ACTIVATION OF PREPHENOLOXIDASE

111. RELEASE OF A PEPTIDE FROM PREPHENOLOXIDASE BY THE ACTIVATING ENZYME

M.Ashida (Department of Biology, College of General Education, University of Tokyo, Komaba, Meguro-ku, Tokyo), K. Dohke (Department of Biology, Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo) and E. Ohnishi (Biological Institute, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya)

Received February 22,1974

Activation reaction of prephenoloxidase (pre-enzyme) was analyzed using a system of homogeneous pre-enzyme and highly purified prephenoloxidase-activating enzyme (PPAE) from the silkworm Bombyx mori. When pre-enzyme was activated by PPAE, release of a peptide was demonstrated. Results of polyacrylamide gel electrophoresis revealed that only a single peptide is liberated from pre-enzyme. Several lines of evidence indicated that the released peptide is inhibitory on PPAE.

Activation of an inactive precursor of phenoloxidase has first been reported by Bodine and Allen in the grasshopper egg (1). Since then, occurrence of prephenoloxidase and its conversion to an active form have been shown in various insect species of different developmental stages (2 - 5), but detailed analysis of the activation reaction at a molecular level has not been worked out.

Recently, prephenoloxidase and prephenoloxidase-activating enzyme (abbreviated as PPAE) have highly been purified from hemolymph and cuticle, respectively, of the silkworm larvae (6 - 7). It was demonstrated that the ester substrate for trypsin can be hydrolyzed by the purified PPAE and that both the activation reaction of pre-enzyme by PPAE and esterase activity of the enzyme are inhibited by a serine protease inhibitor, DFP and also by the specific titrant for tryptic activity, p-NPGB (8). These facts, along with our previous findings (9), strongly suggest that partial proteolysis is involved in the process of the activation reaction of pre-enzyme.

In the following experiments we use a system consisted of homogeneous

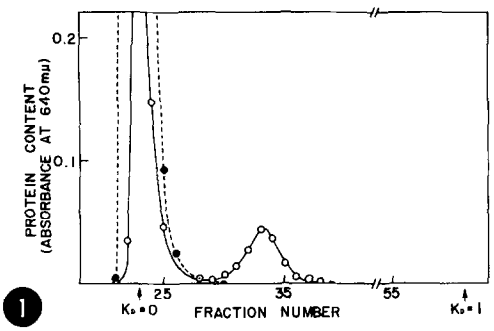
Abbreviations used are: DFP, di-isopropyl phosphorofluoridate; p-NPGB, p-nitrophenyl-p-guanidinobenzoate.

preparation of pre-enzyme and highly purified PPAE and demonstrate that a peptide is released during the activation reaction of pre-enzyme by PPAE and that the released peptide exerts an inhibitory activity on PPAE.

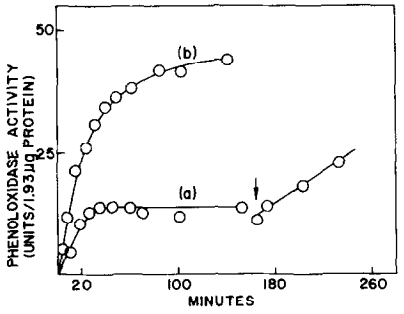
MATERIALS and METHODS. Pre-enzyme was purified from larval hemolymph of the silkworm Bombyx mori, according to Ashida (6). Purified PPAE was prepared by the method of Dohke from cuticle of the silkworm larvae (8).

Phenoloxidase activity was determined colorimetrically after Ashida (6) and PPAE activity was assayed by the method of Dohke (7). Disc-electrophoresis was carried out according to Davis (10), using 7.5 % polyacrylamide gel at pH 8.9 for small pore gel. Dodecylsulfate-polyacrylamide gel electrophoresis (abbreviated as SDS-electrophoresis) was carried out by the method of Weber and Osborn (11) with minor modifications as described previously (6). Protein was determined by the method of Lowry et al (12), using bovine serum albumin as the standard.

