

ACTIVITY OF SELECTED HYDROLASES IN ONTOGENY OF DRONE *Apis mellifera carnica*

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Received 23 March 2007; accepted 18 April 2007

S u m m a r y

An API ZYM test was used to examine the activity of 18 hydrolases in non-sealed and sealed brood and in freshly emerged drones of *Apis mellifera carnica*. In the entire experimental period, 10 enzymes were observed to be active and one to be inactive. The study indicated that the activity of enzymes was changing during drone development, i.e. seven of them were active only in selected developmental stages. The highest activity of most of the enzymes was observed in 4-day-old larvae and in freshly emerged drones, whereas the lowest level was at the stage of prepupa. In the pupae, the activity of hydrolases was subject to only minor fluctuations.

Keywords: *Apis mellifera carnica*, drone brood, drones, enzymes, hydrolases.

INTRODUCTION

Studies into bee metabolism address mainly adult individuals. Most of those studies refer to metabolic changes that accompany age-related and hormonally-mediated changes in worker behavior (Osashi et al. 1999, Costa and Cruz-Landim 2005, Elekonich and Roberts 2005, Roberts and Elekonich 2005). In contrast, there is sparse data depicting changes in metabolism at particular developmental stages of the bee brood (Hepburn et al. 1979, van der Vorst et al. 1983, Ivanova et al. 2000; Schmolz et al. 2005), especially including the drone brood.

Recently, Schmolz et al. (2005) evaluated the energetics and total metabolism rate of workers and drones based on calorimetric analyses and concluded on the nature of metabolic substrates used by their developmental forms in comb cells based on elementary analysis. Due to the research techniques applied and the results obtained, the conclusions reached by these authors

are rather general in character. Hence, they require confirmation with the use of other research methods, including biochemical methods.

In the current study, the authors attempted to determine, by means of an enzymatic test, whether and to what extent the results of calorimetric assays are reflected in the activity of enzymes linked with decomposition of biomolecules as suggested by Schmolz et al. (2005). A API ZYM test was chosen to enable simultaneous measurement of the activity of 18 hydrolases belonging to 3 subclasses: esterases, proteases and glycosidases, i.e. those acting on lipids, proteins and carbohydrates. This test was successively applied in earlier investigations referring to metabolic effects of *A. mellifera carnica* larvae infection with entomopathogenic nematodes (Żółtowska et al. 2003).

MATERIAL AND METHODS

The experimental material was a drone brood of honey bees of the *A. mellifera*

carnica subspecies, collected in the second half of May 2005. The brood originated from a 50-colony private apiary of Dr. Zbigniew Lipiński located ca. 30 km outside Olsztyn. During transportation, the combs were wrapped in slightly moist towels to provide appropriate temperature and humidity.

Directly after reaching the laboratory, drone brood was carefully isolated from drone comb cells and divided based on morphological traits (Jay 1962, 1963) into particular developmental stages. Non-sealed larvae were divided into: two- (L2), three- (L3), four- (L4) and six-day old larvae (L6), and the sealed brood were divided into: seven-day old larvae (L7) and spinning larvae (L8 and L9), prepupae (PP), pupae with white (P1), pale pink (P2), pink (P3), brown eyes and yellow trunk (P4) as well as pupae with dark eyes and dark trunk (P5). Investigations were also carried out for drones freshly emerged from cells (A).

The isolated brood was carefully dried in filter paper and weighed. The mean body weights of larvae at particular developmental stages reached: L2 – 4.6 ± 1.3 mg; L3 – 16.5 ± 5.3 mg; L4 – 37.3 mg; L6 – 293 ± 51 mg; L7 – 314 ± 43 mg; L8 – 336 ± 42 mg; L9 – 323 ± 25 mg; PP – 318 ± 63 mg; P1 – 296 ± 32 mg; P2 – 282 ± 43 mg; P3 – 275 ± 25 mg; P4 – 273 ± 57 mg; P5 – 264 ± 63 mg; A – 253 ± 48 mg. The material was stored at a temperature of -70°C until analysis.

