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Glycosidases from tea-leaf (*Camellia sinensis*) and characterization of β-galactosidase

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*A wide range of glycosidase activities could be detected in the acetone powder extract of tea leaves of Assam variety (*Camellia sinensis*). β-galactosidase (EC3.2.1.23) was found to be the most active among these enzymes at fermentation temperature needed for black tea processing and at acidic pH. Substrate staining showed only one detectable band for this enzyme. A 137-fold purification over acetone powder extract through gel filtration, DEAE-cellulose chromatography, centripep, and HPLC resulted in a preparation that was electrophoretically homogeneous. Substrate staining revealed the absence of any isozymic form of this enzyme. Determination of molecular mass by two independent methods (i.e., by gel filtration and by HPLC) showed the enzyme to be 60 kDa. SDS-PAGE data along with molecular mass data showed the active enzyme to be a monomer of 60 kDa. The enzyme was optimally active at 50°C and at pH 4.0. K_m for lactose was 4.2 mmol/L. Monovalent and divalent cations had no effect on the activity of the enzyme. Galactose, a hydrolytic product of this enzyme was found to be a competitive inhibitor with a K_i of 2.4 mmol/L. Galactose (5 mmol/L) could provide partial, but significant, protection against thermal inactivation. Several thiol modifying agents at low concentrations could rapidly inactivate the enzyme, inactivation could be reversed with dithiothreitol. Significant protection against inactivation by methyl methane thiosulphonate (MMTS) could be observed in presence of galactose indicating the presence of an essential thiol at the active site of the enzyme. Presence of a wide range of glycosidases in tea leaves is indicative of their role in generating the characteristic flavor of black tea.* (J. Nutr. Biochem. 8: 378–384, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Tea (*Camellia sinensis*) is one of the most popular beverages consumed worldwide. Of the estimated 2.5 million metric tons of dried tea that are manufactured annually, nearly 75% is processed as black tea that are mainly consumed in the western world and in many eastern countries, including India. Considering its wide consumption and great commercial value, considerable work has been performed on tea as a health beverage and its constituents as pharmacological entities. Most of this work has been done with green tea and catechin derivatives present therein, the major polyphenolic flavonol constituents of tea leaves. These results have been reviewed periodically.[1–4]

Characteristic color and flavor are the two most important desirable attributes of black tea. Out of the various stages of black tea processing, the so–called fermentation step is the most critical. In this step, after mechanically disrupting the integrity of green tea shoot and leaves, the constituents are allowed to undergo enzymatic biotransformation for several hours in presence of mild aeration and at slightly elevated temperature (32°C ± 3°). Oxidation and partial polymerization of catechin by various polyphenol oxidases, peroxidase, etc. result in formation of bisflavonols

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like theaflavin, thearubigins, and higher oligomers that give the characteristic color and taste for black tea. Considerable work has been performed toward characterization of the polyphenol oxidases.[5–7] Enzymatic biotransformations that lead to the characteristic flavor of black tea, however, remains to be elucidated. Glycosidic derivatives of garaniol have been isolated from Oolong tea[8] and a mixture of volatile monoterpene alcoholic aroma constituents could be shown to be liberated on treatment of nonvolatile material from tea leaves with mixtures of glycosidase preparation added from outside.[9] It is, therefore, likely that various types of glycosidases that may be present in tea leaves can act on nonvolatile mono- or oligoglycoside constituents to generate volatile aglycones that contribute to the characteristic flavor or aroma of black tea. In spite of their obvious importance in generating flavor, no work has so far been reported on the detection and subsequent purification and characterization of various glycosidases that may be present in green tea. Such studies may be useful to improve flavor attributes of black tea by appropriately manipulating the conditions for fermentation process. In this paper, we report that a wide spectrum of glycosidases occur in tea leaves. Further, purification and extensive characterization of β-galactosidase, a major glycosidase of tea leaves has been reported here. It is anticipated that detailed characterization of the various glycosidases will help in manipulating fermentation conditions for production of black tea with desirable aroma.

