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LACCASE-TYPE PHENOLOXIDASE IN THE CUTICLE OF THE SILKWORM BOMBYX MORI

Hiroko I. YAMAZAKI

Atomi Gakuen Women's University,
Niiza-shi, Saitama 352

ABSTRACT

Cuticular-bound phenoloxidase of the silkworm *Bombyx mori* was investigated in relation to the sclerotizing process that occurs following larval-pupal ecdysis. The phenoloxidase, having properties of laccase, seems to play an important role in the hardening and darkening process during the molting course, because of the correlation of these events with the change in the enzymatic activity. The enzyme was the laccase-type phenoloxidase judging from the substrate specificity. The phenoloxidase was in an inactive pro-form in newly ecdysed pupal cuticle and could be isolated with the aid of proteolysis. The pro-laccase could be activated by limited proteolysis. These results suggest that the pro-enzyme is activated by a proteolytic enzyme(s) which is present in the cuticle.

key words: phenoloxidase, insect, cuticle, sclerotization.

Insects belong to the phylum Arthropoda, whose members have an external skeleton. So they need to change the hard cuticle for their growth and development. The cuticle changes at the time of molting(ecdysis). Molting is a dramatic event during post-embryonic development of insects. Especially, larval-pupal and pupal-imaginal moltings are notable ones because of the drastic morphological transformation. The structural and colour changes following larval-pupal ecdysis are most valuable target phenomena to elucidate the mechanism of ecdysis. The hardening of the cuticle after molting has been attributed to quinone tanning, a cross-linking reaction between phenolic compounds and proteins in the cuticular matrix; and phenoloxidase is presumed to catalyze the reaction (Pryor, 1940; Andersen, 1985; Sugumaran, 1987).

In 1953, Ohnishi presented a report on the phenoloxidase in the hemolymph of *Drosophila melanogaster*. In this report, it was clearly shown that phenoloxidase was present in a pro-form and converted to phenoloxidase by a protein-like activator and that the activated enzyme had the properties of tyrosinase-type phenol-oxidase (Ohnishi, 1953). The pro-phenoloxidase from the hemolymph of *Bombyx mori* was completely purified in 1971 and thoroughly investigated (Ashida, 1971). The hemolymphal phenoloxidase is considered to play an important rôle in defense mechanisms of insects (Ochiai and Ashida, 1988). On the other hand, another type of phenoloxidase, one having characteristics like those of laccase, was observed in the cuticle (Ohnishi, 1954; Yamazaki, 1969; Yamazaki, 1972). The role of the cuticular phenoloxidase is suggested to be functional during ecdysis, because the enzyme is active during the time at which the hardening and darkening process is occurring after larval-pupal ecdysis. In this paper I describe some properties of this laccase-type phenoloxidase, its pro-form, and its activation mechanism in *Bombyx mori*.

PROPERTIES OF CUTICLE-BOUND PHENOLOXIDASE

Phenoloxidase activity in the cuticular preparation of pupae was assayed manometrically by using hydroquinone as substrate at various times after larval-pupal ecdysis. The change in the enzyme activity coincided with the darkening and hardening process after ecdysis, as shown in Fig.1. The cuticular phenoloxidase thus seems to play an important role during the process. The phenoloxidase oxidized efficiently hydroquinone, p-phenylenediamine, catechol, and dopamine; however, it could not oxidize tyrosine. Moreover, the activity was completely inhibited with sodium diethyldithiocarbamate, but not with carbon monoxide, which is an effective inhibitor of tyrosinase in hemolymph. So, the phenoloxidase in cuticle was proved to be the laccase-type enzyme. By several attempts to elucidate the biochemical properties of the enzyme, the following results were obtained: The laccase in the cuticle could be solubilized with the aid of trypsin and the solubilized enzyme was purified 930 fold in terms of specific activity. The purified enzyme contained three peptides with phenoloxidase activity, and the molecular weights of the peptides were 70, 66, and 62 Kdaltons, respectively. Interestingly, an inactive pro-laccase was found to be present in newly ecdysed pupal cuticle as a bound form, and the pro-enzyme in cuticle could

