Apidologie 29 (1998) 579–584

Original article

Acid phosphatase activity in the midgut of honeybee (*Apis mellifera* L.) larvae

Ales Gregorc[a*], Ivor D. Bowen[b], Azra Pogacnik[a]

[a] Veterinary faculty of the University of Ljubljana, Gerbiceva 60, 1000 Ljubljana, Slovenia
[b] School of Pure Applied Biology, University Wales, Cardiff, P.O. Box 915, Cardiff, UK

(Received 21 November 1997; accepted 11 May 1998)

Abstract – Acid phosphatase activity has been used to characterise lytic activities within honeybee larvae midgut cells. Significant nascent or free acid phosphatase activity was found in the midgut of 2-, 3-, 3.5- and 5-day-old honeybee larvae. Free acid phosphatase in the cytosol of the midgut cells appeared to be a prelude to cellular autolysis. The source of free acid phosphatase activity was not lysosomal as there was no sign of acid phosphatase activity spreading or leaking from lysosomes. The fine structural localization of acid phosphatase in lysosomes and cytoplasm in honeybee larvae was compared with findings previously reported in other insects. © Inra/DIB/AGIB/Elsevier, Paris

***Apis mellifera* / honeybee larvae / midgut / histochemistry / cytochemistry / acid phosphatase**

1. INTRODUCTION

Acid hydrolases can be used as an indicator of histolysis in a range of insects [4]. Acid phosphatase as a lysosome marker and free acid phosphatase activity have been determined histochemically in haemocytes and salivary glands [1], fat body [6] and salivary glands of metamorphosing *Calliphora erythrocephala* [3], in the midgut of *Drosophila auraria* larvae [7] and in the ventriculus of 5- and 30-day old adult worker honeybees, *Apis mellifera* [13]. Cytochemical analyses of acid phosphatase were also carried out during physiological cell death in the midgut of *Calliphora vomitoria* larvae [20], in developing *Heliothis armigera* midgut [5] and in the salivary glands of *Drosophila melanogaster* larvae [14]. Scanning electron microscope with backscattered electron imaging was also employed to demonstrate the morphology of the honeybee larvae midgut [8]. Programmed cell death in the midgut epithelial cells of *Apis mellifera* larvae was described by showing acid phospatase activity associated with endonuclease released histone groups: H1, H2A, H2B, H3, H4 in

* Correspondence and reprints
E-mail: gregorAl@mail.vf.uni.lj.si

healthy larvae [9] and characteristic acid phosphatase distribution in *Bacillus larvae* infected larvae [10]. The exoplasmic acid phosphatase is associated with programmed cell death as found in salivary glands of developing *D. melanogaster* [14]. Free acid phosphatase is an indicator of cellular autolysis [20].

In this paper, we report the demonstration of acid phosphatase activity in different tissues in honeybee larvae. It is essential to study acid phosphatase activity in honeybee larvae to establish premorphological changes leading to physiological cell death. The pattern of acid phosphatase activity in healthy larvae could be compared to findings in honeybee larvae exposed to different stresses in the environment.

2. MATERIALS AND METHODS

Honeybee, *Apis mellifera* L., larvae were taken from a Buckfast colony at the University of Wales apiary in Summer 1996. A confined queen in a queen excluder cage was allowed to lay eggs in an empty comb. After 1 day the comb with eggs was removed from the cage and larvae were sampled subsequently at intervals of about 12 h.

