

Role of Polyphenol Oxidase and Peroxidase in the Generation of Black Tea Theaflavins

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It has been reported earlier that when macerated tea leaf is fermented at lower pH, the resultant black tea contains increased levels of theaflavin, an important quality marker in black tea. In an attempt to investigate the biochemistry and chemistry underlying this observation, in vitro oxidation experiments using polyphenol oxidase (PPO) from fresh tea leaves, horseradish peroxidase (POD), and tea catechins, precursors for theaflavins, were carried out. In vitro oxidation experiments using crude tea PPO resulted in higher content of theaflavins at pH 4.5 in comparison with pH 5.5, the normal pH of the macerated tea leaf. When purified PPO was used in the in vitro system, surprisingly a reversal of this trend was observed, with more theaflavins being formed at the higher pH. A combination of pure tea PPO and POD led to an observation similar to that with the crude enzyme preparation, suggesting a possible role for POD in the formation or degradation of theaflavin. POD was observed to oxidize theaflavins in the presence of H₂O₂, leading to the formation of thearubigin, another black tea pigment. This paper demonstrates that tea PPO, while oxidizing catechins, generates H₂O₂. The amount of H₂O₂ produced is greater at pH 5.5, the optimum pH for PPO activity, than at pH 4.5. Hence, an observed increase of theaflavins in black teas fermented at pH 4.5 appears to be due to lower turnover of formed theaflavins into thearubigins.

Keywords: *Camellia sinensis*; theaflavins; catechins; black tea; polyphenol oxidase; peroxidase

INTRODUCTION

During black tea manufacture, the mechanical maceration of green tea shoots triggers the enzyme-catalyzed oxidative reactions involving the tea catechins as the substrates (Harler, 1963). Upon disruption of the intracellular compartments, polyphenols present in the cell vacuole are oxidized by the tea oxidative enzymes, leading to the formation of theaflavin and thearubigin pigments characteristic of black tea (Robertson, 1992).

The theaflavins are formed by the enzymatic oxidation and condensation of catechins with di- and trihydroxylated B rings (Brown et al., 1966). The reaction involves the oxidation of the B rings to the quinones followed by a Michael addition of the galocatechin quinone to the catechin quinone, prior to carbonyl addition across the ring and subsequent decarboxylation (Balentine, 1992). The four major theaflavins (TF), namely, theaflavin [TF 1; parent flavanols, (–)-epicatechin and (–)-epigallocatechin], theaflavin-3-gallate [TF 2; (–)-epicatechin and (–)-epigallocatechin gallate], theaflavin-3'-gallate [TF 3; (–)-epicatechin gallate and (–)-epigallocatechin], and theaflavin-3,3'-digallate [TF 4; (–)-epicatechin gallate and (–)-epigallocatechin gallate] are orangish in color with absorbance maxima at 380 nm and impart color, brightness, and astringency to black tea liquors (Mcdowell et al., 1995). Additionally, theaflavins and their gallate esters possess antioxidant properties preventing lipid peroxidation in erythrocyte membranes and microsomes and suppress mutagenic effects induced by H₂O₂, the gallic acid moiety of the theaflavins being essential for this activity (Shivaki et al., 1994; Miller et al., 1996).

On the other hand, thearubigins are heterogeneous polymers, and information about their formation, structures, and contribution to black tea quality is very limited. Recently, a partial structure of thearubigins from black tea was elucidated by employing chemical degradation, which revealed them to be heterogeneous polymers of flavan-3-ols (catechins) and flavan-3-ol gallates having bonds at C4, C6, C8, C2', C5', and C6' in the flavan-3-ol units (Ozawa et al., 1996).

The theaflavins positively correlate with tea quality and, consequently, a considerable amount of research has been carried out on increasing their levels during black tea manufacture. It was observed earlier (Cloughley and Ellis, 1980) that modification of pH during fermentation leads to improvement in tea quality in Central African black teas. The improved quality was ascribed to the enhanced levels of theaflavins in the teas fermented at lower pH. In the present study, during black tea manufacture trials, when the pH of the macerated leaf was lowered to 4.5 from the normal pH 5.5, a higher theaflavin content and a lower thearubigin content were observed in black teas. In the present paper, we report the results obtained from in vitro oxidation experiments carried out at the above-mentioned pH values with highly purified enzymes and endogenous tea catechins to understand the mechanism of formation of theaflavins.

MATERIALS AND METHODS

Materials. (+)-Catechin, bovine liver catalase, catechol, (–)-epicatechin (EC), (–) epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), horseradish peroxidase (POD), polyvinylpyrrolidone (PVPP), benzamidine hydrochloride, phenylmethylsulfonyl fluoride (PMSF), tropolone, and all electrophoresis reagents were

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obtained from Sigma Chemical Co., St. Louis, MO. DEAE-Sephacel, SP-Sephacel, Phenyl-Sepharose CL-4B, Sephacryl S-300, gel filtration, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight marker proteins were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Other reagents and chemicals used were of analytical grade and obtained locally.

Processing of Fresh Tea Leaves to Black Tea. Fresh green leaves (3 kg) were withered for 18 h to ~70% of fresh weight under ambient conditions. The leaves were processed using a minimanufacturing facility consisting of a CTC (curl–tear–crush, 30° teeth profile) machine, a fermenter (controlled temperature and humidity chamber, 2.5 ft × 2.5 ft × 2.5 ft), and a fluid bed dryer (3 L capacity). The leaves were cut four times using the CTC machine. One kilogram of macerated leaf was sprayed with either 200 mL of water or 0.1 M sodium citrate buffer (pH 4.5 or 5.5). Fermentation was carried out for 50 min at 25 °C at 90% relative humidity. The fermented tea leaf was dried at 100 °C for 20 min. The final moisture content of the dried tea was ~5%. The pH was monitored at the beginning and end of fermentation.

