Is gut pH regulated by midgut endocrine system in larvae of *Rhynchophorus ferrugineus* Fab.?

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Midgut of the larvae of *R. ferrugineus* can be divided into anterior saccular midgut, anterior tubular midgut, posterior saccular midgut and posterior tubular midgut. pH of the contents in the anterior saccular midgut was 5.5-5.4. Effect of extracts of midgut epithelium on maintenance of gut pH in the final instar larvae has been studied employing an *in vitro* method. For this, ligated tubes of the anterior saccular midgut filled with pH indicator-buffer solutions having pH either 4 or 7 were incubated with midgut epithelial extract in a bioassay apparatus at 37°C. pH of the contents of midgut preparations changed to normal range following incubation with epithelial extract as indicated by turning of colours. No change in colour was observed in controls incubated with insect saline for 60 min. The effect is both dose and time dependent. The midgut epithelial factor regulating pH in gut lumen could be a hormone.

In insects, secretogogue, endocrine and neural mechanisms are proposed to be involved in the regulation of digestive enzyme secretion. It is known that varying conditions of pH along the alimentary canal influence the activities of different enzymes and facilitate the sequential digestion of food materials. pH of foregut is greatly influenced by the quality of food stuff but pH of the midgut in most insects is stable and remains unaffected by the diet indicating an efficient buffering by midgut secretions. Insects possess a diffuse type of gut endocrine system comparable to that present in vertebrates. In *Oryctes rhinoceros* a peptide hormone from the midgut epithelium is involved in stimulating the release of digestive enzymes from the midgut tissue into the lumen. Other functions, related to digestion, have also been suggested for the midgut endocrine system including maintenance of gut pH, regenerative cell differentiation, absorption of digested food material and secretion of peritrophic membrane. However, these functions are yet to be demonstrated, probably due to lack of suitable bioassay methods. Sreekumar and Prabhu have devised an *in vitro* method to assay the effect of midgut extracts on digestive enzyme secretion in the larvae of *Oryctes rhinoceros*. The same bioassay system with certain modifications has been used in the present study to demonstrate the role of midgut extract in regulation of pH in the midgut of the coconut pest *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae).

**Materials and Methods**

*Material*—Final instar larvae of the red palm weevil *R. ferrugineus* were used. They were collected from diseased coconut trees at Kayamkulam, 110 km from Trivandrum (8° 30' N; 76° 57' E).

*Rearing*—The larvae were reared in the laboratory in plastic troughs (20x40 cm diam.) providing pieces of sugarcane as food.

*Determination of pH within alimentary canal*—For the determination of pH in the alimentary canal, the larvae were allowed to feed for 24 hr on cotton swabs soaked in 0.1% solution of pH indicator dyes (bromophenol blue, methyl orange, bromothymol blue, bromocresol purple and bromo cresol green), placed in plastic cups. The larvae were subsequently dissected to observe the colours in different regions of the gut. To confirm the results, a drop of 0.1% indicator dye solution was mixed with contents collected from each region of the gut and the resultant colour of the mixture was recorded. In both cases, the colours observed were compared with the colour of pH indicator dyes at known pH. Based on these results, the gut was marked into regions having pH greater or lesser than a particular value.

*Preparation of midgut epithelial extract*—The larvae were ether anaesthetised and the alimentary canals dissected in insect saline (7g NaCl, 0.2g KCl,

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0.2g CaCl₂, 0.1g dextrose in 1000 ml distilled water). The anterior saccular midgut was used for preparation of the extract. The contents of the anterior saccular midgut were removed by cutting it open and washing the tissues in 3-4 changes of insect saline. The epithelial tissues were boiled in insect saline for 10 min to denature hydrolytic enzymes present in them, cooled and homogenized in a glass homogenizer by hand for 5 min. The homogenate was centrifuged at 10,000 g at 4°C for 10 min to remove sediments. The supernatant obtained was used as the incubation solution in the bioassay. Varying concentrations of the extract equivalent to 1, 2, 3, 4, and 5 midgut epithelia/10 ml insect saline were prepared for incubating anterior saccular midgut preparations in the bioassay. For other regions of the midgut, the extract of anterior saccular midgut having a concentration equivalent to 3 midgut epithelia/10 ml saline was used.

Preparation of pH indicator - buffer solution for bioassay—Solutions (0.1%) of bromocresol purple and bromocresol green were prepared in phosphate buffer having pH 4 and 7 respectively. This solution (0.05 ml) was injected into midgut preparations for bioassay.

Preparation of midgut for bioassay—The alimentary canal was exposed and the anterior saccular midgut was cut anteriorly behind the foregut and posteriorly slightly above the tubular part of the midgut. The anterior saccular midgut thus separated was taken out and washed thoroughly in 4-5 changes of insect saline. One end of the open tube was ligated using a hair. Through the other end of the tube, 0.05 ml pH indicator-buffer solution was injected. As the needle was withdrawn the loop of hair placed already at this end was tautened and tied. The midgut tube thus containing pH indicator-buffer solution was used for bioassay. For the preparation of anterior tubular, posterior saccular and posterior tubular regions of midgut, the respective regions were isolated, washed and ligated immediately after injecting pH indicator-buffer solution.

