A Protein Factor Inhibiting Activation of Prophenoloxidase with Natural Activator and Its Purification

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Fractionation of the crude prophenoloxidase solution from prepupae of housefly, Musca domestica vicina Maquart, was carried out. The soluble fraction of 40% saturation of ammonium sulfate contained a certain factor being able to inhibit the activation of prophenoloxidase A with natural activator from aged pupae. The factor seemed to be a protein and was named Factor N tentatively.

Purification of Factor N by gel filtrations on Sephadex G-75 and sucrose density gradient ultracentrifugation resulted in an 18.2% of the yield of the activity and a 21.8-fold increase in the specific activity. The purified preparation of Factor N had a sedimentation constant of 5.4 S.

Crude prophenoloxidase from prepupae of housefly, Musca domestica vicina Maquart, was activated either with a crude natural activator from aged pupae or with an anionic detergent such as sodium dodecyl sulfate (SDS), as reported in the previous papers.1-3 The crude prophenoloxidase was also activated while being dialyzed against water or low concentration of buffers. Because of the autocatalytic activation, the enzyme has been difficult to purify prophenoloxidase. However, the finding4-5 that prophenoloxidase was kept intact in a high concentration of buffers such as 0.2 M at limited pH of 6.0 made it possible to progress further the purification of prophenoloxidase. In the partial purification of prophenoloxidase the presence of a certain factor stabilizing prophenoloxidase in the crude prophenoloxidase in the crude prophenoloxidase solution was suggested.

This paper describes the purification and some properties of the factor, which was named Factor N, tentatively.

MATERIALS AND METHODS

Experimental organism. Larvae of housefly, Musca domestica vicina Maquart, were obtained from Kondo Kagaku Co. (Fukuoka) and reared with a moist mixture containing wheat bran and dried yeast powder at room temperature in our laboratory. Prepupae and aged pupae were separated from each other according to the procedure of Funatsu and Inaba.1

Reagents. Catechol of extra pure grade (Merck) was used without further purification. β-(Dihydroxyphenyl)-α-L-alanine (DOPA) was kindly donated by Ajinomoto Co. (Tokyo) and used without further purification. All other chemicals were reagent grade.

Natural activator. Natural activator was prepared from aged pupae by the method described in the previous paper.3

Activation of prophenoloxidase A with natural activator. The activity of phenoloxidase formed by the activation of prophenoloxidase A with natural activator was determined either manometrically or colorimetrically.4-6 The reaction mixture contained a crude prophenoloxidase solution and a crude natural activator solution in a way that the absorbance at 280 nm (A280) of the former was equal to half the absorbance at 280 nm of the latter. The reaction mixture was preincubated for either 20 min or 30 min at 25°C and pH 6.0 for the activation of prophenoloxidase A with natural activator.
Determination of phenoloxidase activity

Method 1. The phenoloxidase activity was determined at 25°C and pH 6.0 with a Warburg manometer according to the procedure of Funatsu and Inaba. The activity was represented as O₂-uptake (µl) per 10 min per A₃₅₀ of the phenoloxidase A solution.

Method 2. The phenoloxidase activity was determined colorimetrically according to the method of Horowitz and Fling, as modified by us, with DOPA as a substrate. The change in the absorbance at 470 nm due to the oxidation of DOPA catalyzed for 10 min at 25°C and pH 6.0 by phenoloxidase was measured with a Hitachi EPO-B colorimeter. The reaction mixture contained 0.5 ml of the crude prophenoloxidase A solution and 0.5 ml of the crude natural activator solution in 0.2 M phosphate buffer, pH 6.0, in a total volume of 3.0 ml. The mixture was preincubated for 20 min at 25°C. After being added with 0.2 ml of 20 mM DOPA, the reaction mixture was incubated for 10 min at the same temperature.

Assay of Factor N. The activity of Factor N for inhibiting the activation of prophenoloxidase A with natural activator was assayed by the following procedure: Prophenoloxidase A was activated with a natural activator in the presence of Factor N, and the phenoloxidase activity was determined by Method 2. The activity of Factor N was represented as the inhibition (%) of the activation of prophenoloxidase A with natural activator. The mixture contained 0.5 ml of the prophenoloxidase A solution (A₃₅₀ was about 5), 0.5 ml of the natural activator solution (A₃₅₀ was about 10) and 1.0 ml of the Factor N solution in 0.2 M phosphate buffer, pH 6.0, in a total volume of 3.0 ml. The mixture was preincubated for 20 min at 25°C. After being added with 0.2 ml of 20 mM DOPA, the reaction mixture was incubated for 10 min at 25°C. The change in A₄₇₀ on the phenoloxidase reaction was measured by Method 2. A control test was run with 1.0 ml of 0.2 M phosphate buffer, pH 6.0, in place of the Factor N solution at the same time. The specific activity of Factor N was defined as the reciprocal of A₂₈₀ of the Factor N solution being able to inhibit the activation at 50%.

