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THE DISTRIBUTION OF POLYPHENOLS, CHLOROGENIC ACID OXIDASE AND PEROXIDASE IN DIFFERENT PLANT PARTS OF TOBACCO, *NICOTIANA TABACUM* L.

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Abstract—Amounts of polyphenols, chlorogenic acid oxidase and peroxidase activities and isozymes in the plant parts of greenhouse-grown burley and flue-cured tobaccos, *Nicotiana tabacum*, have been compared. On a dry weight basis, flower parts, especially the pistil, are about 150-fold higher in phenolic content than the seed and three to five times greater than the leaf. Leaf laminae, either young or senescent, contain more polyphenols than stem, leaf midrib, pith, and root. Three geometric isomers of chlorogenic acid exist in all green tissues as well as in flower parts but only two are detected in the root. The latter contains a high proportion of scopolin and scopoletin, both of which are in low concentrations in photosynthetic tissues and completely absent in pistil. By means of ammonium sulfate fractionation, chlorogenic acid oxidase and peroxidase have been separated from the same tissue extracts. Seeds have no detectable oxidase activity. The activity of chlorogenic acid oxidase is highest in root, lower in pistil, corolla, and stem, and lowest in anther, leaf, and pith. Young leaves exhibit seven times more activity than senescent ones. On the contrary, the leaf peroxidases increase in concentration from upper to bottom stalk positions. Peroxidase content is highest in root but lowest in flower parts. Polyacrylamide gel block electrophoresis has revealed two cathodic and ten anodic isoperoxidases in the leaves varying in physiological age. Two anodic and two cathodic isoperoxidases in slow mobility are lacking in pith and reproductive organs but appear as intensive bands in root. Zymograms of chlorogenic acid oxidase are identical to that of isoperoxidase for all plant parts except pistil and corolla, which have a cathodic band migrating farther away from the origin. Results suggest that the distribution patterns of polyphenols and two oxidases in the plant parts of two tobacco strains coincide with each other, and within a plant the correlation between phenolic quantity and oxidase activity varies depending upon the organs and tissues.

INTRODUCTION

POLYPHENOLS of tobacco leaves have been extensively investigated in the past because of their importance in leaf quality. Several classes of polyphenol, namely, phenolic acids, flavonoids, and coumarins, were found in the leaf of various tobacco types, chlorogenic acid being the predominant compound in green tissues.[1] In contrast to the extensive studies on the change of polyphenols during curing,[2,3] little information is available on their distribution and metabolism in tobacco plants. An earlier study revealed that the root of burley and dark-tobacco seedlings contain a large amount of polyphenols together with strong polyphenol-oxidase (*o*-diphenol: O_2oxidoreductase EC 1.10.3.1) and peroxidase (EC 1.11.1.7) activity.[4] Both oxidases are capable of oxidizing polyphenols to form polymers and brown pigments.[5] Two known polyphenoloxidases, chlorogenic acid oxidase (CAO) and catecholase, are present in tobacco leaves, but the former is present in larger amounts.[6]

[1] S. J. SHEEN and J. CALVERT, *Tobacco Sci.* **13**, 10 (1969).
[2] T. J. WESTON, *Phytochem.* **7**, 291 (1968).
[3] S. J. SHEEN and J. CALVERT, *Plant Physiol.* **44**, 199 (1969).
[4] S. J. SHEEN, J. CALVERT and G. R. REBAGAY, *Crop. Sci.*, in press.
[5] W. W. REID, *Tobacco Sci.* **3**, 109 (1959).
[6] R. A. CLAYTON, *Archs Biochem. Biophys.* **81**, 404 (1959).

In varietal comparisons, burley tobaccos were lower in soluble polyphenol content than dark tobaccos at various stages of growth and after curing.[1] Whether this is due to differences in the rate of biosynthesis or decomposition or both can only be speculative. Although chlorogenic acid is synthesized in the leaf,[7] it may not be catabolized solely in the same organ. It has been reported that chlorogenic acid migrates from leaf to meristem during photoperiodic induction of flowering.[8] Thus, the distribution of polyphenols in tobacco plants may elucidate the sites of phenolic metabolism as well as the physiological roles of these compounds. The present investigation was, therefore, undertaken to compare the qualitative and quantitative makeup of polyphenols and the activity of CAO and peroxidase in the plant parts of mature burley and flue-cured tobaccos. Data are also given on the isozymes of both oxidases in the same plant parts of these tobaccos.

