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TEA CATECHOL OXIDASE: ISOLATION, PURIFICATION AND KINETIC CHARACTERIZATION

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Key Word Index—*Camellia sinensis,* Theaceae, tea, catechol oxidase, peroxidase, galloyl esterase, flavanol oxidation, epimerization

Abstract—Catechol oxidase extracted from tea leaves was purified over 200-fold, using isoelectric focusing The purified catechol oxidase was free of peroxidase and flavanol gallate esterase activities Further, this enzyme was shown to have optimum activity near pH 5 7 and a K_m of 2 3 $\times$ 10^{-3} M (at 25°) for (−)-epigallocatechin gallate The purified enzyme was found to be capable of epimerizing tea flavanols at their C-2 position whether oxidation of the flavanol occurs (aerobic conditions) or not (anaerobic conditions) When oxygen is present, gallic acid is formed as a result of oxidation of either (−)-epigallocatechin gallate or (−)-epicatechin gallate Formation of gallic acid is a side reaction of the oxidation of the flavanol gallates and is named oxidative degallation, no esterase *per se* is involved in this reaction

INTRODUCTION

THE ENZYMES endogenous to tea leaf tissues[1–3] are instrumental in bringing about tea fermentation, i e the biochemical process by which fresh tea leaf is converted to black tea [4,5] It has long been recognized[6,7] that catechol oxidase (*o*-diphenol O_2 oxidoreductase; E C 1 10 3 1) mediates the primary reaction of tea fermentation, namely, the oxidation of tea flavanols (I–IV) to their corresponding reactive orthoquinone intermediates (V–VIII) [4,5] However, peroxidase (donor H_2O_2 oxidoreductase, E C 1 11 1 7) has also been implicated[8] as an enzyme which is important in the fermentation although its role in this process has not been made clear. Further, it has been noted that appreciable amounts of free gallic acid are formed during the oxidation of tea flavanols both in whole tea leaf systems[9 10] and in model tea fermentation systems[11–16] and that some epimerization of the flavanols takes

[1] EDEN, T (1965) *Tea*, 2nd Edn, Longmans-Green, London
[2] HAINSWORTH, E (1969) in *Encyclopedia of Chemical Technology*, 2nd Edn, Vol 19, p 743, Interscience-Wiley, New York
[3] HARLER, C R (1963) *Tea Manufacture*, Oxford University Press, London
[4] ROBERTS, E A H (1962) in *Chemistry of Flavonoid Compounds* (GEISSMAN, T A , ed), p 468, Pergamon Press, Oxford
[5] SANDERSON, G W (1972) in *Recent Advances in Phytochemistry* (RUNECKLES, V C and TSO, T C , eds), Vol 5, p 247, Academic Press, New York
[6] SREERANGACHAR, H B (1939) *Curr Sci* **8,** 13, (1943) *Biochem J* **37,** 661
[7] ROBERTS, E A H (1952) *J Sci Food Agr* **3,** 193
[8] BOKUCHAVA, M A and SKOBELEVA, N I (1969) *Advan Food Res* **17,** 215
[9] ROBERTS, E A H and WOOD, D J (1951) *Biochem J* **49,** 414
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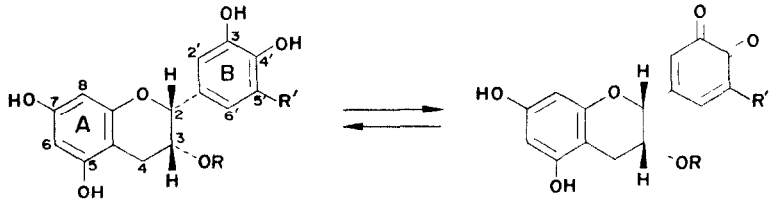
[11] ROBERTS, E A H and MYERS, M (1959) *J Sci Food Agr* **10,** 167
[12] TAKINO, Y and IMAGAWA, H (1963) *Nippon Nogei Kagaku Kaishi* **37,** 417
[13] NAKAGAWA, M and TORII, H (1965) *Agr Biol Chem* **29,** 278
[14] DZHEMUKHADZE, K M , BUZUN, G A and MILESHKO, L F (1966) *2nd International Congress of Food Science and Technology, Warsaw*, Poland, August 22–27
[15] SANDERSON, G W , BERKOWITZ, J E , CO, H and GRAHAM, H N (1972) *J Food Sci* **37,** 399
[16] COGGON, P , MOSS, G A and SANDERSON, G W (1973) *J Agr Food Chem* **21,** in press

place during tea fermentation [16–18] However, in spite of these observations, the relationship between these enzyme activities and their role in tea fermentation is not clear at the present time