Bioassay apparatus—The bioassay apparatus was a modification of previously used one. It is a glass cylinder, (5x1 cm diam.), attached with a slanting side tube (2.5x0.25 cm diam.) near the bottom end (Fig. 1). The side tube was fitted with a rubber stopper through which a hypodermic needle was inserted into the chamber of the bioassay apparatus for delivery of oxygen. A glass rod was placed at the open end of the bioassay apparatus to suspend the midgut preparation with a thread. The bioassay apparatus was kept in a water bath at 37°C.

Bioassay—The preparations of anterior saccular midgut were incubated with 2 ml of the midgut extract solution in the bioassay apparatus, bubbling a small stream of oxygen from an oxygen cylinder. The colour of the gut contents, in the midgut preparation was constantly monitored at 1 min interval and the time required for causing change in colour in the gut lumen was recorded.

Control—In control experiments, ligated preparations of anterior saccular midgut containing pH indicator-buffer solution were incubated with 2 ml insect saline, without epithelial extract.

Results

pH within the alimentary canal—In larvae fed on pH indicator dye solutions for 24 hr, various regions of the gut showed different colours, reflecting the pH of the contents. The alimentary canal was found to be acidic throughout (Fig. 2). Contents of the alimentary canals dissected from the larvae fed on bromophenol blue and bromothymol blue solutions showed purple
and yellow colours, respectively along the entire length of the alimentary canal, thus indicating the lower and upper limits of pH to be 4 to 6, respectively. It was found that pH of the foregut was between 4.6 and 4.7. In the anterior saccular midgut and posterior saccular midgut the pH ranged from 5 to 5.4 and 5.4 to 5.6, respectively. The anterior tubular midgut had pH between 4.5- 5.6. pH of 5-5.4 was recorded in the posterior tubular midgut. The contents of the ileum and the rectum had a pH of 5.6-5.8.

Effect of midgut epithelial extract on pH of the anterior saccular midgut—In midgut preparations filled with buffer-bromocresol purple solution and incubated with epithelial extract having concentration equivalent to 3 midgut epithelia/10 ml saline, the colour of the contents changed from yellow (pH 4) to purple (pH>5) after 26.37±1.68 min of incubation. When buffer solution containing bromocresol green was used, there was a change in colour of contents from blue (pH 7) to green (pH<5.4) after incubation for 22.12±1.12 min. In controls, no change in colour was noticed during 60 min of observation (Table I).

<table>
<thead>
<tr>
<th>pH indicator dye</th>
<th>Change in colour</th>
<th>Time required for change in colour (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromocresol purple (pH 4) to</td>
<td>Yellow (pH 4) to</td>
<td>26.37±1.68* 60.00</td>
</tr>
<tr>
<td>Bromocresol green (pH 7) to</td>
<td>Blue (pH 7) to</td>
<td>22.12±1.12* 60.00</td>
</tr>
</tbody>
</table>

*P<0.001

Table 1—Effect of midgut epithelial extract on pH of contents of midgut preparations of R. ferrugineus.

[Values are mean±SD of 8 determinations in each group]

Time course and dose response of midgut epithelial extract—With an increase in concentration of the midgut epithelial extract from 1 midgut/10 ml saline to 5 midgut/10 ml saline, the time required for attaining normal pH in midgut preparations filled with bromocresol green and bromocresol purple decreased from 35.37±1.84 to 11.87±1.64 min and

Fig. 2—pH within the alimentary canal of the larva of R. ferrugineus

Fig. 3—Changes in pH in anterior saccular midgut preparations of larvae of R. ferrugineus filled with bromocresol green-buffer (pH 7) and incubated with midgut epithelial extracts of varying concentration.
48.37±4.03 to 12.87±1.45 min, respectively (Fig. 3 and 4).

Effect of epithelial extract on regulation of pH in different regions of midgut—The epithelial extracts were effective in maintaining normal pH in various regions of the midgut after being filled with bromocresol purple-buffer solution. The time taken for attaining normal pH in anterior saccular midgut, anterior tubular midgut, posterior saccular midgut and posterior tubular midgut were 26.37±1.68, 28.66±5.95, 29.5±2.16 min and 29.33±2.80 min respectively, this being indicated by change in colour from yellow to purple in the respective regions (Table 2).

Discussion

In insects, the alimentary canal can be distinguished into morphologically distinct and functionally different segments, often revealing differences in pH. In R. ferrugineus different segments of the alimentary canal maintain different pH as in the beetles Phenolia grossa, Neomida bicornis and larvae of O. rhinoceros. In R. ferrugineus the anterior saccular midgut revealed a pH between 5 and 5.4. pH in the anterior tubular midgut, posterior saccular midgut and posterior tubular midgut were 4.5-5, 5.4-5.6 and 5-5.4, respectively; pH of the alimentary canal was found to be acidic in Dysdercus cingulatus, Musca domestica, termites and some fungus feeding beetles. However, majority of insects possess alkaline pH in alimentary canal, e.g. larvae of lepidopterans, detritus feeding larvae of dipterans and scarabaeid beetles. pH conditions in the alimentary canal is found to reflect the quality of food consumed and the nature of digestive enzymes possessed by the insect.

