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Leucine aminopeptidase activity in the midgut of nosema diseased honeybees (*Apis mellifera*)

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ABSTRACT

The aim of this study was to determine leucine aminopeptidase (LAP) activity in the midgut of nosema diseased honeybees. *Nosema* sp. spores were confirmed in 60% of the coprologically examined individual honeybees. For laboratory testing 100 honeybees were collected randomly from three beehives. LAP enzyme activity in the midgut of the honeybees was assessed qualitatively, based on the intensity of staining in histological preparations. In bees in which the midgut coprological examination did not reveal *Nosema* sp. spores, the epithelial cells were red-purple stained, and all the layers of the walls of the midgut were visible. The intensity of honeybees naturally infected with microsporidia *Nosema* sp. increased while the intensity of the staining gradually decreased, showing progressively less pronounced LAP enzyme activity. Also there was an increasing number of damaged epithelial cells. Therefore, nosema disease can be considered as the disease of choice for studying LAP enzyme activity and determining the degree of microsporidium infection.

Key words: leucine aminopeptidase, honeybee (*Apis mellifera*), nosema disease

Introduction

Nosema disease is one of the most important parasitic diseases of adult honeybees caused by the microsporidia *Nosema apis* (ZANDER, 1909) and *Nosema ceranae* (FRIES et al., 1996). Due to the serious honeybee mortality in recent years, interest in the research of honeybee diseases, including nosema disease, is increasing. Pathological changes caused by *N. ceranae* in *Apis mellifera* have not been sufficiently investigated. The disease is

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known to cause metabolic disorders and consequently premature mortality of forager honeybees and rapid reduction in the number of honeybees in the diseased honeybee colonies (HIGES et al., 2007; MARTIN-HERNANDEZ et al., 2007; OLDROYD, 2007). *N. apis* and *N. ceranae* are very similar morphologically, so it is not possible to differentiate their spores using the routine diagnostic method of microscopic examination of native preparations.

Microsporidia of the genus *Nosema* invade the midgut epithelial cells of honeybees as a parasite to multiply and form spores. The honeybee midgut has a digestive function (TOMAŠEC, 1949; SULIMANOVIĆ et al., 1995; SONDGRASS and ERICKSON, 2005). The wall is built of longitudinal and transverse muscle layers, a base membrane and, laterally toward the lumen, a strongly developed layer of cylindrical epithelial cells placed in numerous transverse folds that increase the digestive surface and allow greater expansion (SONDGRASS and ERICKSON, 2005). The epithelial cells are coated with a cross-striped edge formed by plasma layer conversion, which, surrounds the contents of the midgut as a gentle cylindrical sheath and allows the flow of digestive juices and enzymes secreted by the epithelial cells to the content, and the flow of digested food to the wall of the intestine for resorption (TOMAŠEC 1949; SONDGRASS and ERICKSON, 2005).

Ingested with food or water, the spores *Nosema* sp. quickly reach the midgut (KELLNER and JACOBS, 1978), where they germinate, and movable vegetative forms penetrate the perithrophic membrane and enter the epithelial cells. They can multiply in favorable conditions within the cells (Van LAERE, 1977) which leads to cell reduction in terms of calcium phosphate concentration (BAILEY, 1955) and ribonucleic acid synthesis (HARTWIG and PRZELECKA, 1971), indicating the reduced secretion of digestive enzymes. The cells show signs of lysis and vacuoles, and glycogen and ribosomes accumulate (LIU, 1984) and decay in five days (KELLNER and JACOBS, 1978). Aminopeptidases are digestive enzymes that preferentially catalyze the hydrolysis of leucine residues at the N-terminus of peptides and proteins. So they are very important for pollen digestion, which is the main protein source of the honeybee's diet.

The aim of this study was to determine leucine aminopeptidase (LAP) activity in the midgut of nosema diseased honeybees originating from conventional honeybee colonies.

Materials and methods

Honeybee collection for laboratory testing. The samples of gray European honeybees from three randomly selected beehives of the Langsthroot-Rooth type, with apiaries located in the continental part of the Republic of Croatia, were taken in late February to be examined for nosema disease. All bee colonies were fed in the fall (autumn) for the winter with sugar syrup (1:1). Inspections during the winter found a few dead bees on the hive bottom. After opening the hive, we took long tweezers and randomly collected

bees, and placed them, one by one, in plastic 2.5 cm diameter Petri dishes. The covers had pre-drilled holes for the passage of air. We took 100 bees from each hive. Petri dishes with honeybees from the first hive were labeled with the numbers 1-100, from the second hive 101-200 and from the third hive from 201 to 300. We pasted the lids with tape and delivered them to the laboratory in a cardboard box.