Methods and materials

Chemicals and reagents

Fresh tea leaves (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

The glycosidase activities were estimated by measuring the release of p-nitrophenol (pNP) from their respective glycosides as substrates, following the method of Michel et al.[10] in a total volume of 0.5 mL, the reaction mixture contained 100 mmol/L sodium acetate buffer, pH 4, 5 mmol/L of appropriate substrate, and an aliquot of the enzyme. Incubation was for 15 min at 32°C. The reaction was stopped by adding 2.0 mL of 0.1 mol/L NaOH and the absorbance of released pNP was measured at 410 nm. Any change in incubation temperature or pH has been indicated in the text. Because of the uncertainty in molar extinction coefficient of pNP, 1 absorbance was taken as 1 unit of enzyme activity. For purified β-galactosidase, lactose was also used as the substrate. In this case, the released glucose was measured stoichiometrically by taking appropriate aliquots with hexokinase-glucose-6-phosphate dehydrogenase system.

Protein estimation

Protein was routinly estimated by the method of Lowry et al.[11] At later stages of purification when concentration of protein was very low, the method of Bradford[12] was used.

Purification of β-galactosidase

Around 100 gm of fresh tea leaves were homogenised with 80% ice-cold aqueous acetone solution using polytron homogenizer for 2 to 3 min. 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 it completely moisture-free and dry. The dried powder was stored at −20°.

Five grams of acetone powder, along with same amount of polyvinyl-polypyrolidone was taken in a mortar. This was homogenized with 50 mL ice-cold buffer A containing 50 mmol/L K-phosphate buffer, pH 7.0, 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA. The homogenate was strained through two layers of cheese cloth and was then centrifuged at 12,000 × g for 10 min to remove the remaining cell debris. This supernatant was used as the starting material for assay of various glycosidases and also for purification of β-galactosidase activity. Unless otherwise indicated, all subsequent operations were performed at 4°C.

Solid ammonium sulphate was added slowly to the extract with constant stirring to obtain 40% saturation. The mixture was stirred for an additional 30 min and left for 1 hr. The precipitate was removed by centrifugation. The supernatant was further fractionated by adding more solid ammonium sulphate to 60% saturation and left for 2 hr. The precipitated protein containing β-galactosidase activity was collected by centrifugation and dissolved in 2 mL of buffer A. This was applied on a Sephacryl S-200 column (1.0 × 85 cm), earlier equilibrated with buffer A. The fractions having most of the enzyme activity (tube nos. 18 to 26, each containing 2.5 mL) were combined for further purification (*Figure 1*). The total volume of 22.5 mL was reduced to 3.5 mL by passage through a centripep-30 filter (amicon). The concentrated filtrate was applied to a DEAE cellulose column (1.0 × 5.0 cm) equilibrated with 50 mmol/L K-phosphate buffer, pH 7.0. The column was washed with the same buffer and then eluted with a linear gradient of KCl in the buffer. The β-galactosidase activity came out unabsorbed from the column and was pooled together to a volume of 3.0 mL and concentrated to 0.5 mL by passage through a centricon-30 filter (amicon). α-Galactosidase, on the other hand, was absorbed and eluted between 0 and 0.5 mol/L KCl linear gradient (data not shown).

The concentrated and partially purified fraction was passed through an LKB ultropac, TSK G 3000 SW size exclusion HPLC column (0.75 × 30 cm) equilibrated with 25 mmol/L K-phosphate buffer, pH 7.0. The flow rate was 0.5 mL/min. The highest activity fractions of total volume, 1.5 mL was concentrated to 0.2 mL and was subjected to rechromatography on the same column. A single peak was obtained with 137 fold overall purification. It was stored at −20°C and was stable for several months.

