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ACTIVATION OF PRO-PHENOLOXIDASE BY BACTERIAL CELL WALLS OR β-1,3-GLUCANS IN PLASMA OF THE SILKWORM, Bombyx mori

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Silkworm hemolymph plasma contains pro-phenoloxidase* and the activating system for the pro-enzyme. The latter was triggered by elicitors such as Gram-negative or Gram-positive bacterial cell wall, glucans with β-1,3-glycosidic linkages and denatured lipophorin, which is one of silkworm plasma proteins, but not by lipopolysaccharide, dextran sulfate, kaolin or inulin. Calcium was required for the elicitors to activate the system. However, putative pro-PO activating enzyme, which activity is induced in plasma by the action of the elicitors, could activate pro-PO in the absence of the ion, suggesting that at least two reaction steps are involved in the activation reaction of pro-PO in plasma. The activating enzyme was completely inhibited in the presence of p-NPGB, an inhibitor for serine protease.

Melanin formation is often observed around foreign materials such as various invading microorganisms, metazoan parasites and glass beads or nylon threads implanted into the insect hemocoel (1). Generally, melanization has been considered to represent, at least in part, a defence reaction of insects, since quinones, which are intermediates in melanin synthesis from phenolic substances, are cytotoxic and could be bactericidal or fungistatic agents (2); in addition, the reaction of melanin with protein(s) surrounding foreign materials might serve to physically isolate them (3).

Both the inactive precursor of PO and substrate of the enzyme, tyrosine, have been shown to be present in the hemolymph of many insect species (4). As

* Abbreviations: PO, phenoloxidase, o-di-phenol:O_2 oxidoreductase(EC 1.10.3.1); pro-PO, pro-phenoloxidase; hPPAE, hemolymph pro-phenoloxidase activating enzyme; p-NPGB, p-nitrophenyl-p'-guanidinobenzoate; dopa, 3,4-dihydroxy-L-phenylalanine; DFP, di-isopropylphosphrofluoridate; T-M buffer, 0.01 M Tris-maleate buffer, pH 6.5, containing 0.15 M NaCl; CS-plasma, plasma fraction of silkworm hemolymph (see Materials and Methods).

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melanin is synthesized by the action of PO, it is obvious that the activation reaction of pro-PO is somehow triggered by foreign materials in the hemocoel.

All insects thus far examined contain pro-PO and an activating system under normal physiological conditions, suggesting that these substances have important roles, whether or not these are related to immunity. It is desirable to investigate the control mechanism of the pro-PO activating system *in vitro* to know more about its function. However, such studies have been hampered by the fact that the pro-PO activating system is triggered during collection of hemolymph. Recently, we developed a method for obtaining silkworm hemolymph without perturbing these entities (5). We have adopted the method to collect silkworm hemolymph, and have begun to analyze the pro-PO activating system. The results from preliminary studies are reported in the present communication.

MATERIALS AND METHODS. Preparative procedures. Insects: Silkworm larvae (*Bombyx mori*) were raised as reported previously (5) and used between 2nd and 5th day of 5th instar. CS-plasma: The plasma fraction of silkworm hemolymph was collected according to Ashida (5), and designated CS-plasma. CS-plasma was dialysed against physiological saline (6) before use unless otherwise specified. pro-PO: Homogeneous pro-PO was prepared from silkworm hemolymph as reported previously (7). Lipophorin and storage proteins: The method of Chino *et al.* (8) was adopted to purify lipophorin from CS-plasma. The purified preparation was homogeneous as judged by disc poly-acrylamide gel electrophoresis. Homogeneous storage proteins I and II, purified according to Tojo (9), were gifts from Dr. Izumi. Denatured CS-plasma proteins, lipophorin, storage proteins I and II: 10 ml of CS-plasma was dialysed against three changes of 1 liter of T-M buffer for 72 hrs at 4°C. The dialysed CS-plasma was mechanically agitated with 100 up-and-down strokes of a Teflon pestle rotated by a motor. The precipitate formed was collected by centrifugation at 105,000g for 20 min. The precipitate was washed with T-M buffer by several cycles of sedimentation-and-resuspension. The protein concentration of the final suspension was 11.7 mg/ml. Lipophorin (0.8 mg protein/ml of T-M buffer), storage protein I (4.3 mg/ml of sodium acetate buffer, pH 6.1, containing 0.05 M NaCl) and storage protein II (4.2 mg protein/ml of sodium acetate buffer, pH 5.4, containing 0.2 M NaCl) were treated in the same way as CS-plasma; the protein concentrations of their final suspensions were 7.2 mg/ml, 1.7 mg/ml and 1.7 mg/ml, respectively. Suspensions of heat-killed bacteria and bacterial cell wall fractions: Bacteria listed in Table I were cultured in liquid L-broth at 35°C for 20 hrs. Bacterial cells were collected at 1,000g for 20 min and suspended in 0.85% NaCl solution. Sedimentation-and-resuspension of bacteria was repeated twice. The final suspension was incubated at 100°C for 30 min, followed by two cycles of sedimentation-and-resuspension for washing. The concentration of bacteria was adjusted to 10^9 cells/ml. The cell wall fraction of *E. coli* was prepared by the method of Kotani *et al.* (10) in which cells were disintegrated by sonication and the disintegrated cells were treated with trypsin. The cell wall fraction of *S. aureus* was prepared as above except that trypsin digestion was omitted. Both cell wall fractions were lyophilized and then suspended in distilled water (15.7 mg/ml).