RESULTS

Partial Purification and Stability of Oxidases

By ammonium sulfate fractionation, the 20–30 per cent precipitate of tobacco leaf homogenate contained the high CAO activity, whereas the 30–50 per cent fraction was richest in peroxidase content (Table 1). The separation of these oxidases in the extracts of root, stem, pith, and flower parts have been achieved by the same method. Extraction procedure modified by adding insoluble polyvinylpyrrolidone neither enhanced nor reduced oxidase activity. Partially purified oxidases could tolerate 50° temperature for 20 min without an appreciable loss of activity. A 20-min heat treatment at 70° resulted in a nearly total inactivation of both oxidases.

TABLE 1. FRACTIONATION BY AMMONIUM SULFATE OF THE SOLUBLE OXIDASE ACTIVITIES IN TOBACCO LEAVES*

Fraction (% saturation)	Chlorogenic acid oxidase activity measured by		Peroxidase activity measured by spectrophotometer
	Manometric technique	Spectrophotometer	
I 0–20 precipitate	7·38	0·67	0·07
II 20–30 precipitate	21·35	1·39	0·31
III 30–40 precipitate	2·48	0·28	5·11
IV 40–50 precipitate	0	0	29·33
V 50–60 precipitate	0	0	0·11
VI Supernatant from V	0	0	0

* Details of the assay procedures are given under Materials and Methods. Chlorogenic acid activity is expressed as μl O_2 uptake/mg protein/min when measured by manometric technique, and as absorbance change/10 mg protein/min by spectrophotometric measurement. Peroxidase activity is expressed as absorbance change/100 μg protein/min. The results are an average of two experiments using the mature leaves of Ky Iso 4 Hicks as the material.

Qualitative and Quantitative Determination of Polyphenols

Qualitative makeup of phenolic compounds appeared identical between and within the two tobacco strains but different in plant parts. Chlorogenic acid (3-caffeoylquinic acid),

[7] M. ZUCKER and J. F. AHRENA, *Plant Physiol.* **33**, 246 (1958).
[8] M. ZUCKER, C. NITSCH and J. P. NITSCH, *Am. J. Botany* **52**, 271 (1965).

rutin (quercetin 3-rhamnosidoglucoside), scopoletin (7-hydroxy-6-methoxycoumarin), and scopolin (the 7-glucoside of scopoletin) were the major polyphenols in tobacco. Identification and quantitative determination of minor polyphenols were not attempted. The *cis* and *trans* forms of chlorogenic acid and its isomer neochlorogenic acid (5-caffeoylquinic acid) were separable on chromatograms with R_f values approximately the same as to those reported by Walker and Lee.[9] They existed in all tissue extracts except seed and root. In the root extracts of both greenhouse and field-grown plants, chlorogenic acid showed an abundance in quantity but neochlorogenic acid was never present. This is in agreement with the phenolic make up in seedling roots.[4] Polyphenols present in the seed in measurable quantity were scopoletin and one other blue fluorescent spot. The latter may be related to chlorogenic acid because it gave a positive reaction to Arnow's nitrite and molybdate reagent[10] and yielded caffeic acid on acid hydrolysis. Scopoletin and scopolin occurred in all green tissue and root extracts but were not in the pistil. An unknown scopoletin glycoside of the root emitted bright blue fluorescence, had an absorption peak at 345 mμ and yielded scopoletin by acid hydrolysis. A number of scopoletin glycosides have been identified in tobacco pith culture.[11] In the present study, scopolin was the only glycoside detected in pith extracts. The absence of the others may be due to the difference between the systems for polyphenol biosynthesis in the pith and the intact plant. In addition to chlorogenic acids and scopolin, anther and corolla extracts contained rutin and unknown flavonoids. Two of the unidentified flavonoids in corolla yielded kampferol on acid hydrolysis.