(I) L-(−)-epicatechin R = R′ = H
(II) L-(−)-epicatechin gallate R = G, R′ = H
(III) L-(−)-epigallocatechin R = H, R′ = OH
(IV) L-(−)-epigallocatechin gallate R = G, R′ = OH

(V) R = R′ = H
(VI) R = G, R′ = H
(VII) R = H, R′ = OH
(VIII) R = G, R′ = OH

G = Galloyl = 3, 4, 5-trihydroxybenzoyl

SCHEME 1

As part of an investigation of the biochemistry of tea fermentation,[15 16,19] we have studied tea catechol oxidase to determine its role in the above-mentioned reactions In so doing, we have separated tea catechol oxidase from peroxidase and gallic acid esterase (E C 3 1 1 20) enzymes which were also found in tea leaf extracts The properties of our purified tea catechol oxidase are compared with those reported by Bendall and Gregory[20] and by Takeo and Uritani[21,22] and new information on the properties of this enzyme is discussed with particular emphasis on their importance to tea fermentation

RESULTS

Enzyme Extraction

First, a study was made to optimize the extraction of catechol oxidase from fresh green tea leaf tissues The results showed that, as was found earlier,[23] it was necessary to incorporate Polyclar AT in the extraction media in order to obtain any enzyme activity Improvements in the extraction procedure[15,19] include adjustment of extraction media to pH 7 0, and addition of Tween 80 [21] Passage of the extract through a short column of Sephadex G50 significantly increased activity; this treament removed most of the residual inhibiting polyphenol from the enzyme extract Finally, the amount of enzyme activity was doubled by cryomilling the tea leaf material using liquid nitrogen in place of solid CO_2 The clear green extract thus obtained (see Experimental) was designated the crude soluble tea (CST) enzyme preparation

Enzyme Purification

The CST enzyme preparation was further purified by the procedure summarized in Table 1 The first step was the preparation of a 40–60% ammonium sulphate fraction

[17] NAKAGAWA, M (1967) *Agr Biol Chem* **31**, 1283
[18] DZHEMUKHADZE, K M , BUZUN, G A and MILESHKO, L F (1964) *Biokhimiya* **29**, 882
[19] BERKOWITZ, J E , COGGON, P and SANDERSON, G W (1971) *Phytochemistry* **10**, 2271
[20] GREGORY, R P F and BENDALL, D S (1966) *Biochem J* **101**, 569
[21] TAKEO, T (1965) *Agr Biol Chem* **29**, 558
[22] TAKEO, T and URITANI, I (1966) *Agr Biol Chem* **30**, 155
[23] SANDERSON, G W (1964) *Biochim Biophys Acta* **92**, 622

(Fraction No II); this produced only a small purification and little separation of catechol oxidase and peroxidase Gel permeation chromatography on both Sephadex G100 and G150 columns (350 × 25 mm) at this stage failed to give significant separation of catechol oxidase and peroxidase, although tea catechol oxidase has a reported[20] MW between 130 000 and 160 000 and peroxidase a MW *ca* 50 000 [24,25] Further, only about 15% of the enzyme activity was recovered from these columns. These results suggest that tea catechol oxidase and peroxidase are still strongly associated

TABLE 1 PURIFICATION OF TEA CATECHOL OXIDASE

Procedure	Fraction No	Vol (ml)	Enzyme (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Ratio of peroxidase/ catechol oxidase activities
CST enzyme preparation	I	70	9 1	18 5	0 5	23 1
40–60% $(NH_4)_2SO_4$ fractionation	II	100	4 2	6 0	0 7	14 1
Ultrafiltration XM-300 retentate	III	48	7 1	1 5	4 7	12 1
Isoelectric focusing* (Isoelectric point)						
(4 1)	IV-A	25	12 0	0 115	105 0	0 1
(6 8)	IV-B	30	4 0	0 037	110 0	1 2 1
(7 1)	IV-C	20	5 0	0 12	4 6	2 1 1