Assay of PO activity. PO activity was measured colorimetrically using dopa as substrate, and the unit of the enzyme activity was defined after Ashida (7).

Protein determination. Protein concentration was determined by the method of Lowry et al. (11), using bovine serum albumin as the standard.

Chemicals. Chemicals used were as follow: dextran sulfate (M.W. about 500,000), inulin, kaolin and laminarin were purchased from Nakarai Chemical Co. ; zymosan, bovine serum albumin and dopa from Sigma; starch and chitin which was purified before use (12), from Wako Chemical Co.; cellulose (microcrystalline) from Merck; dextran T 10 from Pharmacia Fine Chemicals; p-NPGB from Vega Chemicals; lipopolysaccharide W (E. coli) from Difco Laboratories. All the other chemicals were the highest grade commercially available.

RESULTS. Substances inducing PO activity in CS-plasma. Among substances listed in Table I, both Gram-negative and Gram-positive bacterial cell wall

Table I. Survey of substances inducing phenoloxidase activity in CS-plasma.

Substance[a)]	Activity[b)]
Heat killed bacteria:	
Pseudomonas aeruginosa	+
Escherichia coli	+
Staphylococcus aureus	+
Bacillus thuringiensis	+
Bacillus subtilis	+
Cell wall fraction from	
Staphylococcus aureus	+
Escherichia coli	+
Lipopolysaccharide from	
Escherichia coli	-
Glucans:	
zymosan	+
laminarin	+
starch	-
dextran T 10	-
cellulose	-
Dextran sulfate	-
Inulin	-
Chitin (colloidal)	-
Kaolin	-
Denatured proteins[c)]:	
plasma proteins	+
lipophorin	+
storage protein I	-
storage protein II	-

a) Test substances were dissolved or suspended in distilled water as follows: lipopolysaccharide 0.001 mg to 10 mg/ml (examined successively by ten times dilution); zymosan, 2 mg/ml; laminarin, 5 mg/ml; starch, dextran T 10 and cellulose 15 mg/ml; dextran sulfate, inulin and kaolin, 0.1 to 100 mg/ml (examined successively by ten times dilution); chitin, 3.6 mg/ml. Concentrations of other substances are described in Materials and Methods.
b) 20 µl of test substance was added to 500 µl of CS-plasma and the mixture was incubated for 90 min at 25° C. Then PO activity was assayed. +, PO activity was 1,400 to 1,900 units/ml; -, PO activity could not be detectable.
c) Proteins used did not sediment under the experimental conditions described in Materials and Methods unless they were mechanically agitated. Here, denaturation means the change of property of proteins from soluble forms to precipitable ones under the experimental conditions.

fractions, glucans with β-1,3-glycosidic linkages, denatured plasma protein and denatured lipophorin could induce PO activity in CS-plasma under the experimental conditions employed. Since the denatured plasma protein preparation was shown to contain almost all the proteins present in native CS-plasma (data not shown), it remains to be studied whether proteins other than lipophorin could act as an elicitor upon denaturation.

The elicitors used were not capable of activating purified pro-PO, suggesting that some other factor(s) is involved in the activation of pro-PO in CS-plasma. The time course of the activation reaction proceeded in sigmoidal fashion as shown in Fig. 1; This differs from the hyperbolic time course observed with a previously studied cuticular pro-PO activating enzyme (13).

Effect of divalent cations on the induction of PO activity by zymosan or denatured plasma protein. None of substances listed in Table I could induce PO activity when it was incubated with CS-plasma in the absence of divalent cation, but elicitors resumed activity upon addition of Ca^{2+} (Fig. 1 a and b). The effect of Ca^{2+} was detectable at 10 μM although maximal effect was observed at 4 mM.

