EFFECTS OF FLUORIDE ON THE ACTIVITIES OF ALKALINE PHOSPHATASE, ADENOSINE TRIPHOSPHATASE, AND PHOSPHORYLASE IN THE MIDGUT OF SILKWORM, *BOMBYX MORI* L.

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SUMMARY: Changes in the activities of alkaline phosphatase (ALKP), adenosine triphosphatase (ATPase), and phosphorylase in the midgut of silkworm (*Bombyx mori* L.) larvae exposed to NaF-treated mulberry leaves were investigated. The activity of ALKP was markedly reduced by fluoride compared to the control. In the presence of $Ca(OH)_2$ or $MgSO_4$, the ALKP activity was only slightly affected. The activities of ATPase and phosphorylase in the midgut were also reduced by F treatment. These results suggest that certain midgut enzyme activities of silkworm larvae that are adversely affected by fluoride pollution can be used to monitor the health of silkworms and, in the case of ALKP, can also be countered by Ca or Mg salts.

Key words: Alkaline phosphatase (ALKP); Adenosine triphosphatase (ATPase); Calcium antagonism; Fluoride treatment; Magnesium antagonism; Phosphorylase; Silkworm *Bombyx mori* L.

INTRODUCTION

The silkworm (*Bombyx mori* L.) is an economically valuable Lepidoptera insect with more than five thousand domesticated strains. In China, village brick-kiln industries in rural areas,[1,2] have led to increasingly serious fluoride pollution affecting the silkworm. Because temperatures during brick-making usually range from 900 to 1150 ºC,[3] fluorine is emitted into the air as HF and SiF_4 during the firing process.[4] These gaseous forms of F can be taken up by mulberry trees and are toxic to both mulberry leaves and silkworms that feed on them, leading to serious economic losses, especially in the silkworm-growing plain of Hang-Jia-Hu in Zhejiang Province.[5]

It is important, therefore, to monitor and study the effect of F-pollution on the silkworm and the mechanisms involved in its toxicity. In a previous study[6] we examined biochemical effects of F on the haemolymph of the silkworm. The results suggested that the metabolic changes in the haemolymph could be used as biomarkers to evaluate adverse health effects and the economic impact of F exposure on the silkworm.

The objective of this work was to extend our investigation of the mechanisms of F-induced toxicity in silkworm larvae. For this purpose, the effects of NaF on the activities of alkaline phosphatase (ALKP), adenosine triphosphatase (ATPase), and phosphorylase present in the midgut of the silkworm were studied. In the case of ALKP, antidotal effects of Ca^{2+} and Mg^{2+} were also examined.

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MATERIALS AND METHODS

Experimental colonies and administration of fluoride: Disease-free eggs of the bivoltine silkworm (Hang 8 strain) were used in this study. They were reared as in our previous study and fed the same artificial diet.[6] After the third ecdysis, the larvae were divided into groups and rearing was continued under the same conditions. The procedure used to administer NaF was also the same as in our previous study with each experiment replicated three times with 200 larvae.[6]

Preparation of enzyme extract: Twenty-five larvae were randomly collected from each replication. The midguts from the cold anesthetized silkworms were dissected under a dissecting microscope and placed on filter paper for 5 min to remove surface water. The samples were then weighed and homogenized with a loose-fitting homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min, and the resultant supernatant was used as crude enzyme extract.

ALKP activity: ALKP activity in the extract was determined by the method of Beckman and Johnson[7] with the following reaction mixture: 100 mL 0.01M tris-buffer (pH 8.60), 100 mg α-naphthyl phosphate (as substrate), 100 mg fast blue RR salt (as stain), 223 µL 10% $MnCl_2$, 230 µL 10% $MgCl_2$, 0.5g polyvinylpyrrolidone, and 2 g NaCl. One mL of the crude enzyme extract was transferred to an Eppendorf tube to which 1 mL of the foregoing reaction mixture was added. For the controls, the enzyme extract was replaced with distilled water. The assay mixture was allowed to stand at room temperature for 25 min and the reaction was stopped by the addition of 3 mL 15% trichloroacetic acid (TCA). Absorbency was measured using a Beckman-640U spectrophotometer (Beckman, USA), and the amount of product formed was calculated from a standard curve.

ATPase activity: The activity of ATPase was measured by a standard coupled enzyme assay in which the rate of ATP hydrolysis was measured based on spectrophotometric analysis of NADH oxidation at 340 nm. The activities are expressed as micromoles ATP hydrolyzed per min per mg wet tissue. Enzyme assays were carried out at 37°C in a spectrophotometer with a thermostated multicuvette holder. The percentage inhibition was then calculated.