Estimation of Theaflavins and Thearubigins in Black Tea. Black tea (2.4 g) was infused in 100 mL of boiling water and kept at 90 °C for 20 min. The infusion was rapidly filtered, and the volume was made up to 100 mL with water. The theaflavin and thearubigin (TR) contents in the infusion were determined according to the spectrophotometric method of Roberts and Smith (1961). The spectrophotometric measurement was performed using a Shimadzu UV-1201 instrument. Individual theaflavins, namely, TF 1, TF 2, TF 3, and TF 4, in black teas were determined by using the high-performance liquid chromatography (HPLC) method of Bailey and Nursten (1993).

Separation of Theaflavins from Black Tea. Theaflavins were isolated from black tea as described by Steinhaus and Engelhardt (1989) with modifications. Briefly, black tea (6 g) was boiled in 0.01 M sodium acetate (pH 5.0) for 10 min. The aqueous layer was filtered, and the filtrate (50 mL) was extracted with isobutyl methyl ketone (50 mL). The organic layer was washed with an equal volume of chilled 0.1 M disodium orthophosphate. Subsequently, the organic layer was passed through a Sep-Pak column (Waters), and the theaflavins were eluted with 50% (v/v) methanol. The solvent fraction upon evaporation and freeze-drying yielded an orange powder containing all of the major theaflavins (purity ascertained by HPLC, Nova-Pak C18 analytical column, Waters). The concentration of individual theaflavins is known to vary in black tea (Steinhaus and Engelhardt, 1989), and the purified theaflavin preparation was also found to contain different amounts of TF 1, TF 2, TF 3 and TF 4.

Purification of PPO. Recently, three isoenzymes of PPO have been purified to homogeneity from fresh tea leaves in our laboratory (Subramanian and Sinkar, 1996). Briefly, crude enzyme extract was prepared by grinding the tea shoots snap frozen using liquid nitrogen in a Waring blender and subsequently extracting the soluble, membrane-bound, and cell wall-bound enzymes by including a nonionic detergent (Triton X-100) in the extraction buffer [50 mM MES, pH 5.5/0.2 M Na₂SO₄/0.3% (v/v) Triton X-100/0.5 mM PMSF/1 mM benzamidine hydrochloride/1 mM EDTA containing PVPP (0.4 g of PVPP/g of fresh weight tea leaves)]. After 1 h of stirring, the slurry was centrifuged (30 min, 17600g). The supernatant (crude enzyme extract) was subjected to (NH₄)₂SO₄ fractionation. The proteins precipitated with 70–100% saturation of (NH₄)₂SO₄ had a 5-fold higher PPO specific activity. The precipitate obtained was dissolved in 20 mM Tris-Cl (pH 8.0)/10 mM ascorbate/1 mM EDTA/1 mM benzamidine and dialyzed extensively against the same buffer.

The enzyme sample was loaded on a DEAE-Sephacel column pre-equilibrated with the above buffer. The bound proteins were eluted with a linear gradient of 0–0.5 M NaCl in the buffer. PPO activity was determined (Moore and Flurkey, 1990) by monitoring the *o*-quinone formation at 400 nm using (+)-catechin as the substrate. The assay was carried out over a period of time in a reaction volume of 3 mL using 0.1 M

sodium phosphate–citrate (pH 5.5) as the buffer and (+)-catechin (3 mM) at 40 °C with appropriate enzyme and substrate blanks. The rates were determined from the linear portion of the absorbance (*A*₄₀₀) versus time curves. One unit of enzyme activity is defined as the change in 0.001 absorbance unit per minute in an aliquot of the enzyme used (Moore and Flurkey, 1990). The fractions containing the enzyme activity were pooled, dialyzed against 50 mM sodium phosphate (pH 6.0), and passed through SP-Sephacel pre-equilibrated with the same buffer. The cation-exchanger-bound proteins were eluted with a salt gradient of 0–0.5 M NaCl. The eluted fractions containing PPO activity were dialyzed against 50 mM sodium phosphate (pH 7.0)/1.7 M (NH₄)₂SO₄ and subjected to Phenyl-Sepharose chromatography. The hydrophobic matrix-bound proteins were eluted with a negative gradient of 1.7–0 M (NH₄)₂SO₄ in the buffer. The fractions showing PPO activity were dialyzed against 20 mM Tris-Cl (pH 8.0)/0.25 M NaCl and stored in aliquots at –20 °C. This enzyme preparation has been termed PPO 1.

Additionally, the flow-through obtained from DEAE-Sephacel chromatography was loaded onto the SP-Sephacel column pre-equilibrated with 20 mM Tris-Cl (pH 8.0). After extensive washing, the bound proteins were eluted using a linear gradient of 0–0.5 M NaCl in the buffer. Two pools of activity eluting at different salt concentrations were obtained and termed PPO 2 and PPO 3. The active fractions were subsequently processed for hydrophobic column chromatography and purified to homogeneity.

All of the buffers and reagents used in the entire purification procedure were prepared fresh using Milli Q water (Millipore, Bedford, MA), and benzamidine hydrochloride was included to avoid any proteolysis. All of the above-mentioned steps were carried out at 4 °C except Phenyl-Sepharose column chromatography, which was performed at room temperature. The purity of the enzyme preparations (PPO 1, PPO 2, and PPO 3) was ascertained by silver staining (Merrill et al., 1981) of SDS–PAGE (Laemmli, 1970), and the specificity of the enzyme preparations was confirmed by using the PPO specific inhibitor, tropolone (Kahn and Andrawis, 1985).