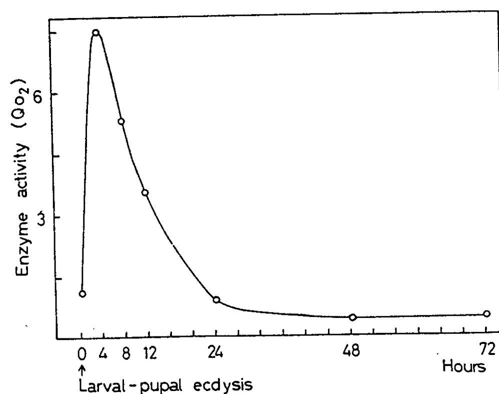

Fig. 1. Change of the cuticle-bound phenoloxidase activity during development.

be activated with the use of trypsin.

ISOLATION OF PRO-LACCASE FROM CUTICLE

Newly ecdysed pupae were collected and dissected on filter paper wetted with 1.15 per cent sodium chloride solution. After removal of alimentary canals, fat bodies, muscles, and tracheas, cuticles were rinsed with the cold saline solution and blotted with filter paper. The isolated cuticles were homogenized with the saline solution and centrifuged at 8,000 *g* for 30 minutes. The pellet was suspended in 0.2 M borate buffer (pH 8.0) and used as cuticle preparation. The cuticles were digested with chymotrypsin for 15 minutes at 32°C and quickly chilled at 0°C. As it was found that chymotryptic digestion of the cuticle brought about solubilization of pro-laccase, the digest was centrifuged at 12,000 *g* for 30 minutes and the supernatant was used as a crude pro-laccase preparation. The pro-laccase activation was analyzed by activity staining after native polyacrylamide gel electrophoresis (PAGE). Phenoloxidase activities on the gel were visualized by incubation with dimethyl-*p*-phenylenediamine or dopa in 0.1 M citrate phosphate buffer (pH 5.5). Proteins on the gel were stained with Coomassie brilliant blue. Laccase activity latent within the pro-laccase preparation was estimated as follows: An aliquot of the preparation was incubated with trypsin at 32°C for 40 minutes. After the treatment, the activated laccase activity was assayed by the use of catechol as substrate in the presence of sulfanilic acid (Yamazaki, 1972).

PURIFICATION OF PRO-LACCASE

The following procedures were performed at 0-4°C. The chymotryptic digest was centrifuged at 20,000 *g* for 30 minutes and the supernatant was applied to a column of Sephadex G-150 equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The pro-laccase

fraction, which was separated from the bulk of the proteins, was loaded onto a column of hydroxylapatite(HA) equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The eluate from the HA column (HA fraction) was applied to a column of DEAE-Toyopearl 650M equilibrated with the same buffer, and the bound material was eluted with a linear gradient of KCl (0-0.45M). The elution profile is shown in Fig.2. The main fractions (No.36 - 40) had been proved to contain pro-laccase by native PAGE, because the preparation following tryptic digestion gave three peptides identical to active laccase. So the fraction was designated as chymotryptic pro-laccase. During the process of the purification, 43.3 % of pro-laccase activity in the cuticlar preparation was found in the finally obtained fraction (chymotryptic pro-laccase), and the specific activity was increased 684 fold.

ACTIVATION OF PRO-LACCASE

The preparation of chymotryptic pro-laccase was analyzed by native PAGE. A single peptide band having Rf value of 0.15 was located on the gel, and it was faintly positive following prolonged