Molecular weight determination

The apparent molecular weight was determined by two independent methods. The sephacryl-S-200, column on which the enzyme was purified after ammonium sulphate fractionation was earlier calibrated with aldolase (Mr. 158,000), bovine serum albumin (Mr. 66,000), ovalbumin (Mr. 45,000) and cytochrome c (Mr. 12,400) as marker proteins. Size exclusion HPLC column was also calibrated with myosin (Mr. 205,000), phosphorylase b (Mr. 97,000), bovine serum albumin (Mr. 66,000), ovalbumin (Mr. 45,000), and carbonic anhydrase (Mr. 29,000) as standards.

Electrophoresis

Polyacrylamide gel electrophoresis and SDS-gel electrophoresis were performed following the methods of Davis[13] and Laemmli,[14] respectively. Silver staining was performed according to Merril et al.[15]

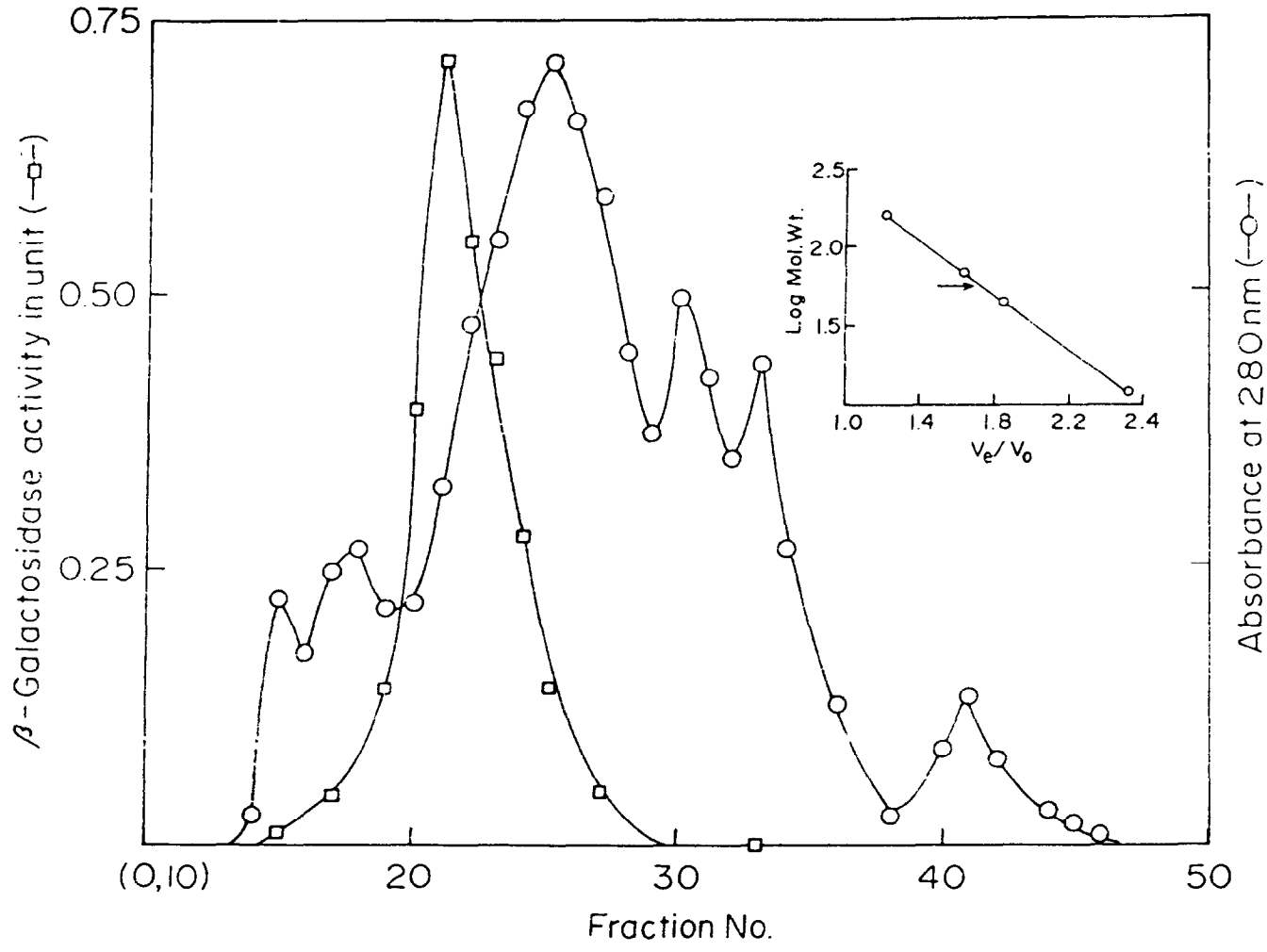

Figure 1 Elution profile on sephacryl S-200 column of β-galactosidase activity. A 2.0 mL solution of the 40 to 60% ammonium sulphate fraction containing 12.5 mg of protein was applied to the column (1.0 × 85 cm). The column was washed with K-phosphate buffer, pH-7.0.–○–○–, absorbance at 280 nm, –□–□– β-galactosidase activity. Inset, Molecular weight calibration curve. Elution position of native β-galactosidase has been marked by arrow in the curve.

Substrate staining

Crude extract was concentrated (10 times) and allowed to run on cellulose-acetate (CA) paper for 45 min, as described by Evans.[16] After electrophoresis, the CA plate was immersed in 10 mL of the staining solution containing 50 mmol/L sodium acetate buffer, pH 5.0, and 1 mmol/L 4-methylumbelliferyl, β-D-galactopyranoside. The incubation time was 30 min at 37°C. The plate was removed from the staining solution and dried. Activity band for β-galactosidase was observed under a UV light (long wave) source.

Results

Spectrum of glycosidase activities in tea leaves

