of life sciences, John Wiley and Sons Ltd.
- HALL M., SCOTT T., SUGUMARAN M., SÖDERHÄLL K., LAW J. H., 1995.- Proenzyme of *Manduca sexta* phenol oxidase: Purification, activation, substrate specificity of the active enzyme, and molecular cloning.- *Proceedings of the National Academy of Sciences of the USA*, 92: 7764-7768.
- HATTORI M., KONISHI H., TAMURA Y., KONNO K., SOGAWA K., 2005.- Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper, *Nephotettix cincticeps*.- *Journal of Insect Physiology*, 51: 1359-1365.
- HAYDAK M. H., 1936.- A food for rearing laboratory animals.- *Journal of Economic Entomology*, 29: 1026.
- HIRUMA K., RIDDIFORD L. M., 1988.- Granular phenoloxidase involved in cuticular melanization in the tobacco hornworm: Regulation of its synthesis in the epidermis by juvenile hormone.- *Developmental Biology*, 130 (1): 87-97.
- HUGHES A. L., 1999.- Evolution of the arthropod prophenoloxidase/hexamerin protein family.- *Immunogenetics*, 49: 106-114.
- HYRŠL P., ŠIMEK V., 2005.- An analysis of hemolymph protein profiles during the final instar, prepupa and pupa of the silkworm *Bombyx mori* (Lepidoptera, Bombycidae).- *Biologia*, 60 (2): 207-213.
- JIRAVANICHPAISAL P., LEE B. L., SÖDERHÄLL K., 2006.- Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization.- *Immunobiology*, 211 (4): 213-236.
- KOPÁČEK P., WEISE C., GÖTZ P., 1995.- The prophenoloxidase from the wax moth *Galleria mellonella*: Purification and characterization of the proenzyme.- *Insect Biochemistry and Molecular Biology*, 25 (10): 1081-1091.
- KRÄMER B., KÖRNER U., WOLBERT P., 2002.- Differentially expressed genes in metamorphosis and after juvenile hormone application in the pupa of *Galleria*.- *Insect Biochemistry and Molecular Biology*, 32: 133-140.
- LI J., ZHAO X., CHRISTENSEN B. M., 1994.- Dopachrome conversion activity in *Aedes aegypti*: Significance during melanotic encapsulation of parasites and cuticular tanning.- *Insect Biochemistry and Molecular Biology*, 24 (10): 1043-1049.
- LI D., SCHERFER C., KORAYEM A. M., ZHAO Z., SCHMIDT O., THEOPOLD U., 2002.- Insect hemolymph clotting: evidence for interaction between the coagulation system and the prophenoloxidase activating cascade.- *Insect Biochemistry and Molecular Biology*, 32: 919-928.
- MANDATO C. A., DIEHL-JONES W. L., MOORE S. J., DOWNER R. G. H., 1997.- The effects of eicosanoid biosynthesis inhibitors on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*.- *Journal of Insect Physiology*, 43 (1): 1-8.
- MEYLAERS K., FREITAK D., SCHOofs L., 2007.- Immunocompetence of *Galleria mellonella*: Sex- and stage-specific differences and the physiological cost of mounting an immune response during metamorphosis.- *Journal of Insect Physiology*, 53: 146-156.
- MÜLLER H. M., DIMOPOULOS G., BLASS C., KAFATOS F. C., 1999.- A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes.- *The Journal of Biological Chemistry*, 274 (17): 11727-11735.
- MUNOZ J. L., GARCÍA-MOLINA F., VARÓN R., RODRIGUEZ-LOPEZ J. N., GARCÍA-CÁNOVAS F., TUDELA J., 2006.- Calculating molar absorptivities for quinones: Application to the measurement of tyrosinase activity.- *Analytical Biochemistry*, 351: 128-138.
- NAPPI A. J., OTTAVIANI E., 2000.- Cytotoxicity and cytotoxic molecules in invertebrates.- *BioEssays*, 22 (5): 469-480.
- SIDERI M., TSAKAS S., MARKOUTSA E., LAMPROPOULOU M., MARMARAS V. J., 2008.- Innate immunity in insects: surface-associated dopa decarboxylase-dependent pathways regulate phagocytosis, nodulation and melanization in medfly haemocytes.- *Immunology*, 123 (4): 528-537.
- STANLEY D. W., MILLER J. S., 2006.- Eicosanoid actions in insect cellular immune functions.- *Entomologia Experimentalis et Applicata*, 119: 1-13.

- SUGUMARAN M., 2002.- Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects.- *Pigment Cell Research*, 15: 2-9.
- SUGUMARAN M., NELLAIAPPAN K., VALIVITTAN K., 2000.- A new mechanism for the control of phenoloxidase activity: Inhibition and complex formation with quinone isomerase.- *Archives of Biochemistry and Biophysics*, 379 (2): 252-260.
- THEOPOLD U., LI D., FABBRI M., SCHERFER C., SCHMIDT O., 2002.- The coagulation of insect hemolymph.- *Cellular and Molecular Life Sciences*, 59: 363-372.
- THEOPOLD U., SCHMIDT O., SÖDERHÄLL K., DUSHAY M. S., 2004.- Coagulation in arthropods: defence, wound closure and healing.- *Trends in Immunology*, 25 (6): 289-294.
- YATSU J., ASANO T., 2009.- Cuticle laccase of the silkworm, *Bombyx mori*: Purification, gene identification and presence of its inactive precursor in the cuticle.- *Insect Biochemistry and Molecular Biology*, 39: 254-262.
- ZHAO P., LI J., WANG Y., JIANG H., 2007.- Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by *Manduca sexta* phenoloxidase.- *Insect Biochemistry and Molecular Biology*, 37: 952-959.

Authors' addresses: Pavel HYRŠL (corresponding author, hyrsl@mail.muni.cz), Jana BENEŠOVÁ, Pavel DOBEŠ, Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic.

Received May 13, 2009. Accepted September 10, 2009.