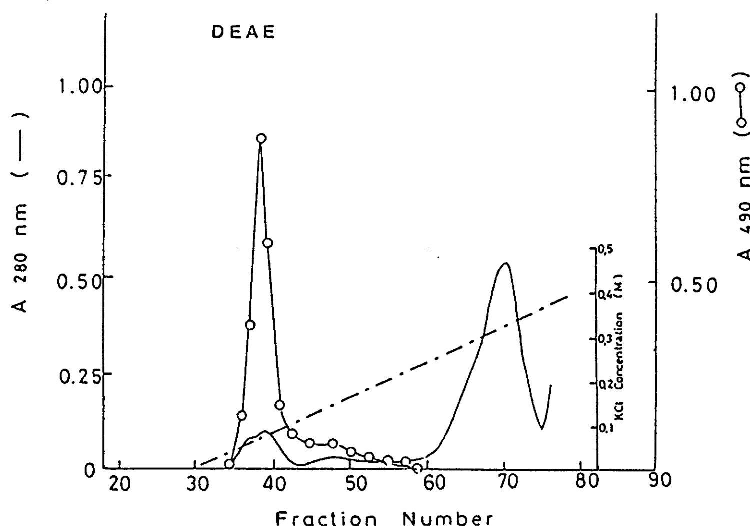

Fig. 2. Column chromatography of pro-laccase on DEAE-Toyopearl.

incubation (4-5 hours) with dimethyl-p-phenylenediamine. So, the pro-laccase was slightly positive for laccase activity. After treatment of the pro-laccase with trypsin, the above peptide band was very faint and some newly formed peptides with reduced molecular weight appeared; and these were positive when stained for laccase activity. The molecular weight of the pro-laccase was analyzed by Sephadex G-150 gel filtration and SDS-PAGE. The molecular size of the pro-laccase was estimated as 81 kdaltons. On the other hand, laccase activated by trypsin treatment contained three peptides on SDS-PAGE and the molecular weights of the peptides were estimated as 70, 66, and 62 kdaltons, respectively. So, the decrease in molecular size during the activation process was confirmed. The activation process was also observed following treatment with other proteolytic enzymes such as thermolysin, pepsin, pronase E, and proteinase K. These results are shown in Fig 3. An interesting result was obtained from activation by pepsin. The chymotryptic pro-laccase produced a peptide with reduced molecular weight by

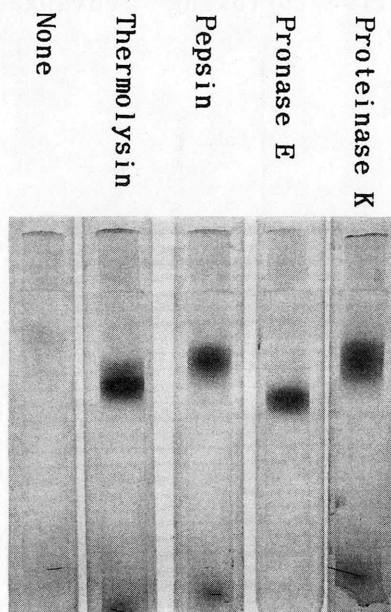

Fig. 3. Disc electrophoretic patterns of the active laccase produced by proteolytic enzymes. Protein bands revealed by incubation of the gel with dimethyl-p-phenylenediamine.

pepsin treatment like with trypsin treatment. However, the substrate specificity of the pepsin-activated enzyme was shown to be similar to the tyrosinase-type phenoloxidase of hemolymph. Results of this experiments are shown in Table 1. The trypsin-treated pro-laccase

Table 1. Comparison of substrate specificity between the products activated by trypsin and pepsin

Compound	Relative activity expressed as percentage relative to that of methylhydroquinone	
	Trypsin-treated	Pepsin-treated
Methylhydroquinone	100	100
Catechol	120	234
Dopamine	100	171
Dopa	36	158

oxidized dopa slightly; on the other hand, the pepsin-activated one oxidized it efficiently and methylhydroquinone was attacked most poorly by the pepsin-treated phenoloxidase. These results suggest that the chymotryptic pro-laccase contains not only the peptide region regulating activity, but also has the site controlling substrate specificity. The chymotryptic pro-laccase was analyzed by Ouchterlony's double diffusion test on an agarose plate with the use of antiserum raised against purified active laccase. This antiserum reacted with chymotryptic pro-laccase, chymotryptic pro-laccase after trypsin treatment, and trypsin-treated cuticular preparation. But non-treated cuticle did not form a precipitin line on the plate, for the laccase portion in the cuticular matrix was not solubilized. Details will be reported elsewhere.