* See Fig 1 for additional information on these fractions

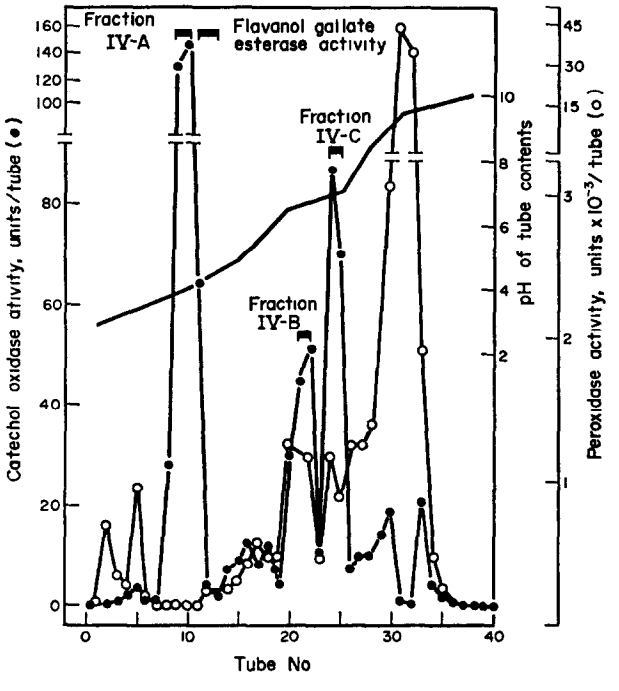


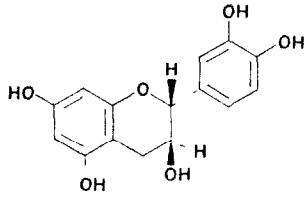
FIG 1 SEPARATION OF TEA ENZYMES; CATECHOL OXIDASE, PEROXIDASE, AND FLAVANOL GALLATE ESTERASE BY ISOELECTRIC FOCUSING

[24] MAEHLY, A C (1955) in *Methods in Enzymology* (COLOWICK, S P and KAPLAN, N. O , eds), Vol II, p 807, Academic Press, New York
[25] HOYLE, M C (1972) *Plant Physiol* **50**, 15

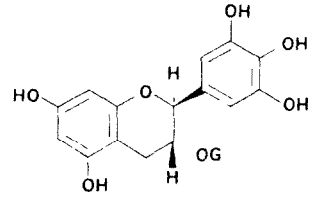
The 40–60% ammonium sulphate fraction was ultrafiltered through an Amicon XM 300 filter which retains molecules with apparent MWs above about 300 000.[26] Both catechol oxidase and peroxidase were recovered in the retentate after this treatment in yields of over 80% which again indicates an association of these enzymes. Approximately a 7-fold purification of catechol oxidase was obtained, with desalting

The retentate from the ultrafiltration step (Fraction No III in Table 1) was purified further by isoelectric focusing The results obtained in a 96 hr run with a column containing LKB pH 3–10 Ampholyte are summarized in Fig 1 This final step completely separated catechol oxidase and peroxidase from each other The major amount of catechol oxidase was found free of peroxidase at isoelectric point 4 1, and most of the peroxidase was found free of catechol oxidase at isoelectric point pH 9 6 The small amount of flavanol gallate esterase present in the CST enzyme preparation was found at isoelectric point pH 4 2 completely separated from any important catechol oxidase or peroxidase activity Smaller amounts of catechol oxidase and peroxidase activity were found together at other isoelectric points Altogether, more measurable catechol oxidase activity was recovered from the isoelectric focusing column than was put onto the column