Three larvae of each ages were sampled and prepared for light histochemistry and electron cytochemistry. Larvae were fixed for light microscopy in a 9:1 v/v mixture of neutral buffered formalin and acetone and then placed into methacrylate (HEMA) monomer and embedded into the mixture of the monomer (5 mL) and activator (0.1 mL) [18]. Sections of 2 and 3 μm were cut on a Bright 5 030 microtome, using a Ralph-type glass knife. Acid phosphatase was demonstrated using the techniques of Hussein et al. [12], Jones and Bowen [4], Kela and Bowen [15] and Gregorc and Bowen [9]. Sections were incubated in a medium where naphthol AS-TR phosphate (Sigma chemicals) was used as substrate and Fast red violet (Sigma chemicals) as a coupler. For demonstrating acid phosphatase, the medium operated at pH 4.8. As a control, 1.25 mL of 0.1 M sodium fluoride was added to the incubation medium to inhibit acid phosphatase. Sections were examined by a Nikon light microscope under 1 000 ×, 1 250 × and 1 500 × magnification.

Midguts of larvae were removed and fixed for electron cytochemistry as described by Ryder and Bowen [19]. The apical and caudal parts of the larvae were cut and released midgut was removed through caudal opening. The midgut tissue was prefixed in 3 % glutaraldehyde in 0.2 M cacodylate buffer. The tissue was then incubated in the medium which consisted of 10 mg of p-nitrophenyl phosphate (Sigma 104 tablets),

Figure 1. In the brush border of midgut epithelial cell, there is membrane-bound acid phosphatase activity (▼). In the cell cytoplasm, there is also vacuolar acid phosphatase activity (➨). Peritrophic membrane (pm) and pollen grains appear in the midgut (P) of 5-day-old larvae. Light micrograph of a methacrylate section; magnification: 1 000 ×.

Figure 2. Membrane-bound (▼) acid phosphatase activity in the columnar epithelial cell. In the cytoplasm of regenerative cells (**O**), there is no enzyme activity. Three-and-a-half-day-old larvae. Light micrograph of a methacrylate section; magnification: 1 500 ×.

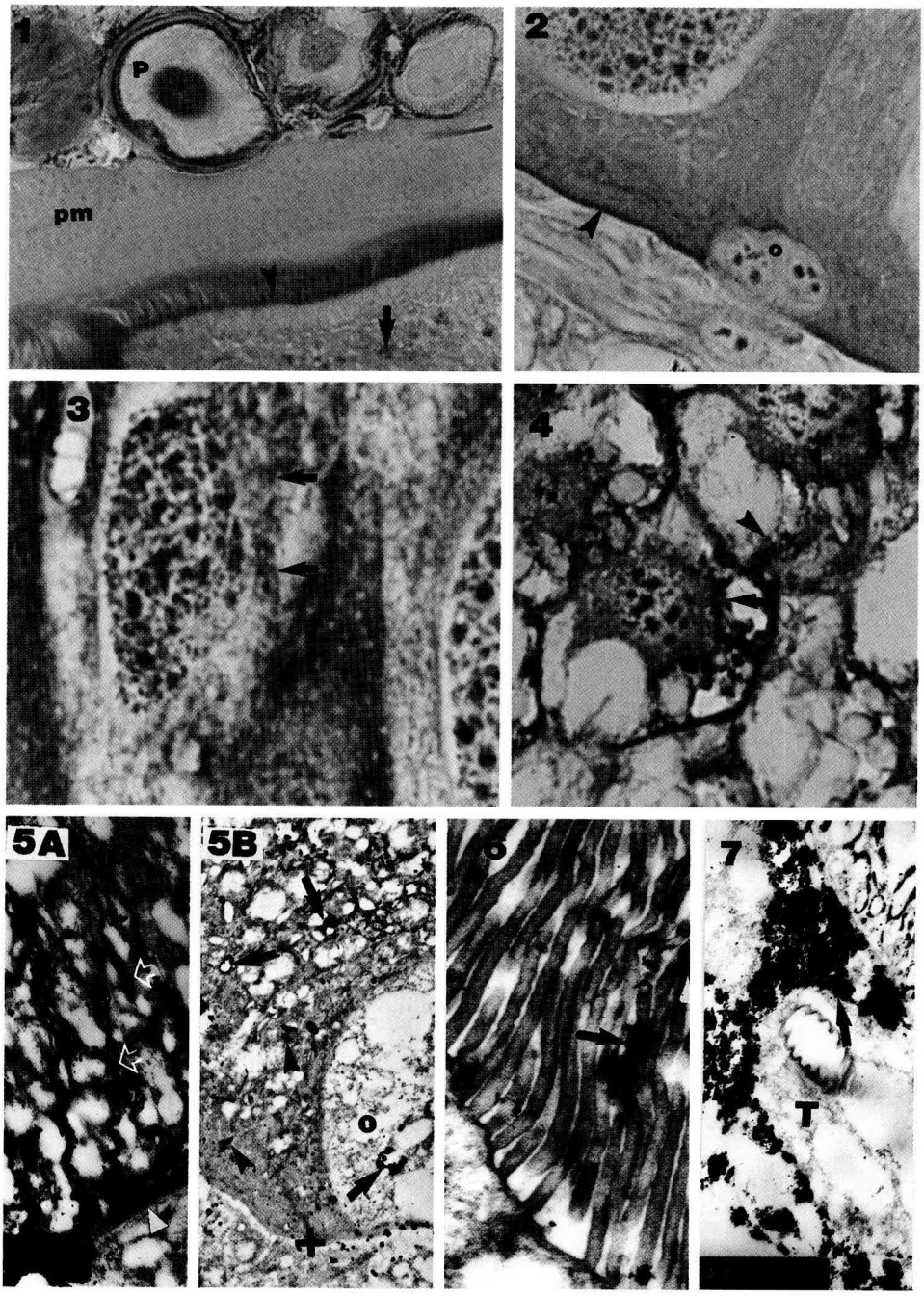
Figure 3. Free acid phosphatase activity (➨) in the cytoplasm of the columnar epithelial cell. Three-day-old larvae. Light micrograph of a methacrylate section; magnification: 1 250 ×.

