JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Fermentation Characteristics of Some *Assamica* Clones and Process Optimization of Black Tea Manufacturing

ANANTA MADHAB BARUAH†

Tocklai Experimental Station, Jorhat, Assam 785 008, India

PRADIP KUMAR MAHANTA*

Hindustan Lever Research Center, 64 Main Road, Bangalore 650 066, India

Changes in the specific activities of polyphenol oxidase (PPO), peroxidase (POD), and protease and in the relative amounts of flavan-3-ols for eight genetically derived cultivated teas at various stages of leaf maturity and in four succescive seasons were examined. A series of investigations were carried out to study the cross-reactivity of complex polyphenols and PPO-generated orange-yellow theaflavins, as well as of POD oxidized substrates, producing brown so-called thearubigins during fermented tea processing. From the estimation of five major catechins, PPO activities in young shoots, and theaflavin and thearubigin contents of crushed, torn, and curled (CTC) black teas, the superior variety and flavorful flush characteristics were refined. Notable protein hydrolysis by endogenous protease as measured from free amino acids and formation of tannin−protein complex (browning products) was obtained for cultivar character and product quality. Results showed that process optimization with respect to time, temperature, moisture, and pH maximizes PPO-catalyzed desirable theaflavin pigments, whereas POD-mediated chemical reaction produces dull color.

KEYWORDS: Clonal teas; phenolics; polyphenol oxidases; protease; amino acids; fermentation; black tea; sensory characteristics

1. INTRODUCTION

The bulk increase of tea, *Camellia sinensis* L., as a garden crop from 970 to 18623 kg/ha, has been due to hundreds of elite vegetatively propagated clonal cultivars, better cultural practices, and the use of agrochemicals including nitrogen, potash, and phosphorus. Unfortunately, inherently inferior old seedling plants and higher yielding hybrid cultivars, namely, *Camellia cambodensis* (a subtype of the variety *assamica*), with inferior fermentation characteristics, have overtaken in a vast majority of plantation gardens of northeastern India, compared to *Camellia assamica* and *C. sinensis* (China variety) varieties (*1*, *2*). Agricultural aspects such as plucking and pruning have been designed to enhance growth of flushes, which are rich in phenolic components or tannins, as well as polyphenol oxidase (PPO) and peroxidase (POD) activities (*3*−*5*). Although the developmental role of enzyme/proteins and secondary metabolites has not yet been elucidated, it is clear that they vary with cultivars and fluctuate with the environment and also with cultural practices (*6*, *7*). The varied oxidation process of polyphenols by polyphenol oxidases during maceration of tea shoots is defined as fermentation.

The first important step for improving the quality of processed tea is withering or partial desiccation; the harvested leaves are heaped in a series of "enclosed/open trough", fitted with perforated trays under forced air circulation. After storage, the leaves are rolled, either by a conventional "three-crank roller" (for orthodox manufacture) or in the McTear rotorvane (RV) (that mimics the effect of a roller), followed by McKercher crushing, tearing, and curling (CTC) machines (*8*−*10*). The mechanical macerated particles (dhool) are shallowly spread onto the factory floor or beds of continuous fermenting machines (CFM) maintaining high humidity (>90%). When the aerated dhool turns coppery red, the fermentation process is stopped by a blast of hot air in a "tray dryer" for orthodox manufacture, whereas a fluid bed dryer (FBD) is used for CTC tea, to reduce the moisture to 2.5%. The most widely used form is CTC tea of smaller particle sizes, whereas orthodox rolled teas are of leaf grades.

Many studies have established that the characteristic volatile flavor compounds (VFC) consisting of terpenoids and fresh green (fatty acid derivatives) and aromatic/benzenoids are developed from the nonvolatile aroma precursors with the leaf softening techniques used during withering (*11*, *12*). In the next important step, during rolling, cytoplasmic flavonoids are progressively oxidized into quinones as a result of chloroplast polyphenol oxidase and cell wall peroxidase generating yellow

* Author to whom correspondence should be addressed (fax +91-80-845-3086; e-mail pk.mahanta@unilever.com).

† Present address: Department of Biochemistry and Agricultural Chemistry, Bishwanath Chariali, A.A.U, Sonitpur, Assam 784176, India.

10.1021/jf030019w CCC: $25.00 © 2003 American Chemical Society
Published on Web 09/27/2003

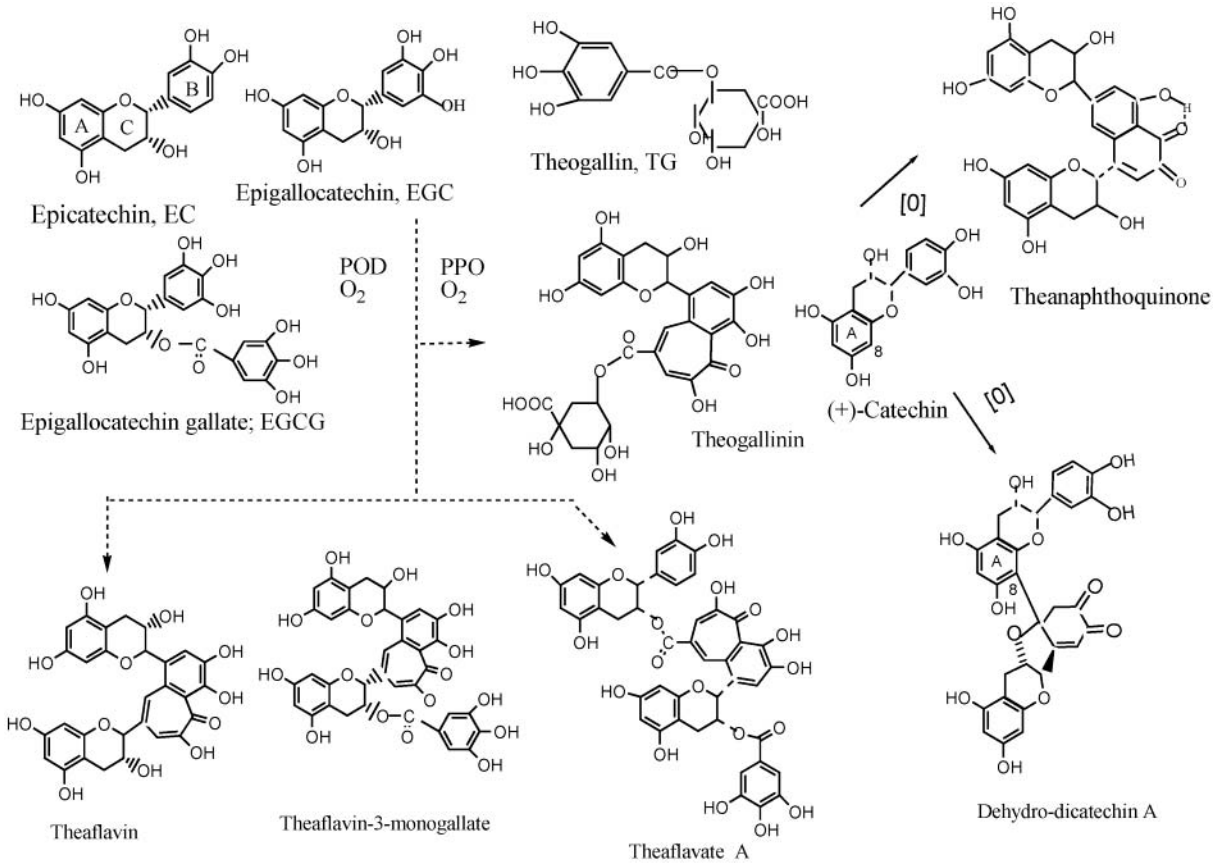

Figure 1. Structures of some orange-yellow compounds found in black tea.

to red-brown colors (*13*). In the fermentation and drying stages, the colorless epicatechin (EC), epigallocatechin (EGC), epicatechingallate (ECG), and epigallocatechin gallate (EGCG) are rapidly transformed into the most visual pigments known as theaflavins and thearubigins, through a series of oxidative condensation reactions (*14*−*16*).

Black teas containing higher ratios of yellow theaflavin species (TF, 2−3 wt %/wt) to dark brown thearubigins (TR, phenol−protein−caffeine precipitate or cream, ∼10−20%) are always in higher demand in the auction market and by global consumers (*17*, *18*). The fascinating structures of the bright pigments [theaflavin (TF-1), theaflavin-3-gallate (TF-2), theaflavin-3′-gallate (TF-3), and theaflavin-3,3′-digallate (TF-4)] and related compounds have been characterized (*19*, *20*). Although thearubigins contribute significantly to the strength and body of the liquor, their mechanism of formation, chemical structure, and even analytical method remained elusive. More recently, additional yellow (bright) compounds from the thearubigin complex, namely, theaflavate, theasinensins, theanaphthoquinone, and dehydrodicatechin A, were isolated and their structures elucidated using HPLC, photodiode array detection, and mass spectrometry (*21*−*24*). (A few structures are shown in **Figure 1**.)