In Vitro Oxidation Experiments. POD activity in the commercial POD (Sigma) as well as in the crude tea enzyme extract was determined spectrophotometrically according to the method of Chance and Maehly (1955) using (+)-catechin (3 mM) and H₂O₂ (8 mM) in 0.1 M phosphate–citrate buffer (pH 5.5) at 30 °C in a reaction volume of 3 mL. One unit of POD activity is defined as the change in 0.001 absorbance unit at 400 nm per minute in an aliquot of the enzyme used (Parry et al., 1996). Equal enzyme units (300 units) of both PPO and POD were used [normalized with respect to their activities against the substrate, (+)-catechin] in a given experiment.

In one set of experiments, green tea (catechin content ~15% w/w) obtained by drying fresh leaves in a hot air oven was used, and in another, pure catechins, namely, EC and EGC (1 mM each), were used as the substrates. Here, green tea refers to a nonfermented product in which the tea catechins are mostly preserved, whereas black tea is a fermented product in which catechins are extensively oxidized during manufacturing processes (Zhu et al., 1997). Oxidation was carried out at the indicated pH values using 0.1 M sodium phosphate–citrate as the buffer in a reaction volume of 3 mL at 40 °C for 1 h unless otherwise mentioned. The reactions were arrested by the addition of 0.2 mL of stop mix solution (Finger, 1994) consisting of acetonitrile, acetic acid, and water (60:10:30, v/v/v). For subsequent HPLC (Waters) analysis, 20 µL of the reaction mixture was injected into an analytical C18 reverse phase column, Nova-Pak (3.9 × 150 mm), connected to a tunable absorbance detector (Waters 486 detector), and the data were processed using Millennium software (version 2.10). HPLC analysis was performed according to the method of Bailey and Nursten (1993) using 2% (v/v) acetic acid in water and acetonitrile as the solvents. When a gradient of 8–29 vol % over 50 min was applied, separation of the individual theaflavins could be achieved. Theaflavins were quantitated using valley to valley integration and represented as HPLC peak areas.

Effect of POD on Theaflavins. Theaflavins purified from black tea were used at a concentration of 1 mM considering an average molecular weight of 600 Da for theaflavins. POD (300 units) was added to the reaction mixture containing theaflavins and various concentrations of H_2O_2 , and the incubations were carried out as mentioned above.

Estimation of H_2O_2 . To quantitate the H_2O_2 levels, the spectrophotometric method reported by Jiang and Miles (1993) was employed with minor modifications. Briefly, the assay was carried out at 40 °C for 5 min using 0.1 M sodium phosphate-citrate (pH 5.5) as the buffer and was found to be linear in the chosen range of H_2O_2 concentrations. To monitor the H_2O_2 generated by PPO, the reaction was carried out in the presence of 1 mM EC, 1 mM EGC, and PPO 1 (300 units) for 1 h at 40 °C, following which PVPP (100 mg) was added, mixed, and centrifuged (1500g, 2 min). To 1 mL of the clarified supernatant in a reaction volume of 3 mL was added catechol (3 mM). The initial absorbance of the solution was monitored at 400 nm. The coupled assay was initiated by the addition of POD (300 units) and carried out at 40 °C for another 5 min and then terminated by the addition of stop mix solution. The absorbance values at 400 nm were corrected for any nonspecific readings using substrate and enzyme blanks. The difference in A_{400} before and after the addition of the enzyme was calculated and used in the standard curve generated for estimation of H_2O_2 in unknown samples.

Polarographic Measurements. In addition to the spectrophotometric measurements of PPO activity and quantitation of the generated H_2O_2 , polarographic measurements were carried out for some of the experiments on a Gilson Oxygraph system (Gilson, Middleton, WI) having a micro-Clark oxygen electrode as the oxygen cathode. The reactions were carried out at 30 °C in a reaction vessel of 1.75 mL volume continuously stirred using a magnetic stirrer. The addition of substrates, enzymes, etc., into the reaction vessel was done by using a microsyringe. The consumption and release of oxygen was recorded using a chart recorder (chart speed = 0.1 mm/s).

The following measurements were carried out under identical conditions: (i) To the reaction vessel containing buffer (0.1 M phosphate-citrate, pH 4.5/5.5) was added (+)-catechin dissolved in absolute alcohol (3 mM) followed by pure PPO isoenzyme, namely, PPO 1 (300 units) or a combination of PPO 1 and POD (300 units each), and the oxygen uptake was monitored (Clark, 1993). Once there was no further uptake of oxygen, bovine liver catalase (160 units, 1 unit will decompose 1.0 μ mol of H_2O_2 at pH 7.0 at 25 °C) was added to the reaction vessel and the release of oxygen was monitored. (ii) In another set of experiments, the activity of catalase in tea leaf crude enzyme extract was monitored by employing the method of Del Rio et al. (1977). Crude enzyme extract containing 300 units each of PPO and POD was taken in the reaction vessel containing 100 mM phosphate-citrate (pH 5.5) or 50 mM sodium phosphate (pH 7.0). H_2O_2 (0.4 mM) was added, and the release of oxygen was monitored. The presence of added H_2O_2 in the reaction vessel was also ascertained by the addition of bovine liver catalase (160 units). To check the integrity of the enzyme preparation, PPO activity in the tea leaf enzyme extract was monitored by the addition of (+)-catechin (1.5 mM).

Statistical Analysis. For each parameter measurement, five samples were analyzed in duplicate and the mean values were obtained. The experimental data were subjected to an analysis of significance using Student's *t* test. Means were significantly different at the level of 0.05.

