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PHOSPHOPROTEIN PHOSPHATASE ACTIVITY DURING DEVELOPMENT OF THE INSECT *Ceratitis capitata*

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SUMMARY

Phosphohistone phosphatase activity was determined in homogenates of the insect *Ceratitis capitata* at several stages of development. Enzyme activity varied differently during development; the highest values were found during larval instars and the lowest values coincided with apolysis. These results are discussed on the basis of the relationship between the cyclic nucleotide-protein kinase system and hormonal regulation previously described in this Diptera.

Cyclic AMP has been suggested to be a second messenger in the action of a variety of hormones in different animal tissues (1). Although the vital role played by cyclic AMP in vertebrates has been studied in detail, its presence and importance in insects has been largely ignored. With regard to the Diptera *Ceratitis capitata*, the existence of cyclic AMP, cyclic GMP, the corresponding cyclases, phosphodiesterases, cyclic AMP-dependent protein kinases and protein binding activities has been demonstrated; a certain relationship between these parameters and hormonal regulation has been suggested (2-7).

A number of enzymes are known to exist in two interconvertible forms which differ in their degree of phosphorylation. Interconversion of these forms by phosphorylation and dephosphorylation is catalyzed by protein kinases and phosphoprotein phosphatases, respectively. Despite the fact that extensive work has been carried out on the kinases (8), little attention has been paid to the phosphoprotein phosphatases.

This paper is concerned with a study of phosphoprotein phosphatase activity during the development of the Diptera *Ceratitis capitata*.

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MATERIALS AND METHODS

Ceratitis capitata (Wiedemann) in the egg, larval, pharate adult and adult stages of development was used. Diet, temperature and humidity conditions of culturing were carefully controlled as previously described (9).

Phosphohistone, labelled with ^{32}P, was prepared from calf thymus histone using γ-labelled ^{32}P-ATP according to the Maeno and Greengard's method (10), with slight modifications. Each ml of incubation mixture contained, 100 µg of protein kinase, 10 µmoles of sodium acetate buffer, pH 6.4, 1 mg histone, 5 nmoles of γ-^{32}P-ATP (5-10 x 10^6 cpm), 10 µmoles of magnesium acetate, 10 µmoles of sodium fluoride, 2.0 µmoles of theophylline and 5.0 nmoles of cyclic AMP. Incubation was performed at 30°C for 45 min. The phosphorylation reaction was terminated by the addition of 0.25 ml of 100% TCA. The resulting precipitate was centrifuged, washed twice by dissolving in water and reprecipitating with 20% TCA and then dialyzed against distilled water for 34 hours. Recovery of histone carried out through the phosphorylation reaction and numerous washings varied from 60-78%. Incorporation of γ-^{32}P-ATP into histone was 36%.

The phosphatase assay was performed essentially according to the method of Khandelwal et al. (11). The reaction mixture for the dephosphotylation of ^{32}P-histone contained 50 mM Tris (pH 7.4), 0.5 mM dithiothreitol, 0.1 M KCl, 10 mM $MgCl_2$, 25-50 µg of ^{32}P-labelled histone (5-10 x 10^5 cpm) and 200 µg of enzyme protein in a total volume of 200 µl.

The reaction was started by the addition of the enzyme preparation to the reaction mixture and stopped by the addition of 0.5 ml of 30% TCA and 0.2 ml of 0.63% albumin. After chilling at 4°C for 10 min, all tubes were centrifuged at 1500 x g for 10 min. Aliquots of 0.5 ml of the clear supernatant were counted with a Packard Tri-Carb liquid scintillation spectrometer using a dioxane-naphthalene solution (12).

In vitro assay for studying the effects of cyclic nucleotides on phosphoprotein phosphatase were done by incubating the enzyme in a 50 mM Tris bufrer, pH 7.5 containing 0.5 mM dithiotreitol in the presence of the nucleotides at different concentrations. Partially purified phosphoprotein phosphatase was prepared from 5-day-old pupae by means of

DEAE chromatography (13). One unit of phosphoprotein phosphatase was defined as the amount of enzyme which releases 1 nmol of P_i from the phosphorylated substrate per min. Specific activity was defined as the number of units per mg of protein. Of the three analytical values, the two extreme results are represented in Fig. 1.

Protein concentration was determined by the method of Lowry et al. (14).

The γ-^{32}P-ATP used (sp. act. 5000 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, England. Calf thymus histone (type IIA) and 3',5'-cyclic AMP-dependent protein kinase from beef heart were obtained from Sigma Chemical Co., St. Louis, USA.

