CYTOCHEMICAL EVIDENCE FOR AN ANOMALOUS DOSE-RESPONSE OF ACID PHOSPHATASE ACTIVITY IN THE BLOOD BUT NOT THE MIDGUT OF FLUORIDE-TREATED SILKWORM LARVAE, BOMBYX MORI

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SUMMARY: Monotonic inhibition of acid phosphatase (ACPase) activity in the midgut of silkworm larvae was observed in response to increasing F exposure, whereas an anomalous dose-response was seen in the blood. In the blood, ACPase activity decreased significantly with oral administration of F at concentrations of 47.6 and 64.2 ppm in the leaf diet, but it returned to the initial value of the control at 99.2 ppm, significantly exceeded the control at 158.9 ppm, and then drastically decreased with further increase in F concentration in the diet. A plausible explanation for this paradoxical effect is proposed on the basis of enzyme cytochemistry of ACPase in the silkworm midgut.

Keywords: Acid phosphatase (ACPase) activity; Fluoride treatment; Midgut cytochemistry; Paradoxical effect; Silkworm Bombyx mori.

INTRODUCTION

The mulberry silkworm, Bombyx mori L, is one of the most sensitive insect species to fluoride pollution. A rapid response of silkworm larvae to oral administration of sodium fluoride, even at fairly low concentrations, shows up as a sharp decrease in certain enzyme activities in the blood and midgut. Fluoride is a well-known inhibitor of many enzymes. Chinoy et al observed a decrease in the activity of succinic dehydrogenase (SDH), adenosine triphosphatase (ATPase), and acid phosphatase (ACPase) in the sperm of rabbits exposed to fluoride. In cocks, fluoride inhibited the semen ATPase activity by as much as 53%. In an investigation of in vitro effects of fluoride on enzyme activities in silkworm blood, Chen found that 0.01, 0.001, and 0.0001 M NaF inhibited the activity of ACPase by 98%, 77.1%, and 4.2%, respectively. Alkaline phosphatase (AKPase) in the midgut and ACPase in the blood of silkworm larvae also displayed marked decreases in activity with oral administration of 16 to 32 ppm F. However, in many cases, paradoxical concentration effects of fluoride in biological systems are also observed, and plausible explanations for some of them are difficult to assign.

The aim of the present investigation was to investigate and confirm the existence of paradoxical effects of fluoride on ACPase in the blood of silkworm larvae and to find a plausible explanation for these anomalies by visualizing the leakage of ACPase into the blood due to structural damage of midgut cells using cytochemical techniques.

MATERIALS AND METHODS

The fluoride-sensitive silkworm variety Hang 8 (LC50 53.03 ppm F) was used as reported previously. Silkworm larvae were reared under standard conditions.

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using mulberry leaves until the fifth instar. A gradient of F concentrations as shown in the table below was prepared to treat mulberry leaves by the same procedure as before. The actual fluoride concentrations in the NaF-treated mulberry leaves were determined by the method of Wu. Newly exuviated fifth instar larvae were fed with NaF-treated mulberry leaves for 24 hr and then sampled for the different experiments.

<table>
<thead>
<tr>
<th>Treatment (with NaF in water)</th>
<th>Control</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>F concentration in water (ppm)</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>80</td>
<td>120</td>
<td>240</td>
<td>320</td>
</tr>
<tr>
<td>F concentration in dried leaves (ppm)</td>
<td>18.7</td>
<td>47.6</td>
<td>64.2</td>
<td>99.2</td>
<td>158.9</td>
<td>292.9</td>
<td>359.4</td>
</tr>
</tbody>
</table>

**Assay of ACPase activity:** Silkworm blood was collected respectively from the different treatments and diluted 25-fold with distilled water for assay of ACPase activity. Midgut tissues of the same silkworms were sectioned, washed and weighed, homogenized and centrifuged, and the supernatants were used for ACPase assay, the activity of which was determined by the method of Bodansky.

**Cytochemical localization of ACPase:** The procedure for ACPase cytochemistry followed Du’s method with modifications. Briefly, midgut tissues of silkworm larvae from the different treatments were sectioned *in vivo* in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 and 4 ºC. The cut midgut tissues from 1 to 5 segments of abdomen, were then sliced into small pieces (12 mm, since the tissue is very thin), and further immersed in the same fixative for 30 min at 4ºC. The tissues were then cut into 60 µm thick sections with a microslicer (Xiangshang ZQP-86, China). These sections were rinsed in buffer, and then examined by Du’s procedure. Ultrathin (70 nm) sections were cut with a Reichert-Jung Ultracut-E, and the silver color sections were observed directly under an electron microscope (JEM-1200EX, Japan).

