Studies on Tyrosinase in Housefly

Part I. Protyrosinase in the Pupae of Housefly and Its Activation

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Occurrence of protyrosinase in the prepupae of housefly, *Musca vicina* MAQUART, and its activation were described. The prepupae possessing no appreciable tyrosinase activity could be separated from the other aged pupae by putting them into water. Homogenate prepared from the prepupae contains protyrosinase and has no activation system for the proenzyme. As has been reported by Ohnishi²⁾ a certain activator occurred naturally in the aged pupae apparently activates the protyrosinase in vitro. However, contrary to Ohnishi's results³⁾ it was found that this protyrosinase can be activated by the treatment with sodium dodecyl sulfate in vitro.

The nature of tyrosinase in the lower animals was first extensively investigated by Bodine et al.¹⁾ Tyrosinase in the eggs of the grasshopper, *Melanoplus differentialis*, was found in an inactive form which was referred to as protyrosinase. The protyrosinase seems to be converted naturally into an active form by a certain unidentified substance which is soluble in oil and glycerol. In vitro, it was found that protyrosinase is possibly activated by treatment of its saline solution with some anionic detergents, such as sodium oleate or sodium dodecyl sulfate (SDS). Treatment with urea or acetone, and heating up to 60°C also brought about the activation. Vigorous shaking with the saline solution was also effective. Thus, the activation was postulated to occur due to the cleavage of some linkages in the protein molecule as a result of the mild denaturation caused by denaturing agents such as those mentioned above.

On the other hand, Ohnishi²⁾ observed that

although tyrosinase in the body fluid of prepupae of *Drosophila melanogaster* or *Musca vicina* MAQUART seems also to exist as a proenzyme as mentioned by Bodine, it is activated by the brei of tissues, but not by SDS. In addition, the activator was assumed to be probably a protein due to its non-dialysability and the loss of activity caused by denaturation with urea or by heating. Thus, depending upon the differences in the types of activator, Ohnishi concluded that there are at least two types of protyrosinase in lower animals, i.e., the one is possibly activated with a certain denaturing agent, and the other is not activated by detergent but by naturally occurring activation system.

Horowitz and Fling⁴⁾ reported that the saline extract of flies, *Drosophila*, six-hours after emergence initially did not possess tyrosinase activity, but it appeared autocatalytically on standing at 0°C. They proposed an

1) J.H. Bodine and T.H. Allen, *J. Cellular Comp. Physiol.*, **12**, 71 (1938).

2) E. Ohnishi, *Japon. J. Zool.*, **11**, 69 (1953).

3) E. Ohnishi, *Annot. Zool. Jap.*, **27**, 188 (1954).

4) N.H. Horowitz and M. Fling, "Symposium on Amino Acid Metabolism", The John Hopkins Press, Baltimore, p. 207 (1955).

activation mechanism depending upon the fact that the fraction containing tyrosinase did not contain an activator, while the supernatant separated from it did:

As mentioned above, notwithstanding the fact that tyrosinase was found to occur in an inactive form in lower animals, there was a difference in the types of activator in the different researches.

As a result of the preliminary study of change in tyrosinase activity in the metamorphosis process from larvae to adult through pupae of *Musca vicina* MAQUART, the authors learned that tyrosinase indeed occurs as an inactive protyrosinase at the beginning of each step of metamorphosis, and that subsequently it is increasingly activated with growth by a certain activation system. Therefore, in order to study the nature as well as the activation mechanism of protyrosinase, prepupae seemed certainly to be favorable as its source. For this purpose, an attempt was first made successfully to separate prepupae from pupae of different ages. In addition, it was detected that protyrosinase in homogenate prepared from prepupae is possibly activated even by SDS in vitro. Thus, it became a necessity to examine over again the type of activator essential to the protyrosinase-activation in vitro.

MATERIALS AND METHODS

Reagents. Catechol of extra-pure grade (Merck) was used without further purification. *p*-Cresol of analytical grade was redistilled at reduced pressure. Commercial sodium dodecyl sulfate (SDS) was recrystallized using absolute ethanol as a solvent. All these reagents were dissolved in M/15 phosphate buffer solution of pH 6.8 which contained 0.9 per cent of NaCl.