To study various types of glycosidase activities that are presumably present in tea leaves, the acetone powder extract of freshly plucked tea leaf (see Methods and materials) was subjected to activity analysis with appropriate substrates at both acid and alkaline pH and also at fermentation (32°C) and at elevated (45°C) temperatures. Glycosidases from various sources are often known to have optimum pH at acidic range. During the processing of green leaf for manufacture of black tea, the oxidative and hydrolytic enzyme activities are initiated by cell rupture through rolling or through crush, tear, and curl (CTC) that presumably shifts the pH to the acidic region. *Table 1* shows that both α- and β-galactosidase activities are very high at pH 4.0 compared with the activity of all the other enzymes tested. Excepting for β-galactosidase, all other glycosidases show significantly lower activity at more elevated temperature (45°C). Interestingly, glucosidase activities are quite low both at pH 4 and pH 7.4. When assayed at its optimum pH of 6.1 and at 37°C, the enzyme showed activity that is comparable to the activity of β-galactosidase at pH 4.0 (data not shown). This preliminary analysis reveals that tea leaves contain a battery of glycosidases that in combination are probably fully capable of releasing volatile flavoring compounds from complex glycosides. For the present work, however, we restricted ourselves to the purification and

Table 1 Spectrum of glycosidase activities in the tea leaf in acidic and alkaline pH at 32°C and 45°C

Substrate (5 mmol/L)	pH 4.0		pH 7.4	
	32°C	45°C	32°C	45°C
pNP-α-D-glucose	0.21	0.12	0.19	0.04
pNP-β-D-glucose	0.12	0.03	0.16	0.06
pNP-α-D-mannose	0.56	0.06	0.17	0.33
pNP-β-D-mannose	0.04	0.02	0.18	0.07
pNPα-D-galactose	1.12	0.56	0.42	0.06
pNP-β-D-galactose	1.28	1.60	0.10	0.15
pNP-β-D-fructose	0.31	0.11	0.16	0.02

The incubation mixture contained in a total volume of 0.5 ml, 100 mmol/L of sodium acetate buffer (pH 4) or 100 mmol/L of potassium phosphate buffer (pH 7.4), 5 mmol/L of indicated substrate and 50 μl of crude extract, 0.8 mg protein ml^{-1}. Incubation was carried out for 20 mins at the above indicated temperature and activity was expressed as absorbancy at 410 nm.

characterization of β-galactosidase that showed considerable activity at acidic pH and enhanced activity at 45°C, which seemed to be enzymologically interesting.