In view of the possible interconversion of chlorogenic acid isomers during extraction and chromatography, the quantity of chlorogenic acid reported here is the sum of all isomers. Likewise, the concentration of flavonoids is expressed in terms of rutin. Amounts of scopoletin and scopolin are determined individually but values for amounts of scopolin in root include the unknown scopoletin glycoside. The results in Table 2 represent the means of six individual plant determinations. The quantity of phenolic compounds in the plant parts of two tobacco strains showed an identical distribution pattern. Seed extracts contained lowest concentrations, whereas the flower parts, particularly the pistil, were highest. On a dry weight basis, the high and low extreme differed almost 150-fold. In agreement with previous results, a higher concentration of soluble polyphenols was found in the flue-cured tobacco (Ky Iso 4 Hicks) than in the burley strain.[1] This varietal difference was also apparent in other plant parts except in corolla where the burley contained about 30 per cent more flavonoids. A trace amount of rutin in the leaf in the present experiment could be attributed to greenhouse conditions. It is known that light quality and duration influence polyphenolic quantity in tobacco leaves.[12, 13] The non-photosynthetic tissues had greater concentrations of scopolin and scopoletin than the leaf.

CAO and Peroxidase Activity

The partially purified CAO did not catalyse the oxidation of catechol; in fact, the latter inhibited the oxidation of chlorogenic acid. This coincides with Clayton's findings on his preparation of polyphenoloxidase from tobacco leaves.[6] The mean activity of CAO and peroxidase in the plant parts of two tobaccos is summarized in Table 3. Neither oxidases were

[9] E. K. WALKER and T. T. LEE, *Can. J. Plant Sci.* **48**, 381 (1968).
[10] L. E. ARNOW, *J. Biol. Chem.* **118**, 531 (1937).
[11] J. A. SARGENT and F. SKOOG, *Plant Physiol.* **14**, 504 (1961).
[12] P. T. PENN and J. A. WEYBREW, *Tobacco Sci.* **2**, 68 (1958).
[13] R. F. DAWSON and E. WADA, *Tobacco Sci.* **1**, 47 (1957).

TABLE 2. MEAN POLYPHENOL CONTENT IN THE DIFFERENT PLANT PARTS OF TWO TOBACCO STRAINS

Plant part	Ky Iso 1 Ky 16 {phenols (mg/g dry weight)}*					Ky Iso 4 Hicks {phenols (mg/g dry weight)}*				
	Chlorogenic acids	Rutin + flavonoids	Scopolin	Scopoletin	Total polyphenol	Chlorogenic acids	Rutin + flavonoids	Scopolin	Scopoletin	Total polyphenol
Seed	0·11 ± 0·01	—	—	0·07 ± 0·02	0·18	0·12 ± 0·02	—	—	0·11 ± 0·03	0·23
Anther	10·29 ± 0·71	3·39 ± 0·10	0·29 ± 0·15	tr.	14·27	13·99 ± 2·10	3·00 ± 0·22	0·71 ± 0·14	tr.	17·70
Pistil	28·40 ± 1·80	—	—	—	28·40	33·96 ± 2·43	—	—	—	33·96
Corolla	4·53 ± 0·80	9·97 ± 1·97	tr.	tr.	14·50	5·31 ± 0·32	6·46 ± 0·71	tr.	tr.	11·77
Young leaf	5·07 ± 0·06	tr.	0·24 ± 0·06	0·20 ± 0·03	5·51	7·62 ± 0·06	tr.	0·53 ± 0·01	0·11 ± 0·02	8·26
Mature leaf	3·15 ± 0·04	tr.	0·27 ± 0·07	0·18 ± 0·05	3·60	9·60 ± 0·89	tr.	0·78 ± 0·18	0·56 ± 0·19	10·94
Senescent leaf	6·62 ± 0·05	tr.	0·36 ± 0·04	0·24 ± 0·08	7·22	12·19 ± 0·87	tr.	0·82 ± 0·09	0·24 ± 0·04	13·25
Young stem	0·44 ± 0·03	—	0·29 ± 0·05	0·27 ± 0·15	1·00	0·37 ± 0·07	—	0·38 ± 0·04	0·39 ± 0·03	1·14
Leaf midrib	0·80 ± 0·06	—	0·20 ± 0·02	0·20 ± 0·01	1·20	2·63 ± 0·57	—	0·38 ± 0·10	0·51 ± 0·14	3·52
Pith	0·23 ± 0·01	—	0·15 ± 0·05	0·18 ± 0·03	0·56	0·18 ± 0·02	—	0·53 ± 0·10	0·49 ± 0·04	1·20
Root	1·26 ± 0·26	—	0·57 ± 0·01	0·49 ± 0·05	2·32	2·64 ± 0·62	—	1·29 ± 0·44	0·44 ± 0·15	4·37

