ELSEVIER

Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camellia sinensis*)

Jyotsnabaran Halder, Prodip Tamuli,* and A.N. Bhaduri

*Division of Enzymology, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Calcutta-700032, India; and *Department of Biochemistry, Tocklai Experimental Station, Jorhat-8, India*

*Polyphenol oxidase (PPO) was purified and characterised from the acetone powder of tea leaves (*Camellia sinensis*). Substrate staining of the acetone powder extract indicated the presence of a maximum of three isozymic forms of this enzyme. The isozymes of PPO were separated on DEAE cellulose column. Two fractions were absorbed and the other was unabsorbed. The unabsorbed fraction was purified up to homogeneity in different chromatographic steps: gel filtration, hydroxyapatite, centricon-30, FPLC. SDS-PAGE data along with molecular mass data showed the active enzyme to be of 72 kD. The pH, temperature, and kinetic parameters were studied. The highly purified enzyme was unable to oxidize monophenols, p-quinol but could oxidize catechol and thus might be regarded as catechol oxidase. Catechin was the best substrate with a K_m of 0.49 mM. The enzyme was completely inhibited by 2 mM tropolone, suggesting it to be a copper-containing enzyme. The enzyme is localized in chloroplast and could be solubilized with Triton-X-100. Trypsinization experiment with freeze-thawed chloroplast further confirms the localization of the enzyme inside the chloroplast.* (J. Nutr. Biochem. 9: 75–80, 1998) © *Elsevier Science Inc. 1998*

Keywords: *Camellia sinensis*; purification; chloroplast; tripsinization

Introduction

Tea (*Camellia sinensis*) is a widely consumed beverage throughout the world. It is estimated that about 2.5 million metric tons of dried tea are manufactured annually. Of this production, about 78% is black tea, mainly consumed in the western world and in many eastern countries, including India.[1] Considering its wide consumption and great commercial value, considerable work has been done in recent years on tea as a health beverage and on its constituents as pharmacologically active compounds. Most of these works have been done with green tea and catechin derivatives present therein, the major polyphenolic flavonol constituents of tea leaves. These results have been reviewed from time to time.[2,3]

The oxidative and hydrolytic enzymes endogenous to tea shoots are crucial for generation of various characteristic quality attributes of black tea. The basic principle underlying black tea manufacture is the controlled chemical transformations that are responsible for the formation of sparkling color, briskness, and aroma that are conventionally associated with taste and quality of made tea. Out of the various stages of black tea processing, the "fermentation" step is the most critical. In this step, after mechanically disrupting the integrity of green tea shoots and leaves, these are allowed to undergo in vivo oxidative and hydrolytic processes in presence of mild aeration and at slightly elevated temperature (32 ± 3°). During this process, a variety of glycosidases present in tea leaf liberate aromatic aglycones from their nonvolatile precursors. Recently, we have reported the presence of an active β-galactosidase in Indian tea leaf.[4] The desirable color and briskness of the made tea, on the otherhand, is dependent on the oxidative polymerization of catechins to theaflavins and thearubigin that is brought about by polyphenol oxidases (PPO) proba-

The work presented in this paper has been fully supported by Tea Research Association (TRA), India. J.B. Halder holds a fellowship from TRA. We are thankful to Dr. S. Sharma, Secretary TRA, for useful discussion and encouragement.

Address correspondence and reprint requests to Dr. A.N. Bhaduri, Emeritus Scientist and Former Director, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Jadavpur, Calcutta-700032, India.

Received July 2, 1997; accepted October 21, 1997.

