Effect of Ionic Strength on Activation of Prophenoloxidase in Its Crude Solution

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We reported previously that protyrosinase in prepupae of housefly, *Musca domestica vicina* Maquart, could be activated either by anionic detergents or by its natural activator extracted from aged pupae.¹⁻³⁾ This enzyme was later demonstrated by Karlson *et al.*⁴⁾ to be phenoloxidase (*o*-Diphenol: oxygen oxidoreductase, EC 1.10.3.1).

Preliminary experiments on prophenoloxidase also showed that it was activated autocatalytically during dialysis against deionized water. The autocatalytic activation was thought to be of significance in connection not only with the purification of prophenoloxidase but also with the chemical nature of the proenzyme.

Prophenoloxidase extracted from prepupae of housefly, *M. domestica vicina* Maquart, with 0.2 M phosphate buffer, pH 6.0, was kept intact in the limited range below pH 6.0, whereas at pH higher than 7.0 it was activated autocatalytically, as shown in Fig. 1. This finding suggests that a change in the charge of prophenoloxidase is involved in the activation. The phenomenon is similar to that in the report of Allen *et al.*⁵⁾ who found that protyrosinase in the extract of eggs of grasshopper, *Melanoplus differentialis*, was activated autocatalytically at pH 9.3 or above. However, no mechanism has been suggested to account for the autocatalytic activation due to the change in pH of the solution. On the other hand, the prophenoloxidase was irreversibly denatured during dialysis against 0.2 M phosphate buffer of pH 5.5 or below.

It was found that the autocatalytic activation of prophenoloxidase also occurred in a low concentra-

FIG. 1. Effect of pH on Activation of Prophenoloxidase.

The crude prophenoloxidase solution was dialyzed against 0.2 M phosphate buffer of different pHs for 24 hr at 3°C. The change in volume of oxygen uptake due to the oxidation of catechol catalyzed by phenoloxidase was measured at 25°C with a Warburg manometer according to the procedures in the preceding paper.¹⁾

○—○, prophenoloxidase; ●—●, prophenoloxidase + natural activator.

tion of buffer even at pH 6.0. Prophenoloxidase in the crude solution was dialyzed against 0.01, 0.05, 0.1 and 0.2 M sodium acetate buffer, pH 6.0, for 24 hr at 3°C, and then the phenoloxidase activity in the dialyzed solution was measured both in the presence and in the absence of natural activator. The natural activator was obtained from the aged pupae of housefly according to the method described in the preceding paper.³⁾ As shown in Fig. 2, prophenoloxidase was kept intact only in 0.2 M buffer without any autocatalytic activation, while at a concentration of lower than 0.2 M it was activated autocatalytically. In 0.2 M buffer, however, prophenoloxidase was activated only by the natural activator. These results show that prophenoloxidase is not irreversibly denatured in 0.2 M buffer but is in an inactive form.

A similar tendency concerning salt concentrations of buffer was observed also in phosphate buffer. In this buffer, prophenoloxidase was kept intact at concentrations of 0.15 and 0.2 M and was activated autocatalytically at 0.1 M or below.

In addition, prophenoloxidase in the crude solution was dialyzed against 0.01 M phosphate buffer, pH 6.0, containing 0.01, 0.05 and 0.1 M NaCl. At the NaCl concentration of 0.1 M, prophenoloxidase was kept stable without any autocatalytic activation.

From these facts it was inferred that the autocatalytic activation of prophenoloxidase depends upon the

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FIG. 2. Autocatalytic Activation of Prophenoloxidase Depending upon Change in Concentration of Sodium Acetate Buffer.

The crude prophenoloxidase solution was dialyzed against different concentrations of sodium acetate buffer, pH 6.0, for 24 hr at 3°C.

○—○, 0.01 M buffer; ○---○, 0.01 M buffer+natural activator; ●—●, 0.05 M buffer; ●---●, 0.05 M buffer+natural activator; △—△, 0.10 M buffer; △---△, 0.10 M buffer+natural activator; ▲—▲, 0.20 M buffer; ▲---▲, 0.20 M buffer+natural activator.

salt concentration or ionic strength of the solution. This seems to be supported also by the following finding. Prophenoloxidase in the crude solution was dialyzed against 0.001 M phosphate buffer, pH 6.0, overnight at 3°C at the first step. At the second step, the above solution was further dialyzed against 0.2 M phosphate buffer of the same pH. At the third step, the solution from the second step was dialyzed under the same conditions as the first step. The phenoloxidase activity in each solution was determined both in the presence and in the absence of the natural activator. During the first dialysis prophenoloxidase

was activated autocatalytically. However, after the second dialysis, although no phenoloxidase activity was detected in the absence of natural activator, the complete activity of phenoloxidase was observed in its presence. In the second dialysis, phenoloxidase formed during the first dialysis appeared to be converted to prophenoloxidase, which was then activated again in the third dialysis. Thus, the activation of prophenoloxidase is reversible depending upon the salt concentration or ionic strength of the solution.

There is another possibility for the activation of prophenoloxidase in the solution of a low salt concentration. Namely, lowering of osmotic pressure of the solution might cause the activation. In order to examine this possibility, prophenoloxidase in the crude solution was dialyzed against 0.05, 0.1, 0.5 and 1.0 M sucrose in 0.01 M phosphate buffer, pH 6.0, and the resulting phenoloxidase activity of each solution was determined. As the result, all of the concentrations of sucrose tested could not restrict the activation of prophenoloxidase. Therefore, the activation of prophenoloxidase occurs independently from the lowering of osmotic pressure of its solution. This finding may exclude the possibility that phenoloxidase is released by destruction of the granules binding phenoloxidase under lower osmotic pressure, as noted by Karlson *et al.*,⁴⁾ and shows that prophenoloxidase in question is a soluble proenzyme.

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