The main catechol oxidase fraction, at isoelectric point pH 4·1 (Fraction No IV-A, Table 1, hereinafter referred to as purified catechol oxidase), contained 48% of the catechol oxidase activity in the CST enzyme preparation, and was 210 times purer The purified catechol oxidase had only limited stability. about 60% of its activity was lost in 2 months at −40°, and thawing and refreezing destroyed virtually all of its activity



(IX) D-(+)-Catechin



(X) G = Galloyl,
L-(−)-Gallocatechin gallate

pH Optimum

The pH optimum of the purified catechol oxidase was determined using catechol as substrate, and was found at pH 5 7 This result must be compared with values of pH 5 5 reported by Takeo,[21] pH 5 1 (Fraction A-I) and pH 4 6 (Fraction A-II) reported by Takeo and Uritani,[22] and pH 5 7 reported by Gregory and Bendall [20]

Kinetics

The reactivity of the purified tea catechol oxidase towards various substrates was studied. The results (Table 2) show clearly that this enzyme is rather specific for the *o*-diphenolic group of the tea flavanols (−)-epicatechin (I), (−)-epicatechin gallate (II), (−)-epigallocatechin (III), (−)-epigallocatechin gallate (IV) and (+)-catechin (IX) and that the K_ms

[26] PORTER, M C and MICHAELS, A S (1971) *Chem Tech* 56, BLATT, W F (1971) in *Methods in Enzymology*, (JAKOBY, W B , ed), Vol 22, p 39, Academic Press, New York

obtained are comparable with those reported earlier [20,22] Gallic acid and chlorogenic acid are not oxidized by our preparations, in spite of the fact that they have *o*-diphenolic groups and that Gregory and Bendall[20] indicate that they were good substrates for their tea catechol oxidase (see Table 2) Gregory and Bendall[20] also report considerably different K_ms for catechol and pyrogallol Possible reasons for these different results are discussed below

TABLE 2 REACTIVITY OF TEA CATECHOL OXIDASE TOWARDS VARIOUS SUBSTRATES

Substrate	Substrate concentration (mM)	Reaction rate V_{max} (μmol/min)	K_m value (M)	K_m values (M) reported by Takeo and Uritani[22]	Gregory and Bendall[20]
(−)-Epicatechin (I)	0–5	25	$1\,4 \times 10^{-3}$	n d *	$9\,8 \times 10^{-4}$
(−)-Epicatechin Gallate (II)	0–5	25	$2\,0 \times 10^{-3}$	n d	n d
(−)-Epigallocatechin (III)	0–5	20	$2\,2 \times 10^{-3}$	n d	$3\,3 \times 10^{-3}$
(−)-Epigallocatechin Gallate (IV)	0–5	17	$2\,0 \times 10^{-3}$	$1\,7 \times 10^{-3}$ (A-I) $4\,4 \times 10^{-3}$ (A-II)	$2\,4 \times 10^{-4}$
(+)-Catechin (IX)	0–5	15	$1\,6 \times 10^{-3}$	$4\,5 \times 10^{-3}$ (A-I) $3\,9 \times 10^{-3}$ (A-II)	$1\,5 \times 10^{-3}$
Catechol	0–5	25	$2\,0 \times 10^{-4}$	n d	$2\,6 \times 10^{-2}$
Pyrogallol	0–5	26	$3\,7 \times 10^{-4}$	n d	$3\,6 \times 10^{-2}$
Phloroglucinol	5	0	No reaction	n d	n d
p-Cresol	4	0	No reaction	n d	n d
Guaiacol	3	0	No reaction	n d	n d
Tyrosine	4	0	No reaction	n d	n d
Gallic Acid	4	0	No reaction	n d	$8\,0 \times 10^{-2}$
Chlorogenic Acid	4	0	No reaction	n d	0†

* Not determined
† V_{max} at infinite concentrations of O_2 reported to be 'very high'