RESULTS AND DISCUSSION

It is well-known that uncontrolled oxidative reactions mediated by PPO and POD are responsible for quality deterioration in several food products derived from plant sources (Whitaker, 1995). In tea processing, unlike other food products, the activity of these oxidative enzymes is indeed essential for the formation of tea pigments, TF and TR, which are responsible for the attributes

Table 1. Effect of pH during Fermentation on the Quality Parameters of Black Tea^a

pH	% TF	% TR
4.5	1.03 \pm 0.1	10.53 \pm 0.15
5.5	0.78 \pm 0.09	12.67 \pm 0.17

^a Theaflavin and thearubigin contents are represented as percentage dry weight of black teas. Data are expressed as mean \pm SD of *n* = 5 samples. Means within the same column are significantly different (*p* < 0.05).

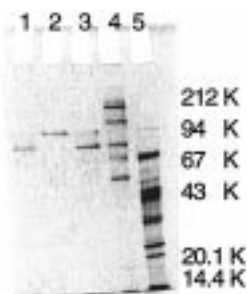


Figure 1. SDS-PAGE analysis. The following samples were electrophoresed on a 10% SDS gel and stained with silver: lane 1, PPO 1 (5 μ g); lane 2, PPO 2 (5 μ g); lane 3, PPO 3 (5 μ g); lane 4, HMW (high molecular weight) markers; lane 5, LMW (low molecular weight) markers.

unique to tea (Robertson and Bendall, 1983). Theaflavins, which contribute to the color, astringency, and brightness of black tea liquor, correlate very well with the price realized at auction (Cloughley and Ellis, 1980), and thearubigins are known to add to the strength of the tea (Mcdowell et al., 1991).

During black tea processing, when the fermentation pH was lowered to 4.5, ~30–40% more theaflavin could be detected in the black teas over the teas made under normal fermentation conditions (Table 1). Additionally, the thearubigin levels (Table 1) were found to decrease upon lowering the fermentation pH to 4.5. It has been well established that PPO is responsible for the oxidation of catechins leading to reactive *o*-quinone intermediates (Gregory and Bendall, 1966; Robertson, 1992), which are then spontaneously converted into black tea pigments, namely, theaflavins and thearubigins (Mayer and Harel, 1991). Tea PPO has an optimum pH of 5.5 toward (+)-catechin (Coggon et al., 1973), and the physiological pH in the tea leaf ranges from 5.4 to 5.6. If PPOs are the only enzymes responsible for major tea pigment formation, it is expected that at pH 5.5 more theaflavins will be formed. On the contrary, more theaflavins were detected at the suboptimal pH of 4.5.

To understand the reason for this intriguing observation, an in vitro oxidation approach was employed in this study. Model fermentations have been carried out earlier (Robertson, 1992; Finger, 1994; Robertson and Bendall, 1983; Opie et al., 1995), but the interpretation of the results obtained from such studies had been impeded owing to the usage of crude enzyme preparations. To avoid this, purified oxidative enzymes were used in the present investigation.

The purification procedure employed resulted in the isolation of three isoenzymes of PPO, namely, PPO 1, PPO 2, and PPO 3. PPO 1 and PPO 2 were isolated to homogeneity, and PPO 3 was purified to near homogeneity as evident from the SDS-PAGE shown in Figure 1. Tropolone, the known competitive inhibitor of PPO (Kahn and Andrawis, 1985), could inhibit the activity of the purified enzymes to >95% even at a concentration

Table 2. Determination of Theaflavins Formed upon in Vitro Oxidation of Tea Catechins by Crude PPO Containing POD^a

theaflavin	pH 4.5	pH 5.5
TF 1	349588 ± 31276	169259 ± 11282
TF 2	174388 ± 15280	83590 ± 5882
TF 3	345095 ± 16961	140843 ± 16548
TF 4	244827 ± 16890	103664 ± 8046

^a Theaflavin amounts are represented as HPLC peak areas. Data are expressed as mean ± SD of *n* = 5 samples. Means within the same row are significantly different (*p* < 0.05).

of 2 mM (data not shown). Purified isoenzymes of PPO, namely, PPO 1 and PPO 2, are homodimers having subunit molecular weights of 75000 and 100000 Da, respectively, whereas PPO 3 is a monomer with a molecular weight of 75000 Da (Subramanian and Sinkar, 1996). *pI* values of the purified isoenzymes are as follows: PPO 1, 5.0; PPO 2, 7.5; PPO 3, 9.6. Tea PPO isoenzymes were found to have an optimum pH of 5.5 and an optimum temperature of 40 °C. All three isoenzymes were found to be capable of forming theaflavins, and qualitatively there was no difference among the isoenzymes (Subramanian and Sinkar, 1996). Recently, Halder et al. (1998) also reported the presence of three isoenzymes of PPO in tea leaf and purified one isoenzyme to homogeneity. The purified enzyme was found to have a molecular weight of 72000 Da and was obtained from the fraction unadsorbed to DEAE matrix as that of PPO 3. The fraction adsorbed to the DEAE matrix had a molecular weight of 164000 Da (Halder et al., 1998), similar to PPO 1 as observed in the present investigation. The highlights of the present study are the in vitro demonstration of the observations made in the large scale manufacture of black tea and delineation of the mechanism of increased formation of theaflavins at the lower pH.

In Vitro Oxidation of Tea Catechins by Crude PPO Enzyme Preparation. In vitro oxidation experiments were carried out using green tea (containing the endogenous catechins and inactivated enzymes) as the substrate and crude PPO enzyme preparation obtained by 70–100% (NH₄)₂SO₄ saturation of the leaf enzyme extract containing PPO (300 units) and POD (300 units) at both pH values. HPLC analysis of the reaction products (Table 2) reveal that at the lower pH, a ~2-fold increase in all four theaflavins could be detected in comparison with that of the oxidation carried out at pH 5.5. This experiment validated the findings obtained earlier in our trials carried out for the manufacture of black tea and those reported by Cloughley and Ellis (1980).