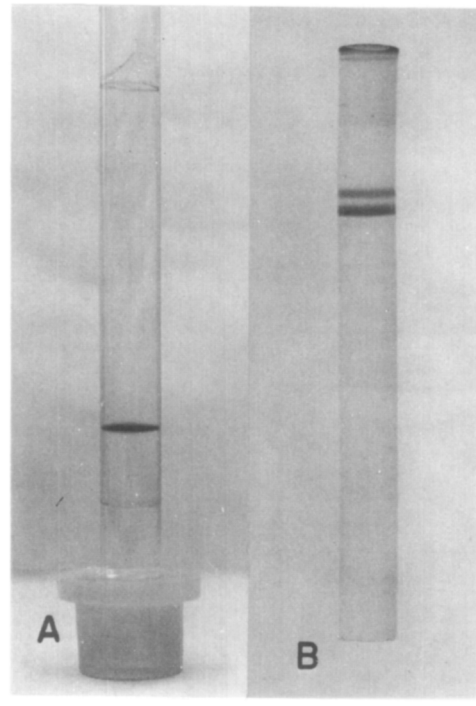
EXPERIMENTAL PROCEDURES and RESULTS. When pre-enzyme was incubated with PPAE, activation reaction took place and phenoloxidase activity appeared (Fig. 3). If any peptide bond of pre-enzyme was hydrolyzed during the activation reaction by PPAE as suggested by our previous findings (8 - 9), peptide(s) might be released from pre-enzyme in the course of activation. This possibility was examined as follows. After 60 min incubation at 0°, the same incubation mixture as described in the legend of Fig. 3a was boiled for 3 min and denatured protein was discarded by centrifugation at 105,000 g for 10 min. Then the supernatant solution (designated as SE) was subjected to column chromatography on Sephadex G-50. As a control, the mixture of an equal volume of both heat-treated pre-enzyme and PPAE was centrifuged and the supernatant solution (designated as SC) was used. Elution profiles are presented in Fig. 1. In the elution pattern of SE a minor peak is seen around fr. No. 33 but is absent in that of SC. In disc-electrophoresis the minor peak fraction (Nos. 31 -35) gave only one band near the front (Fig. 2a). When an aliquot of the minor peak fraction was hydrolyzed with hydrochloric



1



3



2

Fig. 1. Column chromatography of SE and SC on Sephadex G-50.
SE and SC were prepared by methods as described in the text except that pH of the incubation medium was 8.0 Sephadex G-50 column (1.5 x 85 cm) was eqilibrated with 0.01 M K-PO_4 buffer, pH 8.0, and each 2 ml of SE or SC was applied to the column. Protein was eluted with the buffer at the flow rate of 5 ml per hour and 2 ml-fractions were collected. O—O : SE. ●---● : SC.

Fig. 2. Polyacrylamide gel electrophoresis.
(a) About 10 μg of peptide fraction in Fig. 1 (Nos. 31 - 35) was subjected to disc-electrophoresis. An electric current was supplied at 3 mA/tube (gel column size ; 5 x 50 mm), until bromphenol blue migrated to near the end of the anode side. Potein on the gel was stained with Amido Black in 7% acetic acid.
(b) The same reaction mixture as in Fig. 3b was incubated for 60 min at 0° and mixed with the medium for SDS-electrophoresis. About 8.5 μg of protein was applied to a column. Protein was stained with Coomassie Brilliant Blue. Upper and lower bands correspond to pre-enzyme and phenoloxidase, respectively.