Epimerizing Activity of Catechol Oxidase

Since the tea flavanols (I–IV) undergo some epimerization during the conversion of fresh leaf to either green tea or to black tea,[16–18] it was of interest to study the role of the tea catechol oxidase in this reaction Purified catechol oxidase was incubated with (−)-epigallocatechin gallate (IV) and the reaction mixtures were analyzed by PC The results (Table 3) showed that part of the (−)-epigallocatechin gallate was epimerized at the 2-position to produce (−)-gallocatechin gallate (X) under both aerobic and anaerobic conditions No epimerization could be detected at the 3-position, the only other asymmetric center in the tea flavanols (I–IV, IX) Further, epimerizing activity was associated only with those tubes from the isoelectric focusing column (Fig 1) which contained catechol oxidase A smaller amount of epimerization was measured under aerobic than under anaerobic conditions, possibly due to the formation of oxidation products from some of the (−)-gallocatechin gallate under the former conditions

Tea Flavanol Gallate Esterase Activity

The role of tea catechol oxidase in the degallation of tea flavonals which takes place during tea fermentation was studied by incubating the purified tea catechol oxidase with tea flavanol gallates under either aerobic or anaerobic conditions The results (Table 3)

show that some degallation occurs when (−)-epigallocatechin gallate (IV) is undergoing oxidation (aerobic incubation), but that no degallation occurs under anaerobic conditions Similar results were found for (−)-epicatechin gallate (II)

TABLE 3 PRODUCTS FORMED FROM ACTION OF TEA CATECHOL OXIDASE ON (−)-EPIGALLOCATECHIN GALLATE UNDER AEROBIC AND ANAEROBIC CONDITIONS

Incubation conditions	Time (min)	Polyphenols present in reaction mixture* (−)-Epigallocatechin gallate	(−)-Gallocatechin gallate	Gallic acid	(−)-Epigallocatechin	Oxidation products†
A Purified catechol oxidase						
Anaerobic	0	+ + + +	−	−	−	−
Anaerobic (with heat inactivated enzymes)	30	+ + + +	−	−	−	−
Anaerobic	30	+ + +	+ +	−	−	−
Anaerobic	240	+ + +	+ +	−	−	−
Aerobic	0	− + + +	−	−	−	−
Aerobic (with heat inactivated enzymes)	30	− + − +	−		−	−
Aerobic	30	+ +	+	−	−	+
B Crude soluble tea (CST) enzymes						
Anaerobic	0	+ + + +	−	−	−	−
Anaerobic	30	+ + + +	tr	tr	−	−
Anaerobic	240	+ + −	tr	+	+	−
Anaerobic (with heat inactivated enzymes)	240	− + + +	−	−	−	−

All reaction mixtures contained the same amount of substrate and catechol oxidise activity Further details are given in Experimental

* Quantitation of results based on estimation of spot size on paper chromatograms tr = trace (spot just detectable), + = light spot, + + = moderate spot, + + + = strong spot, + + + + = very strong spot

† Bisflavanols and thearubigins detected by PC

A small amount of flavanol gallate esterase activity was found in the CST enzyme preparation (Experiment B, Table 3) as evidenced by the formation of some gallic acid when (−)-epigallocatechin gallate was the substrate This flavanol gallate esterase was found in tube Nos 11–13 obtained from the isoelectric focusing column (Fig 1) separated from the purified catechol oxidase

DISCUSSION

The method used to purify tea catechol oxidase over 200-fold has the advantage of being a simple one. The key step, isoelectric focusing, was effective in disassociating the three enzymes catechol oxidase, peroxidase and flavanol gallate esterase The rather strong association of these enzymes in tea extracts is possibly due to a linking of the proteins via the tea polyphenols which are present in such high concentrations (25–35% of tissue dry weight) [4,5] Certainly, the use of Polyclar AT and Sephadex is essential to success in extracting soluble enzymes from the tea leaf tissues [23,27–29]

Comparison of our purified tea catechol oxidase with those previously reported[20 22,29] brings out some interesting points First, Takeo and Uritani[22] report finding three isozymes in their preparation, our results also indicate this, and it is likely that our Fractions IV-A, IV-B and IV-C (Table 1) correspond to Takeo and Uritani's[22] Components B, A-II and A-I, respectively If this is correct, it is easy to understand our slightly different results from those of Takeo and Uritani[22] since different isozymes (Components A-I and A-II

[27] BUZYN, G A , DZHEMUKHADZE, K M and MILESHKO, L F (1970) *Biokhimiya* **35**, 1002