* Mean ± S.E.

detected in the seed. Roots exhibited more CAO activity than other tissues analyzed. In flower, the pistil and corolla extracts contained four times more CAO activity than the anther. The CAO activity of the leaf increases as stalk position elevates. The difference between young and senescent leaves reached nearly 8-fold in both strains. The flue-cured tobacco possessed higher CAO activities in young leaf, stem, leaf midrib, and pith than the respective parts of the burley.

In contrast to CAO, peroxidase activity was low in young leaf and increased rapidly as the leaf reached maturation and senescence. This is in accord with the increase in peroxidase activity at early stages of air-curing.[3] A strong activity in the mature and senescent leaves of Ky Iso 1 Ky 16 may have indicated an advanced stage of maturation and senescence when compared with that of Ky Iso 4 Hicks on the same stalk positions. Of the other tissues,

TABLE 3. MEAN ACTIVITY OF CHLOROGENIC ACID OXIDASE AND PEROXIDASE IN DIFFERENT PLANT PARTS OF TWO TOBACCO STRAINS*

	Chlorogenic acid oxidase		Peroxidase	
Plant part	Ky Iso 1 Ky 16	Ky Iso 4 Hicks	Ky Iso 1 Ky 16	Ky Iso 4 Hicks
Seed	not detected	not detected	not detected	not detected
Anther	3·54±0·62	2·79±0.63	0·21±0·01	0·24±0·01
Pistil	18·71±1·58	16·44±1·62	0·32±0·05	0·42±0·02
Corolla	16·31±1·32	17·16±2·71	1·40±0·21	1·62±0·11
Young leaf	4·02±0·14	6·10±0·17	10·39±0·88	14·83±1·26
Mature leaf	1·41±0·14	1·33±0·20	40·14±1·04	30·49±1·65
Senescent leaf	0·56±0·06	0·78±0·17	50·18±6·10	33·78±0·64
Young stem	7·34±0·94	17·33±1·53	11·79±0·84	13·71±3·01
Leaf midrib	1·46±0·10	5·54±0·38	9·66±2·64	9·82±1·15
Pith	0·48±0·05	1·62±0·10	6·18±0·99	4·55±0·67
Root	27·80±0·15	30·18±2·00	17·90±1·29	16·13±3·08

* The results are present as Mean ±S.E. The activity of chlorogenic acid oxidase was measured with the 20–30% $(NH_4)_2SO_4$ fraction and expressed as absorbance change/10 mg protein/min. Peroxidase activity was assayed with the 30–50% $(NH_4)_2SO_4$ fraction and expressed as absorbance change/100 μg protein/min. The procedure of preparing soluble oxidase fractions and spectrophotometric assays are given in the Experimental section.

root extracts possessed a high peroxidase content followed in decreasing order by young stem, leaf midrib, and pith. The lowest peroxidase concentration was found in flower parts, especially the reproductive organs. In general, the distribution pattern of peroxidase in the plant parts studied was quantitatively alike for both tobaccos.