In Vitro Oxidation of Tea Catechins by PPO 1. One of the isoenzymes of PPO, namely, PPO 1, was chosen in the present study as this isoenzyme had a higher specific activity (45960 units/mg of protein) and had been purified over 130-fold. When PPO 1 was used instead of the crude enzyme in the above experiment, more formation of theaflavin was found to occur at the higher pH (Table 3). It is pertinent to mention that the trend seen with the purified PPO isoenzyme was in contrast with the observation made with crude PPO. It is also evident that PPO has a relatively higher activity at pH 5.5 with respect to the formation of theaflavins from mixed catechin substrates.

In vitro oxidation reactions carried out using a combination of a simple catechin (EC) and a gallocatechin (EGC) in the presence of PPO 1 at both pH values

Table 3. Determination of Theaflavins Formed upon in Vitro Oxidation of Tea Catechins by PPO 1^a

theaflavin	pH 4.5	pH 5.5
TF 1	201189 ± 14866	378136 ± 16311
TF 2	193763 ± 10860	346234 ± 23089
TF 3	83838 ± 10808	144052 ± 10291
TF 4	108015 ± 9547	193880 ± 10471

^a Theaflavin amounts are represented as HPLC peak areas. Data are expressed as mean ± SD of *n* = 5 samples. Means within the same row are significantly different (*p* < 0.05).

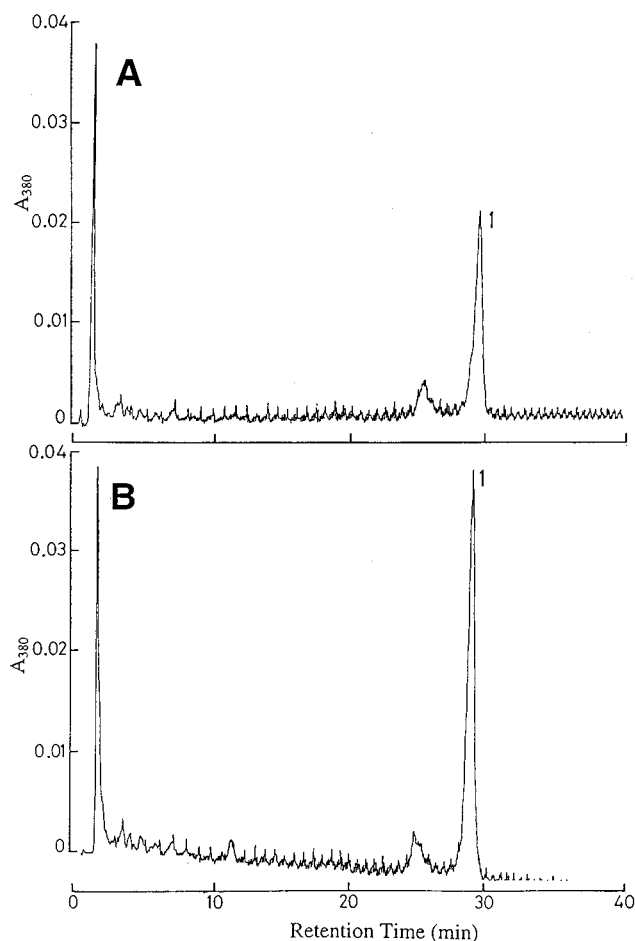


Figure 2. HPLC chromatograms of incubation of catechins, EC and EGC (1 mM each) with PPO 1 (300 units). The oxidation was carried out at 40 °C for 1 h, 0.02 mL of the reaction mixture was injected into the HPLC, and the products were monitored at 380 nm. Shown are the traces obtained at pH 4.5 (A) and at pH 5.5 (B). Peak 1 refers to theaflavin 1 (TF 1).

(Figure 2) also indicated increased (~2-fold) formation of the corresponding theaflavin, namely, TF 1 at pH 5.5 (Figure 2B), as was observed with green tea containing all of the tea catechins (Table 3). The involvement of PPO in the synthesis of theaflavin was also confirmed by using tropolone. When PPO treated with tropolone was added to the reaction mixture containing the above-mentioned pair of catechins, no formation of theaflavin was observed (data not shown).

In Vitro Oxidation of Tea Catechins by PPO 1 and POD. It is clear that when only PPOs are present, formation of more theaflavin occurs under normal fermentation conditions (pH 5.5). However, more accumulation of theaflavin at the lower pH of 4.5 during manufacture of black tea suggests the possible role of

Table 4. Determination of Theaflavins Formed upon in Vitro Oxidation of Tea Catechins by Tea PPO 1 and POD^a

theaflavin	pH 4.5	pH 5.5
TF 1	226533 ± 10689	138383 ± 13055
TF 2	335849 ± 6342	193227 ± 11802
TF 3	111423 ± 9450	75073 ± 8239
TF 4	149826 ± 11228	69686 ± 6891

^a Theaflavin amounts are represented as HPLC peak areas. Data are expressed as mean ± SD of *n* = 5 samples. Means within the same row are significantly different (*p* < 0.05).

other enzymes in the formation of theaflavins. Apart from PPO, as in any other plant source, the other major oxidative enzyme present in tea is peroxidase. A significant amount of POD activity has been detected in fresh tea leaves extract, and POD has been shown to remain potentially active throughout fermentation (Dix et al., 1981; Parry et al., 1996). The role of POD in browning reactions of fruits has also been demonstrated (Richard-Forget and Gauillard, 1997). However, the role of POD during the manufacture of black tea has not been shown conclusively and remains speculative at present.