[28] LOOMIS, W D and BATTAILE, J (1964) *Plant Physiol* **39**, Suppl xxi, (1966) *Phytochemistry* **5**, 423

[29] ANDERSON, J W (1968) *Phytochemistry* **7**, 1973

and Fraction IV-A, respectively) were examined in the two investigations Buzun *et al* [27] also report three isozymes, a dimer (Fraction I), a trimer (Fraction II), and a tetramer (Fraction III) of a monomeric unit with ~30 000 MW From their relative affinity for DEAE-cellulose, it seems likely that these three Fractions I–III correspond to our Fractions IV-C, IV-B and IV-A, respectively In view of the following discussion of Gregory and Bendall's[20] work, it is important to bear in mind that in all these investigations, enzymes have been extracted directly from fresh tea leaf

Gregory and Bendall[20] have reported properties for a purified tea catechol oxidase which vary considerably from those of ours, especially as regards substrate affinities and specificity (Table 2) It seems likely that the explanation for these differences is linked to the methods used to extract and purify the enzyme Gregory and Bendall[20] used an acetone dried powder of fresh tea leaf, a technique which appears to promote co-precipitation, and consequent condensation, of proteins and the tea polyphenols [4,5] In fact, the authors themselves mention the possibility that their enzyme fractions are 'tanned' and that their purified enzymes streak on starch gel electrophoresis Further, the presence of oxidizable flavanols (I–IV) attached to their enzyme would account for the finding[20] that gallic acid and chlorogenic acid can be oxidized by purified tea catechol oxidase In this case, gallic acid and chlorogenic acid would be oxidized by enzyme-attached oxidized flavanols (V–VIII) in much the same way as Berkowitz *et al* [19] describe for the oxidation of gallic acid during tea fermentation The kinetics found[20] for the enzymic oxidation of chlorogenic acid is also consistent with these contentions The rate of chlorogenic acid oxidation appeared to be dependent on oxygen concentration but independent of chlorogenic acid concentration as would be expected in a system with the following properties: (a) The amount of oxidizable tea flavanols per unit of enzyme is fixed by the enzyme purification procedure; (b) the concentration of enzyme-attached oxidized flavanols is determined entirely by the oxygen concentration according to normal Michaelis–Menten kinetics,[30] and (c) the rate of chlorogenic acid oxidation is dependent on the concentration of enzyme-attached oxidized tea flavanols as defined by the laws of mass action Certainly, none of our preparations have shown any activity towards either gallic acid or chlorogenic acid

Finally, in attempting to reconcile the differences in enzyme properties it must be recognized that different isozymes may have been studied and that Gregory and Bendall's purified catechol oxidase preparations all contained peroxidase activity The presence of peroxidase would have a most important effect on the results and would confuse the kinetics of the oxidations observed

Our tea catechol oxidase has a rather high specificity for the tea flavanols (Table 2), unlike that of catechol oxidases reported in other plant tissues, such as apples,[31] potato tubers,[32] grapes,[33] pears,[34] and bananas [35] The ability of tea catechol oxidase to oxidize catechol and pyrogallol, but not phloroglucinol or monophenols (Table 2) indicates that tea catechol oxidase acts specifically on the tea flavanols (I–IV) by oxidizing the 3′,4′-*o*-dihydroxy phenyl B-ring to form the respective *o*-quinones (V–VIII) The tea catechol oxidase also promotes some epimerization of the flavanol molecules at the C-2 position but

[30] DIXON, M and WEBB, G C (1964) *Enzymes*, 2nd Edn, Academic Press, New York
[31] STELZIG, D A , AKHTAR, S and RIBERIRO, S (1972) *Phytochemistry* **11**, 535
[32] PATIL, S S and ZUCKER, M (1965) *J Biol Chem* **240**, 2938
[33] HANEL, E and MAYER, A M (1971) *Phytochemistry* **10**, 17
[34] TATE, J N , LUH, B S and YORK, G K (1964) *J Food Sci* **29**, 829, WALKER, J R L (1964) *Australian J Biol Sci* **17**, 575
[35] PALMER, J K (1963) *Plant Physiol* **38**, 508

not at the C-3 position, probably by extensive delocalization of electrons on the oxidized B-ring [36,37] Since epimerization at the C-2 position appears to depend on the presence of an active catechol oxidase but not actual oxidation (Table 3), it is proposed that the tea catechol oxidase draws electrons from the flavanol substrates strongly enough to allow epimerization at the C-2 position