The Experimental Organism. Pupae of housefly, *Musca vicina* MAQUART, were kindly supplied by Kondo

Kagaku Co. Ltd. Prepupae just after puparium formation and pupae aged one day or four days after puparium formation were employed throughout this experiment.

Separation of Prepupae. Prepupae were separated successfully from the other aged pupae by putting them into water. It was found that prepupae sank to the bottom, while the others floated on the surface of the water.

Preparation of Homogenates. Pupae were weighed and ground in a glass mortar with small quantities of M/15 phosphate buffer solution of pH 6.8 which contained 0.9 per cent of NaCl. The necessary volumes of the same buffer were added to dilute the homogenate three fold, five fold, or seven fold. These diluted preparations were called homogenate A, homogenate B, and homogenate C, respectively. Brei so obtained were filtrated through gauze. The filtrates were used as homogenates throughout this experiment. The homogenates from prepupae, from pupae aged one day, and from pupae aged four days after puparium formation were referred to as E_1 , E_2' , and E_2 respectively. E_1 was yellowish and showed little color change on standing. However, E_2' and E_2 were initially yellowish, but subsequently changed to brownish, particularly E_2 which finally became dark brown even having been kept at 0°C.

Activity Measurements. Measurements were made in a constant temperature Warburg respirometer at 25°C using *p*-cresol or catechol as substrate. The homogenate was placed in the main compartment, the substrate in the side arm, and 0.1 ml of 20 per cent of KOH solution in the center well, in which a piece of filter paper was placed. In some experiment no sodium dodecyl sulfate (SDS) was added and in others 0.1 ml of 0.5 per cent solution of SDS was also added to the main compartment. After temperature equilibration at 25°C for 10 minutes the reaction was started by adding the substrate. Controls were always run for pressure fluctuations with a thermometer and for the activity of the mixture without the homogenate, which were negligibly small or zero in all cases studied.

RESULTS AND DISCUSSION

It has been reported by Ohnishi that prepupae of housefly contained protyrosinase but not tyrosinase in an active form. In addition,

the activation of the proenzyme did not occur merely by the incubation of homogenate prepared from prepupae, but arose by adding a small amount of a homogenate obtained from aged pupae which contained a certain type of activation system. Thus, even if a small amount of aged pupae is together with prepupae in a sample, the activation of protyrosinase must occur in the periods of preparation and incubation of homogenate made from such a sample. Therefore, it seemed to be necessary to separate aged pupae from a sample of prepupae as completely as possible in order to obtain a homogenate containing only protyrosinase. Such a homogenate appears to be available for the study on the nature or the mechanism of activation of protyrosinase *in vitro*. For this purpose, we tried first to separate aged pupae from a sample of prepupae.

It was found that aged pupae were easily separated from prepupae by putting a sample of pupae into water. By this treatment a certain type of pupae sank to the bottom, while the other floated to the surface of the water. The following experiment was accomplished to examine the apparent tyrosinase activity as well as the occurrence of activation system in each group of pupae separated by the above treatment. Tyrosinase activity was determined for each homogenate prepared from a group of pupae sunk to the bottom of the water, from that floated to the top, and from each mixture made of the former and the latter in the ratios 10 : 1, 10 : 5, and 10 : 10 in weight respectively. Each homogenate thus obtained was incubated at 25°C for one hour before catechol was added to the reaction mixture. Results obtained were shown in Fig. 1.

As seen in Fig. 1, it was ascertained that pupae obtained from the bottom of the water possesses neither apparent tyrosinase activity

FIG. 1. Apparent Tyrosinase Activity as well as the Occurrence of Activation System in Each Group of Pupae.