To understand the role of POD in the accumulation of theaflavins, a combination of both the oxidative enzymes (PPO and POD) was used in the in vitro reaction. Upon incubation with green tea, an observation similar to that of the crude PPO enzyme preparation was evident (Table 4) with respect to the accumulation of theaflavins. This observation supports the hypothesis that POD plays a significant role in the accumulation of theaflavins at the lower pH. It may be pertinent to mention that no exogenous H₂O₂ was added to the model fermentation reaction mixture. This suggests that H₂O₂, necessary for the activity of POD, is probably generated during the oxidation reaction catalyzed by tea PPO. It is known that mushroom tyrosinase and potato phenolase generate H₂O₂ while oxidizing catechin (Jiang and Miles, 1993). Recently, pear PPO was also shown to generate H₂O₂ upon oxidation of chlorogenic acid, EC, and 4-methylcatechol (Richard-Forget and Gauillard, 1997).

To ascertain the generation of H₂O₂ by tea PPO, polarographic measurements were carried out using PPO 1 and (+)-catechin. Oxygen uptake by PPO 1 during oxidation of catechin was found to be relatively faster at pH 5.5 than at pH 4.5, and the net oxygen consumption (200 μM O₂) was found to be the same at either of the pH values (data not shown). Addition of bovine liver catalase to the above reaction mixture led to the release of oxygen, supporting the premise of generation of H₂O₂ by tea PPO. To explore further, oxidation of (+)-catechin was performed using a combination of PPO 1 and POD in the absence of any added H₂O₂. It is clearly evident from the slopes of the curves obtained (Figure 3) that relatively more rapid uptake of oxygen occurs at pH 5.5 than at pH 4.5. It may also be mentioned that the combination of PPO and POD led to a ~2-fold greater oxygen consumption (420 μM O₂) at either of the pH values in comparison with PPO alone present in the reaction mixture. A similar observation of enhanced oxidation has been encountered during the oxidation of catechins by a combination of pear PPO and POD (Richard-Forget and Gauillard, 1997). However, the reason for the above observation is not known at present and needs to be investigated. Additionally, lack of release of O₂ (Figure 3A,B) upon

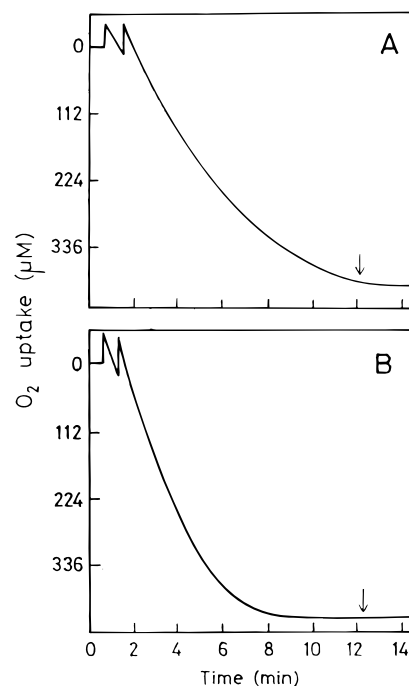


Figure 3. Oxidation of (+)-catechin by tea PPO 1 and POD monitored polarographically. The oxygen uptake was monitored by carrying out the reaction in the presence of both enzymes (300 units each) and (+)-catechin at pH 4.5 (A) and at pH 5.5 (B). Addition of catalase (160 units) is indicated by arrows.

addition of bovine liver catalase to the reaction mixture clearly indicated the consumption of H₂O₂ by POD.

Effect of POD on Theaflavins. We have earlier demonstrated that when tea is fermented at a lower pH, the theaflavin content increases with a decrease in the thearubigin content of the black tea (Table 1). In vitro oxidation experiments using crude PPO enzyme containing POD also resulted in the same observation, but while using purified PPO isoenzyme, more formation of theaflavins was observed at the higher pH. It is known that PPO does not have any effect on the formed theaflavins (Opie et al., 1993; Bajaj et al., 1987). This raises the possibility that under normal fermentation conditions of pH 5.5, POD in the presence of H₂O₂ might convert the formed theaflavins into more complex fermentation products, namely, thearubigins.

To assess this possibility, theaflavins purified from black tea containing all four theaflavins were incubated with POD at 40 °C in the presence of different concentrations of H₂O₂. It may be mentioned that upon addition of the enzyme to the reaction mixture, the original yellowish color of the solution changed to a transient purple color indicative of the quinone formation, which subsequently turned deep orange as was observed earlier (Finger, 1994). In the present experiments, upon stoichiometric addition of H₂O₂ (1 mM), there was almost complete loss of theaflavin (Figure 4B) as was monitored at 380 nm using HPLC. Interestingly, even when the amount of H₂O₂ added was lowered to a concentration of 0.09 mM, a substantial reduction in all of the theaflavins was observed (Figure 4C).

Detection and Quantitation of H₂O₂. To quantify the H₂O₂ generated by tea PPO, oxidation experiments were carried out using EC and EGC (1 mM each) in the presence of PPO 1 (300 units) for 1 h at 40 °C. H₂O₂ content was quantitated using catechol as the substrate (H₂O₂ standards in the range of 0–350 μM).