655 Avenue of the Americas, New York, NY 10010

0955-2863/98/$19.00
PII S0955-2863(97)00170-8

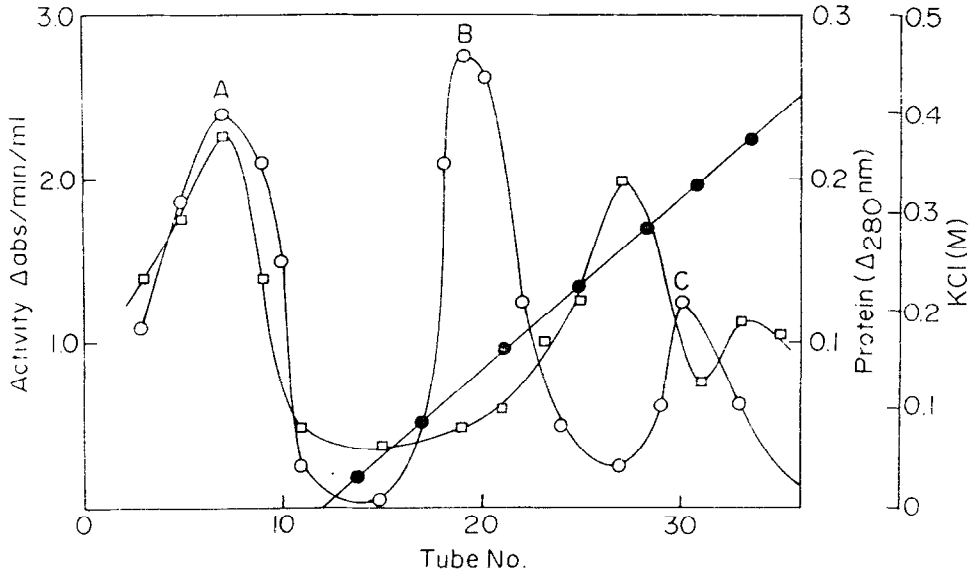

Figure 1 Separation of isoforms of polyphenol oxidase on DEAE-cellulose column. Protein sample was applied to a pre-equilibrated column (2.0 × 20 cm) with 50 mmol/L K-Phosphate buffer pH-7.0. The column was washed with the same buffer and then eluted with linear gradient of 0-0.5 mol/L KCl. The different peak fractions were pooled separately and designated as peak A (unabsorbed) and peak B (absorbed). The small activity at peak C was ignored. □----□ absorbance at 280 nm. ○— —○ polyphenol oxidase activity. ●----● KCl concentration.

bly in conjunction with peroxidase.[5] In view of its obvious importance, considerable effort has been given from time to time to purify and characterize PPO from tea leaf.[6–9] These early works, mostly using ion-exchange chromatography[6–8] and isoelectric focusing,[9] could demonstrate presence of many isozymic forms of the enzyme, though none of these were purified to homogenity for subsequent enzymological studies. In this paper, we have used substrate-staining techniques to demonstrate the presence of a maximum of three isozymic forms. Further, the electrophoretically homogenous major form seems to be a Cu^{2+}-containing monomer of 72 kD that can use only o-diphenols like catechins as substrates. Finally, as in case of most polyphenol oxidases from plant sources,[10] the enzyme is chloroplastic in origin but fails to show any latency toward activating agents.[11]

Methods and materials

Chemicals and reagents

Fresh tea leaf (two leaves and a bud) of different clonal varieties developed by Tocklai Experimental Station, Tea Research Association, India, were collected from different plantation areas and supplied to us. All biochemicals unless otherwise mentioned were from Sigma Chemical Co. (St. Louis, MO USA.). All chemicals were of analytical grade and were purchased from E. Merck (India).

Enzyme assay

PPO was estimated according to the method of Moore and Flurkey.[11] The reaction mixture in a total volume of 1 mL at room temperature, contained 50 mM citrate buffer pH-5.0, 50 mM catechol and an aliquot of the enzyme. The change in absorbance at 410 nm was monitored with time and the velocity of the reaction was determined from the linear portion of the curve. Because of the uncertainty in molar extinction coefficient of oxidized product of catechol, 1 absorbance was taken as 1 unit of enzyme activity.

Protein estimation

Protein was routinely estimated by the method of Lowry et al.[12] At later stages of purification when concentration of protein was very low, the method of Bradford[13] was used, in both cases bovine serum albumin was the standard.

Purification of polyphenol oxidase

One hundred g of fresh tea leaves were homogenized with 80% ice cold aqueous acetone solution using polytron homogenizer for 2 to 3 mins. The suspension was filtered on sintered funnel G-1 and washed repeatedly with ice cold 80% acetone with water until colorless. Finally, it was washed with 100% acetone to make completely moisture free and dried. The dried powder was stored at −20°.

