Protease inhibitor controls prophenoloxidase activation in *Manduca sexta*

Steven J. Saul and Manickam Sugumaran*

Department of Biology, University of Massachusetts, Harbor Campus, Boston, MA 02125, USA

Received 21 July 1986

Prophenoloxidase from the hemolymph of tobacco hornworm *Manduca sexta* can be activated by a specific activating enzyme found in the cuticle. Inhibition studies with benzamidine, diisopropyl phosphofluoridate and *p*-nitrophenyl-*p'*-guanidinobenzoate indicate that the activating enzyme is a trypsin-like serine protease. An endogenous protease inhibitor, isolated from the hemolymph of *Manduca* larvae, inhibits the prophenoloxidase activation mediated by this enzyme. These results indicate that the probable physiological role of endogenous protease inhibitor is to control the undesired activation of prophenoloxidase in the hemolymph.

Prophenoloxidase *Enzyme activation* *Enzyme inhibitor* *Proteolysis* *Insect immunity* (Manduca sexta)

1. INTRODUCTION

Lacking the complicated immunoglobulin system of higher animals, insects have managed to distinguish self from non-self matter by a variety of host defense reactions [1,2]. These include cellular reactions such as phagocytosis, encapsulation and nodule formation, as well as humoral reactions such as agglutination, antibacterial protein synthesis and humoral encapsulation. During invasion, deposition of melanin around the foreign objects is invariably observed [3,4]. Thus phenoloxidase, the enzyme responsible for the production of melanin from catechol derivatives, is considered an essential component of insect immunity [1–4].

The hemolymph phenoloxidase is present in most insects as an inactive proenzyme form under normal physiological conditions [5]. Activation of prophenoloxidase seems to be achieved through limited proteolysis by an enzyme found in the hemolymph as well as cuticle [6,7]. Accordingly, activation of purified prophenoloxidase from the hemolymph of the silkworm, *Bombyx mori*, by the cuticular activating enzyme is accompanied by the removal of a peptide of M_r 5000 from the proenzyme [8]. Recently, Aso et al. [9] have reported the characterization of prophenoloxidase in the hemolymph of *Manduca sexta* larvae and presented evidence for the presence of cuticular activating enzyme in this organism. Here, we demonstrate that a protease inhibitor isolated from the hemolymph of *M. sexta* larvae inhibits prophenoloxidase activation mediated by the cuticular activating enzyme.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

Eggs of *M. sexta* were donated by Dr J.S. Buckner of Metabolism and Radiation Laboratory, Agricultural Research Service, US Department of Agriculture, Fargo, ND. Larvae were reared on a synthetic medium [10] kept at 25°C during a 16 h light–8 h dark photoperiod.

2.1. *Isolation of protease inhibitor*

Protease inhibitor from the hemolymph was isolated using the procedure outlined by Ramesh [11]. Hemolymph of *M. sexta* was subjected to heat treatment at 100°C for 10 min followed by centrifugation at 15000 × *g*. The protease inhibitor in the clear supernatant was adsorbed onto a

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00145793/86/$3.50

trypsin-Sepharose column at pH 7.5 and eluted with 0.1 M HCl containing 0.75 M NaCl. After neutralization, the eluate was dialyzed, concentrated and chromatographed on a Sephadex G-75 column to yield pure protease inhibitor.

2.2. *Isolation of cuticular activating enzyme*

The cuticular activating enzyme was isolated as follows [9]: Cuticles from fifth instar larvae, which were undergoing pupation but before the onset of browning, were collected in ice-cold 0.5 M Tris-HCl buffer, pH 7.5, containing 1 mM phenylthiourea and 5 mM $CaCl_2$. Cuticle was washed in the same buffer and then homogenized in a Waring blender for 1–2 min. After centrifugation, the clear supernatant was brought to 50% saturation with respect to ammonium sulfate and left at 4°C for 1 h. Precipitated proteins were collected by centrifugation at 27000 × *g* for 15 min. The pellets were redissolved in 0.01 M phosphate buffer, pH 7.5, dialyzed overnight against the same buffer at 4°C and loaded onto a hydroxyapatite column equilibrated with the same buffer. The activating enzyme was eluted with 0.1 M phosphate buffer, pH 7.5.