Despite a long history of tea making, the interclonal superiority and developmental and environmental significance of endogenous enzymes/proteins, as well as implications of processing methods on quality, are little investigated and poorly understood. The purpose of this study is to examine in further detail (1) the biochemical basis of the cultivars, (2) standardization of manufacturing technology, and (3) the factors affecting phenolic metabolism vis-à-vis the quality of black tea.

2. MATERIALS AND METHODS

2.1. Plant Materials. Tocklai vegetative clonal series (TVs), TV-1, TV-7 (China hybrids, small leaf), TV-2, TV-12 (Assam hybrids, large leaf), TV-17 (Assam-China hybrid), TV-9, TV-19, TV-25, and TV-26 (Cambod hybrids, similar to Assam variety) were from the model tea garden planted 1961. Fresh shoots were collected at regular intervals of 7−9 days (plucking round) during 1993−1996, during which a 3-year standard pruning cycle was applied.

2.2. Manufacturing Experiments. A single clone (10 kg) was spread over withering troughs (leaf thickness of 5−8 cm) and leaf moisture reduced to ∼68−50%, by blowing hot and cold air (at CFM 770 ft^2/min), for different lengths of time in hours. The leaves (1−5 kg) after withering were "rolled" separately in a "Little Giant Roller" for 30 min and then passed through the CTC machine. Another variation in rolling was that the leaf first was preconditioned at a rotorvane followed by three to four CTC cuts. The macerated particles were spread over trays to undergo aerobic oxidation (fermentation) for ∼45 min and dried in a batch type dryer for 30 min. Each manufacturing experiment was repeated at fortnight intervals for three consecutive years. The samples regularly collected were fresh leaves (FL), deactivated dried green leaf (DADGL), withered leaf (WL), fermented leaf (FL), and drier mouth samples (DMS) for their chemical and biochemical studies.

2.3. Biochemical Assays. *2.3.1. Enzyme*−*Protein Extractions.* Five grams of frozen shoots was homogenized in 20 mL of chilled phosphate buffer (0.1 M, pH 5.6) with polyvinylpolypyrrolidone (PVPP) in a Polytron Kinetic homogenizer for 10 min. The homogenate was then centrifuged at 1200*g* for 15 min. The centrifuged material was passed through a small PVPP column to get rid of remaining polyphenols. The eluent was assayed for PPO and POD activities as already described (*14*). Alternatively, dried acetone powder was prepared by repeatedly homogenizing (250 g) fresh shoots in ice-cold 80% acetone (15000 mL) at 3 °C for 10 min, and the white solid collected was stored in a deep freezer. The enzyme proteins were extracted with standard buffer, detergent, and PVPP.

2.3.2. Partial Purification of Enzymes. Five grams of acetone-washed tea powder was homogenized in 40 mL of ice-cold phosphate buffer (0.1 M, pH 6.0) containing 10 mM cysteine, 3% Tween 80, and 5 g of PVPP, for 10 min. The homogenate after filtering through layers of cheesecloth was centrifuged at 30000*g* for 40 min at 2 °C. To the clear supernatant was added a saturated solution of $(NH_4)_2SO_4$ to precipitate two protein fractions: PPO between 20 and 80% and POD between 30 and 70%. Fractions with PPO and POD activities were pooled (both pellet and supernatant), desalted on a Sephadex G-25 column (3.5 × 30 cm, in a 0.1 M buffer at pH 6.0), and concentrated by dialysis. The amount of protein was estimated using bovine serum albumin (BSA) as standard at 280 nm (Beckman spectrometer model 26).

2.3.3. PPO Assay. PPO activity was measured from the rate of O_2 consumption using a Clark oxygen electrode (Gilson model 5/6). The assay medium contained 0.1 mL of enzyme extract to which 0.1 mL of pyrrogallol solution was added (final concentration = 1 nM), or suitable substrate(s) and 1.6 mL of phosphate buffer (0.1 M, pH 5.6) were added. The activity was determined against blank (without substrate). The total dissolved oxygen was determined by using a strong reducing agent, namely, sodium dithionite. One unit of PPO catalyzes the consumption of 1 μmol of O_2 per gram of dried matter, and the specific enzyme activity was expressed as units per milligram of protein as already described (*14*).

2.3.4. POD Assay. POD activity was determined in a medium containing 3 mL of 0.05 M citrate buffer (pH 5.6), 0.1 mL of *o*-dianisidine (1 μg mL^{-1}), and 0.2 mL of H_2O_2 (1:100 dilution), at 40 °C at 30 min. The color developed was measured at 430 nm (Beckman spectrometer model 26). The unit of POD activity expressed the amount of enzyme that catalyzes 1 μmol of substrate per minute under standard condition.

2.3.5. Thermal Stability and pH Dependence of PPO. A partially purified enzyme extract (0.5 mL) in test tubes (triplicate) was subjected to incubation for 10−60 min at different temperatures (30, 40, 50, 60, 70, and 80 °C). At the end of the required time interval the solutions were cooled by immersing them in crushed ice. The residual PPO activity was determined by measuring the oxygen uptake study (μL/min) under standard conditions at the various time intervals. For individual clones, PPO activity as a function of pH was determined by incubating enzyme preparations at various pH values (in triplicate) ranging from 4 to 6.5 at 40 °C. After incubation, their residual activities were measured by oxygen electrode, using 0.1 M phosphate buffer adjusted with 0.1 M HCl.

2.3.6. Protease Extraction and Assay. One gram of fresh shoots was homogenized with 5 g of PVPP in 0. 01 M phosphate buffer (pH 7.0) containing 0.01% Triton X-100 at 2.5 °C. The supernatant after centrifugation at 1200*g* for 15 min was used for protease activity. Typically 1 mL of the enzyme solution dissolved in water was incubated with 1.0 mL of 1% BSA in a sodium phosphate buffer (pH 6.5) at 40 °C for 90 min. The reaction was terminated with 1.0 mL of 20% trichloroacetic acid (TCA). A portion of the supernatant was diluted 4-fold with water, and the absorbance at 280 nm was determined. One unit of protease activity is expressed as the amount of enzyme that produced a 0.01 increase in absorbency of TCA solution obtained from 1 g of the sample (*25*).

2.3.7. Estimation of Soluble Proteins. The soluble protein fraction was precipitated by 10% TCA in a phosphate buffer (pH 7.0). To 0.5 mL of extracted protein in 0.5 mL of water was mixed 5 mL of alkaline copper sulfate solution (2% Na_2CO_3 in 0.1 N NaOH and 0.3% $CuSO_4 \cdot 5H_2O$ in sodium potassium tartarate mixed at 50:1 ratio), and 1.0 N Folin reagent was added. After 30 min, the violet color developed was measured at 700 nm using BSA as standard (*26*).

2.3.8. Estimation of Amino Acids. One gram of dried leaf was extracted with hot 70% ethanol and further treated with basic lead acetate, and the excess lead was removed by sodium oxalate. One part of the test solution containing phosphate buffer (pH 8.0) and a ninhydrin mixture was heated in a water bath for 10 min. After cooling, the volume was made up to 10 mL with 2-propanol and absorbance was measured at 570 nm using leucine as standard (from Sigma). Another portion of the solution (100 μL) was passed through a 5000mw Millipore filter. A precolumn cyanide derivatization of the free amino acids was carried out by the addition of ethanol, trimethylamine, water, and phenol isothiocynate (7:1:1:1; 40 μL) in a buffer of pH 4 followed by analysis by reverse phase high performance liquid chromatography (RP-HPLC) and monitoring at 254 nm as described (*27*).

2.4. Cemical Analyses. *2.4.1. Catechins.* One gram of steamed-dried, decaffeinated tea powder was extracted with 80% acetone, and the volume was made up to 100 mL with water. The individual catechins were analyzed by RP-HPLC (LKB-Pharmacia, pumps consisting of 501 and 510), and data were calculated by using Millennium software. HPLC conditions: column, μBondapak C18; solvent, acetic acid, acetonitrile, dimethylformamide, and water at the ratio of 3:1:1.5:81; mode, isocratic; flow rate, 0.8 mL min^{-1}; injection volume, 5 μL; UV−visible detector wavelength, 280 nm (*28*). The various catechins were compared against standards obtained from Sigma Chemical Co., St. Louis, MO.

2.4.2. Phenolic Acids. A 5 g decaffeinated sample was hydrolyzed overnight by methanolic 1 M NaOH solution and then acidified with HCl at pH 2.5. After filtration through a membrane (cellulose, 0.45 mm), 20 μL of the solutions was injected directly to an HPLC. HPLC conditions: column, Bondapak C18; mobile phase, water/glacial acetic acid/*n*-butanol (342:1:14 v/v); mode, isocratic; flow rate, 2 mL/min; detector, UV−visible, set at 275 nm; column temperature, 30 °C (*29*). Spiking the retention times under the same analytical conditions with corresponding authentic phenolic acid standards (Sigma Chemical Co.) was performed for each suspected phenolic acid and aldehyde.