Ten g of acetone powder along with same amount of polyvinyl polypyrolidone and double amount of sea sand, was taken in a mortar. This was homogenized with 100 mL ice-cold buffer containing 0.2 M K-phosphate buffer, pH-7.0, 0.35 M KCl, 0.5% Triton-X 100, and 50 μL of a cocktail mixture of protease inhibitors [mixture containing 70 mg phenyl methyl sulphonyl fluoride (PMSF), 1.4 mg leupeptin, and 1 mg pepstatin per mL]. The homogenate was centrifuged at 12,000 × g for 10 min to remove the remaining cell debris. The supernatant was concentrated by dialysis against solid sucrose to 15 mL. The concentrated solution was subsequently dialysed for 6 hr against 50 mM K-phosphate buffer, pH-7.0, with two changes. The dialyzed sample was used as the crude extract for purification of PPO activity and also for substrate staining. Unless otherwise stated, all subsequent operations during purification were performed at 4°.

The dialyzed enzyme (95 mg) was applied to a DEAE-cellulose column (2.0 × 20 cm) equilibrated with 50 mM K-Phosphate buffer, pH-7.0, and was then eluted with a linear gradient of 0 to 0.5 M KCl in the same buffer; 2.5 mL fractions were collected.

Table 1 Purification of polyphenol oxidase (PPO)

Steps	Total protein (mg)	Total activity (A/min)	Specific activity (A/min/mg)	Purification fold
Crude	95.00	62.51	0.65	1.00
DEAE-Cellulose	28.31	26.72	0.99	1.52
Sephacryl S-200	7.83	23.90	3.01	4.63
Hydroxyapatite	1.62	19.73	12.18	18.74
Centricon-30	0.75	18.54	24.72	38.03
FPLC	0.08	16.33	204.13	314.04

The crude extract was separated in isozymic forms of PPO on a DEAE-cellulose column. For details, see Methods and *Figure 1*. The unabsorbed PPO activity or Fraction A was further purified as summarized below. The assay conditions were the same as described in Methods.

PPO activity was separated into two major and one minor fractions as presented in *Figure 1*. The fraction that came out unabsorbed from the column was designated as fraction A. Absorbed fractions were B and C. Fractions A and B were separately concentrated by sucrose dialysis. Fraction C was ignored. The concentrated fraction A was applied on a Sephacryl S-200 column (1.0 × 85 cm) equilibrated with 50 mM K-phosphate buffer, pH-7.0. The fractions having enzyme activity were combined (data not shown) for further purification. Fraction B was also applied separately on the same column. The active eluants of Fraction B were combined seperately for comparative study with Fraction A and was not purified further.

Fraction A was further purified by hydroxyapatite treatment as described by Tiselius et al.[14] Hydroxyapatite was suspended in 10 mM sodium phosphate buffer, pH-6.8, at 4°. Hydroxyapatite 5 mg/mL was centrifuged and the pellet was treated with equal volumes of enzyme solution. The enzyme activity was absorbed and then eluted batchwise with 0.3 M, 0.4 M, and 0.5 M NaCl containing the above buffer. Maximum activities of the enzyme, at a salt concentration of 0.4 M to 0.5 M NaCl were combined to a total volume of 4.0 mL, which was reduced to 1.2 mL by passing through a centricon-30 filter (amicon). This was injected on a FPLC separose column (Pharmacia HKB) equilibrated in 50 mM K-phosphate buffer, pH-7.0. The flow rate was 10 mL/hr. Elution of protein was monitored by measuring the absorbance at 280 nm and the eluted peaks were checked for PPO activity. A single peak was obtained with 314-fold purification. The highest activity fractions of total volume 1.5 mL were concentrated to 0.3 mL. It was stored at −20° and was stable for several months.

Molecular weight determination

A Sephacryl S-200 column was calibrated with myosin (M_r 205,000), aldolase (M_r 158,000), bovine serum albumin (M_r 66,000), and cytochrome c (M_r 12,400) as marker proteins. Appropriate aliquot of Fractions A and B after DEAE cellulose column purification were separately applied to the column for determination of relative molecular weights.