Preparation of brood extracts Weighed portions of particular stages (250 mg each) were homogenized in a Potter glass homogenizer with 2.5 ml of 0.9% NaCl. Homogenate was centrifuged for 15 min at 900 x g, at a temperature of 4°C. In the supernatant, protein was determined with the method of Bradford (1976). Next, 65-μl

portions of the supernatant, containing ca. 100 μg of protein, were applied into wells of the API ZYM test kit by BioMérieux INC Hazelwood, USA. The kit contains substrates enabling a simultaneous analysis of the activity of 18 hydrolases (Table 1). Further procedures followed instructions of the producer. The activity of enzymes was determined after a 3-hour incubation at a temperature of 35°C and expressed in nmoles of the reaction product formed.

RESULTS

In all developmental stages of drone brood, 10 out of 18 hydrolases examined were active, whereas a lack of activity was observed for β-glucuronidase (Table 2). Seven other enzymes displayed activity in selected stages of development specific for them. For instance, the activity of esterase lipase (C8) was not observed only in the stage of L2 larva, whereas in the other stages the enzyme was moderately active. In turn, the activity of trypsin was observed at the end of the period of metamorphosis into stage P4, P5 as well as in freshly emerged drones, whereas chymotrypsin demonstrated weak activity as early as in the stage of four-day-old larva.

In contrast, the activity of cysteine arylamidase¹ was not observed in the two middle stages of development, i.e. spinning larva and prepupa (Table 2). A lack of activities of β-glucosidase and α-fucosidase was reported for the two-day-old larvae. These enzymes appeared to be active in the further stages of larval development, i.e. β-glucosidase was active only till the stage of prepupa (Table 2), whereas α-fucosidase (inactive in sealed brood until the stage of pupa with pale pink eyes (P2) inclusive) was again activated in older pupae (Table 2).

¹ Arylamidases are also known as aminopeptidases

Out of the esterases examined, in the period of larval development, the highest activity was observed for both phosphatases and esterase (C4). Over the entire developmental period of the brood, leucine arylamidase was found to be very active. The other peptidases and proteases usually displayed low and very low activity (Table 2). Out of the group of enzymes metabolizing saccharides, over the entire developmental period high activity was reported in the case of N-acetyl- β -glucosaminidase and β -galactosidase. In the sealed brood, also highly active were α -galactosidase and α -glucosidase (Table 2). The two latter enzymes, after the period of lower activity in the sealed brood, regained a very high activity in freshly emerged drones.

DISCUSSION

In the first ten days after hatching from the egg, drone larvae (Schmolz et al. 2005) grow very intensively (Winston 1987) since they are very intensively fed with food rich in saccharides, proteins and lipids (Schmolz et al. 2005). Hence, the high activity of hydrolases in the non-sealed brood is of no surprise. In the case of this brood, especially active were both phosphatases and leucine arylamidase, an enzyme hydrolysing N-terminal amino acids from peptides and proteins. Intensification of the activity of almost all analyzed enzymes was also observed in the four-day old larvae, which is likely to result from a change in the diet of drone larvae, as with

Table 1

Assayed enzymes and their substrates.

No.	Enzyme	Substrate	pH
Esterases			
1	Alkaline phosphatase	2-naphtyl phosphate	8.5
2	Acid phosphatase	2-naphtyl phosphate	5.4
3	Esterase (C4)	2-naphtyl butyrate	6.5
4	Esterase lipase (C8)	2-naphtyl caprylate	7.5
5	Lipase (C14)	2-naphtyl myristate	7.5
Peptidases and Proteases			
6	Leucine arylamidase	L-leucyl-2-naphtylamide	7.5
7	Valine arylamidase	L-valyl-2-naphtylamide	7.5
8	Cysteine arylamidase	L-cystyl-2-naphtylamide	7.5
9	Trypsin	N-benzoyl-DL-arginine-2-naphtylamide	8.5
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphtylamide	7.5
Glycosidases			
11	α -galactosidase	6-Br-2-naphtyl- α -D-galactopyranoside	5.4
12	β -galactosidase	2-naphtyl- β -D-galactopyranoside	5.4
13	β -glucuronidase	Naphtol-AS-BI- β -D-glucuronide	5.4
14	α -glucosidase	2-naphtyl- α -D-glucopyranoside	5.4
15	β -glucosidase	6-Br-naphtyl- β -D-glucopyranoside	5.4
16	N-acetyl- β -glucosaminidase	1-naphtyl-N-acetyl- β -D-glucosaminide	5.4
17	α -mannosidase	6-Br-2-naphtyl- α -D-mannopyranoside	5.4
18	α -fucosidase	2-naphtyl- α -L-fucopyranoside	5.4