(—●—) prepupae 10 : aged pupae 10, (—○—) prepupae 0 : aged pupae 10, (—△—) prepupae 10 : aged pupae 5, (—▲—) prepupae 0 : aged pupae 5, (—◐—) prepupae 10 : aged pupae 1, (—◑—) prepupae 0 : aged pupae 1, (—◑—) prepupae 10 : aged pupae 0, 60 minutes incubation at 25°C, 12.8 μ M catechol and M/15 phosphate buffer, pH 6.8, in a total volume 1.2 ml.

nor activation system, while pupae from the surface contains tyrosinase in an active form. In addition, the fact that homogenates of the mixtures exhibited rather higher tyrosinase activity than that obtained inherently in pupae floated on the surface, which were added to the mixtures in the different ratios, seems to evidence that pupae sunk to the bottom contains protyrosinase but neither activation system nor active tyrosinase. Thus, pupae sunk to the bottom were accounted as prepupae and employed as a source of protyrosinase throughout this experiment. On the other hand, pupae floated on surface seemed to be available for a source of naturally occurring activation system in terms of aged pupae.

In order to reconfirm the propriety of this treatment the following experiment was additionally carried out: E₁ and E₂ were preincubated together in the manometer flasks at 25°C for 30 minutes and tyrosinase activity

occurred was measured. Controls run for the activity of enzyme systems consisted of solely E_1 , and E_2 alone. The results obtained were given in Fig. 2 for the cresolase activity and Fig. 3 for the catecholase activity.

As shown in Fig. 2 and Fig. 3, although no O_2 -uptake by the E_1 -system was observed within a certain period of reaction, there was a marked increase in the O_2 -uptake by the E_1+E_2 -system as compared with the E_2 -system, so that the activity of the former was approximately two times as large as that of the latter. Therefore, as been done by Ohnishi, it can be concluded that protyrosinase in E_1 is converted into the active form with the aid of an activation system which existed in E_2 . According to the impossibility of the activation of protyrosinase of housefly by SDS, Ohnishi concluded that this protyrosinase was different from that contained in the eggs of the grasshopper which possibly was activated

FIG. 2. Activation of Inactive Tyrosinase in E_1 by E_2 (cresolase activity).

Manometric method, (—○—) E_1 0.2 ml + E_2 0.2 ml, (—●—) E_2 0.2 ml, E_1 (—□—) E_1 0.2 ml, after 30 minutes incubation at 25°C, 12.8 μ M *p*-cresol and M/15 phosphate buffer, pH 6.8, in a total volume 1.2 ml.

FIG. 3. Activation of Inactive Tyrosinase in E_1 by E_2 (catecholase activity).

Manometric method, (—○—) E_1 0.2 ml + E_2 0.2 ml, (—●—) E_2 0.2 ml, (—□—) E_1 0.2 ml, after 30 minutes incubation at 25°C, 12.8 μ M catechol and M/15 phosphate buffer, pH 6.8, in a total volume 1.2 ml.

by a certain detergent such as SDS as mentioned by Bodine et al.

Thus, in order to ascertain the type of protyrosinase contained in prepupae of the housefly, *Musca vicina* MAQUART, it became necessary to reexamine the possibility of the activation of this protyrosinase by a detergent such as SDS. Prepupae obtained by the procedure mentioned above were employed as a source of protyrosinase. As a result of the experiment described below, we found that protyrosinase contained in prepupae of the housefly is also activated by detergent in vitro distinctly in contrast to the observation made by Ohnishi.

Tyrosinase activities of E_1 , E_2' , and E_2 were measured in the presence (SDS-system) as well as in absence of SDS (non SDS-system) respectively. Results obtained are indicated in Fig. 4.

As shown in Fig. 4, E_1 , homogenate pre-

FIG. 4. Activation of Protyrosinase Contained in Prepupae of Housefly by SDS.

Manometric method, (—△—) E_1 0.2 ml + SDS 0.1 ml (0.5 mg), (—○—) E_2 0.2 ml + SDS, (—○—) E_2' 0.2 ml + SDS, (—●—) E_1 0.2 ml, (—●—) E_2 0.2 ml, (—●—) E_2' 0.2 ml, after 30 minutes incubation at 25°C, 12.8 μ M catechol and M/15 phosphate buffer, pH 6.8, in a total volume 1.2 ml.

pared from prepupae, possessed practically no tyrosinase activity in the non SDS-system. However, on addition of SDS tyrosinase activity toward either *p*-cresol or catechol as substrates developed to a considerably great extent. This fact apparently indicates that a principle contained in E_1 can be affected by SDS.