2.3. *Enzyme assays*

Phenoloxidase activity was monitored using a 1 ml reaction mixture containing varying amounts of enzyme and 5 mM dopamine in 0.025 M sodium phosphate buffer, pH 6.0. The reaction was initiated by the addition of enzyme and followed by measuring the increase in absorbance at 410 nm due to the product formation. *M. sexta* activating enzyme was assayed for activity using H-D-isoleucyl-L-propyl-L-arginine-*p*-nitroanilide dihydrochloride as the substrate. A reaction mixture containing 0.5 ml of 0.1 mM substrate in water and 0.5 ml of 0.1 mM sodium phosphate buffer, pH 7.5, is mixed with 50 Il of *Manduca* activating enzyme (4.5 mg/ml) and incubated at 30°C. The increase in absorbance due to the release of *p*-nitroaniline is monitored at 410 nm. Protease inhibitor activity was assayed using a reaction mixture (1 ml) containing 2 Ig trypsin, 20 mM $CaCl_2$, 1 mM *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride and varying concentration of inhibitor in 0.1 M Tris-HCl buffer, pH 8.3. The reaction was initiated by the addition of substrate. After incubation at 37°C for 30 min, it was arrested with 0.5 ml of 30% acetic acid. The absorbance of *p*-nitroaniline formed was measured at 410 nm using a Gilford model 260 spectrophotometer. One unit of protease inhibitor activity is defined as that amount of inhibitor which caused 50% inhibition of trypsin activity measured under above assay conditions.

Protein content of various preparations was assayed by the Coomassie dye binding assay [12].

3. RESULTS

To isolate native prophenoloxidase, *Manduca* larvae were injected with the anticoagulant buffer described by Leonard et al. [13] and the hemolymph was collected. After lyophilization, the hemolymph proteins were chromatographed on a Sepharose 6B column. Fig.1 illustrates a typical Sepharose 6B chromatographic elution profile of hemolymph proteins from *M. sexta*. The major peak observed at fraction 48 was identified as manducin and the minor peak at fraction 54 was characterized to be insectacyanin. The peak around fraction 80 was due to the hemolymph phenols. Prophenoloxidase eluted at fraction 54 and protein inhibitor eluted at fraction 70. The chromatographic pattern of hemolymph contain-

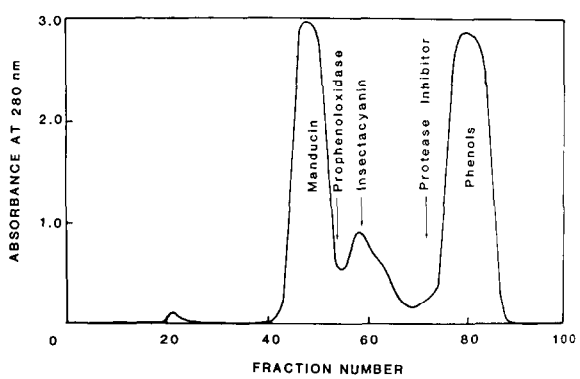

Fig.1. Sepharose 6B column chromatography of hemolymph proteins from *M. sexta* larvae. Fifth instar *M. sexta* larvae were injected with the anticoagular buffer described by Leonard et al. [13] and the hemolymph collected. It was lyophilized, dissolved in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2 M sodium chloride, and chromatographed on a Sepharose 6B column (2.5 × 90 cm) equilibrated with the same buffer at 4°C. A flow rate of 28 ml per h was maintained and fractions of 5.6 ml were collected.

ing activated phenoloxidase was essentially the same as that of fig.1 except for the appearance of a new peak at fraction 26 corresponding to active phenoloxidase. From these results, it can be concluded that phenoloxidase is present as a proenzyme form in the hemolymph and, upon activation, forms higher molecular mass aggregates.

The prophenoloxidase isolated from the Sepharose column remained quite stable and resisted spontaneous activation. Since proteases such as chymotrypsin are known to activate silkworm prophenoloxidase [14], we tested a number of proteases for their ability to activate prophenoloxidase. However, none of the following proteases tested could activate prophenoloxidase from *Manduca* larvae: pronase, thermolysin, chymotrypsin, subtilisin and trypsin. The activating enzyme from *M. sexta* cuticle, on the other hand, readily activated prophenoloxidase, demonstrating that this limited proteolysis is a specific reaction.