Purification of β-galactosidase

Some of the salient features of the purification procedure are mentioned here. Details have been given in the Methods and materials section. A summary of the purification process is provided in *Table 2*. Treatment with cold acetone and subsequent extraction with polyvinyl-polypyrolidone provided a convenient method of extracting the glycosidases from tea leaves free of coloring materials. This procedure was used earlier successfully for purifying polyphenol oxidases from tea leaves.[5,6] The β-galactosidase activity that came out unabsorbed from the DEAE-cellulose column was separated completely from α-galactosidase activity at this step. Size exclusion HPLC resulted in several-fold purification and rechromatography on this column yielded a pure β-galactosidase fraction that was free from all glycosidase activities.

Molecular characterization

The purified β-galactosidase was apparently homogenous (>95%) as revealed by native gel electrophoresis both at 5.5 and 7.5 (data not shown). The apparent molecular mass was

Table 2 Purification of β-Galactosidase. The activity of the enzyme was measured at pH 4 and temperature 32°C

Steps	Total activity (A/min)	Total protein (mg)	Specific activity (A/min/mg)	Purification (fold)
Acetone powder extract	1500	45.00	33.33	1.00
40–60% A.S. precipitation	1200	12.50	96.00	2.88
Sephacryl S-200	1090	5.20	209.62	6.29
Centripep-30	1000	1.96	510.20	15.31
DEAE cellulose	675	1.00	675.00	20.25
HPLC	622	0.30	2073.33	62.21
Rechromatography on HPLC	550	0.12	4583.33	137.51

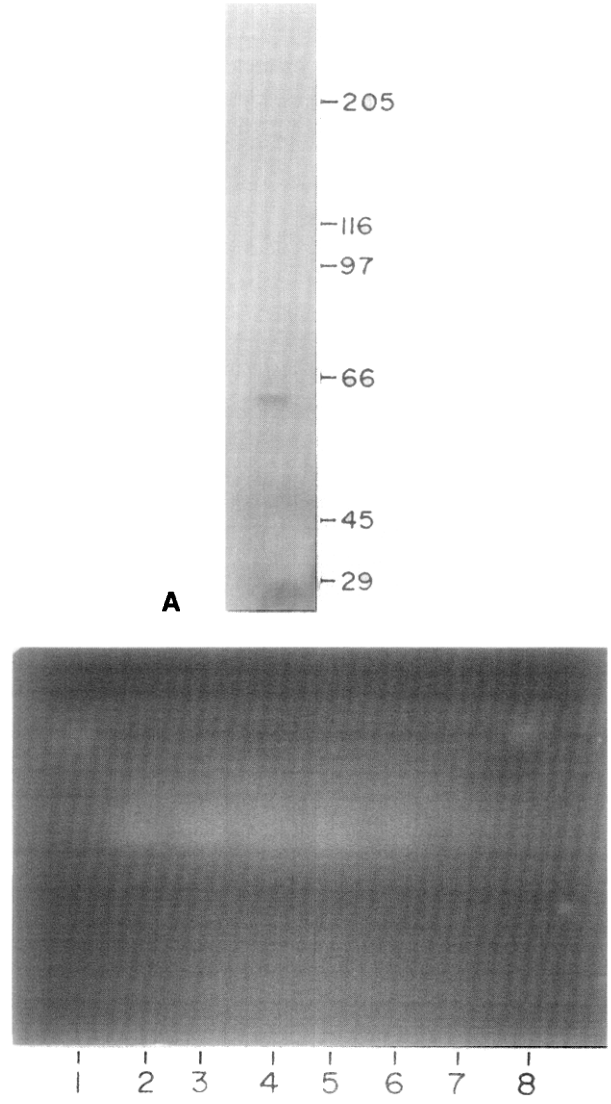

Figure 2 *A*, SDS-PAGE of purified protein (7.5%) *B*, Substrate staining with 4-methyl umbelliferyl β-D-galacto-pyranoside as substrate. For details, see experiment. *Lanes* 1 and 8 are *E. coli* β-galactosidase, in sodium acetate buffer, pH-5.0. *Lanes* 2–6, five μL of the acetone powder extract from strains TV1, TV25, TV2, TV18, and TV20, respectively, in the same buffer and *lane* 7 without any acetone powder extract.