Electrophoresis

Polyacrylamide gel electrophoresis and SDS-gel electrophoresis were carried out following the methods of Davis[15] and Laemmli,[16] respectively. Silver staining was performed according to Merril et al.[17]

Activity staining

After electrophoresis, PPO activity was visualized with catechol as substrate following the method of Vanloon.[18] The gel was equilibrated for 30 min at room temperature in 50 mM citrate buffer, pH-5.0 and was then incubated for 30 mins in 50 mM catechol in the same buffer under vigorous aeration. Black bands indicated sites of PPO activity.

Isolation of chloroplast

Chloroplast from tea leaves were isolated by following the method of Robinson and Barnett.[19] Twenty g fresh tea leaves were minced and mixed with 100 mL semifrozen grinding medium (0.35 M sucrose, 25 mM Hepes-KOH buffer, pH-7.6, 2 mM EDTA). The leaves were homogenized twice in 5-sec bursts at full speed of a grinder homogenizer (locally made). The homogenate was strained through eight layers of cheese cloth to remove debris. The chloroplast was pelleted by centrifugation at 4000 × g for 1 min at 4°. The pellet was washed twice by centrifugation in the same medium. The resulting pellet contained 40 to 50% intact chloroplast, as estimated by phase-contrast microscope. The crude chloroplast was subjected to percoll gradients by layering successively 80%, 65%, 45%, 25%, and 10% (vol/vol) percoll in 50 mM Hepes-KOH pH-7.5, 0.33 M sorbitol. The chloroplast pellet that banded at 50% percoll region was resuspended in the above Hepes-sorbitol buffer and centrifuged at 1500 g for 15 min at 4°. Intact chloroplast migrated further down the gradient than lysed organelles. The intact chloroplast band was removed using a wide-bore pipette and mixed with five volumes of Hepes-sorbitol buffer. It was centrifuged at 4000 g for 2 min at 4°. The supernatant was discarded and the pellet was washed again with

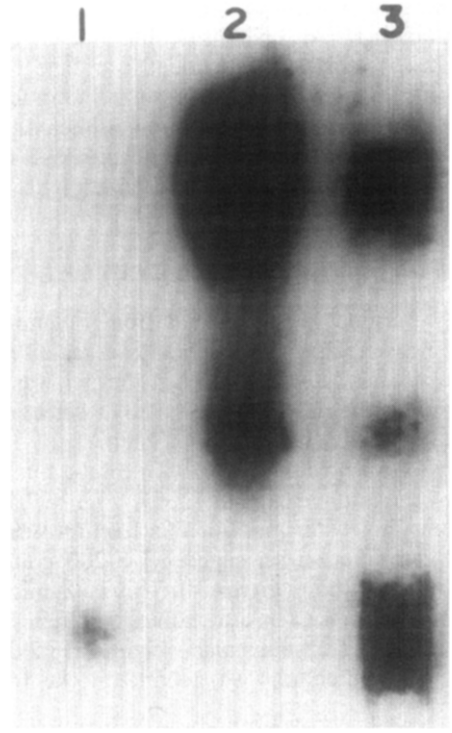

Figure 2 Substrate staining with catechol as substrate. Lane 1: PPO activity in unabsorbed fraction from DEAE-cellulose column (Fraction A). Lane 2: Total PPO activity eluted after absorption from the same column (Fractions B and C, combined). Lane 3: Crude extract. Aliquots were not matched for total enzymatic activities.

Hepes-sorbitol buffer to remove percoll from chloroplast preparation. The resulting pellet contained purified intact chloroplast, free of percoll.

Results

Purification of polyphenol oxidase

A summary of the purification process is provided in *Table 1*. Some of the salient features of the purification procedure are mentioned here. Treatment with cold acetone and subsequent extraction with polyvynyl polypyrolidone provided a convenient method of extracting the polyphenol oxidase from tea leaf, free of coloring materials. Triton X-100 (1.0%) was necessary for full recovery of activity from acetone powder extract. A battery of protease inhibitors were used prior to substrate staining to avoid nonspecific proteolysis of PPO activity. FPLC column at the final stage, resulted in several-fold purification. The enzyme at this stage was completely free of catalase and peroxidase activities.