RESULTS

Partial purification of prophenoloxidase and separation of Factor N

Prophenoloxidase was partially purified from the homogenate of prepupae in 0.2 M phosphate buffer, pH 6.0, by the procedures summarized in the following scheme. The homogenate was centrifuged at 9000 rpm for 30 min at 3°C. The supernatant was called a crude extract, and was saturated to 100% with solid ammonium sulfate. The precipitate formed was dissolved in 0.2 M phosphate buffer, pH 6.0, and dialyzed against the same buffer. The dialyzed solution was called a prophenoloxidase A solution. Prophenoloxidase A became less stable than prophenoloxidase N, and was activated while being dialyzed against water. A preliminary examination on the unstability of prophenoloxidase A showed that a certain factor stabilizing prophenoloxidase A was contained in the supernatant on the fractionation of prophenoloxidase A from the prophenoloxidase N solution, and was named Factor N tentatively. The mode of its inhibitory action for the activation of prophenoloxidase A with the natural activator was first examined.

Effect of Factor N on activation of prophenoloxidase with natural activator

Prophenoloxidase A was activated with a natural activator in the presence and in the absence of Factor N, respectively. The resulting activities of phenoloxidase were measured.
Purification of Factor Inhibiting Prophenoloxidase Activation

by Method 1. The result is shown in Fig. 1. In the presence of Factor N, a strong inhibition of the activation was observed.

Effect of Factor N on natural activator
A mixture containing 0.2 ml of the natural activator solution and 0.5 ml of the Factor N solution (A$_{280}$ was 0.56) was allowed to stand for one hr at 3°C. After being added with the prophenoloxidase A solution, the mixture was preincubated and then the activity of phenoloxidase formed in the mixture was measured by Method 1. Figure 2 shows the result. No difference in the phenoloxidase activity was observed in the preincubation of natural activator between with and without Factor N.

Effect of Factor N on phenoloxidase activity
The activity of phenoloxidase formed by the activation of prophenoloxidase A with the natural activator was measured in the presence and in the absence of 0.5 ml of the Factor N solution (A$_{280}$ was 0.56). The result is shown in Fig. 3. No inhibition of the phenoloxidase reaction was detected even in the presence of Factor N.

Purification of Factor N
The Factor N was purified by gel filtration on Sephadex G-75 followed by sucrose density gradient ultracentrifugation. The crude Factor N solution in 0.2 M phosphate buffer, pH 6.0, was dialyzed against 0.01 M phosphate buffer of the same pH for one day. The precipitate formed during dialysis was removed by centrifugation. The supernatant was applied to a column (4.0 x 138 cm) of Sephadex

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**Fig. 1.** Effect of Factor N on Activation of Prophenoloxidase A by Natural Activator.
Prophenoloxidase A was activated with natural activator in the presence and in the absence of Factor N. ○—○, prophenoloxidase A; ●—●, natural activator; △—△, prophenoloxidase A+natural activator; ▲—▲, prophenoloxidase A+natural activator+Factor N.

**Fig. 2.** Effect of Factor N on Natural Activator.
○—○, prophenoloxidase A+natural activator+Factor N; ●—●, prophenoloxidase A+a preincubated mixture of natural activator and Factor N.

**Fig. 3.** Effect of Factor N on Phenoloxidase.
○—○, phenoloxidase; ●—●, phenoloxidase+Factor N.

**Fig. 4.** Gel Filtration of Crude Factor N on Sephadex G-75.
G-75 which had been equilibrated with 0.01 M phosphate buffer, pH 6.0, and eluted at a flow rate of 48 ml per hr with the same buffer. Figure 4 shows a typical pattern of the gel filtration. The crude Factor N was separated into three fractions, each of which had the activity for inhibiting the activation of prophenoloxidase A with the natural activator. The second fraction, S-2, had the highest specific activity that was 23 times higher than that of the crude Factor N solution.