determined both by gel filtration on Sephacryl S-200 column of the ammonium sulphate fraction obtained during purification (*Figure 1*), and also by size-exclusion HPLC method of the purified enzyme (data not shown). The apparent molecular weights determined by these methods were 59,000 and 60,500, respectively. The excellent agreement between the two values shows the β-galactosidase to have an apparent molecular weight of approximately 60,000. Electrophoresis on 7.5% SDS polyacrylamide gel of the final purified enzyme showed only one band of 60 kDa (*Figure 2A*), indicating the purified β-galactosidase to be a monomer of 60 kDa.

To detect the possible presence of any other isozyme form of the enzyme, the acetone powder extract was subjected to analysis by substrate staining. Using umbel-

liferyl β-galactoside as the substrate, the native gel that was run at pH 5.0 (100 mmol/L sodium acetate buffer) was scanned for the possible presence of isozymes of the enzyme. Only one single fluorescence band could be detected (*Figure 2B*) eliminating the presence of any other form of the enzyme. *Lanes* 1 and 8 are *E. coli* β-galactosidase (Sigma) used as controls. This enzyme has optimum pH at alkaline pH, but is fairly active at pH 5.0. This result suggests strongly that unlike for β-galactosidase from some other plant sources[17] only one form of β-galactosidase is expressed in tea-leaf.

Kinetic characterization of the enzyme

The highly purified β-galactosidase from tea leaves does not show even trace activity for other glycosidases. The enzyme has a broad substrate specificity. When assayed at pH 4 and at 32°C, it could hydrolyze both *p*-nitrophenyl β-D-galactoside (pNP-gal) and *o*-nitrophenyl-β-D-galactoside with K_m of 0.53 mmol/L and 2.7 mmol/L, respectively. Lactose is not a natural substrate in plant system. It could, however, be hydrolyzed with a K_m of 4.2 mmol/L and the specific activity under these conditions was recorded to be 5 μmol min^{-1} mg^{-1} protein.

Because the enzyme was very active at acidic pH and at unusually high temperatures, we decided to monitor its temperature and pH profile. The enzyme shows its highest activity at 50°C. It has an optimum pH at 4.0 and is very weakly active at alkaline pH.

Effect of potential inhibitors and modulators

A series of potential inhibitors and modulators were tested for their effect on the activity of the enzyme. Except for Hg^{2+}, none of the monovalent or divalent cations that were tested had any effect. Hg^{2+} is often a potent inhibitor of thiol groups in proteins. All the three specific thiol-modifying reagents [i.e., *p*-chloromercuribenzoate (pCMB)], O-iodosobenzoate and methyl methane thiosulphonate (MMTS) had profound inhibitory effects on the enzyme, strongly suggesting the essential role of one or more thiols in the overall activity of the enzyme. 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) could not be used for these studies because of overlap of generated colors. Interestingly, galactose, an obligatory product of β-galactosidase activity had strong inhibitory effect at high concentrations. Galactose was found to inhibit the β-galactosidase activity competitively. *Figure 3* presents a Dixon plot[18] of the obtained data. K_i for galactose was calculated to be 2.4 mmol/L. For this assay pNP-gal was used as the substrate.