Isozyme profile

Multiple forms of PPO in tea leaf have been reported from time to time either by ion exchange chromatography or by isoelectric focusing. Takeo and Baker[8] had earlier reported at least six forms of PPO activity with varying isoelectric points by isoelectric focusing. Sanderson[6] had also reported a large number of bands by the same technique. In contrast, in our hand repeated substrate staining experiments with the crude extract revealed the presence of only three bands for this activity. A typical profile is shown in *Figure 2*. Fractionation on DEAE cellulose column resulted in separation of these isoforms as is evident from lane 1 and lane 2 of the same figure. Lane 3 represents the crude extract showing the presence of three isoforms of the enzyme. It is likely, that addition of a battery of protease inhibitors allowed us to prevent nonspecific proteolytic cleavage giving rise to artifacts.

Molecular characterization

The highly purified PPO obtained from Fraction A was found to be essentially homogenous as revealed by native gel electrophoresis at pH-7.5. When the gel was scanned with catechol as the substrate, we failed to detect any other band with PPO activity (*Figure 3*. Panel A).

Electrophoresis on 7.5% SDS-PAGE of the final purified enzyme showed a single band of 72 kD. (*Figure 3*, Panel B). The apparent molecular mass of this fraction when determined by gel filtration on sephacryl S-200 column also turned out to be 72 kD (*Figure 4*). This isozymic form of PPO, therefore, seems to be a monomeric protein of 72,000 molecular weight. The molecular weight of Fraction B on the same calibrated column was recorded to be 164,000.

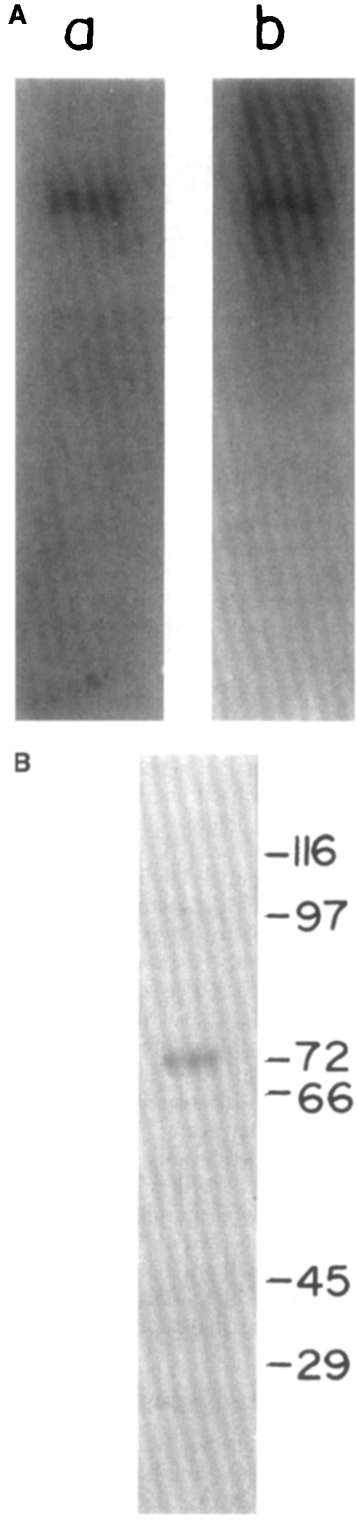

Figure 3 Purity of enzyme. Panel A. Lane a: Non-denaturing PAGE-silver staining band. Lane b. Non-denaturing PAGE-substrate staining. Panel B. 7.5% SDS-PAGE of purified protein with standard markers.

Substrate specificity of the enzyme

Polyphenol oxidase activity comprises of a broad range of activities that include diphenol oxidase or catechol oxidase (EC 1.10.3.2), laccase (EC 1.10.3.1), and often monophenol mono-oxygenase or tyrosinase (EC 1.14.18.1) activities. Most of these activities are widely distributed and often overlap in the same plant tissue or fungi.[10] To ascertain the exact nature of tea enzyme, the highly purified PPO activity was screened for substrate specificity against a variety of