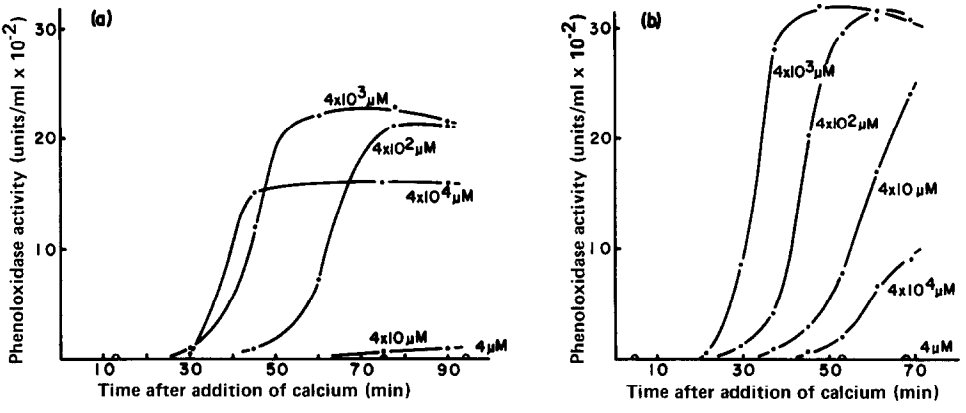

Fig. 1. Effect of calcium ion concentration on induction of PO activity by zymosan or by denatured plasma protein.
20 ml of CS-plasma was dialysed against 3 changes of 2 liter of T-M buffer over 72 hrs before use.
a) CS-plasma (500 μl) was added to the mexture of 20 μl of zymosan suspension (1 mg/ml) and 4 μl of $CaCl_2$ solution.
b) CS-plasma (5 ml) was mechanically agitated at 0°C by homogenization with 20 up-and-down strokes of a Teflon pestle rotated by a motor to produce denatured plasma protein. To 500 μl of the mechanically agitated CS-plasma, 4 μl of $CaCl_2$ solution was added.
The reaction mixtures were incubated at 25°C and PO activity was assayed at intervals. Final concentrations of $CaCl_2$ in the reaction mixtures are indicated in the figures.

Other than Ca^{2+}, chloride salts of Cd^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} had about the same effect as Ca^{2+} at 4 mM. Mg^{2+} could replace Ca^{2+} in the induction of PO activity by zymosan, but not in the case of denatured plasma protein. This may suggest that mechanisms of pro-PO activation by zymosan and denatured protein are not entirely the same.

Requirement of divalent cation for hPPAE activity. When purified pro-PO was added to CS-plasma which had been mechanically agitated and incubated at 25°C for 60 min, PO activity resumed increasing as shown in Fig. 2. This indicates that the pro-PO added was converted to PO. p-NPGB inhibited completely the activation of exogenous pro-PO at 50 µM. The same phenomena were observed after CS-plasma was incubated with zymosan.

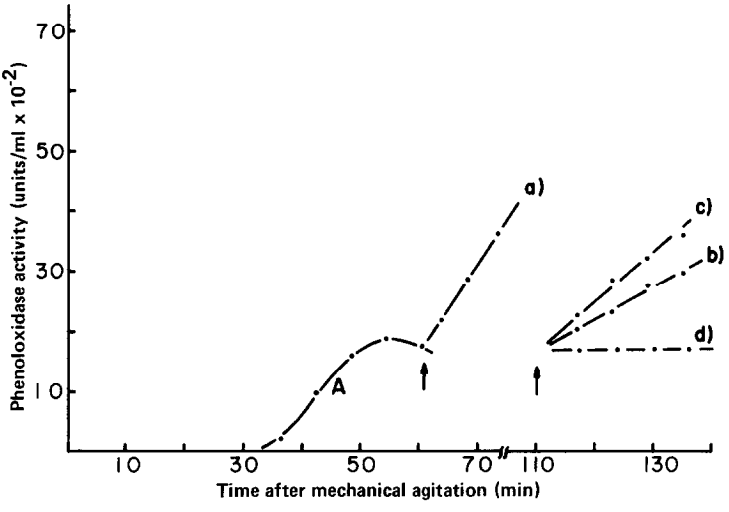

Fig. 2. Effect of calcium ion on pro-PO activating enzyme(hPPAE). CS-plasma was mechanically agitated as in Fig. 1b and incubated at 25°C. After the increase of PO activity leveled off (curve A), 2 ml of the CS-plasma(Activated CS-plasma) was passed through a Sephadex G-25 column (2 cm x 20 cm) equilibrated with T-M buffer at 4°C. Fractions appearing at void volume were pooled (Sephadex fraction). Activated CS-plasma and Sephadex fraction were used for the preparation of the following mixtures; a): 200 µl of Activated CS-plasma and 200 µl of pro-PO (200 µg protein/ml of T-M buffer). b): 200 µl of Sephadex fraction, 200 µl of pro-PO. c): 200 µl of Sephadex fraction, 200 µl of pro-PO and 4 µl of 0.4 M $CaCl_2$. d): Same as c) except that 0.01 M p-NPGB was added to final concentration of 50 µM.
At the time indicated by arrows, the above mixtures were prepared. Reaction mixtures were incubated at 25°C and assayed for PO activity at intervals. PO activities of b), c) and d) are presented in the figure after they were multiplied by a factor of 4.4, by which CS-plasma was diluted due to Sephadex G-25 column chromatography and addition of pro-PO. Those of a) are presented after they are multiplied by a factor of 2.