Figure 4. Vacuolar (➨) and free (▼) acid phosphatase activity in fat body cells. Three-day-old larvae. Light micrograph of a methacrylate section; magnification: 1 250 ×.

Figure 5. Three-and-a-half-day-old larvae. Transmission electron micrograph. Panel A: reaction product denoting intense acid phosphatase activity outside the endoplasmic cisternae (▼). Basal lamina (▼) of the columnar midgut epithelial cell. Panel B shows vacuolar (➨) and free acid phosphatase activity (▼) in the midgut epithelial cell. In the regenerative cell (**O**), there is no free enzyme activity. Basal lamina of the midgut (✚) magnification: 6 400 ×.

Figure 6. Acid phosphatase activity (➨) in the microvillar membrane of the columnar cell brush border of a 2-day-old larvae. Transmission electron micrograph; magnification: 6 400 ×.

Figure 7. Acid phosphatase activity (➨) in the haemocoel surrounding the tracheoblast cell (T). Two-day-old larvae. Transmission electron micrograph; magnification: 8 000 ×.


1
P
pm
2
o
3
4
5A
5B
o
6
7
T

10 mL of 0.1 M acetate buffer at pH 4.8, and 10 mg of lead acetate.

After incubation for 0.5 h, the tissue was post-fixed in Millonig's osmium tetroxide (Taab Laboratories). The material was dehydrated in a series of alcohols of 30 to 100 % each for 0.5 h, and embedded in Araldite. Control tissue was incubated in a medium without the substrate and also in a medium where 0.1 M sodium fluoride was added as an inhibitor. Ultrathin sections were cut on LKB ultramicrotome with glass knife and were placed on copper grids. Sections were then counterstained for 1.5 h in uranyl acetate in alcohol and then for 2 min in lead citrate. Sections were examined by transmission electron microscope (JEOL 100S) under 6 400 × and 8 000 × magnification.

3. RESULTS

Acid phosphatase activity was found in the midgut of honeybee larvae of all examined ages, from 2- to 5-day-old larvae. Extensive enzyme activity was found in the area of the brush border (*figure 1*) and in the basal area of the midgut epithelial cells (*figure 2*).