2.4.3. Theaflavins and Flavonol Glycosides. A 2.4 g tea sample was allowed to infuse at 95 °C for 20 min in 100 mL of boiling distilled water, and after filtration, the volume was made up to 100 mL. A 20 μL aliquot was injected to an HPLC with the following analytical conditions: column, Novapak (C18, 3.9 × 150 mm, 4 m); mobile phase, A = 2% acetic acid; B = 100% acetonitrile. Gradient mode was used—from 92% of acetic acid, A, at the start to 69% of acetonitrile, B, at the end of 50 min. The flow rates were 1 mL/min, and the detector was kept at 380 nm (*28*). The authentic (isolated) major theaflavins were compared with their UV−visible spectral scan data and HPLC retention times, whereas flavonol glycosides were compared with Sigma Chemicals. Using classical chemical quality parameters, namely, theaflavins, thearubigins, total water-soluble solids (TSS), total polyphenol contents, and caffeine were analyzed (*11*).

3. RESULTS AND DISCUSSION

3.1. Enzyme Activities and Flavanol Contents with Maturity. The distribution of PPO, POD, protease, and the soluble protein contents of shoot components used for black tea manufacture is illustrated in **Figure 2A**. In general, PPO and POD exhibited the highest activities in the internode portion followed by the first and second leaves, the bud, and the third leaf of eight different cultivars. In the case of protease activity, it was highest in the second leaf, followed by the first leaf, the apical bud, and the stem. However, soluble protein content was higher in the first and second leaves, which decreased from the third, and was least in the stem, irrespective of cultivar type. PPO and POD are ubiquitous plant proteins, and proteolysis is essential in all varieties, providing a means for cells to change their protein content during development and adaptation to environmental conditions. An overall 10-fold higher POD activity, compared to PPO, showed its key roles in morphogenesis, development, and phenol metabolism (*30*−*32*).

We then compared the five major flavan-3-ols (total of catechin, epicatechin, epigallocatechin, epicatechingallate, and epigallocatechin gallate) and caffeine contents in different parts of the shoots (**Figure 2B**). There was a significant increase of secondary metabolites, namely, flavanols and caffeine at the early stage of organ growth, and thereafter a continued decrease as the tissue matured, which is a common phenomenon in a number of fruits and leafy vegetables (*7*, *33*). On the other hand, the polyphenol composition was lowest in the stem, but enriched with fiber components or cell wall matrix (*34*). Also, due to genetic variations of the different cultivars, different amounts

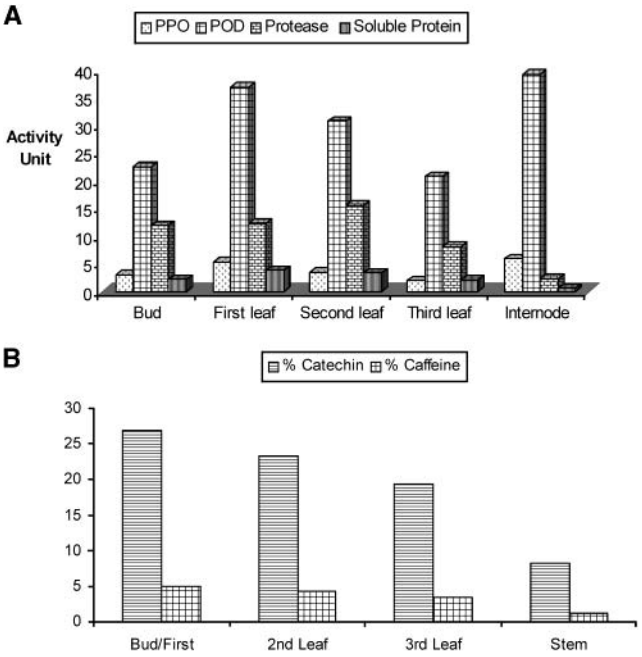

Figure 2. (A) PPO, POD, and protease activities and the soluble protein content of different shoot components; (B) total catechin and caffeine contents (percent) in different parts of a shoot (average of three repeated experiments).

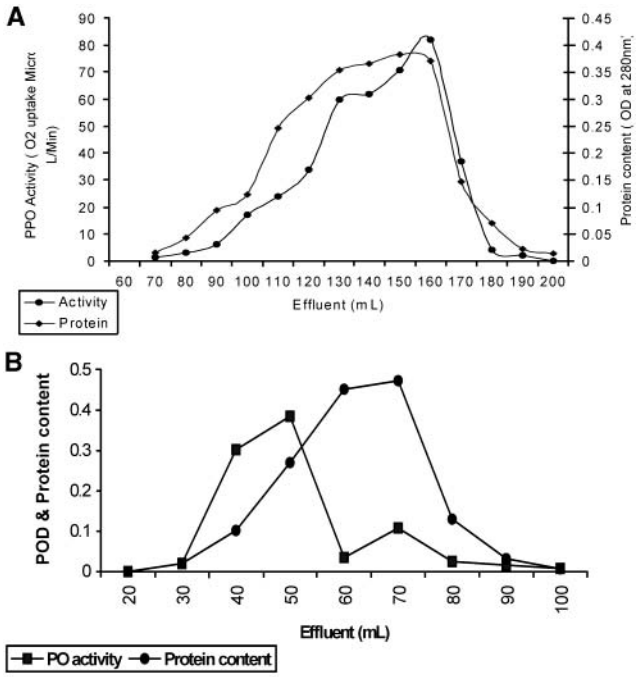

Figure 3. (A) PPO activity and protein from $(NH_4)_2SO_4$ PPT gel fractionation; (B) POD activity and protein from $(NH_4)_2SO_4$ PPT gel fractionation.

of proteins can be synthesized, as a result of which variations in the enzymatic activities exist (table not shown, but CD between 0.64 and 0.9 is a statistically significant difference) (*35*, *36*).

Generally, plant PPOs involve hydroxylation and dioxidases, yielding catechol moieties (as in catechin and gallocatechin), whereas PODs are known to be involved in phenol coupling reactions such as lignification (*37*, *38*). Recently, genetically modulated enzymatic biosynthesis of many pigments and scents that occur in flowers and leafy plants, for example, pathways of flavonoids ($C_6-C_3-C_6$), have been reported (*39*, *40*). Thus, a similar argument could be proposed to explain the flushing of young shoots (the active photosynthetic tissues) and fluctuation in the phenolic metabolism being controlled by the key metalloenzyme functionality such as PPO and POD (*41*, *42*).

3.2. General Characteristics of Enzyme-Proteins. Results of fresh shoots PPO, POD, and protein profiles are shown in **Figure 3**. The protein precipitated [20−80% $(NH_4)_2SO_4$ cut] showed excellent yield for PPO (12-fold higher than the crude enzyme) and was fully latent, both in mature and in immature shoots (**Figure 3A**). On the other hand, POD [30−70% $(NH_4)_2SO_4$ cut] exhibited two distinct brands in terms of activity and protein (**Figure 3B**). Because substrate specificity of PPO and POD is still obscure, we report degree of oxidation for some common phenolic compounds such as catechol, pyrrogallol, (−)-epicatechin, (+)-catechin, chlorogenic acid, caffeic acid, gallic acid, and others. Substrate specificities of these compounds were tested at various concentrations from 0.5 μM to 0.01 M. The enzyme activities were expressed in relation to catechol (100%) and guaiacol (100%) through an in vitro assay (*42*).

Tea PPO showed high catalytic activity toward all of the flavan-3-ols; it especially preferentially oxidized epicatechin and its gallated forms (di- or trihydroxyl groups) during fermentation, whereas phenolic acids are practically inactive (**Table 1**). In contrast, POD exhibits wider organic substrate specificity and highest activity toward *o*-dianisidine followed by pyrogallol (3-hydroxyphenols), phenolic acids, and related compounds and

Table 1. Substrate Specificity of Tea Polyphenol Oxidase (Average of Three Repeated Experiments)

substrate specificity (0.02 M)	% reactivity in relation to catechol
catechol	100
pyrogallol	86
(−)-epicatechin	72
(+)-catechin	42
tyrosine	2
caffeic acid	8
chlorogenic acid	6
gallic acid	0

Table 2. Substrate Specificity of Tea Peroxidase (Average of Three Repeated Experiments)

substrate specificity (0.01 M)	% reactivity in relation to guaiacol
guaiacol	100
o-dianisidine	87
chlorogenic acid	65
pyrogallol	62
caffeic acid	55
gallic acid	50
(−)-epicatechin	31
(+)-catechin	22
tyrosine	25

catechins (**Table 2**). It is noteworthy that this enzyme exhibits activity regardless of orientation, ascorbate, phenolic acids, flavonol glycosides, etc. Unlike many plants, tea PPO did not exhibit tyrosinase activity; ascorbic acid showed highest inhibition, but POD remains active (**Table 3**).