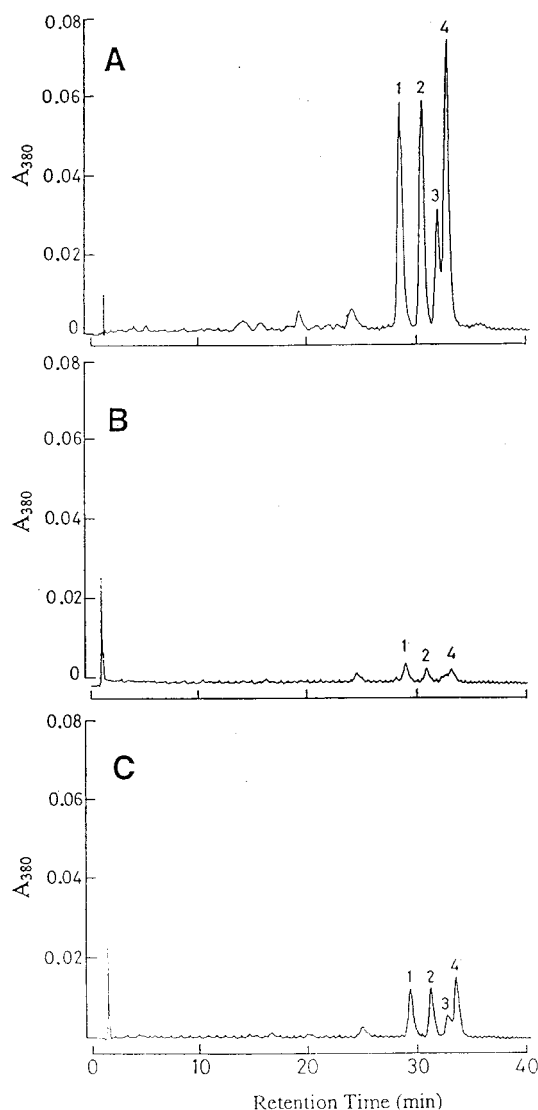


Figure 4. HPLC chromatograms of incubation of theaflavins with various concentrations of H_2O_2 in the presence of POD (300 units). Shown are the traces obtained by injecting 0.02 mL of a 3 mL reaction mixture into a reverse phase HPLC column: (A) theaflavins (1 mM in 3 mL) in the absence of POD; (B) theaflavins treated with 1 mM H_2O_2 and POD; (C) theaflavin treated with 0.09 mM H_2O_2 and POD. Peak 1 refers to theaflavin 1 (TF 1), peak 2 to TF 2, peak 3 to TF 3, and peak 4 to TF 4.

Table 5. H_2O_2 Production during Oxidation of Catechins by Tea PPO 1^a

catechin	amount of H_2O_2 generated (μM)	
	pH 4.5	pH 5.5
EC, EGC (1 mM each)	20 \pm 1	30 \pm 1.2
(+)-catechin, EC, ECG, EGC, EGCG (1 mM each)	35 \pm 2.1	60 \pm 2.7
green tea (80 mg)	290 \pm 5.9	390 \pm 5.2

^a In a given set of experiment, samples were analyzed for H_2O_2 content in duplicate and data are expressed as mean \pm SD of $n = 5$ samples. Means within the same row are significantly different ($p < 0.05$).

Micromolar amounts of H_2O_2 could be detected in the tea system (Table 5). PPO was capable of generating approximately 20 μM (pH 4.5) and 30 μM (pH 5.5) H_2O_2 from a combination of EC and EGC. The amount generated was comparable to that of tyrosinase-catalyzed

oxidation of catechin (Jiang and Miles, 1993). When all of the major tea catechins were present in the reaction mixture, higher amounts of H_2O_2 (35 μM at pH 4.5 and 60 μM at pH 5.5) were found to be generated. It may also be noted that at the higher pH of 5.5, more amounts were generated both in the case of paired catechins as well as in the total catechin mixture. This observation of increased production of H_2O_2 at the higher pH goes very well with PPO having enhanced activity at pH 5.5. Additionally, when PPO 1 (300 units) was added at a concentration present in vivo (activity in the enzyme extracts from fresh leaves determined using catechin as the substrate) to the corresponding amount of green tea (reaction mixture containing green tea was found to contain 0.8 mM EC, 3.4 mM EGC, 0.5 mM ECG, and 2.6 mM EGCG), levels of H_2O_2 generated (290 μM at pH 4.5 and 390 μM at pH 5.5) were found to be much greater as compared to paired catechins (Table 5).

It may be mentioned that the steady-state level of H_2O_2 determined during tea slurry fermentation was found to be in the range of 70–90 $\mu\text{M/g}$ of fresh weight (Parry et al., 1996). The relatively higher level of H_2O_2 reported in the present study while using green tea in the in vitro oxidation reaction is not the steady-state level, and H_2O_2 generation by other means (enzymatic/nonenzymatic) has not been investigated.

When a time course study of theaflavin conversion into thearubigin-like products by POD was carried out using the steady-state level of H_2O_2 (100 μM) in the presence of green tea catechins, significant loss of theaflavin was observed as early as 5 min, which progressed further to a greater extent when the incubation was continued for 1 h (data not shown). Considering the above-mentioned findings, it is evident that the amount of H_2O_2 (not necessarily a stoichiometric amount) generated during fermentation under normal conditions is adequate to bring about conversion of theaflavins into thearubigin-like products. As the amount of H_2O_2 required for POD to act on the formed theaflavins is in very small amounts, probably it could be a free radical mediated reaction. However, further extensive work is needed to unravel this complex phenomenon.