On the other hand, tyrosinase activity of E_2' increased to a considerably great extent similarly to E_1 in SDS-system, whereas E_2' possessed inherently a low tyrosinase activity. In contrast to the above two homogenates, E_2 possessed a considerably high tyrosinase activity even in the non SDS-system and scarcely increased its activity by addition of SDS.

This seems to mean that protyrosinase occurs in prepupae and is increasingly activated in vivo with aging of pupae by an activation system, which seems to be lacking

in prepupae but becomes more important with the aging of pupae.

Furthermore, we tried to examine the effect of SDS on the activation of protyrosinase by natural activator. E_1 and E_2 were preincubated together in the manometer flasks for 30 minutes at 25°C in the presence as well as absence of SDS and the tyrosinase activity occurred was measured. Controls run for the activity of enzyme systems consisted of E_1 alone, and E_2 alone. The results obtained were given in Fig. 5 for the cresolase activity and Fig. 6 for the catecholase activity.

As shown in Fig. 5 and Fig. 6, there was a remarkable increase in the reaction rate in $E_1 + E_2$ -system on addition of SDS as compared with that in the absence of SDS (Figs. 2 and 3). This evidence suggested that the spontaneous activation with the aid of naturally occurring activator in the homogenate did not occur completely in vitro in a short

FIG. 5. Activation of Protyrosinase in E_1 by E_2 and SDS (cresolase activity).

Manometric method, (—○—) E_1 0.2 ml + E_2 0.2 ml + SDS 0.1 ml (0.5 mg), (—●—) E_2 0.2 ml + SDS, (—△—) E_1 0.2 ml + SDS, (—▲—) E_1 0.2 ml, after 30 minutes incubation at 25°C, 12.8 μ M *p*-cresol and M/15 phosphate buffer, pH 6.8, in a total volume 1.2 ml.

FIG. 6. Activation of Prottyrosinase in E_1 by E_2 and SDS (catecholase activity).

Manometric method, (—○—) E_1 0.2 ml + E_2 0.2 ml + SDS 0.1 ml (0.5 mg), (—●—) E_2 0.2 ml + SDS, (—△—) E_1 0.2 ml + SDS, (—▲—) E_1 0.2 ml, after 30 minutes incubation at 25°C, 12.8 μ M catechol and M/15 phosphate buffer, pH 6.8, in a total volume 1.2 ml.

period, but was promoted by SDS. This seems to suggest strongly additionally to the evidence mentioned above that SDS is an activator for the conversion of prottyrosinase of housefly into the active form in vitro.

Consequently, it seems that prottyrosinase in the prepupae of housefly can be activated by either SDS or the natural activation system in vitro. The effect of SDS on the activation of prottyrosinase will be described in detail in a subsequent paper. However, the fact described above renders a question on the existence of a different type prottyrosinase in prepupae of housefly from that in the eggs of grasshopper at least concerning to the type of activator in vitro, although it is not important whether prottyrosinase in the different

insects are always identical protein or not.

Concerning the function of SDS as an activator seemingly there are at least two possibilities. On the one hand, it is well known that a detergent like SDS acts on protein as a denaturing agent. Therefore, there is a possibility that prottyrosinase is activated owing to a configurational change caused in its molecule by SDS. On the other hand, it is well known that SDS attacks the microsomal membrane causing the liberation of enzymes from the particle. Recently, Karlson and Schmidt⁵⁾ reported that tyrosinase in the larvae of the blowfly, *Calliphora erythrocephala*, was linked to the microsome.

It is not yet clear whether prottyrosinase in the prepupae of housefly is linked to the microsome. The mechanism of action of SDS on the prottyrosinase activation is still obscure but further studies are going on and will be discussed in a subsequent paper.

SUMMARY

1. Prepupae of housefly, *Musca vicina* MAQUART, possessing tyrosinase activity were separated from the aged pupae by putting into water.

2. In prepupae tyrosinase occurred in an inactive form, i.e., as a prottyrosinase which could be activated in vitro by the natural activation system existed in aged pupae.

3. Prottyrosinase also could be activated by SDS in vitro, whereas the mechanism of its activation is still obscure.

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5) P. Karlson and H. Schmidt, *Z. Physiol. Chem.*, **300**, 35 (1955).