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CHANGES IN MULTIPLE FORMS OF POLYPHENOL OXIDASE DURING MATURATION OF TEA LEAVES

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Key Word Index—*Camellia sinensis*; Theaceae; polyphenol oxidases; changes with leaf maturation.

Abstract—Changes in polyphenol oxidase activity during development of tea leaves were investigated by CM-cellulose column chromatography and isoelectric focusing. Polyphenol oxidase activity decreased about 70% during maturation. Of three components I–III separated by CM-cellulose chromatography, II and III decreased markedly during maturation. Polyphenol oxidase activity was separated into at least six main components, with varying isoelectric points, by isoelectric focusing. The quantities of these components changed during development of the tea leaves.

INTRODUCTION

MULTIPLE forms of tea-leaf polyphenol oxidase (E.C. 1.10.3) have been demonstrated in studies utilizing ion-exchange chromatography and disc electrophoresis.[1–4] In addition, it has been shown that a remarkable increase in polyphenol oxidase activity occurs in tea leaves after plucking.[5] Column chromatography (CM-cellulose) showed that enzyme protein of the stored leaves after plucking was different from that of fresh leaves. Available evidence indicated that new enzymic protein was synthesized in stored leaves.[6] The object of the present investigation was to study the multiple forms of polyphenol oxidase during maturation of tea leaves in order to cast light on the processes of aging and senescence in plant tissues.

RESULTS AND DISCUSSION

Isoelectric Focusing of Enzymes

The enzyme was adsorbed on CM-cellulose and subsequently eluted with 0·02 M Na–phosphate–0·01 M citric acid buffer, pH 5·0, containing 1·0 M NaCl. Polyphenol oxidase activity was separated into three fractions I–III by CM-cellulose chromatography (Fig. 1). Isoelectric focusing of these three fractions yielded characteristic patterns as shown in Fig. 2. Fraction I showed an isoelectric focusing pattern with a major activity peak at pH 7·8 and minor peaks at pH 6·8, 8·3 and 9·2. In the case of fraction II, an activity peak at pH 7·5 predominated, but there were also peaks at pH 6·5, 8·9 and 9·7. Fraction III showed a major peak at pH 9·5, with lesser peaks evident at pH 4·4 and 5·3.

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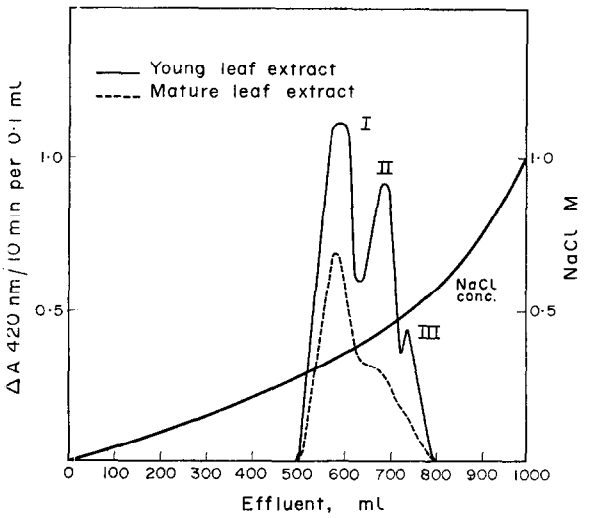

FIG. 1. CM-CELLULOSE COLUMN CHROMATOGRAM OF TEA LEAF POLYPHENOL OXIDASE.
The buffer was 0·02 M Na–phosphate—0·01 M citric acid, pH 5·0, and the flow rate was 0·5 ml/cm²/min.

Electrophoretic Patterns

The R_f values of the main enzyme components separated by iso-electric focusing at pH 4·4, 5·3, 7·5, 7·8, 8·3 and 9·5 were as follows: 0·4, 0·45, 0·50, 0·38, 0·35 and 0·39. The combined chromatographic and electrophoretic data indicate strongly that the multiple forms of polyphenol oxidase differ not only in electric charge but also in size.

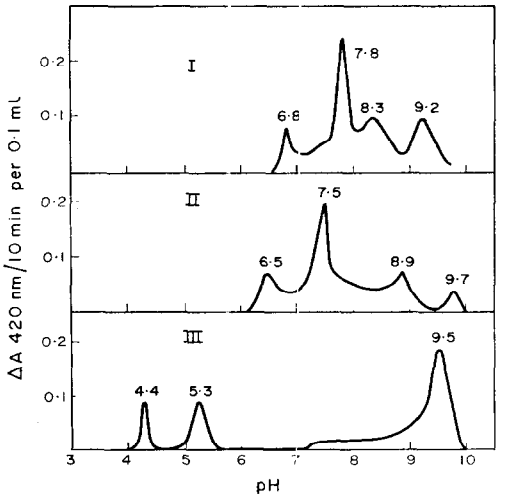

FIG. 2. ISOELECTRIC FOCUSING PATTERNS OF THREE FRACTIONS OBTAINED AFTER CM-CELLULOSE CHROMATOGRAPHY OF YOUNG LEAF EXTRACTS.