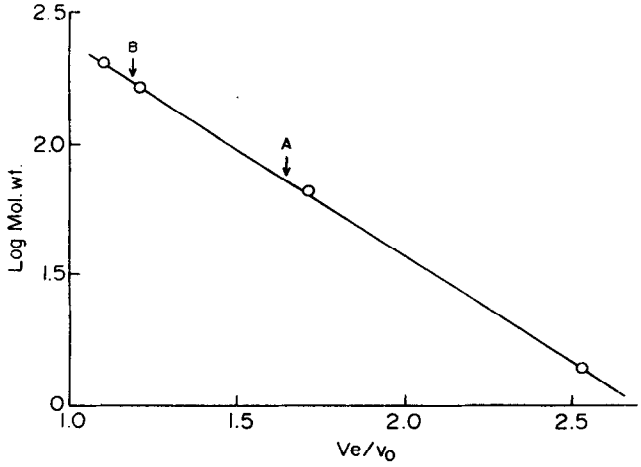

Figure 4 Determination of molecular weight of column.

mono and diphenols. The results are summarized in *Table 2*. Quite clearly, the enzyme is totally unable to utilize monophenols and *p*-diphenols as substrates but is very active against O-dephenols, including the tea flavonols. Interestingly, both pyrogallol (1,2,3 trihydroxy benzene) and gallic acid (2,3,4 benzoic acid) could be used as substrates with considerably reduced efficiency. Functionally, therefore, this enzyme should be regarded as catechol oxidase. Coogan et al.[9] had earlier reached similar conclusions from their studies with an enzyme fraction purified by isoelectric focusing.

The enzyme was optimally active at pH 5.0 (data not shown). Studies on K_m values clearly show that both catechin and epicatechin are preferred substrates when compared with other artificial substrates (*Table 2*). We had also determined the substrate specificity for the partially purified Fraction B of PPO. This enzyme also appears to be a catechol oxidase and is probably a true isoform of the purified form A.

Effect of activators and inhibitors

One intriguing property of PPO from some plant sources is its latency and activation by unusual treatments, such as

Table 2 Substrate specificity of purified polyphenol oxidase

Substrate	K_m (mM)	Relative activity (%)
Catechin	0.49	100
Epicatechin	0.81	95
Catechol	12.52	92
Pyrogallol	17.81	57
Gallic acid	19.33	40
p-Quinol	—	0
p-Cresol	—	0
Tyrosine	—	0

The incubation mixture contained in a total volume of 1 ml, 50 mmol/L of sodium citrate buffer (pH 5.0), an appropriate aliquot of purified enzyme and varying concentrations of the substrates. One hundred-percent activity corresponds to specific activity of 20 A/min/mg protein. For this purpose, the homogenous enzyme (Fraction A) was used.

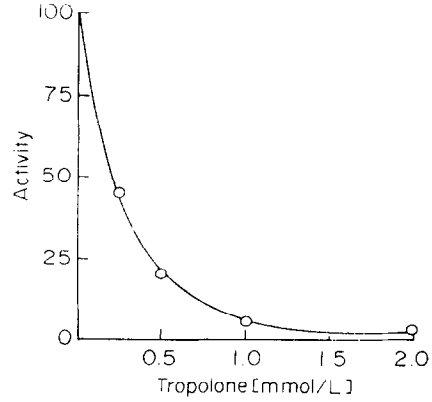

Figure 5 Inhibition of catechol oxidase activity with tropolone.

acid shock,[20] exposure to urea,[21] or SDS or other anionic detergents.[11,21] Careful analysis by Moore and Flurkey[11] with purified broad bean polyphenoloxidase has shown that a limited conformational change at the active site because of binding of small amounts of SDS is responsible for this activation process. Using both the highly purified enzyme and also the crude extract, we were unable to detect any activation of the enzyme in presence of SDS (0.1 to 5 mM) and urea (0.5 to 2 M).