Table 2
The activity of hydrolases in extracts of drone broods (nmol/mg⁻¹ proteins).

No.	Enzyme	Stage of development													
		L2*	L3	L4	L6	L7	L8	L9	PP	P1	P2	P3	P4	P5	A
Esterases															
1	Alkaline phosphatase	10	20	40	20	30	20	10	5	5	5	5	5	5	15
2	Acid phosphatase	10	20	30	10	20	25	25	5	5	5	10	10	20	40
3	Esterase (C4)	20	15	35	10	20	10	20	20	10	15	20	20	30	30
4	Esterase lipase (C8)	ND ^a	2.5	5	5	5	2.5	5	5	5	5	5	5	5	10
5	Lipase (C14)	ND	ND	2.5	5	10	10	15	5	10	10	10	10	10	10
Peptidases and Proteases															
6	Leucine arylamidase	40	30	40	40	40	40	40	20	20	20	20	20	30	40
7	Valine arylamidase	5	5	10	5	5	5	5	2.5	2.5	2.5	5	5	5	5
8	Cysteine arylamidase	5	5	10	5	5	5	ND	ND	2.5	5	5	5	5	5
9	Trypsin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	5	5	5
10	Chymotrypsin	ND	ND	2.5	2.5	5	5	5	5	15	10	10	10	5	5
Glycosidases															
11	α -galactosidase	10	20	30	10	20	25	25	5	5	5	10	10	20	40
12	β -galactosidase	10	10	20	10	15	20	15	20	20	15	30	20	30	10
13	β -glucuronidase	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	α -glucosidase	10	20	40	10	10	15	10	5	2.5	2.5	2.5	10	15	30
15	β -glucosidase	ND	5	5	15	20	20	20	ND	ND	ND	ND	ND	ND	ND
16	N-acetyl- β -glucosaminidase	20	30	30	25	20	30	20	40	30	25	20	20	20	30
17	α -mannosidase	2.5	10	20	5	5	5	5	2.5	2.5	ND	2.5	2.5	2.5	2.5
18	α -fucosidase	ND	2.5	2.5	2.5	ND	ND	ND	ND	ND	ND	2.5	2.5	5	10

a – Not detected, * - see in Material and Methods section

bee larvae, from royal jelly into honey-pollen brood food richer in proteins and lipids. (Pollen, as demonstrated by among others Loidl and Crailsheim (2001), is an important source of lipids to bees.) In this period, enzymes decomposing lipids (being esters of high fatty acids (lipase C14)), as well as chymotrypsin (decomposing proteins) are activated for the first time. Their lack in an earlier phase of development may indicate that specific substrates for those enzymes do not occur in drone larvae, which has been confirmed for lipids (Van der Vorsta et al. 1983). Another explanation may be the assumption that royal jelly contains components partly digested

by enzymes of hypopharyngeal glands of nurse bees, hence the larvae do not require such a rich set of digestive enzymes (Ohashi et al. 1999, Loidl and Crailsheim 2001, Santos et al. 2005).