3.3. Flushing and Cultivar Variations. The 3-years' month-wise average variations in the four distinct flushes [first, March−April; secone, May−June; third or rain, July−September; and fourth or autumn flush, October−November] in PPO and POD activities as shown in **Figure 4A**. Both of the enzymes exhibited

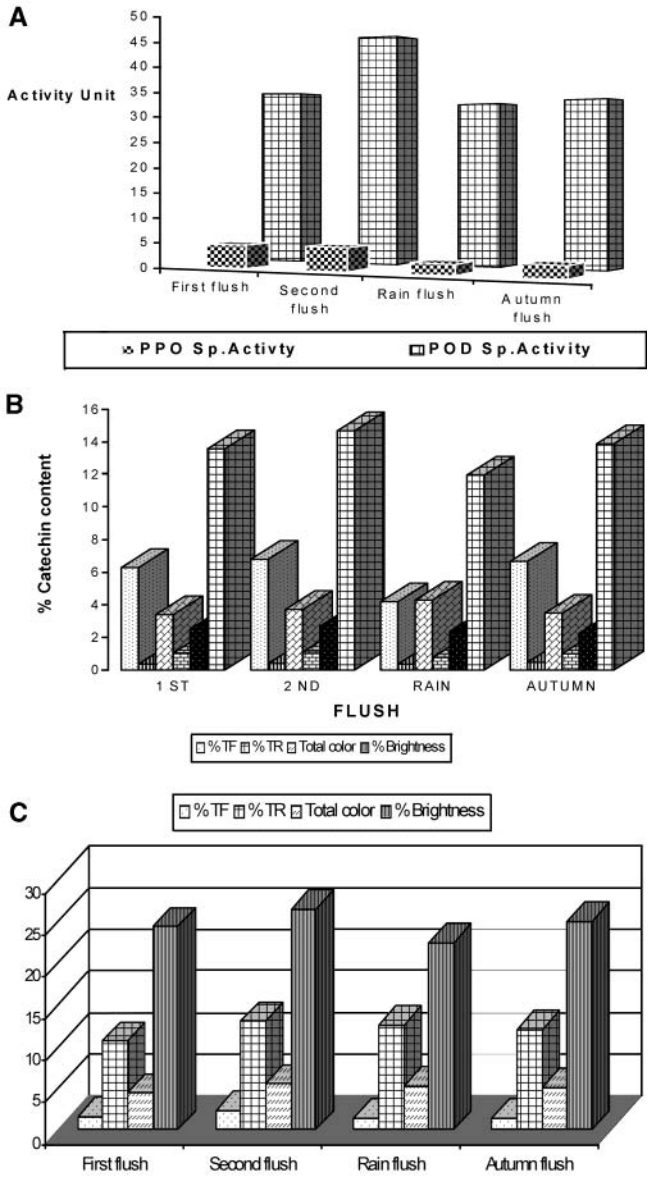

Figure 4. (A) Seasonal variations of PPO and POD in different flushes; (B) flush-wise mean variations in the catechin contents; (C) flush-wise mean seasonal variations in the quality parameters of tea liquors (3-years' mean seasonal average).

Table 3. Effect of Some Inhibitors on the Activity of Tea Polyphenol Oxidase (Average of Three Repeated Experiments)

	% inhibition		
concn of inhibitors (μmol)	ascorbic acid	β-mercapto-ethanol	sodium metabisulfite
0.5	10	18	7
10	27	32	26
20	38	48	51
30	51	70	62

highest activities in the flavorful second-flush teas and lowest in the monsoon flushes in all eight of the clones studied. From the changes in the levels of five major catechins, namely, EGCG, ECG, EGC, EC, and C (monthly average from 3-years' cycle of eight individual hybrid plants), it was observed that clonal and seasonal variations are highly significant (**Table 4**, with SD variations).

Table 4. Flush and Clonal Variation of Individual Catechins (Monthly Average Grouped Together during 1993–1996)

	flavanols					
clone	EGC	+C	EGCG	EC	ECG	total
			First Flush			
TV1	4.608	0.419	7.730	0.977	2.040	15.774
TV2	3.184	0.492	9.487	0.768	2.563	16.494
TV7	2.289	0.281	6.971	0.574	1.242	11.965
TV9	3.938	0.211	7.503	0.791	2.418	14.861
TV12	3.658	0.424	5.044	1.320	2.460	12.906
TV19	3.995	0.384	4.482	1.146	3.294	13.301
TV25	3.107	0.412	4.201	1.205	3.566	12.491
TV26	3.819	0.218	5.890	1.482	2.544	13.953
mean	3.401	0.355	6.289	1.033	2.516	13.593
SD	1.042	0.105	2.883	0.309	0.714	1.712
			Second Flush			
TV1	5.12	0.460	8.41	1.050	1.910	16.950
TV2	4.076	0.547	8.660	0.853	2.717	16.853
TV7	3.184	0.293	8.331	0.525	1.480	14.813
TV9	4.376	0.3	9.662	0.862	2.429	17.329
TV12	4.110	0.471	5.689	1.280	2.740	14.290
TV19	4.217	0.399	4.560	1.278	3.300	13.763
TV25	3.377	0.453	5.616	1.349	3.968	14.763
TV26	4.291	0.227	6.475	1.551	2.936	15.480
mean	3.719	0.399	6.800	1.094	2.685	14.780
SD	1.230	0.122	3.430	0.334	0.775	2.083
			Rain Flush			
TV1	3.564	0.545	4.140	0.777	3.119	12.145
TV2	5.154	0.240	2.981	0.598	1.762	11.735
TV7	2.758	0.273	3.829	0.825	1.859	9.544
TV9	4.332	0.02	6.242	0.25	1.285	12.859
TV12	3.966	0.179	6.137	0.508	2.856	13.646
TV19	5.111	0.234	2.167	0.831	2.303	10.646
TV25	3.399	0.599	5.614	0.851	3.285	13.748
TV26	5.060	0.441	2.425	0.923	2.499	11.348
mean	4.293	0.359	4.192	0.759	2.371	11.834
SD	0.729	0.168	1.699	0.150	0.703	1.217
			Autumn Flush			
TV1	2.630	0.629	11.600	0.528		15.387
TV2	3.733	0.393	8.308	0.985	1.234	14.653
TV7	2.650	0.382	6.652	0.740	2.084	12.508
TV9	3.336	0.466	3.712	2.134	2.109	11.757
TV12	3.552	0.617	7.300	1.143	1.823	13.735
TV19	2.580	0.12	5.397	1.346	1.842	12.165
TV25	3.144	0.333	4.854	1.357	2.545	12.233
TV26	4.325	0.24	6.567	0.736	2.418	13.264
mean	3.44	0.470	7.674	0.996	2.267	14.735
SD	0.711	0.126	2.919	0.623	0.846	1.843

Figure 4B presents a summary of the results (percentage mean value) of EGCG, ECG, EGC, EC, and C and their total composition, harvested in the four plucking seasons from April to November. The second flush, with mellow sunshine and relatively cold weather, helps biosynthesis of secondary metabolites (polyphenols, caffeine, and terpenoids), whereas the higher moisture of rain teas dilutes the quality attributes in all of the cultivars. Similarly, analyses of black tea quality parameters revealed that teas made from the second flush were the best followed by the autumn and first flush teas, which is well-known within the tea trade (**Figure 4C**). Because of higher enzymatic activity and higher amounts of major catechins, the greenish yellow second flush is well correlated to taster's brightness and organoleptic briskness.

A further important feature is that tea containing the highest amount of catechins and their gallated esters (20−25% dry wt)

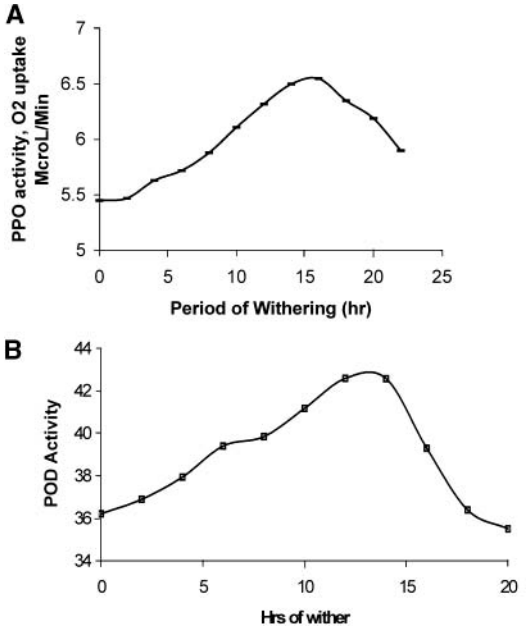

Figure 5. (A) PPO activity and (B) POD activity with progress of withering (repeated mean of eight clones).

bears its own fingerprint, which differs for individual clones, and their balance influences the characteristic taste and flavor. For example, higher levels of polyphenols, especially EGC, EGCG, ECG, and EC in the Assam-China hybrids, showed superior tea-making potential compared to other varieties. EGCG and EGC with low redox potentials were shown to undergo rapid oxidation compared to ECG and EC with high redox potential, which is critical in the development of theaflavin and thearubigin pigments, hence, varietal difference (*43*−*45*).

4. BIOCHEMICAL CHANGES DURING PROCESSING

4.1. Changes during Withering. Under the controlled conditions of withering, PPO, POD, and protease exhibited maximum activities within 12−16 h and thereafter declined as shown in **Figures 5A**,**B** and **6A**. **Table 5** shows the increased amount of total phenolic components (or precursors), caffeine, and total soluble solids (TSS) with the degree of withering as compared to fresh leaf. This stage is similar to the senescence process, where the endogenous multienzyme system might facilitate de novo biosynthesis of phenolic metabolites (*46*); purines (*47*) also released some of the cell wall bound lignin precursors from the leaf tissues (*48*, *49*).