When a series of potential inhibitors and activators of catechol oxidase[10] were tested for their effect on the activity of the enzyme only tropolone and phenylhydrazine were found to have profound inhibitory effects on the enzyme (data not shown). In a separate experiment we could demonstrate that inhibition of oxidase activity by tropolone takes place in a dose-dependent manner (*Figure 5*). Tropolone is known to be a powerful chelator of Cu^{2+}, suggesting the presence of Cu^{2+} in the active holoenzyme form of the enzyme. Extensive studies with catechol oxidase isolated from various plant and fungal sources had earlier shown the

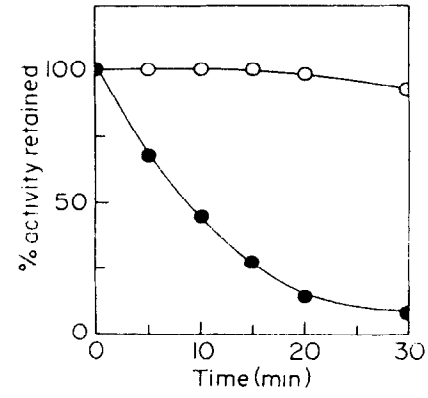

Figure 6 Effect of Trypsin on chloroplast polyphenol oxidase activity. After repeated freezing and thawing, purified chloroplast was incubated in a total volume of 0.5 mL, containing 175 μmol sucrose, 12.5 μmol Hepes buffer pH-6.1, 1 μmol EDTA, and trypsin (5 μg/mg chloroplast protein). At incubated time intervals, suitable aliquots were transferred to the same incubation mixture containing ten times more soyabeen trypsin inhibitor than trypsin. The enzyme activity was assayed after solubilization with Triton X-100 (1%). A control of chloroplast that did not undergo freezing and thawing was run parallely. (●----●): Freeze-thawed chloroplast, (○----○): control.

involvement of bound Cu^{2+} in the catalytic activity of the enzyme.[10,20]

Localization of polyphenol oxidase activity

Using purified chloroplast (see Methods and materials), we could demonstrate that the PPO activity is largely localized in the chloroplast. This activity could not be detected in absence of some detergent treatment. The PPO activity could be fully solubilized in presence of 0.7 to 0.8% Triton X-100 [data not shown]. Interestingly, after repeated freezing and thawing of the chloroplast preparation and subsequent treatment with a proteolytic enzyme, a progressive loss of PPO activity was observed. For this experiment, purified chloroplast was frozen at −20° and slowly thawed to 10°. The process was repeated thrice. The treated chloroplast was then incubated with trypsin for indicated time intervals and the reaction was stopped by addition of trypsin inhibitor in excess. In a typical experiment, after 30 min of incubation the enzyme lost 90% of its initial activity. In contrast, the purified chloroplast that did not undergo freezing and thawing but was exposed to trypsin retained most of its activity during this period (*Figure 6*). Quite clearly, polyphenol oxidase is localised inside the chloroplast and its catalytic site becomes accessible to trypsin only after the integrity of the plasma membrane is lost.

Discussion

The characteristic colour and brightness of black tea is largely dependent on the relative proportions of theaflavin and thearubigin.[22] The biotransformation of catechins and their gallates to these compounds is obviously a complex process and almost certainly involves participation of enzymatic and nonenzymatic reactions other than that carried out by polyphenol oxidases. To delineate the steps, it is imperative that each individual enzyme be purified, product characterized, and stoichiometry be established. It is anticipated that the convenient method of purification of one of the major forms of polyphenol oxidase reported here (*Table 1*) will be of considerable help in this direction.

The polyphenol oxidase in tea leaf is clearly a o-diphenol oxidase or catechol oxidase with distinct preference for catechin and epicatechin as substrates (*Table 2*). Catechol oxidase is supposed to play important roles in baking, in acceptability of yams or sunflower products, and in ensuring quality of wines.[10] In most of these cases the enzyme has only been partially purified. Recently, the enzyme has been purified from broad bean[23] and from sunflower seed.[24] Unlike the broad bean enzyme,[11] the tea enzyme could not be activated by SDS treatment (see text). As in the case of the tea enzyme (*Figure 3*), the sunflower enzyme is also monomeric (42 kD) but shows optimum pH at alkaline range. Like catechol oxidase from various other sources, this enzyme has several isozymic forms (*Figure 2*) in tea leaves. This enzyme, along with other housekeeping enzymes, can probably be used as a marker for identifying elite varieties of tea by isoenzyme profile analysis. Our biochemical demonstration of the chloroplastic localization of the PPO (*Figure 6*) is in conformity with earlier electron microscopic cytochemical observations[25,26] about the localization of the enzyme. Finally, like most other phenol oxidases studied from both fungal and plant sources, the tea enzyme also appears to be a Cu^{2+} enzyme, as is evidenced by its complete inhibition by tropolone (*Figure 5*). It will be interesting to study the micronutrient status of Cu^{2+} in relation to production and quality of tea.

