Tea Leaf Polyphenol Oxidase

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Tea leaf polyphenol oxidase (O-diphenol: O_2 oxidoreductase. 1.10.3.1) plays an important role in black tea fermentation. Anent this enzyme many investigations had been conducted in Ceylon, India, England, U.S.S.R. and Japan. Recently, this enzyme, known as a water-insoluble enzyme, was solubilized into water-soluble form and many new information about the enzymological properties of the solubilized enzyme has been reported by three groups in Japan,[1-6] Ceylon[7] and England.[8]

Following are some results of the investigation concerning tea leaf polyphenol oxidase reported by the author:

Solubilization of tea leaf polyphenol oxidase

As shown in Table 1, the solubilizing effect of some surface-active agents on the polyphenol oxidase was investigated. By the addition of Tween-80 (non-ionic surface active agent) to the homogenate in a final concentration of 5%, about 80% of the enzyme activity in tea leaf homogenate appeared in the supernatant. On addition of other agents, however, the enzyme activity nearly remained in the sediment after centrifugation. In case of the acetone powder suspension, the enzyme

Table 1. Effect of surface-active agents on solubilization

Samples	Agents		Activities of the original samples	Ratios of activities after treatment to those of the original samples	
				Total	Supernatant
Leaf homogenate	Tween-80	1V%	$O_2\mu l$/10min./g of fresh weight	106%	20%
		2		105	35
		4		105	63
		5	810	122	81
	Span-80	1V%		100	3
	Cholate	0.2W%		100	2
	Duponol	2W%		105	18
	Digitonin	1W%		100	4
Acetone powder suspension	Tween-80	2V%	976	101	7

could not be solubilized by the addition of Tween-80.

Purification of the solubilized enzyme

The solubilized enzyme was purified and fractionated to three components by the procedure shown in Fig. 1.

Solubilized protein from tea leaves
↓
Gel-filtration by Sephadex G-25
↓
Fractionation with $(NH_4)_2SO_4$ ($0.3\sim0.9$ $(NH_4)_2SO_4$ saturation-ppt. fraction)
↓
DEAE cellulose column chromatography at pH 7.0.
↓
CM cellulose column chromatography at pH 5.0.
↓
CM cellulose column rechromatography at pH 5.0.
↓
Polyphenol Oxidase
↓ A-I ↓ A-II ↓ B

Fig. 1. Purification procedure of the solubilized enzyme.

After DEAE cellulose column chromatography, there appeared two components of polyphenol oxidase (A and B). The activity of A was about ten times as much as that of B.

Component A applied to a CM cellulose column was absorbed on the top of the column and formed a blue colored band. It was divided into two components, A-I and A-II (Fig. 2). Component B was also separated into two components. Main component of polyphenol oxidase in B was eluted at an early stage of elution, and a small component was eluted at a later stage at which A was eluted (Fig. 2).

A-I and A-II were individually subjected to rechromatography using CM cellulose column. Under this procedure, both A-I and A-II were almost purified, and new blue colored protein, not containing the enzyme activity, was separated from A-II.

Specific activities of A-I and A-II were increased respectively about 200 and 140 times as much as that of tea leaves homogenate.

Properties of the purified enzyme

Starch Gel Electrophoresis: The starch gel electrophoresis of three components A-I, A-II