The other notable changes are the hydrolysis of proteins by protease as measured from free amino acids. **Figure 6A** illustrates significant changes in protease activity resulting from breakdown of proteins into soluble form at up to 12 h of withering followed by a decrease (**Figure 6B**) (*26*, *50*). As withering progresses, the concomitant release of free amino acids goes up to 3.50% (total and dry wt basis) from 2.65% from fresh leaf (**Figure 6C**). The highest rises are in aspargine (Asn), glycine (Gln), threonine (Thr), and isoleucine (Ile), compared to aspartic acid (Asp), glutamic acid (Glu), serine (Ser), leucine (Leu), methionine (Met), alanine (Ala), phenylalanine (Phe), tryptophan (Trp), histidine (His), and lysine (Lys) (HPLC profile, figure not shown). Importantly, for theanine (Thea; γ-*N*-ethylglutamine), comprising 50% of the total, the sweet flavor amino acid in tea, the highest decrease was observed due to hydrolases (*51*, *52*).

4.2. Changes during Roll Fermentation. During rolling, there is a sharp increase in PPO and POD activities up to 60−75 min (higher rate of O_2 consumption); however, in overfermented leaves, PPO declines linearly but POD remains active (**Figure 7A**,**B**). In the case of free amino acids, their amounts marginally increased followed by a decrease during fermentation (**Figure 7C**), because the *o*-quinones, being very reactive, might

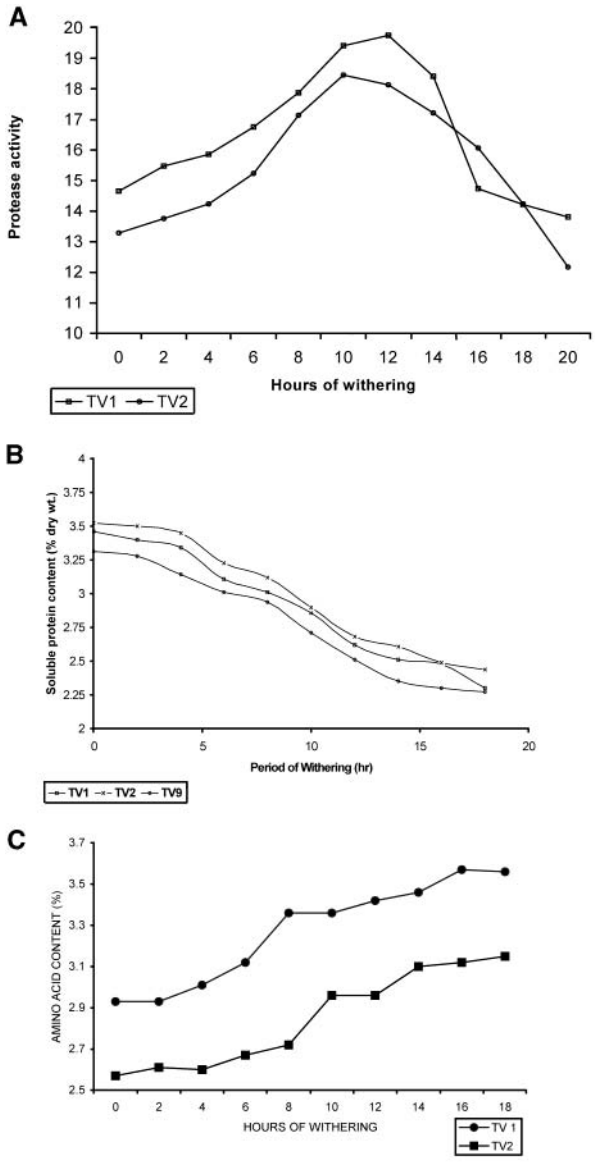

Figure 6. (A) Effect of withering on protease activity; (B) changes of soluble proteins with progress of withering; (C) total free amino acids with progress of withering (repeated means of six experiments).

Table 5. Effect of Withering on Total Polyphenols, Caffeine, and Total Soluble Solids (TSS) (3-Years' Mean Average Value)

	% total polyphenols		% caffeine		% TSS	
withering time (h)	Assam hybrid	China-Assam	Assam hybrid	China-Assam	Assam hybrid	China-Assam
fresh	25.87	24.37	4.26	4.07	42.46	41.28
6	25.91	24.38	4.32	4.09	42.46	41.38
8	25.90	24.54	4.45	4.11	42.78	41.39
10	26.41	25.46	4.38	4.13	42.88	41.38
12	26.25	25.22	4.21	4.42	43.12	41.42
14	25.97	24.87	4.78	4.22	43.32	41.40
16	26.02	24.77	4.88	4.21	42.79	41.38
18	25.88	27.78	4.87	4.24	42.69	41.36
mean ± SD	1.09	1.68	0.32	0.30	1.39	0.43

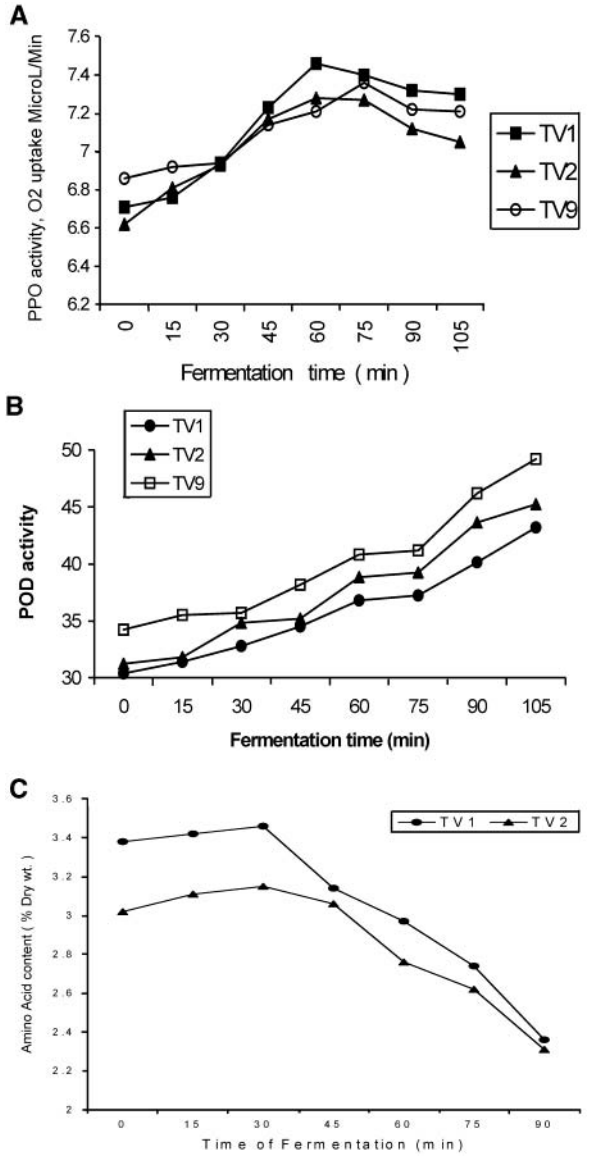

Figure 7. (A) PPO activity, (B) POD activity, and (C) changes in total free amino acids with progress of fermentation (repeated means of six experiments).

bind them with tea polyphenolics and also nucleophilic attack on them can precipitate enzymes/proteins. Most importantly, some of the free amino acids changed into volatile carbonyls in GC peaks such as heptanal, 6-methylhepten-2-one, benzaldehyde, and phenylacetaldehyde, and 2-phenylethanol with a roselike aroma could be observed due to oxidase and heat treatment (table not shown) (*53*, *54*). Therefore, the nitrogen source and processing methods were both found to have profound effects on the texture of made tea and amounts of flavors formed.

The initial stage of the fermentation process is accompanied with a large increase in the O_2 consumption, but as fermentation progresses, O_2 consumption declines linearly as a function of time and temperature. **Table 6A** shows the gradual lowering in the leaf cell sap pH from 5.5 to 3.7, when the fresh leaves were progressively withered, macerated by CTC, and fermented. The heat generated is the result of frictional heat by crushing, cutting, curling, and the exothermic oxidation reactions (**Table 6B**). The temperature stability profile for PPO is presented in the form of the residual percentage activity shown in **Figure 8A**. When the PPO preparation was heated for 20 min at 30 °C, it did not

Table 6. (A) Change of pH of Cell Sap during Manufacturing and (B) Temperature Profile[a] during Processing (Average of Three Repeated Experiments)

	pH	
stage of manufacture	cultivar A	cultivar B
fresh tea shoot	5.5	5.6
withered shoot	5.2	5.1
first CTC cut	4.5	4.5
second CTC cut	4.5	4.6
third CTC cut	4.6	4.5
middle of fermentation	4.3	4.1
fermentation termination	3.8	3.7
made tea extract	5.45	5.47

	leaf temp (°C) at different units					
stage of CTC processing	A	B	C	D	E	F
rotorvane	27.8	28.7	27.4	27.4	27.8	27.5
first cut	34.8	36.8	31.3	31	31	31.5
second cut	36.8	36.5	33.5	29.3	32.3	34.0
third cut	33.4	32.3	34.5	33	31.0	33.5
fourth cut	NC[b]	33.5	NC	34	NC	NC

[a] Temperature used: Solomat, MPM 500e (made in U.K. by Neotronics Co.). [b] No change.