Acid phosphatase activity was bound to apical and basal membrane and found also in the cell cytoplasm. This was largely non-vacuolar or free acid phosphatase activity (*figures 3, 5A*). In the columnar epithelial cells of 3.5-day-old larvae, specific lead reaction product was free in the cell cytoplasm. In those cells, vacuolar acid phosphatase activity and also non-vacuolar activity or free acid phosphatase activity were found (*figure 5A, B*). The cells with free acid phosphatase activity were vacuolated, and some were clearly autophagic. No free acid phosphatase was found in the cytoplasm of regenerative cells of all larval ages (*figures 2, 5B*). There, the enzymatic activity was confined to the vacuolar system (*figure 5B*). Vacuolar and free acid phosphatase activity was found in the fat body cells of the haemocoel in 3-day-old honeybee larvae (*figure 4*).

In midgut epithelial cells, the vacuolar enzymatic activity was most intensive in the apical cell region (*figure 1*), where there was more intensive phagocytic cell activity and also enzymatic activity in secondary lysosomes. An apparent intensive exchange of digestive and excretory material was recorded across the apical and basal membrane of epithelial cells.

Specific lead phosphate reaction product was detected membrane-bound in the brush border of the midgut epithelium in the larvae of all ages (*figure 6*). Extracisternal reaction product was found predominantly surrounding the rough endoplasmic reticulum (*figure 5A*). Free acid phosphatase activity was established also in the basal area of the midgut epithelial cells and in the haemocoel (*figure 7*).

4. DISCUSSION

In most individual epithelial cells, acid phosphatase activity was enclosed within the lysosomal system. The function of epithelial cells of the midgut is secretion of digestive enzymes and absorption of nutritive components from the midgut [11]. The cytoplasm of these cells abounded with rough endoplasmic reticulum and free ribosomes. Different types of secretion are described in the midgut epithelial cells of adult worker honeybees [13] and in *Stomoxys calcitrans* [17]. Previously published results of cytochemical analysis show that the process of epithelial cell secretion and extrusions are physiological and not an artefact [16]. The epithelial cells with indicative extrusion from the epithelial surface show almost no membrane-bound acid phosphatase activity. But in the cytoplasm of those cells, there is significant vacuolar hydrolase activity. Histochemical and cytochemical findings of acid phosphatase activity in epithelial brush border is indicative for intensive metabolic function of the midgut epithelial cells of honeybee larvae. Secretory granules are mainly in the apical part of the epithelial cell, and secretion takes place over all the epithelial surface and also

at the apical part of intercellular junctions [2].

The activity of free acid phosphatase in the cytosol is apparently not the result of injured lysosomal membranes, but is synthesized de novo and may be genetically determined [4]. Incidence of free acid phosphatase activity in the midgut was not found to be associated with ecdysis, which occurs about every 24 h during larval development. Cell death was shown associated with free acid phosphatase activity and the endonuclease released - histone group proteins [9]. Appearance of free acid phosphatase associated with cell lysis derives from physiological processes in the midgut epithelial cells. Our histochemical and cytochemical observations demonstrated that the occurrence of acid phosphatase in honeybee larvae was lysosomal and extracisternal. Acid phosphatase activity as a marker of lytic activity could be used for further investigation of pathogen and non-pathogen effects on honeybee larvae in the field.

ACKNOWLEDGEMENTS

The authors thank Mrs Carole Winters, Mr Gareth Lewis and Mr Mike Turner for their technical assistance. Thanks are also due to professors Milan Pogacnik and Andrej Bidovec from Veterinary Faculty University of Ljubljana for support during the research and to Ministry for Science and Technology for a research grant.