DISCUSSION

Studies on the dynamics of cuticular phenoloxidase activity have suggested that the enzyme is intimately involved in the process that follows larval-pupal ecdysis. The phenoloxidase activity in cuticle was not detectable immediately after molting; however, the activity increased rapidly and reached a maximal level after 4 hours in the silkworm Bombyx mori (Yamazaki, 1972).

The phenoloxidase responsible for the above activity was isolated and characterized in the present study. Some of its most important properties are as follows: 1)it is bound tightly to the cuticular matrix such that enzymatic proteolysis is essential for its isolation; 2)it is a polypeptide whose molecular weight is 70 kdaltons; 3)it is the laccase-type of phenoloxidase judging from its substrate specificity usually found in sap from lacquer tree. These findings support the idea that the phenoloxidase is the key enzyme for the hardening and darkening process in the cuticle following larval-pupal ecdysis and give the molecular bases for further approaches to an understanding of these events.

The results described above bring to question the mechanism underlying the increase in enzyme activity, i.e., de novo synthesis versus activation of inactive precursor during the process. A successful isolation of pro-laccase (chymotryptic pro-laccase) supports the possibility of the involvement of an activation process during molting. The chymotryptic pro-laccase could be activated by limited proteolysis. The portion of the peptide chain released in the process was estimated to be about 10 kdaltons in molecular weight. In the released peptide portion, the two regulatory sites for the function of the phenoloxidase might be present. The first one masks the active centre and the second is the regulatory site for determining substrate specificity.

The experimental results described above clearly show that the pro-laccase is bound in the cuticular matrix. And the pro-laccase

may be activated by activator(s) in concert with the hardening and darkening process of the newly formed cuticle. The data suggest the natural activator(s) resides in the cuticle and might be a proteinase(s). From such a working hypothesis, proteolytic activity was investigated in the newly formed cuticle and a factor causing activation of the chymotryptic pro-laccase was detected in the insoluble fraction obtained from the homogenate (Yamazaki, 1987). In order to study the dynamics of the pro-laccase peptide in the cuticular matrix, immuno-blotting analysis was attempted with the use of antiserum raised against purified laccase. From the experiment, an immunologically active peptide, presumably native pro-laccase, could be detected in the pupal cuticle of 5th instar larvae; and this peptide might be transferred immediately to the cuticular matrix (Yamazaki, 1988).

Phenoloxidases are a rather more common enzyme group in plants than in animals, and their phenolic substrates are similarly distributed. In mammals, tyrosine, a monophenol amino acid, is noted as the parent compound for adrenalin, a well-known catecholamine with hormonal activity; otherwise, the functional roles of phenolic compounds and phenoloxidases are known in only a few cases such as metabolic diseases or necrosis due to some abnormality. However, in insects, phenolic compounds and phenoloxidases are involved in additional essential biochemical events including the building of the external skeleton, defense mechanisms against parasitic invaders, cure of injury, and in the formation of some important biologically active molecules such as pheromones .

The relationship between insects and plants or microorganisms has often been noticed. For instance, trehalose, known as blood sugar in insects, is also found in yeast and fungi. As described above, many phenolic compounds are commonly found in insects, plants, and fungi. And laccase has been known for some time as the functional enzyme of the tanning process of sap from the lacquer tree involved

in the making of japan. It is very interesting that the tanning of this sap and the hardening of the silkworm cuticle share a similar biochemical reaction. As laccase is a protein, the amino acid sequence can be determined by DNA sequencing of the laccase gene. The techniques for gene cloning and base sequence analysis are now in our hands. Thus the structural analysis of laccase should not be difficult. It is an interesting approach to determine the DNA base or amino acid sequences of the two laccases. As a result, a more detailed discussion of the relationship between plants and insects might be possible.

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