Fig. 3. Activation of prephenoloxidase by purified PPAE.
(a) A mixture of an equal volume of pre-enzyme (11.6 mg protein/ ml of 0.01 M K-PO_4 buffer, pH 7.0) and PPAE (30 μg protein/ml of 0.01 M K-PO_4 buffer, pH 7.0) was incubated in an ice bath. An aliquot of the incubation mixture was diluted 300-fold with cold 0.01 M K-PO_4 buffer, pH 7.0 at intervals just before assay of phenoloxidase and 0.1 ml of the diluted reaction mixture was pipetted into the assay mixture of phenoloxidase. At the time indicated by the arrow, the reaction mixture was diluted 30-fold with 0.01 M K-PO_4 buffer, pH 7.0, and 10 ul of the diluted reaction mixture was assayed for phenoloxidase at intervals.
(b) Same as in (a) except that the concentrations of pre-enzyme and PPAE were 386 μg protein/ml and 5 μg protein/ml, respectively and 10 μl of the mixture was pipetted into the assay mixture of phenoloxidase. In each case, an equal amount of pre-enzyme (1.93 μg) was pipetted into the assay mixture of phenoloxidase.

acid, only amino acid were detected in the hydrolyzate, of which amino acid composition was presented in Table 1.

From above results it can be inferred that a peptide is released from pre-enzyme by PPAE in the course of activation reaction. If peptide is released from pre-enzyme during activation, the molecular weight of active phenol-oxidase might be smaller than that of pre-enzyme. This was examined by means of SDS-electrophoresis. As shown in Fig. 2b, after incubation of pre-enzyme with PPAE, a new band appeared just beneath the pre-enzyme one. The result indicates that pre-enzyme is transformed to a slightly smaller molecule upon activation.

Another feature of the activation reaction is that an inhibitor for PPAE might be produced in the course of activation of pre-enzyme by PPAE. This is suggested by the following observations: (1) As shown in curve a of Fig. 3, when the incubation mixture is diluted after the increased activity leveled off, re-increase of the phenoloxidase activity is induced; (2) If the pre-enzyme concentration is lowered to one-thirtyth that in curve a, the final yield of phenoloxidase attained is elevated to about 3-fold compared with that of curve a (Fig. 3, curve b). These phenomena are understandable if we assume that the activation reaction could be terminated half-way by the inhibitor produced in the presence of higher concentration of pre-enzyme. To examine the above possibility, effects of SE and SC on the activation reaction were tested. Results are presented in Fig. 4. SE inhibited completely the activation reaction of pre-enzyme under the experimental conditions. SC seems also to have some inhibitory activity, when the activation velocity in Fig. 4b is compared with that in Fig. 4c. This inhibitory activity of SC may be explained by assuming that denatured protein remained in SC has some affinity to PPAE and compete with native pre-enzyme for PPAE. The activation reaction of pre-enzyme by PPAE and esterase activity of PPAE were inhibited by the minor peak fraction in Fig. 1.

DISCUSSION. The results described above indicate that a step of limited

Table 1. Amino acid composition of peptide released from prephenoloxidase by PPAE.

Amino acid	Amount (g/100 g peptide)	Amino acid	Amount (g/100 g peptide)
Lys	2.26	Ala	2.59
His	0.23	Cys/2	0.18
Arg	4.44	Val	3.58
Asp	15.12	Met	0.00
Thr	5.15	Ileu	3.53
Ser	2.75	Leu	12.65
Glu	19.18	Tyr	2.48
Pro	5.41	Phe	8.93
Gly	2.35		

Peptide fraction (Nos. 31 - 35) in Fig. 1 was lyophilized and then hydrolyzed with 6 N hydrochloric acid for 24 hours at 105°. Amino acids in the hydrolyzate were analyzed according to the method of Spackman et al (13).

proteolysis is involved in the activation reaction of pre-enzyme and that a peptide, which acts as an inhibitor for PPAE, is released from pre-enzyme. This conclusion is also supported by our following observations. Firstly, highly purified PPAE has peptidase or esterase activity and the activation of pre-enzyme by PPAE is inhibited by some serine-enzyme inhibitors such as DFP (7). Secondly, the release of peptide from pre-enzyme by PPAE was inhibited by p-NPGB (unpublished observtion). Thirdly, amino acid analysis of pre-enzyme showed that the protein dose not contain appreciable amount of carbohydrate or lipid (6). Coincidentally, the substance released from pre-enzyme is only composed of amino acids (Table 1).