Thermal stability of the enzyme. Because the β-galactosidase was found to be maximally active at 50°C, its stability at elevated temperatures was investigated. *Figure 4* summarizes results of such a study. The enzyme is fully stable at 50°C for 60 min, but rapidly looses its activity beyond 60°C. Thus, at 70°C, it loses nearly 90% of its activity within 5 min. Interestingly, the presence of galactose, a competitive inhibitor for the enzyme as shown earlier, and hence a ligand for the active site, could slow down significantly the rate of inactivation of the enzyme

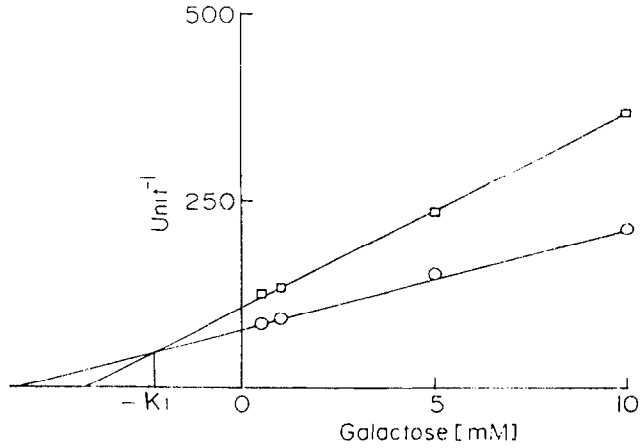

Figure 3 Determination of K_i (competitive inhibition). Enzyme samples in 100 mmol/L acetate buffer, pH 4.0 were incubated for 10 min. with different concentrations of galactose in the presence of 1 mmol/L pNP-gal. Activities at various concentrations of inhibitor were assayed (1 absorbance = 1 unit).

both at 60°C and at 70°C. Quite clearly, the active site is partially protected against thermal inactivation in the presence of galactose.

Presence of essential thiol in β-galactosidase

We had already observed that the enzyme rapidly lost its activity in presence of various thiol modifying reagents. Pursuing this further, we could demonstrate that the activity could be restored completely under appropriate conditions. Thus, the pCMB-inactivated enzyme (0.1 mmol/L, incuba-

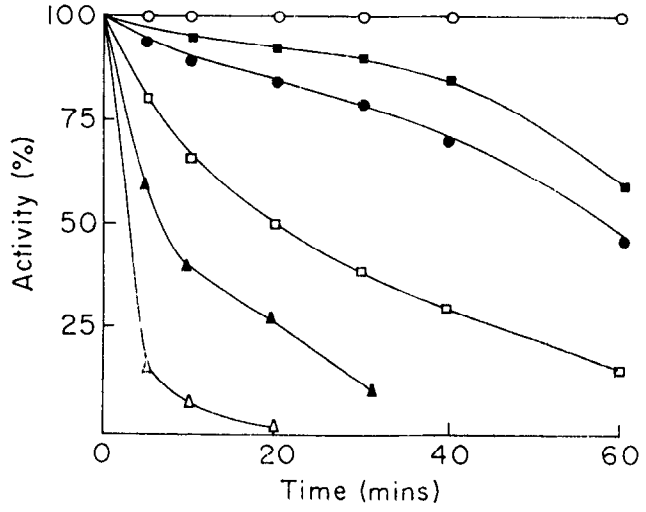

Figure 4 Thermal stability in presence of galactose. Enzyme samples in 100 mmol/L sodium acetate buffer, pH 4.0, were preincubated at 50°C, 60°C, and 70°C for different time periods. Suitable aliquots were then transferred to assay buffer and assayed for activity by adding pNP-gal as substrate. Activities at various times are expressed as a % of the appropriate zero time control. -○-○- indicates activity at 50°, -□-□- at 60°, -▷-▷- activity at 70°C, -●-●- activity at 60°C in the presence of galactose (1 mmol/L). -■-■- activity at 60°C in the presence of galactose (5 mmol/L) -▲-▲- activity at 70°C in the presence of galactose (5 mmol/L).