References

1. Zongmao, C. (1995). Tea in 21st century. *Proceedings of '95 International tea-quality-human health symposium.* 3–6. Shanghai, China
2. Katiyar, S.K. and Mukhtar, H. (1996). Tea in chemoprevention of cancer: Epidemiologic and experimental studies (review). *Int. J. Oncology.* **8,** 221–238
3. Yang, C.S. and Wang Z.Y. (1993). Tea and cancer (review). *J. Nat. Can. Inst.* **85,** 1038–1049
4. Halder, J. and Bhaduri, A. (1997). Glycosidases from tea-leaf (*Camellia sinensis*) and characterization of β-galactosidase. *J. Nutr Biochem.* **8,** 378–384
5. Roberts, E.A.H. (1962). In *Chemistry of Flavonoid Compounds* (T.A. Geismann, ed), p. 468, Pergamon Press, Oxford
6. Sanderson, G.W. (1964). Extraction of soluble catechol oxidase from tea shoot tips. *Biochem. Biophys Acta.* **92,** 622–624
7. Gregory, R.P.F. and Bendall, D.S. (1966). Purification and some properties of polyphenol oxidase from tea. *Biochem. J.* **101,** 569–581
8. Takeo, T. and Baker, J.E. (1972). Changes in multiple forms of polyphenol oxidase during maturation of tea leaves. *Phytochemistry,* **12,** 21–24
9. Coggon, P., Moss, G.A., and Sanderson, G.W. (1973). Tea catechol oxidase: Isolation, purification and kinetic characterization. *Phytochemistry* **12,** 1947–1955
10. Mayer, A.M. (1987). Polyphenol oxidases in plants (review). *Phytochemistry,* **26,** 11–20
11. Moore, B.M. and Flurkey, W.H. (1990). Sodium dodecyl sulfate activation of a plant polyphenol oxidase. *J. Biol. Chem.* **265,** 4982–4988
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275
13. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem* **72,** 248–254
14. Tiselius, A., Hierten, S., and Levin, O. (1956). Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys* **65,** 132–140
15. Davis, B.J. (1964). Disk electrophoresis-II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci* **121,** 404–427
16. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage. *Nature* **227,** 680–685
17. Merril, C.R., Dunau, M.L., and Godman, D. (1981). A rapid sensitive sillver stain for polypeptides in polyacrylamide gels. *Anal. Biochem.* **110,** 201–207
18. Vanloon, L.C. (1971). Tobacco polyphenol oxidases: A specific staining method indicating non-identity with peroxidases. *Phytochemistry* **10,** 503–507
19. Robinson, C. and Barnett, L.K. (1988). *Plant molecular biology: A practical approach,* p. 67, IRL Press, Oxford
20. Solomon, E.I. (1981). In Copper Protein: *Metals Ions in Biology* (T.G. Spiro, ed.) p. 3, 41. Wiley, New York, NY USA
21. Lerch, K. (1981) In *Metal Ions in Biological Systems* (H. Sigel, ed.), p. 13, 143, Dekker, New York
22. McDowell, I. and Owuor, P. (1992). The taste of tea. *New Scientist* **11,** 30–33
23. Flurkey, W.H. (1989). Polypeptide composition and amino-terminal sequence of broad bean polyphenol oxidase. *Plant Physiol.* **91,** 481–483
24. Raymond J., Rakariyathan, N., and Azanza, J.L. (1993). Purification and some properties of polyphenol oxidases from sunflower seeds. *Phytochemistry* **34,** 927–932
25. Mayer, A.M. and Harel, E. (1979). Polyphenol oxidase in plants (review) *Phytochemistry* **18,** 193–215
26. Bar-Nun, N. and Mayer, A.M. (1983). Supression of catechol oxidase by norleucine in plant suspension cultures. *Phytochemistry* **22,** 1329–1333