PPAE seems to be highly specific for activation of prephenoloxidase, since PPAE produces one protein molecule (phenoloxidase) from pre-enzyme with concomitant release of a peptide and the phenoloxidase seems not to be degraded further (Fig. 2b). This situation becomes apparent when the activation reaction by PPAE is compared with that by α-chymotrypsin. α-Chymotrypsin produces several fragments along with an active enzyme and the active

fragment is degraded further as judged by SDS-electrophoresis during the activation process (unpublished observation).

In the activation reaction by PPAE, a single peptide seems to be released from the pre-enzyme as evidenced by the following facts: (1) In SDS-electrophoresis protein band other than pre-enzyme and phenoloxidase did not appear (fast running peptide band was not visible due to low content and diffusion in the gel under the experimental conditions.). This indicates that large fragment other than phenoloxidase is not produced during the activation of

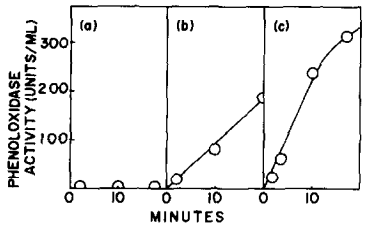


Fig. 4. Effect of SE and SC on the activation reaction of pre-enzyme by PPAE. SE and SC were prepared by the method as described in the text. Each 0.45 ml of SE (202.8 µg protein/ml) (a), SC (202.7 µg protein/ml) (b) and 0.01 M K-PO_4 buffer, pH 7.0 (c), was preincubated with 0.5 ml of PPAE (5 ug protein/ml of 0.01 M K-PO_4 buffer, pH 7.0) for 10 min, respectively. Then, 0.05 ml of pre-enzyme (386 µg protein/ml of 0.01 M K-PO_4 buffer, pH 7.0) was added to (a), (b) and (c), respectively. An aliquot of each reaction mixture (0.1 ml) was assayed for phenoloxidase activity at intervals.

Since original protein concentration of SC was 681.2 µg protein/ml, SC was diluted to the same protein concentration as SE in order to compare the inhibitory effect of SE and SC at the equal amount of protein.

pre-enzyme by PPAE. The distance between the band of pre-enzyme and that of phenoloxidase roughly accounts to molecular weight of several thousand for the peptide (unpublished observation). (2) In Sephadex G-50 column chromatography only one peak appeared between $K_d = 0$ and $K_d = 1$ (Fig. 1). (3) The minor peak fraction in Fig. 1 gave a single band near the front in the disc-electrophoresis (Fig. 2a).

As far as we know, there is no example that an inactive precursor of oxidase or oxygenase is activated by a specific hydrolytic enzyme. In this respect, the activation of prephenoloxidase in the silkworm seems to be the

first case in this category. To confirm unambigously the above conclusion, more detailed analyses such as determination of the terminal amino acids of the peptide, prephenoloxidase and phenoloxidase seem to be necessary. The work along this line is now in progress.

REFERENCES.

1. Bodine, J.H., and Allen, T.H., J. Cell and Comp. Physiol., 12 71 (1937)
2. Ohnishi, E., Jap. J. Zool., 11 69 (1953)
3. Schweiger, A., and Karlson, P., Z. Physiol. Chem., 28 206 (1962)
4. Ashida, M., and Ohnishi, E., Arch. Biochem. Biophys., 122 411 (1967)
5. Hyneman, R.A., and Vercauteren, R.E., J. Insect Physiol., 14 409 (1968)
6. Ashida, M., Arch. Biochem. Biophys., 144 749 (1971)
7. Dohke, K., Arch. Biochem. Biophys., 157 210 (1973)
8. Dohke, K., Arch. Biochem. Biophys., 157 203 (1973)
9. Ohnishi, E., Dohke, K., and Ashida, M., Arch. Biochem. Biophys., 139 143 (1970)
10. Davis, B.J., Ann. N. Y. Acad. Sci., 121 Art. 2., 404 (1964)
11. Weber, K., and Osborn, M., J. Biol. Chem., 244 4406 (1969)
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193 265 (1951)
13. Spackman, D.H., Stein, W.H., and Moore, S., Anal. Chem., 30 1190 (1958)