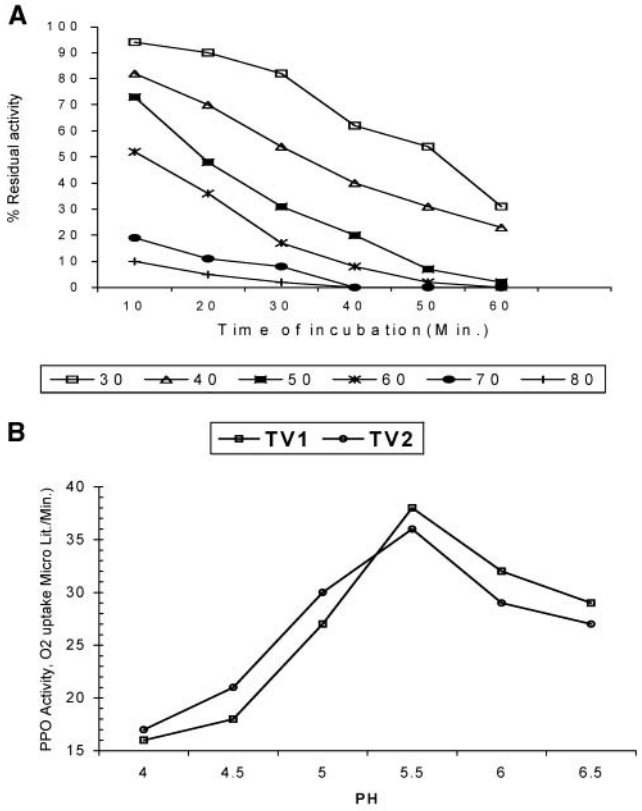

Figure 8. (A) Thermal inhibition of PPO activities (repeated means of six experiments); (B) effect of pH on PPO activity (means of repeated experiments).

cause a significant loss, but above 40 °C activities decreased markedly. The rise in temperature might greatly affect the protein stability in primary and secondary structures through conformational entropy (*55*).

The pH optimum for PPO activity was found around 5.5, and thereafter a gradual fall was noticed for the clones studied (TV1 and TV2, **Figure 8B**). Therefore, a rise in temperature (**Figure 8A**) and a fall of pH during processing (**Figure 8B**) results in a lowering of oxidative fermentation of EGCG, ECG,

Table 7. Theaflavin (TF), Thearubigin (TR), Total Color (TC), and Sensory Quality of Processed Teas under Different Conditions (Consistent Mean of Experimental Data)[a]

type of manufacture	% TF	% TR	total color	taster's av score	taster's comments
		Withering Variations			
cold wither CTC	1.41 ± 0.52	11.15 ± 0.46	4.3 ± 0.35	6. 5	bright and aromatic
hot wither CTC	1.23 ± 0.23	12.92 ± 0.56	3.9 ± 0.52	5.0	reddish cup
		Fermentation Variations			
control: RV-3 CTC, ferm	1.03 ± 1.25	12.75 ± 0.76	4.43 ± 0.52	6.0	brownish cup
RV + 1 CTC 25% ferm + 2 CTC-75% ferm	1.28 ± 0.52	12.18 ± 0.88	4.70 ± 0.06	8.0	bright, brisk, yellowish cup
RV − 50% ferm + 3 CTC-50% ferm	1.45 ± 0.49	13.25 ± 0.76	5 ± 0.06	8.5	almond bright and aromatic
		Table Roller and CTC versus Rotorvane and CTC			
roll-CTC	2.12 ± 0.03	13.28 ± 1.00	4.60 ± 0.52	7	bright and aromatic
rotorvane-CTC	0.76 ± 0.66	14.87 ± 0.76	4.22 ± 0.90	5.5	dull, poor aroma

[a] Same sources of materials were used for paired comparison.

Table 8. Chemical and Sensory Evaluation at Various Levels of Withering and Fermentation (3-Years' Average Mean Value of Manufacturing Experiments)

fermentation time (min)	12 h of withering				14 h of withering				16 h of withering			
	TF	TR	TC	B	TF	TR	TC	B	TF	TR	TC	B
0	1.49	7.14	3.32	38.34	0.87	7.47	2.42	31.96	0.86	7.46	2.22	30.42
10	1.96	7.92	4.30	40.12	1.91	9.36	4.52	39.23	1.82	8.79	3.04	32.64
20	2.34	10.15	5.30	38.91	2.29	9.88	5.00	42.5	2.14	9.20	3.27	34.27
30	2.74	14.09	5.10	46.68	2.62	12.24	5.77	40.69	2.46	12.63	5.63	37.26
40	2.40	14.08	6.97	32.26	2.90	12.35	6.62	42.07	2.89	13.77	5.72	36.20
50	2.14	11.45	5.42	35.25	2.60	13.71	6.67	39.14	2.74	14.14	6.44	37.21
	tasters score[a] 5				tasters score[a] 6.5				tasters score[a] 7.5			
correlation												
TF			0.70	0.30			0.79**	0.89*			0	0.94**
TR			0.82*	0			0	−0.71			0.99**	0.93**

[a] Scores from 0 to 10, depending upon the comparison of several attributes such as aroma, color, flavor, body, and taste. *, significance at 5%; **, significance at 1%.

ECG, EC, and C by endogenous PPO, whereas autoxidation or coupled oxidation deteriorates quality in overfermented tea (*15*, *56*). As enzyme inactivation is well-known during tea processing, use of commercial enzymes, namely, cellulase and pectinase, yielded quantitative improvements of theaflavins, total polyphenols, and total soluble solids compared to untreated controls (table not shown).

4.3. Process Optimization Attempts. In commercial manufacturing, withering is mostly uneven or excessive; such leaves when rolled generate more heat and leaf breaks, oxygen uptake is reduced, and the yield and quality of the product suffer. To improve enzymatic activity and to enhance the yellow color of the brewed liquor, we studied modified processing. These experiments were (1) lowering the temperature in the withering trough, (2) rolling table versus rotorvane, and (3) two-stage fermentation. In the "split fermentation", the aerobic oxidation was given in two stages: the first after preconditioning in the rotorvane machine and the second after three CTC rolls, followed by drying in an FBD. **Table 7** shows consistent increases in theaflavins and total color (TC), as well as taster's quality, compared with than tandemly manufactured teas.

A number of studies using grape (tea as well) catechins and PPO (and POD) have shown that at higher pH values (at 6 and 7), oxidation products formed are yellow (λ_{max} 385−415 nm), whereas at lower pH values (at 3−4), products are colorless (λ_{max} 280 nm) (*56*, *57*). Therefore, attempts were made to produce more yellow species by maintaining the pH in the range of 5.6−7 (by application of buffer or salt, not shown in the table). In general, enhancement in the theaflavin levels up to 20−30% and often lower levels of thearubigins have been observed, a factor that imparts flavor and visual brightness.

4.4. Effect on Color and Flavor. Chemical and organoleptic characteristics of brewed black teas produced from different withering times (in hours) and fermentation times (in minutes) are shown in **Table 8**. Besides these, PPO showed higher activity at high moisture in the fermented leaf, hence enhancing total color due to theaflavins and therubigins (**Figure 9**). In the overwithered and overfermented leaves, reduced amounts of theaflavin (also aroma) were found because tannins had high affinities for proteins, forming melanin-type of complex precipitation (*58*, *59*). Furthermore, POD inhibits PPO and affects primary formation of quinones and semiquinones, resulting in the loss of theaflavins and flavonol glycosides, producing secondary products, the so-called thearubigins (**Figure 9B**).

The development of four major theaflavins (TF1, TF2, TF3, and TF4), with the progress of withering and fermentation time, is presented in the HPLC profile (**Figure 10A**). Interestingly, we could observe fast formation of TF1 (EC + ECG) and TF2 (EC + EGCG) followed by increases in the amounts of TF3 (ECG + EGC) and TF4 (ECG + EGCG). **Figure 10B** summarizes flavonol glycosides (FG) compositions: rutin (RG), quercetin glycosides (QG), myricetin glycosides (MG), and kaempferol glycosides (KG). It is possible that primary fermentation and rapid oxygen uptake due to PPO-catalyzed oxidation of EGCG, ECG, EGC, and EC produced higher levels of the four major theaflavins, whereas on longer fermentation, PPOs are thought to produce POD, which can use any catechins, flavonol glycosides, also theaflavins formed are further transformed to phenol−protein complex precipitates or haze (*15*, *44*).