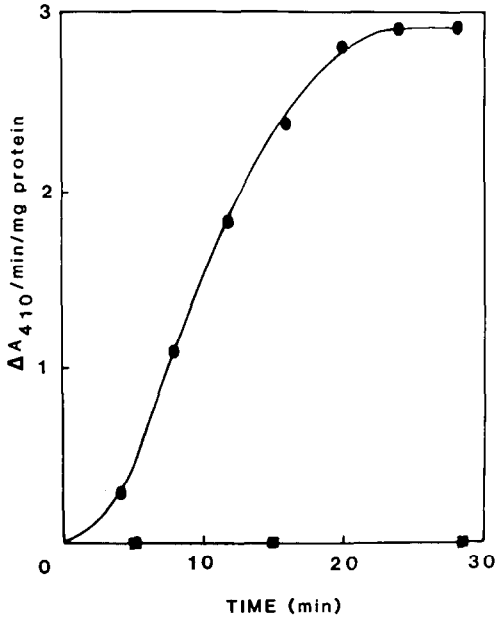

Fig.2. Effect of serine protease inhibitors on prophenoloxidase activation catalyzed by cuticular activating enzyme. Prophenoloxidase fractions (tubes 53,54) collected from Sepharose 6B chromatography were dialzyed against insect saline, pH 6.5, with four changes for 2 h. Activation of prophenoloxidase (300 μl) was initiated by the addition of *M. sexta* cuticular activating enzyme (100 μl) at time 0. Aliquots (50 μl) were withdrawn at the indicated time intervals and assayed for phenoloxidase activity as described in section 2. Inhibitors were incubated with prophenoloxidase for 5 min before the addition of activating enzyme. Controls showed no phenoloxidase activation. (●---●) Prophenoloxidase + cuticular activating enzyme, (■---■) prophenoloxidase + cuticular activating enzyme + 10 mM benzamidine or 30 μM *p*-nitrophenyl-*p'*-guanidinobenzoate or 20 mM diisopropyl phosphofluoridate.

Fig.2 illustrates the time course of appearance of phenoloxidase activity in a reaction mixture containing prophenoloxidase and cuticular activating enzyme. This activation process is completely inhibited by serine protease inactivators such as diisopropyl phosphofluoridate and *p*-nitrophenyl-*p'*-guanidinobenzoate, or by benzamidine which is a competitive inhibitor of trypsin-like enzymes, indicating that the activating enzyme is indeed a serine protease with a trypsin-like active site.

We then tested the effect of endogenous protease inhibitor isolated from *Manduca* hemolymph on the activation process. Fig.3 gives a typical time course study of prophenoloxidase activation by cuticular activating enzyme in the absence and

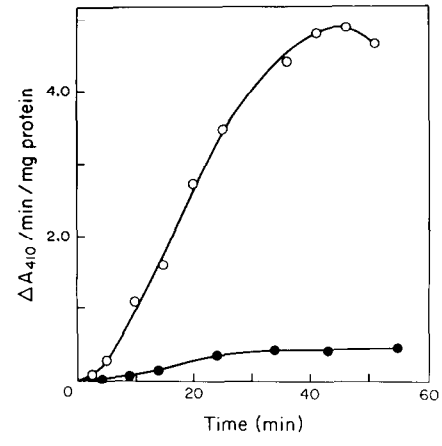

Fig.3. Effect of endogenous protease inhibitor on prophenoloxidase activation catalyzed by cuticular activating enzyme. Prophenoloxidase was prepared and activated as described in the legend to fig.2. *Manduca sexta* protease inhibitor (100 units) was incubated with prophenoloxidase 5 min before the reaction was initiated by the cuticular activating enzyme. Control experiments showed no activity. (○---○) Prophenoloxidase + cuticular activating enzyme, (●---●) prophenoloxidase + cuticular activating enzyme + *Manduca* protease inhibitor.

presence of endogenous protease inhibitor. As demonstrated by the graphs, it is clear that endogenous protease inhibitor drastically inhibits the appearance of phenoloxidase activity by inhibiting the activating enzyme.