CAO and Peroxidase Isozymes

Comparisons on the same polyacrylamide gel block for the isozyme pattern of crude extracts and partially purified oxidases have revealed no preferential selection of isozymes by purification procedure. Neither the addition of polyvinylpyrrolidone, Triton X100 (1%), nor deoxycolate (1%) for enzyme preparation has altered the zymogram of both oxidases. On the contrary, with the use of purified enzyme fraction a streaking phenomenon on the gel was reduced and consequently, the resolution of weak isozyme bands increased. Excluding the

seed which contained no measurable oxidase activity, four samples of each plant part of both tobaccos were studied with gel block electrophoresis. Identical zymograms were produced with chlorogenic acid and 3,4-dihydroxyphenylalanine as substrates. When tyrosine was the substrate, no bands developed. Similarly, no qualitative differences resulted when benzidine-2HCl or guaiacol were used as alternative electron donors in developing isoperoxidases. Zymograms obtained from different plants of the same tobacco strain were identical. Between strains, no qualitative but quantitative contrast in isozyme bands was noticeable. In most cases, the isozyme activity coincided with enzyme assay results. For this reason, the CAO and peroxidase zymograms of Ky Iso 4 Hicks, as shown in Figs. 1 and 2 respectively, will be used for illustration. The polyacrylamide gel block may be arbitrarily divided into three zones: zone I containing cathodic isozymes; zone II for anodic isozymes having R_f values less than 0·5; and zone III for anodic isozymes having R_f values larger than 0·5. For isozyme designation, the Roman numerals stand for the zones and the alphabetic order of subscripts indicates the relative distance of isozymes migrating away from the origin.

At least three cathodic CAO isozymes, namely I_a, I_b, and I_c were present in tobacco plants. Bands I_a and I_b occurred in common in leaf, stem, pith, and root but were not in flower parts. Pistil and corolla tissues possessed band I_c which may have also been present in young leaf. Anodic bands II_a and II_b showed a strong CAO activity in root but absent in flower and pith. In leaf, their activities diminished sharply from bottom to upper stalk positions. Six sharp isozyme bands III_a, III_b, III_c, III_d III_e, and III_f were found in all green tissues as well as in pith. The CAO fraction from pistil has shown a weak III_a band. By applying an increased quantity of enzyme preparation in disc electrophoresis, bands III_a and III_c appeared universally in all tissues including anther.

A similarity between polyphenoloxidase and peroxidase zymograms developed from the crude extract of senescent tobacco leaves has been previously noted.[3] The purification procedure employed in the present study yielded the same results. Since the leaf isoperoxidases appeared in the same bands with CAO, the symbols used for CAO bands will be followed. The isoperoxidase pattern of ten plant parts had several features deviating from CAO zymogram. First, band I_c did not present in any organ or tissue. Secondly, two additional but weak isoperoxidase bands appeared in zone II. One is located between the origin and band II_a, and the other is between bands II_a and II_b. While these bands may be individual isoperoxidases, it is also possible that they are the dissociated products of major bands. However, a repeating freeze-thawing process did not modify the isoperoxidase pattern. Thirdly, there were differences in isozymic activity in zone III. Bands III_a and III_c were the strongest isoperoxidases in all green tissues, while band III_f was an important one in stem and pith. The lack of qualitative differences in the isoperoxidase pattern of young, mature and senescent leaves observed in the present study supports Novacky and Hampton's findings.[14] The heat treatment of leaf extracts resulted in a corresponding loss of isoperoxidase band intensity with an exception that band I_b retained a strong activity even after a treatment of 70° for 20 min under which the remainders were completely inactivated.

DISCUSSION

Although the occurrence of polyphenols in the plant parts of two tobacco strains was qualitatively unique, their quantities were low because of greenhouse conditions. Since light is a limiting factor for polyphenol biosynthesis,[12, 13] the extraordinarily high concentration of

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chlorogenic acid found in the flower bud may require a different explanation. Zucker *et al.*[8] postulated a photoperiodically induced migration of chlorogenic acid from leaf to meristem in connection to flower induction. This upward translocation may have taken place during the development of inflorescence. Phenolic accumulation in pistil and anther is of interest. Tobacco is an often open-pollinator and the flavonoids in its corolla may function as insect attractors. Whether the polyphenols in pistil and anther have the same function or perform other physiological roles such as regulating enzymic activity essential for flower development and/or sexual reproduction awaits future experimental evidence. The high chlorogenic acid content in flower parts may account for the low peroxidase activity because chlorogenic acid has inhibitory effect upon peroxidase.[15] On the other hand, the coincidence of high chlorogenic acid content and strong CAO activity in pistil suggests an inductive nature of this oxidase *in vivo*. The CAO-catalyzed oxidation of *o*-dihydric phenols to *o*-quinones which in turn become a portion of high molecular weight constituents in the cell probably occur in the pistil in view of the fact that soluble polyphenol content is extremely low in the seed extract.

