Affinity Chromatography of Phenoloxidase in the Larvae of Housefly

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Although efforts to purify phenoloxidase or tyrosinase from various organisms have been made by a number of investigators, many difficulties due to a high molecular weight and a tendency of agglutination of the enzyme and occurrence of color substances arose in the course of purification process. Recently, affinity chromatography is considered to have a very bright prospect of purifying enzymes or proteins. In the purification of tyrosinase from mushroom affinity chromatography has been employed by Lerman¹⁾ and O'Neill *et al.*²⁾ An attempt to employ affinity chromatography in the purification of phenoloxidase from the larvae of housefly, *Musca domestica vicina* Maquart, has been also made in our laboratory. This paper describes the utility of affinity chromatography for a step of the purification of phenoloxidase.

Crude phenoloxidase employed throughout this experiment was prepared from the larvae by the procedure described below. The homogenate prepared with 0.01 M phosphate buffer containing 0.05 M sodium ascorbate, pH 7.0, was filtered through gauze, and the filtrate was centrifuged at 8000 rpm for 20 min at 4°C. The supernatant was brought to 34% saturation with ammonium sulfate. The resulting precipitate was collected by centrifugation and dissolved in ice-cold

0.05 M carbonate buffer, pH 9.5. The solution was dialyzed against the same buffer with several changes for 48 hr at 10°C. The dialyzed solution was centrifuged for removal of precipitate. The fractionation with ammonium sulfate was repeated again. The supernatant thus obtained was used as crude phenoloxidase solution.

Measurement of the phenoloxidase activity was made by a modification of Horowitz and Fling's method.³⁾ The change in optical density at 470 nm due to the oxidation of 3,4-dihydroxyphenylalanine (DOPA) catalyzed by phenoloxidase for 10 min at 25°C was measured with a Hitachi photoelectric spectrophotometer, type EPO-3. The reaction mixture consisted

TABLE I. INHIBITION OF PHENOLOXIDASE BY DIFFERENT AROMATIC COMPOUNDS

To 0.2 ml of enzyme solution (O.D.₂₈₀=0.03) was added 0.2 ml of inhibitor solution of given concentration in the table and 1.6 ml of 0.1 M phosphate buffer, pH 6.0. The mixture was incubated for 10 min at 25°C. The determination of remaining activity of phenoloxidase was made in the same manner as described in the text.

Inhibitor	Concentration of inhibitor	Per cent of inhibition	Type of inhibition
L-Phenylalanine	10 mM	9.3	
N-Acetyl-L-phenylalanine	1	19.4	
L-Tyrosine	0.5	8.1	
Benzoic acid	1	63.1	Competitive
Salicylic acid	1	15.0	
Cinnamic acid	1	91.3	Competitive
Benzamide	1	7.5	
Aniline	1	25.6	
Acetanilide	1	1.6	
p-Aminobenzoic Acid	1	84.0	Competitive

TABLE II. ADSORPTION OF PHENOLOXIDASE ON SEPHAROSE 4B DERIVATIVE COLUMN

Ad-sorbent ^{a)}	Column size (cm)	Flow rate (ml/hr)	Applied total protein (O.D. ₂₈₀)	Applied total activity (O.D. ₄₇₀)	Relative recovery of phenoloxidase activity non-adsorbed	Relative recovery of phenoloxidase activity adsorbed	Relative recovery of protein non-adsorbed	Relative recovery of protein adsorbed
I	0.4×19	—	4.2	19.1	81.0	0	114.0	0
II	0.3×15	—	6.5	23.4	62.4	0	63.4	0
III	0.7×10	21	24.6	101.0	80.7	1.4	77.2	21.4
IV	0.7×12	15	24.6	101.0	87.5	1.6	90.7	3.2
V	1.0×19	23	16.2	210.0	2.3	90.8	47.9	37.0

^{a)} Sepharose 4B derivatives:

I, Sepharose; II, Sepharose-NH-CH(CH₂-C₆H₅)-COOH;

III, Sepharose-NH-(CH₂)₂-NH-CO-CH(CH₂-C₆H₅)-NH-COCH₃;

IV, Sepharose-NH-C₆H₄-COOH;

V, Sepharose-NH-(CH₂)₂-NH-CO-CH₂-NH-C₆H₄-COOH.

FIG. 1. Elution Profile of Larvae Phenoloxidase from Sepharose 4B Derivative V Column and Disc Electrophoretic Pattern of Enzyme Fraction.

The phenoloxidase solution was applied to a column (1.0×1.9 cm) of Sepharose 4B derivative V equilibrated with 0.05 M phosphate buffer, pH 7.0 at 8°C. After washing the column with about 100 ml of the same buffer, elution was carried out with 0.05 M carbonate buffer, pH 9.5. Phenoloxidase was eluted in fraction F-3.

of 0.2 ml of the enzyme solution, 2.0 mM DOPA in 0.1 M phosphate buffer, pH 6.0, in a total volume of 2.0 ml. The activity was expressed in O.D._{470nm} in 10 min.

Table I shows the effect of inhibitory compounds on the phenoloxidase activity. The phenoloxidase activity was strongly inhibited by cinnamate, *p*-aminobenzoate and benzoate. The inhibition was competitive. It seems to be inferable that the inhibitory effect

is attributed to a carboxyl and an amino group of these compounds and particularly to a dissociated state of the carboxyl group.

Taking into account the above findings, Sepharose 4B derivatives of the inhibitors such as *p*-aminobenzoate and L-phenylalanine were synthesized according to the method of Cuatrecasas.⁴⁾ The utility of the Sepharose 4B derivatives in affinity chromatography of phenoloxidase from the larvae was examined, and the results were summarized in Table II. A typical chromatographic pattern of the crude phenoloxidase solution on a Sepharose 4B derivative V column was shown in Fig. 1. Phenoloxidase was eluted from the column by a stepwise change of pH from 7.0 to 9.5. The purified enzyme appears to be homogeneous, when tested by disc electrophoresis at pH 8.3. The specific activity of the purified enzyme solution was three times over that of the crude phenoloxidase solution. The derivative could be repeatedly used without any loss of its adsorption capacity.

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