Laboratory tests for Nosema sp. spores and the midgut sampling. In the laboratory, we examined whether the honeybees were alive, and then, after they had defecated in the Petri dishes, we undertook coprological testing of the contents of dung. The time to excrete feces was different for each bee, varying from a few minutes to two hours.

We used an Olympus CX21 microscope, using phase contrast and 400x magnification. We labeled samples in which the spores were not found negative (-) and where they were found we marked them positive, depending on the number of spores in the visual field, with one (+) to three crosses (+++). One cross (+) meant 5-10 spores of *Nosema* sp. in one visual field, two crosses (++) medium-strong infection with 11-25, and three crosses (+++) severe infection with 26-50 spores in one visual field. Honeybees with feces were sedated by ether, and then one by one had their intestine slowly and carefully pulled out. We held their thorax and abdomen with larger tweezers, while holding and pulling the last visible abdomen scale with small anatomical tweezers.

Preparation of histological preparations to determine leucine aminopeptidase enzyme activity. We prepared frozen sections of the intestinal tissue of medium thickness of 6-9 microns to determine the enzyme LAP, in accordance with the procedure of HRAPCHAK and SHEEHAN (1980). We incubated the bees' midgut tissue in a nutrient medium for 60 minutes at 37 °C, washed it in a 0.85% solution of sodium chloride (2-3 min), placed it for two minutes in 0.1 M copper sulfate, and again washed it in 0.85% sodium chloride solution. Samples were then dehydrated in increasing concentrations of alcohol, cleared in xylene, and fixed in Canada balsam to obtain the histological preparations. Control sections were incubated in the same medium without substrate. Also, separate sections of intestinal tissue of the coprologically negative bees were used as a control. LAP enzyme activity was determined by qualitative microscopic examination (Olympus BX41 microscope) with 10 to 20x magnification, and photographed with an Olympus DP12 U-TVO camera. Sites of LAP activity in the preparations stained reddish-purple. Sites of greater activity of this enzyme stained darker, while the sites of weaker activity manifested themselves in brighter shades of reddish-purple.

Results

All the honeybees were alive upon their delivery to the lab. Coprological tests for nosema disease in winter bees from three hives from apiaries located in the continental part of the Croatia found 40% negative samples among the individual honeybees, and *Nosema* sp. spores were confirmed in 60% from mildly to severely infected (positive) individual honeybees, as shown in Table 1.

Table 1. The number and percentage of honeybees examined for nosema disease. The numerator indicates the number of negative or positive, and the denominator the total number of honeybees examined for nosema disease.

Honeybees examined for nosema disease										
	Negative		Positive							
			+		++		+++		Total	
Hive	Number	%	Number	%	Number	%	Number	%	Number	%
1	30/100	30.00	40/100	40.00	30/100	30.00	0/100	0.00	70/100	70.00
2	40/100	40.00	40/100	40.00	20/100	20.00	0/100	0.00	60/100	60.00
3	50/100	50.00	40100	40.00	00/100	0.00	10/100	10.00	50/100	50.00
Total 1+2+3	120/300	40.00	120/300	40.00	50/300	16.67	10/300	3.33	180/300	60.00

Upon examination of the histological preparations of the midgut of honeybees, in which coprological examination did not find *Nosema* sp. spores (-), all the layers of the walls of the midgut were visible (Fig. 1). Epithelial cells were stained reddish-purple. In the histological preparations of the midgut of mildly infected honeybees, we found preserved muscle and some preserved as well as some damaged epithelial cells (Fig. 2). In the midgut of the moderately infected bees (++) epithelial cells were stained a lighter shade of reddish-purple, and the muscle was also preserved (Fig. 3). On histological preparations of the midgut of severly infected honeybees (+++) we found thin muscle, lightly stained and entirely destroyed epithelial cells and individually lighter stained regeneratory cells (Fig. 4).

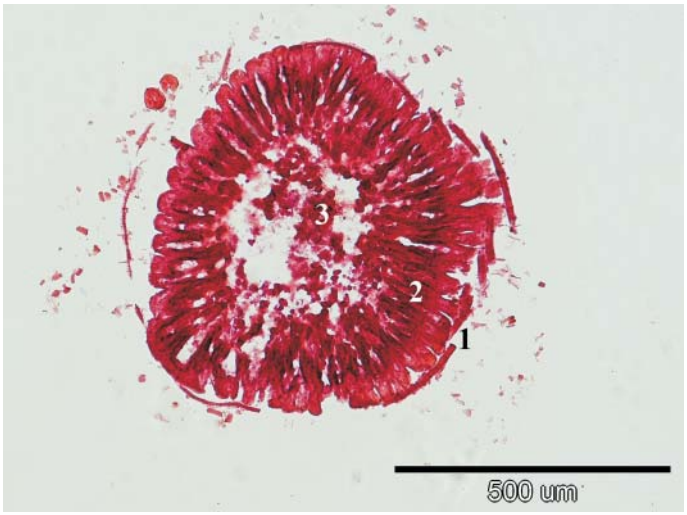

Fig. 1. Leucine aminopeptidase enzyme activity in the midgut of honeybees with no *Nosema* sp. spores found (-), enlarged ×10 (1 - activity of the enzyme in the muscle layer, 2 - enzyme activity in epithelial cells, 3 - content of the midgut)