As noted above, some deesterification of the gallated tea flavanols (II, IV) has been recognized to be a consequence of tea fermentation,[4,5] although the nature of this 'esterase', or 'tannase', activity has not been clarified The results of our investigation have shown that there is indeed a low level of flavanol esterase activity in tea leaf but that tea catechol oxidase also catalyzes some degallation of tea flavanols as a consequence of its oxidizing activity This latter reaction has been named oxidative degallation, and it is entirely dependent on the activity of catechol oxidase under aerobic conditions These results (Table 3) also indicate that oxidative degallation must account for most of the gallic acid formed during tea fermentation since the flavanol gallate esterase activity is present in tea leaf at such low levels As with the epimerizing activity, this catechol oxidase-mediated oxidative degallation of tea flavanols must depend on extensive delocalization of electrons on the enzymically oxidized B-ring of these molecules (II, IV) The mechanism of this reaction is further discussed elsewhere [16]

Two recent papers have appeared which have an important bearing on our results First, Takeo and Baker[38] have reported on the fractionation of tea catechol oxidase by CM-cellulose chromatography and isoelectric focusing Three fractions were obtained on CM-cellulose chromatography with at least six main components three have isoelectric points that are very close to those reported here The finding that the presence, and relative amount, of individual components of this enzyme vary with the age of tea leaf may explain some of the differences in results reported by different investigations Finally, Illingworth[39] has reported that isoelectric focusing of isocitrate dehydrogenase causes isozymes to form that were not present in the original purified enzyme preparation. This work suggests that great caution must be exercised in interpreting results obtained with enzymes which have undergone isoelectric focusing

EXPERIMENTAL

Materials (a) *Green tea leaf* Fresh tea flush, i e the tea shoot tips to just below the second leaf, was air-freighted to us from Lipton's Experimental Tea Garden near Charleston, South Carolina, U S A , and stored at −40° until required (b) *Chemicals* (−)Epigallocatechin gallate and (−)-epicatechin gallate were prepared from fresh green tea leaf as described previously [40] (−)-Gallocatechin gallate was a gift from Dr C K Wilkins, Unilever Research Laboratory, Vlaardingen, The Netherlands Other chemicals were purchased (c) *Isoelectric focusing column* LKB 8102 (440 ml) column and Ampholine (carrier ampholyte), pH range 3–10, from LKB Instruments, Rockville, Maryland (d) *Ultrafiltration* Amicon stirred cell ultrafiltration unit, Model 52, and filters from Amicon Corporation, Lexington, Massachusetts

Extraction of enzymes from fresh tea leaf All operations in extraction and purification of enzymes were carried out at 4° unless stated otherwise Fresh frozen tea leaf (25 g at about 75% moisture content) was added to enough liquid N_2 to cover the tea leaf The liquid N_2 was decanted off, and the frozen leaf was immediately transferred to a cold base Waring blender and homogenized below 4° After the initial blending, 130 ml of 0 05 M phosphate buffer, pH 7 0, 12 g Polyclar AT and 7 5 ml of Tween 80 were added, and the

[36] PELTER, A , BRADSHAW, J and WARREN, R F (1971) *Phytochemistry* **10,** 835
[37] JURD, L (1972) in *The Chemistry of Plant Pigments* (CHICHESTER, C O , ed), p 123, Academic Press, New York, JURD, L (1972) in *Structural and Functional Aspects of Phytochemistry* (RUNECKLES, V C and TSO, T C , eds ,), p 135, Academic Press, New York
[38] TAKEO, T and BAKER, J E (1973) *Phytochemistry* **12,** 21
[39] ILLINGWORTH, J A (1973) *Biochem J* **129,** 1125
[40] CO, H and SANDERSON, G W (1970) *J Food Sci* **35,** 160

slurry was blended for 5 min at 4° The resultant homogenate was centrifuged at 15 000 × *g* for 10 min to remove cellular debris The supernatant was passed through a short Sephadex column (5 × 3 cm) to remove the last traces of polyphenol The eluate (70 ml) from this column was designated the crude soluble tea (CST) enzyme preparation