**RESULTS**

**Effects of NaF on ACPase activity in blood and midgut tissues of silkworm larvae:** Figure 1 shows the variation of ACPase activity in the blood and midgut tissues of silkworm larvae treated with different concentrations of NaF in the leaves. In the case of the midgut tissues, significant inhibition in comparison with the control was observed, and the ACPase activity decreased drastically as the F concentration in the food increased, disappearing almost completely after treatment V. On the other hand, lower F concentrations in the blood—up to 64.7 ppm F—had a significant inhibitory action in comparison with the control. However, a sudden increase occurred at higher F concentrations and even exceeded that of the control; then it abruptly decreased again when the F concentration exceeded 292 ppm.
Effect of NaF on subcellular distribution of ACPase activity in midgut tissues: Distribution of ACPase-positive reactions could be successfully demonstrated in ultrastructure as shown in the six photos in Figure 2. In the midgut cells of silkworm larvae, ACPase-positive reactions were mainly located in the nucleus of the cells, and the most significant variations were also observed there. Hence only this cell organelle was excised and is illustrated here. Photo (6) in Figure 2 is that of a normal control structure of a nucleus without substrate or added inhibitor in the incubation solution for ACPase reaction in the sliced tissue.

Figure 2. Cytochemical localization of ACPase in midgut of silkworm larvae.
(3) ACPase-positive reactions condensed in the nucleus and leaking out through the membrane at 64.2 ppm F.

(4) Massive reaction deposits released from the nucleus at 99.2 ppm F.

(5) ACPase activity completely inhibited with very little remaining in the broken nucleus membrane and metachromatin at 292.9 ppm F.

(6) A control cell showing normal nucleus structure without cytochemical localization of ACPase activity.

Figure 2 continued. Cytochemical localization of ACPase in midgut of silkworm larvae.
Photo (1) in Figure 2 shows the small black deposits of ACPase-positive reactions located mainly in the euchromatin that were evenly distributed in clusters or granules in the whole nucleus of a midgut cell in the control. Photo (2) reveals how normal distribution was affected when the F concentration increased to 47.6 ppm, and the reaction products conglomerated into large black chunks. From photo (3), it can be clearly seen that ACPase is leaking out through the nucleus membrane due to the structural damage from exposure to a concentration of 64.2 ppm F, which is the turning point as sharp increase of ACPase activity in blood. Photo (4) shows the massive release of ACPase-positive products from the nucleus when the F concentration reached 158.9 ppm. When the F concentration was as high as 292.9 ppm, ACPase activity almost completely disappeared in the nucleus of midgut cells, and small ACPase-positive reacting granules were scattered all over with complete destruction of the normal cell ultrastructure as seen in photo (5).

**DISCUSSION**

ACPase activities in the blood and midgut tissues are strongly related to silk protein synthesis, digestion, and absorption of phosphorylating substances in silkworm larvae. Therefore, intense enzyme activity can be observed in both the blood and midgut. In cytochemical studies, ACPase has been traditionally used as a lysosomal marker enzyme. However, in the present work it was found to be significantly located in the nucleus of midgut cells. Positive ACPase-activities were found in the endoplasmic reticulum surrounding the nucleus and a few lysosomes; however, the focus of our cytochemical analysis was on the variation of ACPase activity in the nucleus since the majority of the reaction products were found there.

Since the midgut is the first organ of the silkworm to be attacked by excessive ingestion of fluoride by the larvae, it is understandable that the ACPase activity in it was significantly inhibited in a fairly regular monotonic way by oral administration of increasing F concentrations. On the other hand, ACPase activity in the blood displayed a paradoxical response as shown in Figure 1. Although paradoxical effects of F in biological systems are not always understood, in the present investigation it appears that the large increase of ACPase activity in the blood at 64.2 ppm F was caused by the massive release of ACPase from midgut cells due to cell structure damage caused by the high F concentration. Evidently, the inhibiting action of F on the enzyme in the blood was not strong enough to cover the massive increase of enzyme activity leaking out from the midgut cells.

Afterward, the sharp decrease of ACPase activity in the blood by exposure to 292.9 ppm F can be explained by the essentially complete inhibition of ACPase activity in the midgut cells with very little ACPase being released from the midgut cells into the blood. Thus with less ACPase in the blood, the higher F concentration resulted stronger inhibition of ACPase activity in the blood.

In conclusion, the present study found that ACPase was dominantly present in the nucleus of silkworm larvae midgut cells, and, on the basis of cytochemical evidence, the anomalous dose-response of ACPase activity in the blood can be
explained by the massive release of the enzyme caused by structural damage to the midgut cells from the higher F concentrations.

REFERENCES