Phosphorylase activity: Glycogen phosphorylase was measured by a modification of the method of Siegert.[8] The determination is based on the conversion of glucose-1-phosphate to glucose-6-phosphate, and the rate was determined fluorimetrically using $NADP^+$ and glucose-6-phosphate dehydrogenase. An aliquot of the enzyme extract was incubated in the presence of 1 mM AMP (total activity) and in the absence of AMP (active phosphorylase, phosphorylase-a). The absorbency at 340 nm was measured with a Beckman-640Ua spectrophotometer. Total phosphorylase activity was expressed as nmol/min/mg, while phosphorylase-a was given as percentage of total phosphorylase activity.

Data analysis: Assay measurements in each experiment were performed in triplicate. Bars in Figures 1-–5 represent standard errors, $n = 5$.

RESULTS

Change of ALKP activity: Changes in ALKP activity in the midgut of silkworm after exposure to F are presented in Figure 1. Compared to the control, ALKP activity in the midgut of F-treated worms was significantly reduced after 48 hr of treatment. At the end of the 5th instar, the activity was about 30% lower. As also seen in Figure 1, NaF and CaF_2 affected the ALKP activity differently. NaF produced marked inhibition, but CaF_2 had almost no effect.

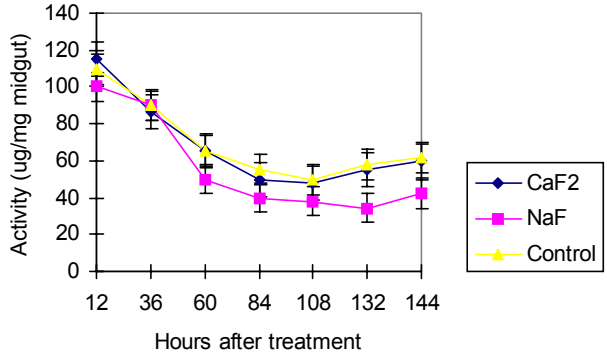

Figure 1. Changes of ALKP activity in the midgut of silkworms exposed to F compounds. Fluoride was administered at 32 mg F (as NaF)/kg dry diet. The worms were reared in the spring season.

Further experiments were conducted in a different worm rearing season to study the effect of NaF on ALKP in the presence of added Ca or Mg salts. As shown in Figure 2, results of these experiments indicated that the midgut activity of ALKP showed an even greater decrease with NaF after 108 hr compared with the results shown in Figure 1. But in the presence of $Ca(OH)_2$ very little decrease in activity occurred with NaF, and only a small decrease occurred in the presence of $MgSO_4$ after prolonged exposure.

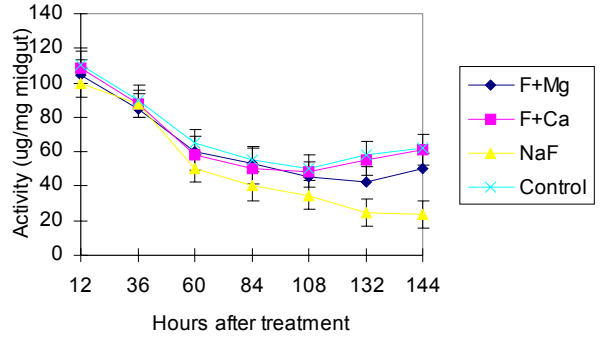

Figure 2. Changes of ALKP activity in the midgut of silkworms exposed to F and different chemicals. F+Ca: a mixture of 32 mg F/kg dry diet and 0.01M $Ca(OH)_2$; F+Mg: a mixture of 32 mg F/kg dry diet and 0.01M $MgSO_4$; NaF: 32 mg F (as NaF)/kg dry diet. The worms were reared in the summer season.

ATPase activity: As shown in Figure 3, ATPase activity in the midgut was inhibited by increasing concentrations of NaF in the diet and was already evident at 16 mg F/kg dry diet.

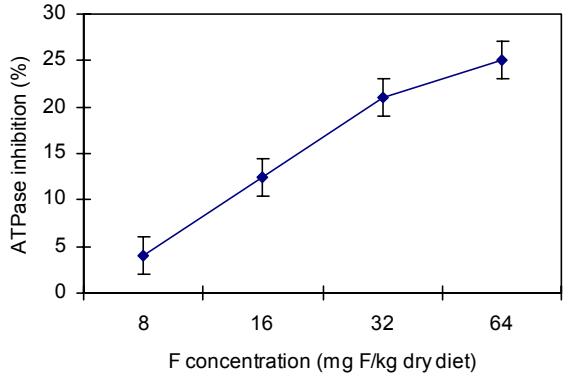

Figure 3. Percent inhibition of ATPase activity in the midgut of silkworms exposed to F (as NaF).