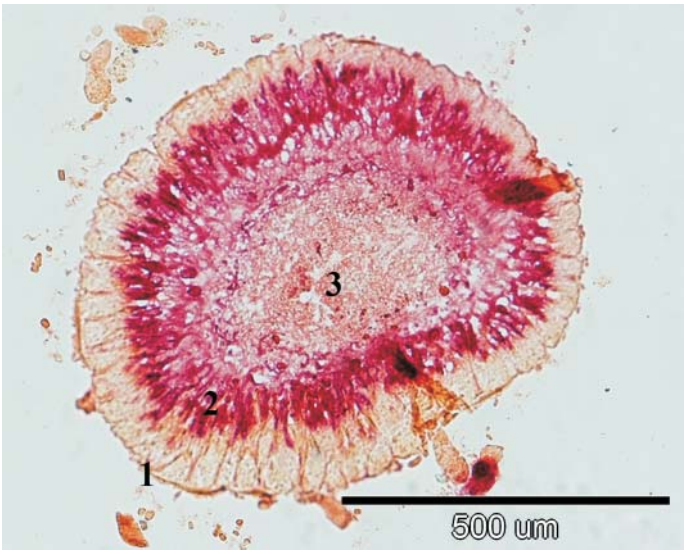

Fig. 2. Leucine aminopeptidase enzyme activity in the midgut of honeybees with *Nosema* sp. spores confirmed (+), enlarged ×10 (1 - activity of the enzyme in the muscle layer, 2 - enzyme activity in epithelial cells, 3 - content of the midgut)

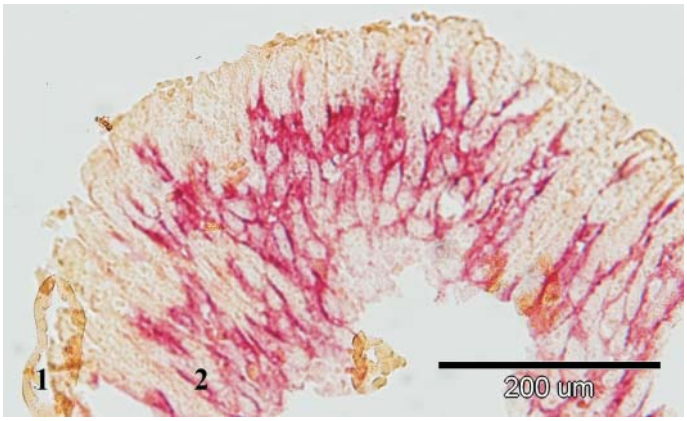

Fig. 3. Activity of leucine aminopeptidase the midgut of honeybees infected with *Nosema* sp. spores confirmed (++), increased ×20 (1 - activity of the enzyme in the muscle layer, 2 - enzyme activity in epithelial cells)

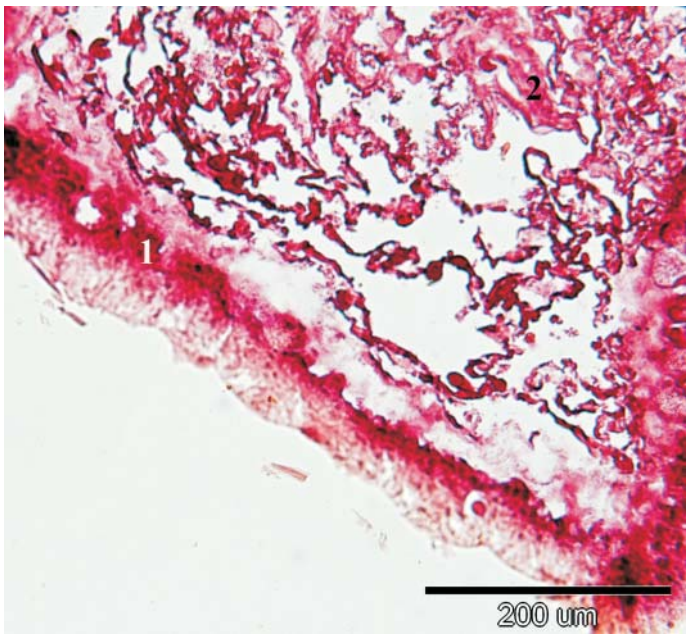

Fig. 4. Leucine aminopeptidase enzyme activity in the midgut of honeybees with *Nosema* sp. spores confirmed (+++), enlarged ×20 (1 - enzyme activity in epithelial cells, 2 - content of the midgut)