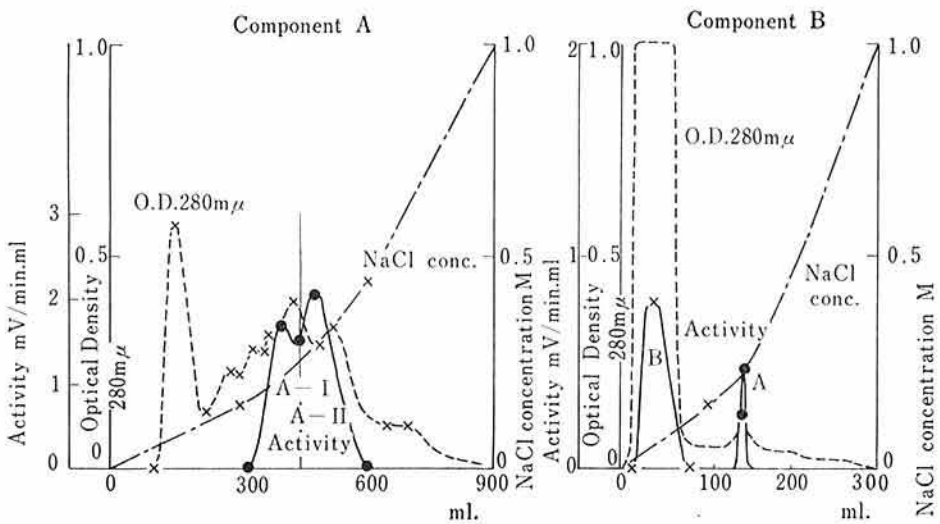

Fig. 2. CM-cellulose column chromatograms of components A and B.
Column. 2.8 × 18 cm (A), 1 × 15 cm (B).
Starting buffer: 0.02 M Na_2HPO_4+0.01 M citric acid (pH 5.0).
Limiting buffer: above buffer containing I MNaCl.

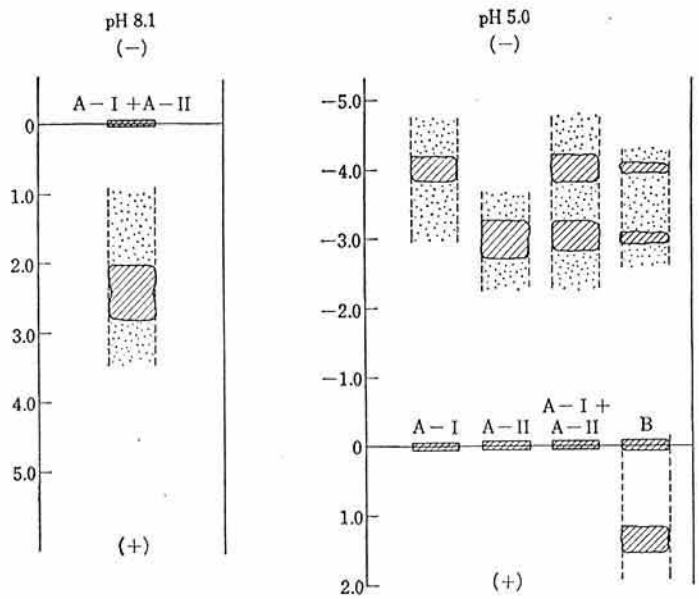

Fig. 3. Starch-gel electrophoregrams of solublized polyphenol oxidase.
(+), (−): Anode and cathode.
the ordinate: migration in cm.
Voltage of electrophoresis: 4 V per cm.
Period of migration: 24 hrs. at pH 8.1.
Period of migration: 16 hrs. at pH 5.0.

and B are shown in Fig. 3. A-I and A-II migrated from anode to cathode on starch gel at pH 5.0, and both components were separated distinctly. Component B migrated from cathode to anode at pH 5.0, and was separated from A-I and A-II, which were present as contaminants. Each component migrated slightly at pH 8.1.

The results of starch gel electrophoresis and CM cellulose chromatography indicated that A-I and A-II were basic protein, but B was neutral protein.

Sedimentation Analysis of A-I and A-II: Two components were ultracentrifugically homogenous and the sedimentation constants of A-I and A-II were 4.0 S and 4.5 S, respectively.

Copper Contents in A-I, A-II and Blue Protein: Copper content in A-I, A-II and blue protein was determined by neutron activation analysis using JRR-1 reactor at Tōkai-mura. After neutron irradiation, gamma-ray energy of ^{64}Cu in samples was directly measured by 256-channel gamma-ray scintillation spectrometer without the chemical separation.