Purification of CST enzyme preparation The CST enzyme preparation was treated with $(NH_4)_2SO_4$ so as to collect the 40–60% fraction This precipitate was dissolved in 100 ml of 0 05 M phosphate buffer, pH 7 0 This enzyme solution was dialyzed in an Amicon ultrafiltration unit[26] using 50 ml of 0 05 M phosphate buffer, pH 7 0, and an XM 300 filter The retentate (48 ml) was treated for 96 hr in an LKB isoelectric focusing column[41] using the LKB pH 3–10 ampholyte and 400 V At the end of the run, the column contents were drained off and collected 11 ml/tube After each tube was assayed (Fig 1), the major catechol oxidase containing tubes were bulked and dialyzed against 0 05 M phosphate buffer, pH 5 6, in the Amicon ultrafiltration unit using a PM 10 filter to remove the sucrose and the Ampholyte

Catechol oxidase assay Catechol oxidase activity was measured by a modification of the method of El-Bayoumi and Friden [42] This method is dependent on following spectrophotometrically the oxidation of ascorbic acid which is chemically linked to the enzymic oxidation of the phenolic compounds, no ascorbic acid oxidase could be detected in any enzyme preparation made from tea leaf material The reaction mixture consisted of 0 5 ml of 0 3 mM ascorbic acid, 0 5 ml of $1\ 5 \times 10^{-5}$ M EDTA, 0 3 ml of 0 1 M phosphate–citrate buffer, pH 5 5, 0 2 ml of 7 5 mM catechol, and enzyme solution plus H_2O to make up to a total of 3 0 ml The reaction mixture was held at 30° and the reaction measured by following the decrease in absorbancy at 265 nm One unit of activity is defined as that amount of enzyme which will oxidize 1 μmol of substrate/min in the above-described system The pH of 0 1 M phosphate–citrate buffer used in the above assay was varied to obtain the pH/activity profile of the enzyme Enzyme kinetic data were obtained at pH 5 7 and 25° by standard procedures [43]

Peroxidase assay Peroxidase activity was determined spectrophotometrically using guaiacol as the hydrogen donor [44] One unit of activity corresponds to the decomposition of 1 μmol H_2O_2/min at 30° calculated from ΔA_{470nm} assuming 4 mol H_2O_2 are required to form 1 mol tetraguaiacol (λ_{max} 470 nm, $\epsilon\ 2\ 66 \times 10^4$) [45]

Determination of epimerizing and gallate esterase activity These enzyme activities were determined by analyzing appropriate reaction mixtures by PC The reaction mixtures consisted of 2 5 ml of 8 7 mM tea flavanol in 0 1 M phosphate–citrate buffer, pH 5 7, plus 1 0 ml of enzyme preparation (1 unit) Incubations were carried out at 30° in either open shaking test tubes (aerobic incubation), or in evacuated Thunberg tubes (anaerobic incubation) Reactions were terminated by immersing the reaction mixtures in boiling H_2O for 5 min Any formation of C-2 epimer, i e (−)-gallocatechin gallate (X) from (−)-epigallocatechin gallate (IV), was taken to be an indication of epimerase activity Any formation of gallic acid in reaction mixtures containing a gallated flavonol (II or IV) under anaerobic conditions was taken to be an indication that flavanol gallate esterase was present in the enzyme preparation

Protein determination The protein content of solutions free of phenolic material was measured spectrophotometrically [46]

Paper chromatography Phenolic compounds present in the reaction mixtures were studied by PC[7,19] on Whatman No 1 paper The solvents used were 1st direction–*n*-BuOH–HOAc-H_2O (4 1 2 2), 2nd direction –2% HOAc. The spots were visualized under UV and with $FeCl_3$–$K_3Fe(CN)_6$ They were identified by comparison with authentic compounds and by reference to reported R_fs [17]

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