Discussion

Of all amino peptidases (proteolytic enzymes), the LAP enzyme secreted by midgut epithelial cells, has a particularly important role in the digestion of proteins in the honey bee (MALONE and GATEHOUSE, 1998). Proteolytic enzymes are most active during the first seven days of life of honeybees (young bees), and are necessary for the digestion of proteins. Of the food consumed by honeybees, pollen is the main source of protein. Since microsporidia genus *Nosema* parasites in the honeybees' midgut epithelial cells destroy them, and healthy cells secrete the enzyme LAP, our research had to examine honeybees by coprological tests for *Nosema* sp. spores, and, based on the intensity of staining of histological preparations, had to determine LAP activity in the negative (healthy) honeybees and in those infected with varying intensity.

The research was conducted in winter, on long-living honeybees (collected in February). According to CHRISTELLER and SHAW (1989) and MALONE and GATEHOUSE (1998), the activity of proteolytic enzymes and LAP in these honeybees should be less than that in young, healthy honeybees during the first seven days of age. Our findings showed most pronounced LAP activity in coprologically confirmed, negative honeybee samples (free of *Nosema* sp. spores), based on the most intensely reddish-purple stained epithelial cells, when compared to infected honeybees.

While MALONE and GATEHOUSE (1998) assessed the level of the enzyme LAP (using the CHRISTELLER and SHAW (1989) method) quantitatively, we assessed it qualitatively, based on the intensity of staining of histological preparations. In the available literature, we found information on different LAP activity in healthy and diseased honey bees (MALONE and GATEHOUSE, 1998), but we did not find data on the intensity of LAP activity according to the degree of infection of individual honeybees, or within honeybees naturally infected by *Nosema* sp. Our findings of a gradual decrease in intensity of the staining of epithelial cells from mildly (+) to severely (+++) infected honeybees, also show progressively less pronounced LAP enzyme activity, which is consistent with the results of CHRISTELLER and SHAW (1989) and MALONE and GATEHOUSE (1998).

Namely, the histological preparations of the mildly infected honeybees (+) showed that both darker and brighter reddish-purple staining indicated stronger and less pronounced LAP enzyme activity, i.e. the existence of undamaged and damaged parts of cells, which was not expressed in the severely infected honeybees (+++), due to the complete destruction of epithelial cells.

In conclusion, the results of our study show that LAP enzyme activity decreases inversely with the degree of the honeybees' infection with the micriosporidia genus *Nosema*. Therefore, nosema disease, regarding the site of parasitism and the destruction of epithelial cells in the midgut of honeybees, and thus decreased LAP activity, may be considered as a disease to choose to study LAP enzyme activity and determine the degree of infection of honeybees.

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MATAŠIN, Ž., S. NEJEDLI, I. TLAK GAJGER: Aktivnost leucin-aminopeptidaze u srednjem crijevu nozemoznih medonosnih pčela (*Apis mellifera*). Vet. arhiv 82, 599-607, 2012.

SAŽETAK

Cilj ovog istraživanja bio je odrediti aktivnost leucin-aminopeptidaze (LAP) u srednjem crijevu nozemoznih medonosnih pčela (*Apis mellifera*). Koprološkom pretragom na nozemozu, spore *Nosema* sp. potvrđene su u 60% pretraženih pojedinačnih medonosnih pčela. Za laboratorijske pretrage slučajnim odabirom skupljeno je po 100 medonosnih pčela iz triju košnica. Aktivnost enzima LAP u srednjem crijevu pčela određena je kvalitativno na osnovi jačine obojenja u pripremljenim histološkim preparatima. U pčela u kojih koprološkom pretragom u srednjem crijevu nisu utvrđene spore *Nosema* sp. epitelne stanice bile su crveno-purpurno obojene i svi su slojevi stijenke srednjega crijeva bili vidljivi. Jačina prirodne invazije invadiranih medonosnih pčela mikrosporidijama *Nosema* sp. se povećavala, dok se jačina obojenja postupno smanjivala što ukazuje na postupno slabije izraženu aktivnost enzima LAP. Također je utvrđeno i sve više propalih epitelnih stanica. Zbog toga se nozemozu može smatrati bolešću od izbora za utvrđivanje aktivnosti enzima LAP i utvrđivanje stupnja invadiranosti mikrosporama.

Ključne riječi: leucin-aminopeptidaza, medonosna pčela, *Apis mellifera*, nozemoza