Figure 11 shows the effect of processing on phenolic acids and related compounds such as gallic, ferulic, caffeic, coumaric, and chlorogenic acid, vanillin, and others from fresh leaf. The most significant increase in vanillin, homovanillic acid, and vanillic acid during withering and rolling indicated that these compounds are hydrolyzed from the soft portion of cell wall tea fibers (*48*, *49*). It was shown that some of these phenolic precursors could undergo oxidative transformation during dry-

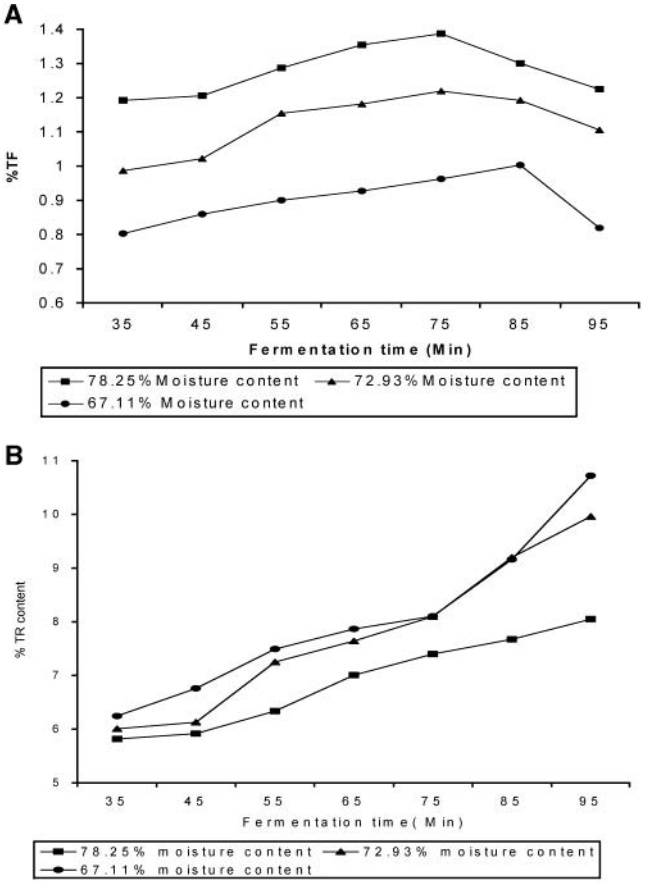

Figure 9. (A) Percent TF formation and (B) percent TR formation with progress of fermentation and moisture level (repeated mean value).

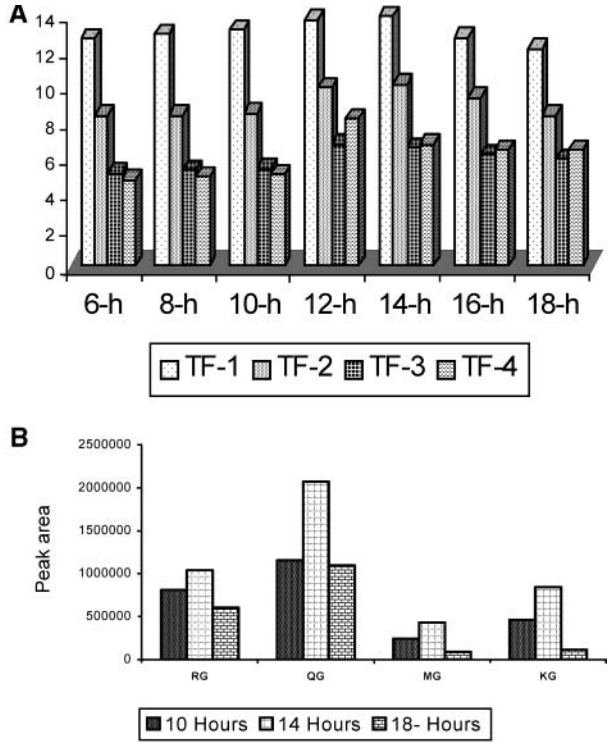

Figure 10. (A) HPLC theaflavins profile and (B) HPLC flavonol glycosides profiles with progress of withering at 30 min of fermentation time (repeated mean value).

ing, due to peroxidase, contributing significantly to the flavor and bright color of the brewed liquor (*16*, *21*−*24*).

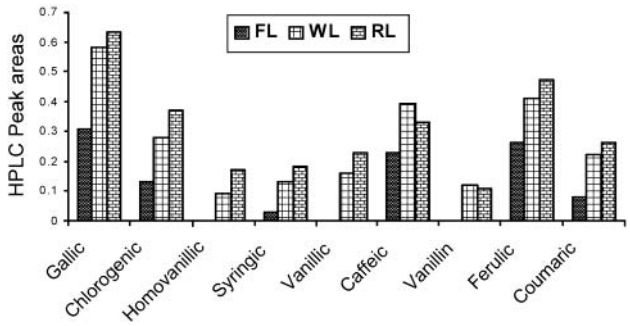

Figure 11. HPLC phenolic acids and related compounds during processing (repeated mean value of three experiments).

In conclusion, this paper contains initial information on genetic characteristics of some common tea cultivars (superiority: Assam-China > China hybrid > Cambod variety) and their tea-making potentials. The study resulted in optimized manufacturing in terms of organoleptic profiles, namely, theaflavins and thearubigins, based on flavan-3-ols, proteins and PPO activities processed under different conditions. We determined the effects of temperature, optimum pH, moisture, and rolling versus rotorvaned CTC on PPO-generated yellow color, whereas POD leads to enhanced formation of phenol−protein complexes, deteriorating tea quality.

ACKNOWLEDGMENT

We are grateful to the Council of Scientific and Industrial Research (CSIR, New Delhi) for a fellowship (A.M.B.) and to the Director, TRA, Tocklai Experimental Station, for his permission to conduct experiments.

LITERATURE CITED

(1) Barua, D. N. *Science and Practice in Tea Culture*; Tea Research Association: Calcutta, India, 1989.
(2) Bezbaruah, H. P.; Dutta, A. C. Tea germplasm collection at Tocklai Experimental. *Two Bud* **1977**, *24*, 22−30.
(3) Roberts, E. A. H. The chemistry of tea fermentation. *J. Sci. Food Agric.* **1952**, *5*, 193−198.
(4) Singh, H. P.; Ravindranath, S. D. Occurrence and distribution of PPO activity in floral organs of some standard and local cultivars of tea. *J. Sci. Food Agric.* **1994**, *64*, 117−120.
(5) Kvaratskhelia, M.; Winkel, C.; Thorneley, N F. Purification and characterization of a novel class III peroxidase isoenzyme from tea leaves. *Plant Physiol.* **1997**, *114*, 1237−1245.
(6) Pichersky, E.; Gang, D. R. Genetics and biochemistry of secondary metabolites in plants: An evolutionary perspective. *Trends Plant Sci.* **2000**, *5*, 439−445.
(7) Dakora, F. D. Plant flavonoids: Biological molecules for useful exploitation. *Aust. J. Plant Physiol.* **1995**, *22*, 87−99.
(8) Baruah, T. C. Common problems with the withering trough. *Two Bud* **1977**, *24*, 35−37.
(9) Sen, M. The CTC process. *Proc. Tocklai Eng. Semin.* **1975**, 20−26.
(10) Barbora, D. N. Use of Rotorvane. *Two Bud* **1964**, *10*, 41−44.
(11) Mahanta, P. K. Color and flavor characteristics of made tea. In *Modern Methods of Plant Analysis. Analysis of Nonalcoholic Beverages*; Linskens, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, Germany, 1988; Vol. 8, pp 221−295.
(12) Saikia, P.; Mahanta, P. K. Specific fluctuations in the composition of lipoxygenase- and glycosidase-generated flavors in some cultivated teas of Assam. *J. Sci. Food Agric.* **2002**, *50*, 7691−7699.
(13) Harries, N.; Ellis, R. T. Black tea manufactures. Effects on leaf structure of different processing systems. *Ann. Appl. Biol.* **1981**, *99*, 359−366.

(14) Mahanta, P. K.; Baruah, S. K.; Baruah, H. K.; Kalita, J. K. Changes of polyphenol oxidase and peroxidase activities and pigment composition of some manufactured black teas (*Camellia sinensis* L.). *J. Agric. Food Chem*. **1993**, *41*, 272−276.

(15) Mahanta, P. K.; Baruah, H. K. Theaflavin pigment formation and polyphenol oxidase activitiy as criteria of fermentation in orthodox and CTC teas. *J. Agric. Food Chem*. **1992**, *40*, 860−863.

(16) Tanaka, T.; Mine, C.; Watarumi, S.; Fujioka, T.; Mihashi, K.; Zhang, Y.-J.; Kouno, I. Accumulation of epigallocatehin quinone dimers during tea fermentation and formation of theanaphthoquinone. *J. Nat. Prod.* **2002**, *65*, 1582−1587.

(17) Mahanta, P. K.; Baruah, S. Changes in pigments and phenolics and their relationship with black tea quality. *J. Sci. Food Agric.* **1992**, *59*, 21−26.

(18) Sava, V. M.; Yang, S.-W.; Hong, M.-Y.; Yang, P.-C.; Hung, G. S. Isolation and characterization of melanic pigments derived from tea polyphenols. *Food Chem.* **2001**, *73*, 177−184.