Various factors influence pH in the lumen of alimentary canal of insects. In Acheta domesticus, midgut luminal pH may change with feeding conditions and chemical quality of food. pH of the crop is unbuffered and fluctuates with the pH of the food in M. domestica, Phormia regina and Calliphora vicina. In Gonoccephalum the midgut pH remains unaffected by starvation. In Apis midgut pH is maintained constant by the interplay of two different buffering system, one contains organic acids and their salts and the other mono- and di-hydrogen phosphates. These two-systems tend to maintain the pH at about 6.3 (ref. 3). High alkalinity in lepidopteran larvae is mediated by active secretion of K onto the midgut lumen. Secretion of HCO₃⁻ may provide the main anion for both balancing K⁺ concentration and titrating the luminal contents to normal pH. In the present study ligated tubes of the anterior saccular midgut filled with pH indicator-buffer solutions having pH either 4 or 7 have been incubated with epithelial extracts of the same region in a bioassay apparatus at 37°C. The time required for attaining normal pH in gut lumen as indicated by change in colour of the solution is recorded as a measure of activity of the epithelial extract in

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Table 2—Regulation of pH in in vitro preparations of different segments of midgut of larvae of R. ferrugineus filled with bromocresol purple (pH indicator-buffer) and incubated with midgut epithelial extract

<table>
<thead>
<tr>
<th>Midgut segments</th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior saccular midgut</td>
<td>26.37±1.68*</td>
<td>60.00</td>
</tr>
<tr>
<td>Anterior tubular midgut</td>
<td>28.66±5.95*</td>
<td>42.0±2.0</td>
</tr>
<tr>
<td>Posterior saccular midgut</td>
<td>29.5±2.16*</td>
<td>44.2±2.3</td>
</tr>
<tr>
<td>Posterior tubular midgut</td>
<td>29.33±2.80*</td>
<td>43.8±2.3</td>
</tr>
</tbody>
</table>

*P < 0.001
regulating gut pH. The results suggest that in larvae of *R. ferrugineus* a factor present in the midgut epithelium is effective in maintaining normal pH in the midgut lumen in the event of altered pH and thus chemical conditions in the gut. In the anterior saccular midgut preparations filled with bromocresol purple-buffer solution regulation of pH of the contents took place after 26.3±71.68 min of incubation with the epithelial extract. Meanwhile, in bromocresol green-buffer filled midgut preparations, the time required for regulation of luminal pH was 22.12±1.12 min. The controls have shown no change in pH in other regions of the midgut as well viz, anterior tubular midgut, posterior saccular midgut and posterior tubular midgut.

Midgut epithelial extract of larvae of *R. ferrugineus* is equally effective in stimulating digestive enzyme release from midgut tissue into the gut lumen19. A peptide hormone from the midgut tissue is involved in stimulating digestive enzyme secretion in *O. rhinoceros* and it is non-specific in action20. Furthermore, endocrine cells have been identified in the midgut epithelium of insects employing electron microscopy, immunohistochemistry and conventional staining. Thus, midgut endocrine cells showing ultrastructural characteristics of the mammalian gastro-entero-pancreatic system are known to be present in *Periplaneta americana*, *Oryctes nasicornis*, *Aedes aegypti* and *Blaberus craniifer*. Immunochemical studies reveal that these cells contain several vertebrate-like gut peptides such as gastrin, cholecystokinin, insulin, and pancreatic polypeptide. Presence of granular cells stainable with lead haematoxylin have been reported in the midgut of the larvae of *O. rhinoceros*22. It has been demonstrated in the present study that in the larvae of *R. ferrugineus* a factor present in the midgut epithelium is capable of regulating gut pH possibly by stimulating epithelial cells to release secretions of buffering nature into the lumen of the gut. In *M. domestica* the middle acidic region of the midgut is provided with cup shaped cells resembling the oxytic cells of mammalian stomach in morphology and probably secreting hydrochloric acid containing fluid. The effect of midgut epithelial factor on maintenance of gut pH in larvae of *R. ferrugineus* is both time and dose dependent as may be seen from Figs. 3 and 4. This observation indicates that the midgut epithelial factor regulating pH in larvae of *R. ferrugineus* could be a hormone. The midgut epithelium is both the source and target of this putative hormone. It appears that, in larvae of *R. ferrugineus*, the pH of the midgut lumen is regulated probably by the action of a hormone present in the midgut itself. This is a new finding and may open up prospects for several lines of investigation, for effecting changes in efficiency of digestion through manipulation of gut pH.

References