The activity of enzymes decomposing carbohydrates, including: α -glucosidase, α -galactosidase, β -glucosidase, was especially high in larval stages, which is consistent with the findings of Schmolz et al. (2005) and the remarkably earlier work of Bishop (1925) that the metabolism of larvae of this developmental period is mainly linked with metabolism of carbohydrates. The activity of this group of enzymes increases in spinning larvae, which may be

associated with higher energy expenditure on cocoon shell generation and necessity of expressing stereotypical spinning moves. No significant changes were observed, in turn, between the sealed and non-sealed larvae in the activity of enzymes from the group of peptidases and proteases (except for cysteine arylamidase) and lipases (C8 and C14).

In the prepupa stage, the activity of most of the determined hydrolases was low, which is consistent with the results reported by Schmolz et al. (2005) who in the same period observed a considerable decrease in the rate of metabolism, measured by an energy production index. The period of metamorphosis is connected with complete reconstruction of internal structures (Bishop 1923 b). A substantial increase was then expected in the activity of histolytic enzymes linked mainly with proteolysis of larval tissues, yet the results of the test applied only partially confirmed the current results. Only a 2-3-fold increase was observed in the activity of chymotrypsin, indicating the appearance of trypsin activity. α -fucosidase appeared to be re-activated, whereas high activity was observed in the case of N-acetyl- β -glucosaminidase and β -galactosidase, i.e. enzymes linked with the removal of saccharide chains from glycoproteins and glycolipids.

In the final stage of metamorphosis, especially in freshly emerged drones, the level of hydrolases activity was elevated in most cases. It points to adaptation of their enzymatic organ to independent life and is consistent with the findings of Schmolz et al. (2005) referring to a considerably higher rate of metabolism of an adult drone as compared to brood.

In observing the distribution of the activity of hydrolases examined, it can be noticed that most of the enzymes reach the maximal activity in larvae of stage L4. A number of hydrolases display diminished activity just before sealing: for as many as

eight enzymes it was the lowest in the stage of prepupa. Usually, in the sealed larvae and pupae, the level of hydrolases activity was subject only to slight fluctuations. A re-increase in the metabolic activity occurs at the end of metamorphosis, hence enzymes of the emerged drones display appropriately high activity. These observations reflect changes in brood feeding as well as in transformation of its metabolism from changes of carbohydrates (the main source of energy in the larval stage) into lipids and proteins in the period of metamorphosis (Bishop 1925, Schmolz et al. 2005).

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AKTYWNOŚĆ WYBRANYCH HYDROLAZ W ROZWOJU OSOBNICZYM TRUTNIA *Apis mellifera carnica*

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S t r e s z c z e n i e

Badano aktywność 18 hydrolaz rozkładających białka, tłuszcze i cukry u rozwijającego się czerwiu oraz u świeżo wygryzionych trutni *Apis mellifera carnica*. Do badań zastosowano test API ZYM. W całym badanym okresie ontogenezy aktywne było 10 hydrolaz, brak było α -glukuronidazy. Siedem enzymów ujawniało swoją aktywność tylko u niektórych stadiów rozwojowych. Należą do nich lipazy C8 i C14, chymotrypsyna i tripsyna, mannozydaza, fukozydaza i β -glukozydaza. Zauważono, że większość enzymów uzyskuje maksymalną aktywność u larw stadium L4. Wiele hydrolaz ma obniżoną aktywność tuż przed zasklepieniem, aż dla ośmiu enzymów była ona najniższa w stadium przedpoczwarki. Na ogół u zasklepiionych larw i poczwarek poziom aktywności hydrolaz podlegał tylko niewielkim wahaniom. Ponowny wzrost aktywności metabolicznej następuje pod koniec metamorfozy, tak że wygryzione osobniki mają enzymy odpowiednio wysoko aktywne. Spostrzeżenia te współgrają ze zmianami w odżywianiu czerwiu oraz w przedstawieniu jego metabolizmu z przemian węglowodanów, będących głównym źródłem energii w okresie larwalnym, na lipidy i białka w czasie metamorfozy.

Słowa kluczowe: *Apis mellifera carnica*, czerw trutowy, trutnie, enzymy, hydrolazy.