The inconsistent correspondence between oxidase activity and polyphenol content in the plant parts of two tobaccos may be explained by the variation in physiological state and function of the organs and tissues and/or due to a possible spacial separation of enzymes and substrates. The increase of peroxidase activity in the senescent leaf reflects an enzymic induction evoked by the direct contact of substrates with enzymes after the rupture of cell membrane. Peroxidase synthesis in the senescent tobacco leaf disks has been recently reported by Parish.[16] Aside from the participation of peroxidase in respiration and degradation of cellular components, the high peroxidase activity in young stem, leaf midrib and root possibly relates to lignification. This is supported by the histochemical localization of peroxidase in the xylem and endodermis of these tobacco organs (unpublished data). The high content of polyphenols as well as both oxidases in the extract of mature and seedling roots[4] deserves attention. Questions needed to be answered are: Are polyphenols synthesized *in situ* in tobacco root; and what are the functions of these oxidases, CAO in particular, in the root other than lignification?

Molecular forms of a given enzyme in different organ and tissue may relate to development and differentiation of an organism. This is, in part, supported by the striking difference in isoperoxidases and CAO isozymes between root and other tissues analyzed (Figs. 1 and 2). Organs with a similar ontogenetic origin shared more isozymes in common. The thermostability of isoperoxidase band I_b in leaf extracts is of interest. By histochemical means, DeJong[17] recently observed the presence of pseudoperoxidase in the middle lamella between cortex and endodermis in onion roots, which was also heat resistant. The presence of isoperoxidase I_b in most of the organs having vascular differentiation may suggest a similarity in its cellular localization.

Phenolic constituents in leaf extracts have been used as biochemical criterion in checking species relationship. The similarity of polyphenolic pattern in a given organ or tissue of the two tobacco types studied verifies its usefulness in taxonomical purposes. Since pistil and anther are rich in polyphenol content and are directly participating in sexual reproduction by which the course of evolution in plants is determined, these organs may be desirable materials in the chemotaxonomical viewpoint. Moreover, the involvement of more than one organ or tissue for comparison may increase the precision of distinguishing closely related species.

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MATERIALS AND METHODS

Chemicals

All chemicals used in extraction, paper chromatography, spectrophotometry and enzyme assays were analytical grades purchased from Fisher Scientific Company, Nutritional Biochemicals Corporation, and Aldrich Chemical Company. Reagents for polyacrylamide gel block and disc electrophoresis were purchased from Canalco, Rockville, Maryland. All solutions were prepared using de-ionized glass distilled water.

Plant Materials

Six plants each of two tobacco, *Nicotiana tabacum* L., strains, Ky Iso 1 Ky 16 (burley) and Ky Iso 4 Hicks (flue-cured), were cultured in Hoagland's solution[18] during early spring in the greenhouse where temperatures were maintained at 24° during the day and 18° at night with supplementary fluorescent lights providing a photoperiod of 16 hr each day. Hoagland's solution was changed once a week. At flowering, the anthers, pistils and corolla tissues of mature flower buds were collected daily for 2 weeks. The flower parts of the same plant were bulked as a single sample. By the end of 2 weeks the plants were separately harvested for the following organs and tissues: young leaf (16th–20th node), mature leaf (9th–12th node), senescent leaf (2nd–4th node), young stem (16th–20th node), leaf midrib (from mature leaves), pith, and root. Roots of mature field-grown Ky Iso 1 Ky 16 were used to compare the results of the greenhouse-grown plants. The deveined leaves and other plant parts were stored at −90° prior to lyophilization. Seeds of both strains were collected from field-grown plants whose inflorescences had been bagged to avoid outcrosses. All plant parts were ground into 40-mesh particles and stored in amber bottles at −20°.