Résumé – Activité de la phosphatase acide dans l'intestin moyen des larves d'abeilles (*Apis mellifera* L.). L'activité de la phosphatase acide en tant qu'indicateur de la destruction des cellules a été utilisée dans des études d'histochimie et de cytochimie chez des larves d'abeilles saines. Une technique d'inclusion dans le méthacrylate, avec le phosphate AS-TR de naphtol comme substrat pour l'activité de la phosphatase acide, a été utilisée pour l'étude en microscopie optique et le phosphate de *p*-nitrophényle a été utilisé comme substrat pour les études cytochimiques en microscopie électronique. La localisation structurale fine de la phosphatase acide dans les lysosomes et dans le cytoplasme des larves d'abeilles a été comparée avec les résultats d'études antérieures portant sur d'autres insectes en cours de développement. Une activité phosphatase acide libre (PAL) se manifeste à des âges variées de la vie larvaire dans le cytoplasme des cellules de l'épithélium de l'intestin moyen et plus particulièrement dans les lysosomes. Cette activité est plus intense à proximité de la lame basale séparant l'épithélium de l'hémocœle. La PAL dans le cytosol des cellules épithéliales semble précéder l'autolyse cellulaire et la mort cellulaire physiologique. La source de l'activité de la PAL ne se trouve pas dans les lysosomes puisqu'il n'y avait aucun signe d'activité de la PAL diffusant à partir des lysosomes. L'activité de la phosphatase acide ainsi décrite pourrait être utilisée comme modèle de la distribution physiologique de l'enzyme dans l'intestin moyen des larves d'abeilles saines.

***Apis mellifera* / larve / intestin moyen / phosphatase acide / histochimie / cytochimie**

Zusammenfassung – Aktivität der sauren Phosphatase im Mitteldarm von Larven der Honigbiene (*Apis mellifera* L.) Die Aktivität der sauren Phosphatase wurde als Indikator des Zellzerfalls in histo – und zytochemischen Untersuchungen an gesunden Honigbienenlarven genutzt. Für Lichtmikroskopie wurde eine Technik mit Methacrylateinbettung angewendet, wobei Naphtol AS-TR Phosphat (Sigma Chemikalien) als Substrat für die saure Phosphatase benutzt wurde. Bei der zytochemischen Untersuchungen im Elektronenmikroskop diente *p*-Nitrophenylphosphat als Substrat. Die Lage der sauren Phosphatase in den Feinstrukturen von Lysosomen und Zellplasma der Honigbienenlarven wurde mit Befunden verglichen, die bereits für Entwicklungstadien anderer Insekten beschrieben wurden. Deutliche Aktivität von freier

saurer Phosphatase wurde im Mitteldarm von Honigbienenlarven unterschiedlicher Alterstufen in den basalen und parabasalen Bezirken, unter der Basalmembran des Epithels im Hämocoel nachgewiesen. Freie saure Phosphatase im Cytosol der Epithelzellen schien auf den Beginn der Zellauflösung und des physiologischen Zelltods hinzuweisen. Die Quelle der Aktivität der freien sauren Phosphatase lag nicht in den Lysosmen, da es keine Anzeichen für eine von den Lysosomen sich ausbreitende oder durchsickernde Aktivität der sauren Phosphatase gab. Die beschriebene Aktivität der sauren Phosphatase könnte als Modell für die physiologische Verteilung von Enzymen in gesunden Larven genutzt werden.

***Apis mellifera* / Honigbienenlarven, Mitteldarm / Histochemie / saure Phosphatase**

REFERENCES

[1] Armbruster L., Levy M., Mathieu M.N., Bautz A.M., Acid phosphatase activity in the hemolymph, hemocytes, fat body and salivary glands during larval and prepupal development in *Calliphora erythrocephala* (Diptera: Calliphoridae), Comp. Biochem. Physiol. 84B (1986) 349–54.

[2] Bignell D.E., Oskarsson H., Anderson J.M., Formation of membrane-bound secretory granules in the midgut epithelium of a termite, *Cubitermes severus*, and a possible intercellular route of discharge, Cell. Tissue Res. 222 (1982) 187–200.