Cu contents in A-I, A-II and blue protein were 0.27, 0.25 and 0.25%, respectively. From this result, it was shown that the blue protein in tea leaf was Cu-containing protein, but it did not contain the activity of polyphenol oxidase.

Absorption Spectra of A-I, A-II and Blue Protein: Each component showed the very intense and sharp protein band with a maximum at 278 mμ and a shoulder at 290 mμ.

A-I, A-II and blue protein showed green, yellow-green and blue colors, respectively, and the three components had a shoulder at near 406 mμ and a peak of the absorption at near 605 mμ in the visible range:

Optimum pH of A-I and A-II: Polyphenol oxidase activity was measured by a polarographic equipment at 30°C using oxygen electrode. Two components had maximum activi-

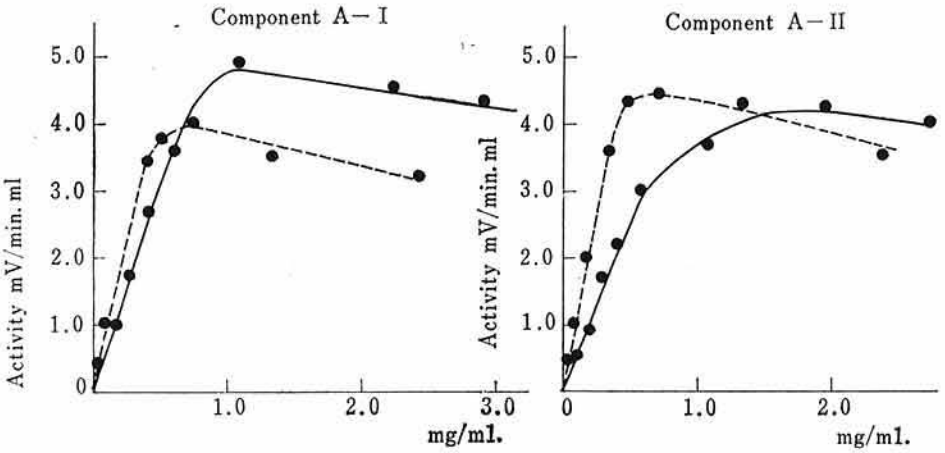

Fig. 4. Effect of substrate concentration on the enzyme activity. Continuous line and dotted line: (+)-catechin and (−)-epigallocatechin-gallate. Reaction mixture: 0.1 ml of the enzyme preparation +2.8 ml of substrate solution +0.8 ml of 0.2 M Na-phosphate and 0.1 M citric acid buffer (pH 5.0 in the case of A-I. pH 4.6 in the case of A-II).

ties at pH 5.1 and 4.6, respectively.

Effects of Substrate Concentration: The reaction velocity of each component for (+)-catechin and (−)-epigallocatechin-gallate obeyed the Michaelis-Menten law in the substrate concentration less than 5.5×10^{-4} M and 1.6×10^{-4} M, respectively, whereas it did not obey over the above concentration (cf. Fig. 4). Therefore, it was shown that the reaction velocity of each component was inhibited by a high substrate concentration.

Km values of A-I and A-II for (+)-catechin, obtained from the Lineaweaver and Burk plot, were 4.5×10^{-3} M and 3.9×10^{-3} M, and that for (−)-epigallocatechnin-gallate were 1.7×10^{-3} M and 4.4×10^{-3} M, respectively.

The ratio of the maximum velocity of A-I and A-II for (+)-catechin to that for (−)-epigallocatechin-gallate were 1.3 and 0.95, respectively. A-I was found to oxidize O-diphenol rather well, whereas, A-II oxidized well both vicinal-triphenol and O-diphenol.

Effect of Temperature: The enzyme activity increased at temperatures ranging from 20 to 35°C and began to drop at 40°C.

The activation energy of the reaction velocity, determined by Arrhenius equation, was found to be 5.0 Kcal per 1 M on A-I and 3.5 Kcal per 1 M on A-II in the range of 20 to 35°C.

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