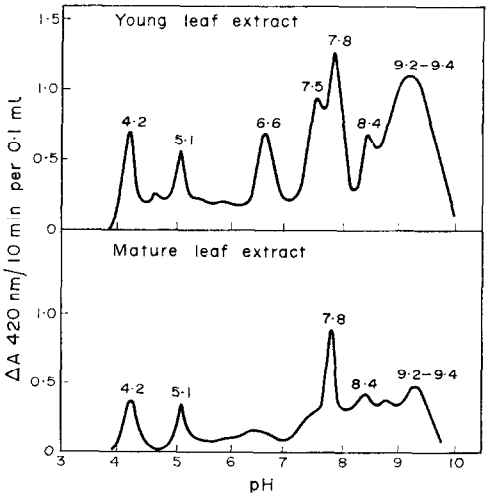

FIG. 3. ISOELECTRIC FOCUSING PATTERNS OF POLYPHENOL OXIDASE ACTIVITY OF YOUNG AND MATURE LEAF EXTRACTS.

Comparison of Polyphenol Oxidases in Tea Leaves of Different Ages

Polyphenol oxidase activity was much higher (*ca.* 3-fold) in extracts of young leaves than in extracts of fully mature leaves (Table 1). This was reflected in the elution profile from CM-cellulose (Fig. 1). Also, there was a striking difference in CM-cellulose chromatographic patterns of the two extracts. Of the three fractions I–III obtained from extracts of young leaves, II and III were greatly reduced in extracts of mature leaves, and only I was present in any sizeable quantity. A further study of differences in polyphenol oxidase components in young and mature leaves was made by isoelectric focusing (Fig. 3). In comparing the patterns of young and mature leaf extracts, special attention should be paid to the broad peak at pH 9·2–9·4 and the peak at pH 7·5. These components decreased remarkably in mature leaves, judging from the isoelectric focusing pattern resulting from mature-leaf extracts. The activity peak at pH 7·8 was, however, about equal in magnitude in both extracts of young and mature leaves. A third component with activity peak at pH 6·6 also was considerably lower in extracts of mature leaves, judging from isoelectric focusing patterns. These results agree closely with those obtained by CM-cellulose chromatography. The observed decreases in components at pH 7·5 and 9·2–9·4 in isoelectric focusing patterns of mature leaf extracts agree especially well with the reductions of components II and III on CM-cellulose chromatograms.

TABLE 1. POLYPHENOL OXIDASE ACTIVITIES IN EXTRACTS OF YOUNG AND MATURE LEAVES

	Fraction	Young leaves	Mature leaves		Fraction	Young leaves	Mature leaves
Total activity*	Extract	17	5	Specific activity†	Extract	3	0·2
	Effluent from CM-cellulose	9·5	3		Effluent from CM-cellulose	450	28

* Total activity is expressed in μmol of (+)-catechin used per g of fr. wt per min at 30°. The extinction coefficient of (+)-catechin at 420 nm: 2·95/μmol.

† Specific activity is expressed in μmol substrate used per mg of nitrogen per min at 30°.

The results show that polyphenol oxidases change unequally during maturation in tea leaves. In a previous paper,[5] one of the authors reported that the activity of polyphenol oxidase in tea leaves increased during storage and this change might be induced by *de novo* synthesis of the enzyme protein. In this case, it was also observed that one component in enzyme protein was remarkably activated. Therefore, it is possible that each component in the polyphenol oxidase complex may vary with physiological changes in the leaf.

EXPERIMENTAL

Preparation of enzyme. Extraction of tea (*Camellia sinensis*, L.) leaves, variety Benihomare and CM-cellulose column chromatography were carried out according to procedures previously reported.[2]

Assay for polyphenol oxidase. Polyphenol oxidase activity was determined colorimetrically by measuring the increase in absorbance (A) at 420 nm using (+)-catechin as the substrate. The reaction mixture contained citric acid 0·1 M, Na–phosphate, 0·2 M, (+)-catechin 0·003 M, and 0·1 ml enzyme extract in a total volume of 1·1 ml at pH 5·0. This mixture was incubated at 35° for 10 min in a water bath. The reaction was stopped by the addition of 2 ml 5% trichloroacetic acid, the mixture was centrifuged at 2100 *g* for 10 min, and absorbance of the supernatant was measured at 420 nm. Enzyme activity was suppressed as ΔA 420 nm/10 min/0·1 ml enzyme extract or as μmol of substrates used per min. Ampholines did not interfere in this assay.

Isoelectric focusing. Isoelectric focusing was carried out in an LKB Electrofocusing column. Gradients

of pH were established by using Carrier Ampholytes of a pH range 3–10. Electrofocusing took place for 72 hr at 700 V and 20°. The run was made at 20° since the enzyme activity was relatively stable at this temperature, and there was more tendency for precipitates to form at lower temperature. Fractions of 2 ml each were collected at a rate of 1 ml/min. The pH of each fraction was measured with a pH meter at room temp.

Polyacrylamide gel electrophoresis. Enzyme samples containing 40% sucrose were added directly to the top of a 7.5% acrylamide gel column, pH 4·2. As electrode buffer, 0·01 M glycine-HOAc buffer, pH 4·2, was used. The electrophoretic run was carried out with 3-mA per tube to the cathode at the bottom. After electrophoresis, the gels were stained by dipping them into staining solution containing 3×10^{-3} M (+)-catechin.