Phosphorylase activity: Compared to the control larvae, the activity of phosphorylase-a in the midgut of silkworms exposed to NaF was reduced from the second day of exposure in the 5th instar (Figure 4).

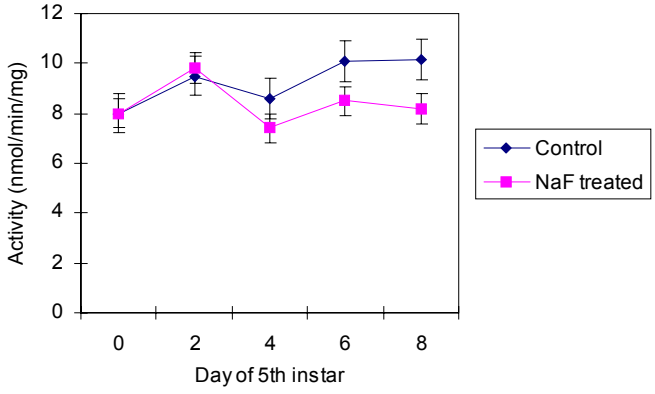

Figure 4. Change of phosphorylase-a activity in the midgut of silkworms exposed to F. Fluoride was administered at 32 mg F (as NaF)/kg dry diet.

Similarly, the total phosphorylase activity was also reduced compared to the control, and the reduction began shortly after exposure to the F-treated diet in the 5th instar (Figure 5).

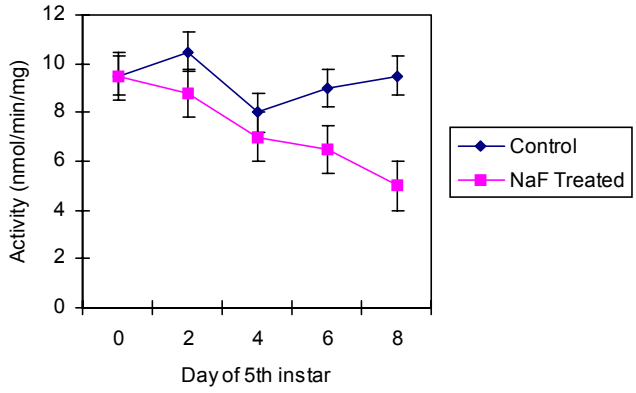

Figure 5. Changes of total phosphorylase activity in the midgut of silkworms exposed to F. The F was administered at 32 mg F (as NaF)/kg dry diet.

DISCUSSION

The biochemical profile of the midgut and haemolymph of silkworms poisoned by F compounds should theoretically serve as an indicator of a toxicological mechanism and a biochemical index for evaluating the health effects of F exposure. Changes in enzyme activity in the midgut imply that the metabolism in the midgut cells was affected by F, and these effects have been associated with histolysis.[9]

ALKP is a set of hydrolytic enzymes which hydrolyze phosphomonoesters under alkaline conditions. The enzyme is located in the midgut, Malpighian tube, muscles, nerve fibers, and silk glands of the silkworm.[10] Significant differences in resistance to F exist among different silkworm species.[11,12] Several factors affect the activity of ALKP in the midgut of silkworm, such as viral and bacterial infection and administered chemicals.[13] The activity of the enzyme is related to the physiological condition of silkworms and reflects the digestion, absorption, and positive transport of nutrients in the midgut.[14,15] The changes in enzyme activity observed here indicate that various chemicals affected ALKP differently. This may imply that Ca^{2+} and Mg^{2+} ions combine with F^{-} ions from NaF to form relatively insoluble and much less toxic CaF_2 and MgF_2. This observation is in agreement with the results reported by others.[16,17] Our results also suggest a possible use of calcium compounds such as lime to reduce F toxicity in the silkworm.

ATPase activity was also reduced by exposure to F and was dependent on the concentration of NaF in the diet. Our study suggests that under low F concentrations the worm exhibited some tolerance to F, but at high concentrations ATPase activity was markedly inhibited. This implies disturbance of the energy metabolism in the exposed organism. Differences in tolerance to F among different silkworm strains have been reported [12] and deserve further study.

In insects phosphorylase exists in two forms: phosphorylase-a and phosphorylase-b. Glycogenolysis by phophorylase is a key step for insect trehalose bio-

synthesis and energy metabolism.[18] The reduction of phosphorylase-a and total phophorylase activity in the midgut of silkworm exposed to NaF indicates that glycogenolysis and carbohydrate metabolism in the larvae midgut tissues exposed to F were inhibited and that energy supply in the cells of larval midgut was decreased.

In conclusion, F pollution in silkworm larvae was found to have adverse effects on the activity of some enzymes and metabolism in their midgut. It is possible that these changes can be used as a biochemical index to evaluate the health and economic characteristics of the silkworm and could provide basic knowledge for protection against F toxicity.

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