After being concentrated by the ultrafiltration with a collodion bag under reduced pressure, the S-2 fraction was filtered through the same column of Sephadex G-75 under the same conditions. Figure 5 shows the elution pattern. Most of the activity of Factor N was found in the second peak, S'-2. By a subsequent gel filtration of the S'-2 fraction on Sephadex G-75 Factor N was eluted as a single peak, S'-2-1 (data not shown). The S'-2-1 fraction was centrifuged at 41,000 rpm for 18 hr at 4°C by a Spinco L-50 ultracentrifuge with an SW41 Ti rotor with a linear gradient of sucrose concentration from 5 to 10% in 0.01 M phosphate buffer, pH 6.0. Figure 6 shows the ultracentrifugal pattern, which gave a single peak of protein corresponding to the activity of Factor N.

The purification steps are summarized in Table I, showing that the yield of activity is 18.2% and the rate of purification is 21.8-fold.

### Sedimentation constant of Factor N

The sedimentation constant of Factor N was determined by 5 to 20% linear sucrose gradient centrifugation in 0.01 M phosphate buffer, pH 6.0. Lysozyme (1.9 S), ovalbumin (3.5-3.6 S), transferrin (5.5 S) and γ-globulin (7.12 S) were used as standard proteins. Factor N and those proteins were ultracentrifuged separately at 41,000 rpm for 16 hr at 4°C. From the plot shown in Fig. 7, a sedimentation constant of Factor N was calculated to be 5.4 S.
Thermal stability

The purified Factor N was incubated for 30 min at different temperatures shown in Fig. 8. The remaining activity of Factor N was determined by Method 2. Figure 8 shows that Factor N was stable at 50°C or below, whereas it lost its activity at higher temperature than 50°C.

![Fig. 8. Thermal Stability of Purified Factor N.](image)

The purified Factor N was allowed to stand for 30 min at pH 6.0 and different temperatures.

pH-Stability

Factor N was allowed to stand for 24 hr at 6°C in the aqueous solutions of different pHs, and then the pH of the solutions was adjusted to 6.0. The remaining activity of Factor N was determined by Method 2. Figure 9 shows that Factor N was stable over the pH range from 5 to 10.5.

![Fig. 9. pH-Stability of Purified Factor N.](image)

The purified Factor N was allowed to stand for 24 hr at different pHs and 6°C.

DISCUSSION

To characterize prophenoloxidase and to elucidate the mechanism of its activation, the enzyme is necessary to be purified. However, the purification of prophenoloxidase in its intact form has been difficult because of its autocatalytic activation in low concentrations of buffers. Examinations on the autocatalytic activation of prophenoloxidase showed that it was kept stable in the crude solution at limited pH of 6.0, and that it was kept intact when equilibrated with 0.2 M phosphate buffer, 0.2 M sodium acetate buffer and 0.1 M sodium chloride in 0.01 M phosphate buffer, pH 6.0.

The following findings suggested a possibility of the purification of prophenoloxidase further. When the prophenoloxidase N solution was saturated to 40% with ammonium sulfate, it was found that the resulting precipitate contained a labile form of prophenoloxidase, prophenoloxidase A, which was activated more easily in low concentrations of buffers at pH 6.0 than prophenoloxidase N. Another finding emphasized in this study was that the resulting supernatant contained a certain factor inhibiting the activation of prophenoloxidase A with the natural activator. The possibility that the Factor N is a protein would be deduced from the evidences that it showed a typical UV-spectrum of proteins, that it was inactivated at higher temperatures than 60°C and that it was not permeable through the membrane of a Visking tube (#18/32) during dialysis. The mode of inhibitory action of the Factor N for the activation of prophenoloxidase A with the natural activator may be explained by the following three possibilities:

1. The Factor N may bind to prophenoloxidase A so as to stabilize it.
2. Factor N may inhibit the natural activator.
3. Factor N may inhibit phenoloxidase formed by the activation of prophenoloxidase A with the natural activator.

Although the Factor N did not affect both the natural activator and phenoloxidase, it strongly inhibited the activation of prophenoloxidase.
A with the natural activator. The results may exclude the second and the third possibilities, and may support the first possibility. The evidence that prophenoloxidase A became less stable than prophenoloxidase N also supports the first possibility.

The Factor N was purified from the crude extract of prepupae. The gel filtration of Factor N on Sephadex G-75 seemed to be effective for its purification. The Factor N behaved as a single component of protein on the gel filtration and on the linear sucrose gradient ultracentrifugation. However, on the third gel filtration the specific activity of Factor N decreased. Indeed, the Factor N was less stable and was inactivated while it was kept precipitating in 100% saturation of ammonium sulfate and kept soluble at low temperatures. The instability of Factor N will be discussed in a subsequent paper.

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REFERENCES

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