Partial Purification of CAO and Peroxidase

Dry tissues (2–5 g) were ground in 40–100 ml of 0·1 M phosphate buffer (pH 7·2) containing 0·1% cysteine–HCl with a mortar and pestle at 4°. Cell debris were homogenized again in the same volume of the buffer. Ammonium sulfate was added to the combined filtrates to make a final concentration of 20%. The precipitates were discarded by centrifugation and the supernatant fluid was made to 30% $(NH_4)_2SO_4$ to yield precipitates which were dialyzed in two changes of 0·05 M phosphate buffer (pH 7·2) for 16 hr at 4°. This yielded the CAO fraction. The supernatant fluid from 30% $(NH_4)_2SO_4$ saturation was increased to a final concentration of 50% $(NH_4)_2SO_4$. The resultant precipitates rich in peroxidase activity were dialyzed as for CAO. The protein content of two oxidase fractions was determined by the method of Lowry *et al.*[19] using bovine serum albumin as a standard.

Assay of Enzyme Activity

The determination of CAO activity by spectrophotometric and manometric techniques using 3,4-dihydroxyphenylalanine (0·014 M) and chlorogenic acid (0·02 M) as substrates respectively has been described in a previous paper.[3] Peroxidase activity was assayed at 485 nm of a colored complex produced by a mixture of H_2O_2 (0·045 M), *p*-phenylenediamine (0·5%), and the enzyme preparation after the method of Luck.[20] Absorbancy increase in

[18] D. R. HOAGLAND and D. I. ARNON, *California Agri. Exp. Sta. Cir.* **347**, 1 (1950).
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[20] H. LUCK, in *Methods of Enzymatic Analysis* (edited by H. M. BERGMAYER), p. 895, Academic Press, N.Y. (1963).

reaction mixtures was followed using a Beckman DB-G double-beam spectrophotometer equipped with a potentiometric recorder. The initial reaction velocity in both spectrophotometric and manometric measurements was calculated from the tangent drawn to the reaction time course curve. The enzymic activity was expressed in absorbance change or μl O_2 uptake/amount of protein/min under the defined experimental conditions.

Quantitative and Qualitative Determination of Polyphenols

Soxhlet extraction, evaporation *in vacuo*, and two-dimensional paper chromatography with *n*-butanol:acetic acid:water (BAW, 10:1:3 v/v/v) as the first solvent and isopropanol: formic acid:water (IFW, 5:0·1:95 v/v/v) as the second solvent have been described elsewhere.[1] Identification of polyphenols was based on a comparison of R_f values with authentic compounds, u.v. spectral analysis, fluorescence emission, acid hydrolysis (boiling in 2 N HCl for 30 min), and reaction to specific reagent. Quantity of individual polyphenols expressed in mg/g dry weight was determined by eluting off the spots in 30% methanol and absorbancy was recorded at 328 nm or 345 nm for chlorogenic acid or scopolin and scopoletin, respectively. For rutin and other flavonoids the spots were eluted in 0·1 N $A.C._3$ in 30% methanol and measured at 416 nm according to Weaving's method.[21] Total polyphenol content is the sum of the major phenolic compounds.

Polyacrylamide Gel Electrophoresis and Detection of CAO and Peroxidase Isozymes

Davis' method[22] of disc electrophoresis was adapted to the Buchler starch gel vertical electrophoresis apparatus by which ten samples can be compared on the same gel block (26 × 12 cm). About 100 μg protein of peroxidase fraction and 300 μg protein of CAO fraction were delivered into the sample slots of stacking gel. The polyacrylamide gel block (7·5% gel, pH 8·6) was subjected to 50 mA current at 150V for 16 hr at 4°. Isoperoxidases were visualized by a solution of benzidine-2HCl and H_2O_2 (0·015%) according to Ornstein[23] and by the incubation of gel in 0·02 M guaiacol for 30 min followed in 0·1% H_2O_2 until the completion of color development. A 0·02 M concentration of chlorogenic acid, tyrosine or 3,4-dihydroxyphenylalanine in 80% ethanol was used to develop CAO isozymes after the method of Constantinides and Bedford.[24] Due to an extremely low enzymic activity in certain tissues, an increased quantity of enzyme preparation has been applied to check the anodic isozymes by disc electrophoresis.

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