The observation that PPO leads to more theaflavin formation at the higher pH with green tea or pure catechins in a model fermentation system is in accordance with tea PPO having maximal activity at the higher pH. The higher PPO activity can result in more H_2O_2 generation, facilitating POD to act on the formed theaflavins. Additionally, the amount of H_2O_2 generated could vary with different raw materials (clones) having different PPO isoenzymes. It was also observed earlier in the case of mushroom tyrosinase (Jiang and Miles, 1993) and pear PPO (Richard-Forget, 1997) that the H_2O_2 produced by the same enzyme varies with different phenolic compounds. It may be mentioned that during tea fermentation, the possibility of other means of generation of H_2O_2 cannot be ruled out and the levels of H_2O_2 could primarily be governed by the activity of catalase.

In an attempt to determine the activity of catalase in tea leaves, crude enzyme extract obtained from fresh leaves was used as the enzyme source. Measurements were carried out at pH 5.5, the physiological pH in the tea system (Robertson, 1992), and at pH 7.0, the optimum pH for most of the catalases (Aebi, 1962). The reaction was initiated at zero time by the addition of H_2O_2 to the reaction vessel, and the release of O_2 was

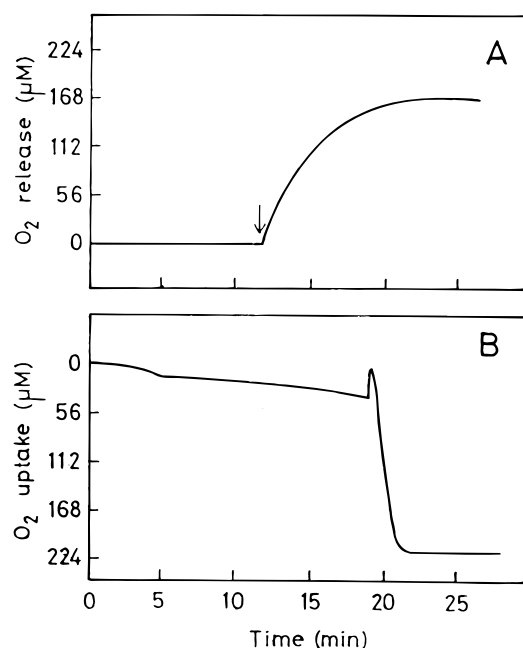


Figure 5. Catalase activity in tea leaves. Polarographic measurements were carried out using fresh leaf enzyme extract and H₂O₂: (A) Fresh leaf enzyme extract (1 mL) was taken in 0.1 M phosphate-citrate buffer, pH 5.5, and the reaction was initiated at zero time by the addition of H₂O₂ (0.4 mM). Release of O₂ was monitored, and after 12 min, catalase (160 units) was added (indicated by the arrow) to confirm the presence of H₂O₂ in the reaction mixture. (B) Fresh leaf enzyme extract (1 mL) was taken in 0.05 M sodium phosphate buffer, pH 7.0, and the reaction was initiated at zero time by the addition of H₂O₂ (0.4 mM). As there was no release of O₂, after 18 min, (+)-catechin was added to the reaction vessel (seen as a blip due to dissolution of catechin in absolute alcohol), and the uptake of O₂ was monitored; the oxygen consumed is indicative of the presence of PPO in the leaf enzyme extract.

monitored. Surprisingly, no release of O₂ was observed either at pH 5.5 (Figure 5A) or at pH 7.0 (data not shown), indicating the absence of significant amounts of catalase activity in the fresh leaf enzyme extract. Bovine liver catalase was added after 12 min to the reaction vessel, and immediate release of O₂ confirmed the presence of H₂O₂ (Figure 5A). It was considered important to monitor PPO activity of the leaf enzyme extract already present in the reaction vessel to ascertain the integrity of the enzyme preparation. Toward this end, (+)-catechin was added after 18 min and the consumption of O₂ was monitored. As evident from Figure 5B, there was a rapid uptake of O₂ indicating the presence of PPO activity in the leaf enzyme extract. The reason for the baseline drift in Figure 5B could be due to oxidation of the residual catechins present in the crude enzyme extract.

The results obtained from the above experiments indicate that the enzyme extract did contain active PPO (used as a reference to ascertain the enzyme extract). The lack of release of O₂ upon addition of H₂O₂ indicates the absence of catalase activity. This observation favors the view that the H₂O₂ generated by PPO is available for subsequent POD-mediated oxidations. The results presented for the absence of catalase activity are only preliminary in nature. A thorough investigation using different raw materials employing probably different extraction conditions or some additional treatments of samples prior to assay is required to ascertain the presence or absence of catalase in tea leaves.

From the results obtained by employing the in vitro oxidation approach, it is clear that PPO generates H₂O₂ during oxidation of catechins and POD utilizes the formed H₂O₂ for subsequent oxidation of the products of PPO-catalyzed reaction. The turnover of the formed theaflavins to thearubigins by POD appears to be more under normal black tea manufacturing conditions, probably due to greater availability of H₂O₂. The lesser availability of H₂O₂ at the lower pH in turn leads to an apparent increase in theaflavins.

ABBREVIATIONS USED

EC, (–)-epicatechin; ECG, (–)-epicatechin gallate; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin gallate; MES, morpholinoethanesulfonic acid; pI, isoelectric pH; PMSF, phenylmethylsulfonyl fluoride; POD, peroxidase; PPO, polyphenol oxidase; PVPP, polyvinylpyrrolidone; TF, theaflavin; TR, thearubigin.

ACKNOWLEDGMENT

We thank Dr. C. V. Natraj for helpful discussions and constant support throughout the course of this investigation and Prof. T. Ramasarma, Indian Institute of Science, Bangalore, for polarographic measurements. N.S. thanks Unilever Research for a postdoctoral fellowship.

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Received for review September 21, 1998. Revised manuscript received February 22, 1999. Accepted April 14, 1999.

JF981042Y