CS-plasma, which had been incubated with denatured plasma protein, was applied to a Sephadex G-25 column to remove divalent cations from the medium. Fractions eluted at void volume were able to activate purified pro-PO in the absence of divalent cation (Fig. 2). This result seems to indicate that activation reaction of pro-PO by elicitor consists of at least two reaction steps. Ca^{2+} slightly enhanced hPPAE activity at 4 mM. This may partly be ascribed to a stabilizing effect of Ca^{2+} on hPPAE, since the enzyme lost more than 70% of its activity for 12 hrs at 0°C in the absence of Ca^{2+}, but less than 20% in the presence of Ca^{2+} at 4 mM.

DISCUSSION: The pro-PO activating system present in the plasma of silkworm hemolymph could be triggered by elicitors such as those substances listed in Table I. As for lipophorin, it may be useful to determine whether the plasma membranes of cells could trigger the pro-PO activating system, since lipophorin has been shown to be a complex molecule which is similar to plasma membrane in terms of protein, phospholipid, carotenoid and cholesterol content (8).

Intensive studies seem to be necessary to elucidate the pro-PO activating system. However, it should be noted that the pro-PO activating system of silkworm plasma has some similarities to cascade reactions such as the complement system of mammals (14) and the blood coagulation system of the horse shoe crab (15): some elicitors are common among them, all are known to have multi-step properties, all require divalent cation, and proteolytic enzymes in each case seem to be activated by the action of elicitors. Considering the myriad physiological functions of the complement and blood coagulation systems, these similarities suggest that the pro-PO activating system may play as yet unknown, but important, roles in defence mechanisms of insects.

Pye reported that the pro-PO of wax moth larvae immunized with lipopolysaccharide from Shigella flexneri could be activated by zymosan, heat-killed Pseudomonous aeruginosa, or mechanical agitation of plasma (16, 17). In the crayfish (Astacus astacus), it has been reported that β-1,3-glucan could enhance PO activity in hemocyte lysates and that the enhancement was inhibited by soybean trypsin inhibitor or DFP (18, 19). Recently, results suggesting the

presence of a multi-step activation mechanism of pro-PO in the crayfish have been obtained (Ashida and Söderhäll, unpublished). A pro-PO activating system with the properties reported here may thus be widely distributed among arthropods.

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REFERENCES

1. Ratcliffe, N.A., and Rowley, A.F. (1979) Insect hemocytes, pp331-414. Cambridge Univ. Press.
2. Graham, D.G., Tiffany, S.M., and Vogel, F.S. (1978) J. Invest. Dermatol. 70, 113-116.
3. Salt, G. (1970) The Cellular Defence Reactions of Insects. Cambridge Univ. Press. London and New York.
4. Wyatt, G.R., and Pan, M.L. (1978) Ann. Rev. Biochem. 47, 779-818.
5. Ashida, M. (1981) Insect Biochem. 11, 57-65.
6. Jungreis, A.M., Jatlow, P., and Wyatt, G.R. (1973) J. Insect Physiol. 19, 225-233.
7. Ashida, M. (1971) Arch. Biochem. Biophys. 144, 749-762.
8. Chino, H., Murakami, S., and Harashima, K. (1969) Biochim. Biophys. Acta. 176, 1-26.
9. Tojo, S., Nagata, M., and Kobayashi, M. (1980) Insect Biochem. 10, 289-303.
10. Kotani, S., Kitaura, T., Hirano, T., and Tanaka, A. (1959) Biken's J. 2, 129-141.
11. Lowry, O.H., Rosebrough, H.J., Farr, A.L., and Randall, R.J. (1951) 193, 265-275.
12. Lunt, M.R., and Keny, P.W. (1960) Biochim. Biophys. Acta. 44, 371-373.
13. Ashida, M., and Dohke, K. (1980) Insect Biochem. 10, 37-47.
14. Götze, O., and Müller-Eberhard, H.J. (1976) Adv. Immunol. 24, 1-26.
15. Morita, T., Tanaka, S., Nakamura, T., and Iwanaga, S. (1981) FEBS Letter. 129, 318-321.
16. Pye, A.E. (1974) Nature, Lond. 251, 610-613.
17. Pye, A.E. (1978) Insect Biochem. 8, 117-123.
18. Söderhäll, K., and Unestam, T. (1979) Can. J. Microbiol. 25, 406-414.
19. Söderhäll, K. (1981) Develop. Comparative Immunol. 5, 565-573.