[3] Bowen I.D., Laboratory technique for demonstrating cell. death, in: Davies I., Sigee D.C. (Eds.), Cell Ageing and Cell Death, Cambridge University Press, Cambridge, UK, 1984, pp. 1–38.

[4] Bowen I.D., Bowen S.M., Programmed Cell Death in Tumours and Tissues, Chapman and Hall, London, UK. 1990

[5] Bowen I.D., Worrill N.A., Winters C.A., Mullarkey K., The use of backscattered electron imaging, X-ray microanalysis and X-ray microscopy in demonstrating physiological cell death, Scan Microsc. 2 (1988) 1453–1462.

[6] De Priester W., Van Pelt-Verkuil E., De Leeuv G., Demonstration of acid phosphatase activity induced by 20- hydroxyecdysone in the fat body of *Calliphora*, Cell Tissue Res. 200 (1979) 435–442.

[7] Dimitriadis V.K., Kastritsis C.D., Ultrastructural analysis of the midgut of *Drosophila auraria* larvae – distribution of alkaline phosphatase, acid phosphatase, leucine aminopeptidase, and glycogen, Cytologia 50 (1985) 689–700.

[8] Gregorc A., Bowen I.D., Scanning electron microscopy of the honeybee (*Apis mellifera* L.) larvae midgut, Zb. Vet. Fak. Univ. Ljubljana 33 (1996) 237–244.

[9] Gregorc A., Bowen I.D., Programmed cell death in the honey-bee (*Apis mellifera* L.) larvae midgut, Cell Biol. Int. 21 (1997) 151–158.

[10] Gregorc A., Bowen I.D., The histopathological changes in honeybee larvae after infection with *Bacillus larvae* the causative agent of american foulbrood disease, Cell Biol. Int. (1998) (in press).

[11] Harvey W.R., Blankemeyer J.T., Epithelial structure and function, in: Maramorosch K., Shope R.E. (Eds.), Invertebrate Immunity, Academic Press, New York, USA, 1975, pp. 3–22.

[12] Hussein M.A., Bowen I.D., Lewis G.H., The histochemical localization of ATPase, cholinesterase and acid phosphatase activity in *Culex pipiens* (Diptera, Culicidae) larvae using a methacrylate embedding technique, Cell Biol. Int. 14 (1990), 775–781

[13] Jimenez D.R., Gilliam M., Ultrastructure of the ventriculus of the honey bee, *Apis mellifera* (L.): cytochemical localization of acid phosphatase, alkaline phosphatase, and nonspecific esterase, Cell Tissue Res. 261 (1990) 431–443.

[14] Jones H.E., Bowen I.D., Acid phosphatase activity in the larval salivary glands of developing *Drosophila melanogaster*, Cell Biol. Int. 17 (1993) 305–315.

[15] Kela SL, Bowen ID, The histopathological effect of *Detarium microcaprum* extract, a naturally occurring plant molluscicide on the mid-gut and digestive gland of *Limnaea stagnalis*, Cell Biol. Int. 19 (1995) 175–181.

[16] Lehane M.J., Quantitative evidence for merocrine secretion in insect midgut cells, Tissue Cell 19 (1987) 451–461.

[17] Lehane M.J., Evidence for secretion by the release of cytoplasmic extrusions from midgut cells of *Stomoxys calcitrans*, J. Insect Physiol. 34 (1988) 949–953.

[18] Lewis G.H.J., Bowen I.D., A methacrylate embedding technique for combined autoradiography and acid phosphatase histochemistry, Histochem. J. 17 (1985) 467–75.

[19] Ryder T.A., Bowen I.D., A method for the fine structural localization of acid phosphatase activity using p-nitrophenyl phosphate as substrate, J. Histochem. Cytochem. 23 (1975) 235–237.

[20] Skelton J.K., Bowen I.D., The cytochemical localisation and backscattered electron imaging of acid phosphatase and cell death in the midgut of developing *Calliphora vomitoria* larvae, Epithelia 1 (1987) 213–223.