4. DISCUSSION

In agreement with published literature [9], prophenoloxidase was found to exist as the proenzyme form in the hemolymph of *M. sexta* larvae. Hemolymph is also known to have the activating protease. If suitable physiological control mechanisms are not present, this co-occurrence could result in spontaneous activation of prophenoloxidase and destruction of self matter. Confinement of prophenoloxidase in hemocytes was suggested to be a mechanism by some workers [13,15], but we observed its presence both in plasma and hemocytes. Our attempts to assay the activating enzyme in the hemolymph failed. Hence we examined the larval hemolymph for the presence of protease inhibitor and found as much as 60–70 units protease inhibitor activity per ml hemolymph. This, coupled with the fact that protease inhibitor binds to proteases on a mole to mole basis [11], accounts for our failure to observe endogenous protease activity in the whole hemolymph. To evaluate the role of protease inhibitor, we partially purified the activating enzyme from cuticle. The cuticular activating enzyme readily activated the hemolymph prophenoloxidase while none of the following proteases tested could activate it: pronase, thermolysin, chymotrypsin, subtilisin and trypsin. Since the activation caused by cuticular enzyme was specifically inhibited by the serine protease inactivators diisopropyl phosphofluoridate and *p*-nitrophenol-*p'*-guanidinobenzoate and the trypsin inhibitor benzamidine, it was clear that the activating enzyme is a serine protease with trypsin-like specificity (fig.2). Phenoloxidase activation catalyzed by this enzyme was drastically inhibited by the hemolymph protease inhibitor (fig.3), confirming our contention that protease inhibitor prevents undesired activation of prophenoloxidase in the hemolymph.

To our knowledge, this is the first report of an endogenous protease inhibitor controlling prophenoloxidase activation in insect hemolymph. In this context, it is interesting to note the report of the presence of an uncharacterized heat-stable factor which prevents melanization in hemocyte cultures [16]. It is most likely that this factor could be the protease inhibitor isolated in our study. Further studies on the molecular mechanism involved in control of the prophenoloxidase activation process are in progress in our laboratory.

ACKNOWLEDGEMENTS

This work was supported in part by a Biomedical Research Support Grant and NIH grant 2R01-AI-14753. We thank Mr Liu Bin and Ms Debra Swanson for their help.

REFERENCES

[1] Coombe, D.R., Ey, P.L. and Jenkin, C.R. (1984) Qu. Rev. Biol. 59, 231–255.
[2] Gotz, P. and Boman, H.G. (1985) in: Comprehensive Insect Physiology, Biochemistry and Pharmacology (Kerkut, G.A. and Gilbert, L.E. eds) vol.3, pp.453–485, Pergamon, Oxford.
[3] Nappi, A.J. (1975) in: Invertebrate Immunity (Maramorosh, K. and Shope, R.E. eds) pp.293–326, Academic Press, New York.
[4] Ratcliffe, N.A., Leonard, C. and Rowley, A.F. (1984) Science 226, 557–559.
[5] Brunet, P.C.J. (1980) Insect Biochem. 10, 467–500.
[6] Dohke, K. (1973) Arch. Biochem. Biophys. 157, 203–221.
[7] Yoshida, H. and Ashida, M. (1986) Insect Biochem. 16, 539–545.
[8] Ashida, M. and Dohke, K. (1980) Insect Biochem. 10, 37–47.
[9] Aso, Y., Kramer, K., Hopkins, T. and Lookhart, G. (1985) Insect Biochem. 15, 9–17.
[10] Reinecke, J.P., Buckner, J.S. and Grugel, S.R. (1980) Biol. Bull. 158, 129–140.
[11] Ramesh, N. (1986) PhD Thesis, University of Masssachusetts, Worcester, MA.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Leonard, C., Soderhall, K. and Ratcliffe, N.A. (1985) Insect Biochem. 15, 803–810.
[14] Ohnishi, E., Dohke, K. and Ashida, M. (1970) Arch. Biochem. Biophys. 139, 143–148.
[15] Leonard, C., Ratcliffe, N.A. and Rowley, A.F. (1985) J. Insect Physiol. 31, 789–799.
[16] Silverwyatt, S. (1956) J. Gen. Physiol. 39, 841–852.