(19) Collier, P. D.; Bryce, T.; Mallows, R.; Thomas, P. E. The theaflavins of black tea. *Tetrahedron* **1973**, *29*, 125−142.

(20) Kiehne, A.; Engelhardt, U. H. Thermospray-LC-MS analysis of various groups of polyphenols in tea II. Chlorogenic acids, theaflavins and thearubigins. *Z. Lebensm. Unters. Forsch*. **1996**, *202*, 299−302.

(21) Lewis, J. R.; Davis, A. L.; Cai, Y.; Davis, A. P.; John, P. G. W. Theaflavine-3′-*O*-gallate and neotheaflavin-3-*O*-gallate: three polyphenolic pigments from black tea. *Phytochemistry* **1998**, *49*, 2511−2519.

(22) Hashimoto, F.; Nonaka, G.-I.; Nishioka, I. Tannin and related compounds. LXIX. Isolation and structure elucidation of B,B′-linked bisflanoids, theasinensins D−G and oolongtheanine from oolong tea. *Chem. Pharm. Bull*. **1988**, *36*, 1676−1684.

(23) Tanaka, T.; Bestsumiya, Y.; Mine, C.; Kouno, I. Theanaphthoquinone, a novel pigment oxidatively derived from theaflavin during tea-fermentation. *Chem. Commun*. **2000**, 1365−1366.

(24) Hashimoto, F.; Nonaka, G.-I.; Nishioka, I. Structures of novel fermentation products, theogallin, theaflavonin and desgalloyl theaflavonin from black tea, and changes of tea leaf polyphenols during fermentation. *Chem. Pharm. Bull*. **1992**, *40*, 1383−1389.

(25) Cruz, L. J.; Cagampang, G. B.; Julian, B. O. Biochemical factors affecting protein accumulation in rice grain. *Plant Physiol*. **1970**, *46*, 743−747.

(26) Bhatia, I. S.; Deb, S. B. Nitrogen metabolism of detached tea shoots: Changes in amino acids and amides of tea shoots during withering. *J. Sci. Food Agric*. **1965**, *16*, 759−769.

(27) Lamikanra, O.; Kassa, A. K. Changes in the free amino acid composition with maturity of the noble cultivar of *Vitis rotundifolia* Michx. Grape. *J. Agric. Food Chem.* **1999**, *47*, 4837−4841.

(28) Bailey, R. G.; McDowell, I.; Nursten, H. E. Use of an HPLC phtodiode-arry detector in a study of the nature of a black tea liquor. *J. Sci. Food Agric*. **1990**, *52*, 509−525.

(29) Hartley, R. D. HPLC for separation and determination of phenolic compounds in plant cell walls. In *Modern Methods of Plant Analysis, New Series*; Linskens, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, Germany, 1987; Vol. 5, pp 92−101.

(30) Sherman, T. D.; Vaughn, K. C.; Duke, S. O. A limited survey of the phytogenic distribution of polyphenol oxidase. *Phytochemistry* **1991**, *30*, 2499−2506.

(31) Xu, F.; Shin, W. S.; Brown, S. H.; Wahleithner, J. A.; Sundaram, U. M.; Solomon, E. I. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochim. Biophys. Acta* **1996**, *1292*, 303−311.

(32) Mulligan, R. M.; Houtz, R. L.; Tolbert, N. E. Reaction-intermediate analogue binding by ribulose biphosphate carboxylase/oxygenase causes specific changes in proteolytic sensitivity: The amino-terminal residue of the large subunit is acetylated proline. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1513−1517.

(33) Wilson, M. F.; Blunden, C. A. Changes in the levels of polyphenols in three pear varieties during bud development. *J. Sci. Food Agric*. **1983**, *34*, 973−978.

(34) Mahanta, P. K.; Hazarika, M.; Baruah, S. Influence of plucking and processing on cell wall and soluble components in black tea. *Two Bud* **1990**, *37*, 17−19.

(35) McMullen, M. D.; Byrne, P. F.; Snook, M. E.; Wiseman, B. R.; Lee, E. A.; Widstrom, N. W. Quantitative trait loci and metabolic pathways. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1996−2000.

(36) Haslam, E. In *Practical Polyphenolics. From Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: Cambridge, U.K., 1998.

(37) Smith-Backer, J.; Huguet, E. J.; Midland, S. L.; Sims, J. J.; Keen, N. T. Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by transient increase in phenylalanine ammonia-lyase activity in petiole and stems. *Plant Physiol*. **1998**, *116*, 231−238.

(38) Smith, C. E.; Ruttledge, T.; Zeng, Z.; O'Malley, R. C.; Lynn, D. G. A mechanism for inducing plant development: The genesis of a specific inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6986−6991.

(39) Nakayama, T.; Sakakibara, K.; Sato, T.; et al. Auresidin synthase: A polyphenol oxidase homologue responsible for flower coloration. *Science* **2000**, *290*, 1163−1166.

(40) Susan Marles, M. A.; Gruber, M. Y.; Scoles, G. J.; Muir, A. D. Pigmentation in the developing seed coat and seedling leaves of *Brassica carinata* is controlled at the dihydroflavonol reductase locus. *Phytochemistry* **2003**, *62*, 663−672.

(41) Van Assche, F.; Clijsters, H. Effects of metals on enzyme activity in plants (commissioned review). *Plant Cell Environ.* **1990**, *13*, 195−206.

(42) Miller, A. R.; Kelley, T. J.; Mujer, C. V. Anodic peroxidase isoenzymes and polyphenol oxidase activity from cucumber fruit: Tissue and substrate specificity. *Phytochemistry* **1990**, *29*, 705−709.

(43) Robertson, A. The chemistry and biochemistry of tea production—the nonvolatiles. In *Tea Cultivation to Consumption*; Wilson, K. C., Clifford, M. N., Eds.; Chapman and Hall: London, U.K., 1992; pp 556−601.

(44) Balentine, D. A.; Wiseman, S. A.; Bouwens, L. C. M. The chemistry of tea flavonoids. *CRC Crit. Rev. Food Sci. Nutr*. **1997**, *37*, 693−704.

(45) Kumamoto, M.; Sonda, T.; Nagayama, K.; Tabata, M. Effects of pH and metal ions on antioxidant activities of catechins. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 126−132.

(46) Tushida, T.; Takeo, T. The hydroxylation of *p*-coumarate to caffeate by the tea enzyme. *Study Tea* **1981**, *60*, 29−33.

(47) Suzuki, T.; Hiroshi, A.; Waller, G. R. Purine and purine alkaloid metabolism in *Camellia* and *Coffea* plants. *Phytochemistry* **1992**, *31*, 2575−2584.

(48) Gasson, M. J.; Kitamura, Y.; McLauchlan, W. R.; et al. Metabolism of ferulic acid to vanillin. *J. Biol. Chem*. **1998**, *273*, 4163−4170.

(49) Peterson, A.; Lundquist, K. Radical breakdown of lignin. *Nature* **1985**, *316*, 575−576.

(50) Nieri, B.; Canino, S.; Versace, R.; Alpi, A. Purification and characterization of an endoprotease from alfalfa senescent leaves. *Phytochemistry* **1998**, *49*, 643−649.

(51) Tsushida, T.; Takeo, T. An enzyme hydrolysing L-theanine in tea leaves. *Agric. Biol. Chem*. **1985**, *49*, 2913−2917.

(52) Ekborg-Ott, K. H.; Taylor, A.; Armstrong, D. W. Varietal differences in the total and enantiomeric compounds of theanine in tea. *J. Agric. Food Chem.* **1997**, *45*, 353−363.

(53) Saijo, R.; Takeo, T. The formation of aldehydes from amino acids by tea leaves extracts. *Agric. Biol. Chem*. **1970**, *34*, 227−233.

(54) Aaslyng, M. D.; Martens, M.; Poll, L.; Nielsen, P. M.; Flyge, H.; Larsen, L. M. Chemical and sensory characterization of hydrolyzed vegetable protein, a savory flavoring. *J. Agric. Food Chem*. **1998**, *46*, 481−489.

(55) Danial, R. M.; Dines, M.; Petach, H. H. The denaturation and degradation of stable enzymes at higher temperature. *Biochem. J*. **1996**, *317*, 1−11.

(56) Opie, S. C.; Clifford, M. N.; Robertson, A. The formation of thearubigin-like substances by in-vitro polyphenol oxidase-mediated fermentation of individual flavan-3-ols. *J. Sci. Food Agric*. **1995**, *67*, 501−505.

(57) Guyot, S.; Vercauteren, J.; Cheynier, V. Structural determination of colorless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279−1288.

(58) Sekiya, J.; Kajiwawa, T.; Monma, T.; Hatanaka, A. Interaction of tea catechins with proteins: Formation of protein precipitate. *Agric. Biol. Chem*. **1984**, *48*, 1963−1967.

(59) Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanism of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4 → 8) catechin (procyanidin). *J. Agric. Food Chem*. **1998**, *46*, 2590−2595.

Received for review January 9, 2003. Accepted August 10, 2003.

JF030019W