RESULTS AND DISCUSSION

In Fig. 1 the enzyme activity values are plotted against the various stages of development of the insect. The values of specific activity of phosphoprotein phosphatase in *Ceratitis capitata* are much higher than those found in both soluble and particulate fractions of nerve cords of *Manduca sexta* (15), to our knowledge, the only data on insects described to date. It is difficult to establish a direct comparison between the values found for *Ceratitis capitata* and those reported for mammals, due to the wide range of values described for mammalian organs or tissues (11, 16-28).

As it can be seen in the figure, the highest enzyme activity was present in the first instars of larval stages, while the lowest activity coincided with apolysis. Three matamorphosis hormones play vital roles in the regulation of post-embryonic development: the brain hormone (BH), the juvenile hormone (JH) and the moulting hormone (MH). During larval development JH and MH are both secreted, thus initiating larval apolysis; transformation to the pupa occurs when JH levels are low enough and MH is secreted alone. In view of this fact, it is possible that the peak of phosphohistone phosphatase activity exhibited during larval moults is related to JH action.

To provide a better understanding of the relationship between phosphorylation and dephosphorylation events during the development of the insect, the pattern of the protein

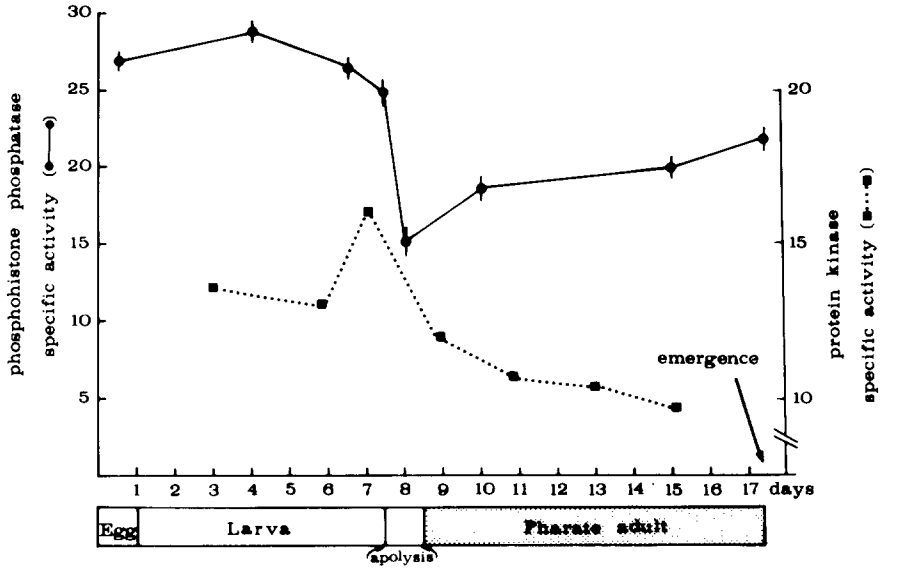

Figure 1. Phosphohistone phosphatase activity of homogenates of the insect *Ceratitis capitata* at different stages of development. Protein kinase activity (7) is also given.

kinase activity is also included. In general, opposite effects were observed on the activity of the two enzymes, which result fits in well with the established interpretation of the phosphorylation phenomenon.

The lowest activity of phosphoprotein phosphatase coincides with the highest levels of cyclic AMP found during the development of the insect, as has been previously shown (3). This fact seems to suggest that the enzyme plays an important role in such critical stage of development as lipolysis. In order to determine whether insect phosphatase is modulated by cyclic nucleotides, the effect of different cyclic nucleotides on partially purified insect phosphoprotein phosphatase was measured during in vitro incubations. The results, given in Table 1, showed no significant variations in enzyme activity. It should be pointed out that although some studies on phosphorylase phosphatase report cyclic AMP-induced inactivation (29,30), most research on the subject has found that cyclic AMP (15,31) and cyclic GMP (15,32,33) , appear to have no effect on phosphoprotein phosphatases.

Table 1

In vitro effect of cyclic nucleotides on phosphoprotein phosphatase activity from *Ceratitis capitata*

Addition	Concentration	Phosphatase activity*
None		13818
Cyclic AMP	10^{-7} M	13887
"	10^{-6} M	13048
"	10^{-5} M	12613
"	10^{-4} M	12169
Cyclic GMP	10^{-7} M	12951
"	10^{-6} M	13194
"	10^{-5} M	13213
"	10^{-3} M	13527
Cyclic IMP	10^{-7} M	13194
"	10^{-6} M	12774
"	10^{-5} M	11868
"	10^{-3} M	12409

*Values are given in arbitrary units (cpm/50 µl)

Studies on the purification of the enzyme and its role in the hormonal regulation of development of the insect are now in progress.

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