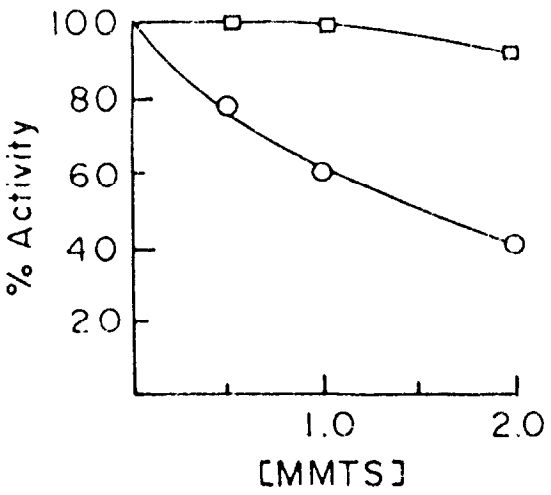

Figure 5 Protection of MMTS inhibition by galactose. Enzymes samples were incubated in 200 μL of 50 mmol/L K-phosphate buffer, pH 8.0, for 5 min in different concentrations of MMTS and in presence or absence of galactose (5 mmol/L). Suitable aliquots were then transferred to the assay mixture, containing in a total volume of 0.5 mL, 50 μmol of sodium acetate buffer, pH 4.0, 0.5 μmol of pNP-Gal and then activity was assayed. (–O–O– activity expressed in the absence of galactose; –□–□– activity in the presence of 5 mmol/L galactose).

tion for 8 min) could completely (100%) recover its activity when subsequently incubated with dithiothreitol (20 mmol/L) for 30 min (data not shown). MMTS methylates reactive thiol groups and stimulates minimal steric changes on the protein surface.[19] Inactivation of the enzyme by MMTS could also be reversed by dithiothreitol. The reactive thiol(s) appears to be located in the vicinity of the active site. This became evident when the enzyme was exposed to varying concentrations of MMTS both in the presence and absence of galactose. Because MMTS is active at alkaline pH, the inactivation with MMTS was performed at 50 mmol/L K-phosphate buffer, pH 8.0, although the activity of the enzyme was finally measured at pH 4.0 by transferring suitable aliquots. *Figure 5* shows that galactose (5 mmol/L) provides nearly complete protection against modification by MMTS, indicating the presence of the thiol group at the active site of the enzyme.

Discussion

The generation of desirable color and flavor in black tea is a complex process. Enzymatic biotransformations that lead to such properties are very little understood. Theaflavin and thearubigins are complex polyphenols that are generated from catechins in large quantities during the fermentation process and are assumed to be responsible for the characteristic color and briskness of black tea.[2] Even in this case the exact enzymatic steps that are involved remain to be elucidated. Except for linalool and geraniol,[9] most of the flavor generating water-soluble constituents have not yet been chemically identified and characterized. Our study clearly demonstrates that the battery of glycosidases that are present in tea leaves are probably capable of generating volatile flavoring constituents from their nonvolatile glycoside precursors (*Table 1*). In view of the varying temperature and pH profile of these enzymes, the determination of optimum condition for fermentation may be a difficult task. Characterization of each of these enzymes in their purified form is probably a prerequisite to get a better understanding of the fermentation process.

β-galactosidase from tea leaves shows some interesting enzymological properties. The unusual thermal stability (*Figure 4*) is broadly reminiscent of the properties of the enzyme, as isolated from jack-bean. The jack-bean enzyme was found to be stable at 60°C, but rapidly lost its activity at 65 to 70°C.[20] Unlike the case of germinating *Vigna* seeds[17] and *Petunia* shoot tips,[21] the tea leaf does not show the presence of any isozyme form in detectable quantity (*Figure 2B*). The possibility of proteolytic cleavage for these earlier observations can not be ruled out totally. Strong competitive inhibition by galactose (*Figure 3*), a product of this enzymatic reaction, is an interesting feature of this enzyme. The tea β-galactosidase seems to have an essential thiol at the active site. Sensitivity to various thiol reagents and regeneration of full activity with dithiothreitol (see text) is indicative of a critical role of thiol in the overall catalytic function. Significant protection provided by galactose against chemical modification by MMTS (*Figure 5*) further suggests that the thiol is located at the active site. Apart from their putative role in biotransformations for generation of flavor in black tea, these enzymes along with polyphenol oxidase and other housekeeping enzymes can be useful for zymodeme analysis to identify elite clones of tea being generated by agronomic techniques. Our laboratory is presently involved in such analysis.

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