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Influence of Genotype and Manufacturing Process on the Activity of β-D-Glucosidase and β-Galactosidase in Tea

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Abstract: Tea flush shoots comprising three leaves and a bud were harvested from 20 different cultivars planted in a single area of UPASI Tea Research Farm and analyzed for β-D-glucosidase and β-galactosidase activities. Among the cultivars studied a range of variability of 0.63 to 5.45 units of β-galactosidase activity and 0.06 to 1.91 units of β-D-glucosidase activity was observed. This result suggested the influence of genotype on these enzymes. The activity was higher in actively growing tea tissues (bud) and lowest in bark of the tea plant. At any given stage of black tea manufacture β-galactosidase activity was higher than that of β-D-glucosidase. During manufacturing process the activity of both enzymes varied widely and remained maximum in withered leaves. The insignificant correlation coefficient obtained for these two enzymes with productivity suggest that they cannot be used as one of the biochemical tools to predict the yield potential of tea cultivar. The flavour analysis carried out in green leaf, withered leaf, cut dhool, fermented dhool, made tea and tea brew indicated that not even a single flavour compound was found maximum in green leaf. This provides important information that most of the flavour compounds are produced after harvest.

Key words: β-D-glucosidase, β-galactosidase, black tea manufacture, flavours

INTRODUCTION

Phytochemists have identified about 7000 plant chemicals, many thousands of which are glycosylated (Williams and Harborne, 1994). The glycosylated compounds are often considered as transportable storage compounds or detoxification products assumed to lack physiological activity. The *o*-glycoside hydrolases are widespread group of enzymes of significant biological, biomedical and industrial importance. Among the 82 families of glycoside hydrolases (Henrissat, 1991; Henrissat and Davis, 1997), β-glycosidases have been the subject of importance because of its role in numerous biological processes including flavour enhancement in beverages (Gunata *et al*., 1993). As far as tea is concerned, the enzymes glucosidase and galactosidase play a key role in breaking the glycoside forms of several important aroma compounds of black tea. Several glucosides have been isolated and reported as tea aroma precursors in fresh tea leaves and have been identified as β-D-glucopyranosides, 6-*o*-β-D-xylopyranosyl-β-D-glucopyranosides, 6-*o*-β-L-arabinopyranosyl-β-D-glucopyranosides and 6-*o*-β-D-apiofuranosyl-β-D-glucopyranosides with aglycons of (Z)-3-hexenol, geraniol, linalool and four linalool oxides, benzyl alcohol, 2-phenylethanol and methyl salicylate. The enzymes glycosidases were characterized by Halder and Bhaduri (1997) from tea leaves. Most of the works on glucosidase and galactosidase were limited to manufacturing stages of black tea. There is hardly any published data on distribution of these two enzymes over tea plant, hence we made an attempt to study the

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distribution of these enzymes in various parts of the harvestable shoots and also tea plant. Earlier the activity of nitrate reductase which involves in nitrogen metabolism was proved as the productivity index of tea, under south Indian conditions (Venkatesan and Ganapathy, 2004; Venkatesan, 2005). Similarly the current study aims to check the feasibility of using glucosidase and galactosidase activity as biochemical parameter to predict tea productivity, basically because these two enzymes are involved in carbohydrate metabolism. The varieties of cultivars planted in south India belong to three different species such as *Comellia sinensis* (L.) O. Kuntze, *Comellia assamica* (Masters) Wight and *Comellia assamica* spp. *lasiocalyx* (Planch. Ex. Watt) Wight and their hybrids. However, their bio-chemistry with reference to enzymology has not yet been studied. Since an understanding in this field would be helpful in clonal selection and breeding programmes in future, an attempt has been made to study their genotypic variation towards the enzyme β-D-glucosidase and β-galactosidase. The other objective includes 1) determination of these enzymes in various tea cultivars 2) documentation of changes in activity in various steps of tea manufacture and 3) to find out the relationship between these two enzymes in tea leaves.

MATERIALS AND METHODS

Tea shoots consisting of three leaves and a terminal bud were harvested from twenty different tea cultivars including one Assam seedling located in a single area at UPASI Experimental Farm to estimate β-D-glucosidase and β-galactosidase activity. The enzyme assay was repeated for five consecutive harvests during the year 2005. The fields received uniform quantities of nutrients through fertilizers and were kept under similar cultivation practices as per the standard recommendation of UPASI (Verma and Palani, 1997; Venkatesan, 2004) throughout the year 2005. The samples were analyzed for enzyme activities using the method described below.

Preparation of Enzyme Acetone Powder

Around 20 g of fresh tea leaves (three leaves and a bud) were homogenized with ice cold aqueous acetone (800 mL L^{-1}) and the suspension was filtered on sintered funnel (G-1) and then washed repeatedly with ice cold acetone of same concentration until colourless (Halder and Bhaduri, 1997). Finally it was washed with acetone to make it completely moisture free and dry. The dried powder was stored at -20°C.

Preparation of Crude Enzyme Solution

To 0.8 g acetone powder, 0.4 g polyclar AT, 20 mL of 50 mM sodium citrate buffer (pH-5.0) were added. The mixture was centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was used as the crude enzyme solution for the assay of β-D-glucosidase. For β-galactosidase sodium acetate buffer (pH-4.0) was used.

Assay of β-D-glucosidase

Substrate (*p*-NP-β-D-glucopyranoside) was prepared in 50 mM sodium citrate buffer pH-5.0. Exactly 200 µL of the substrate was incubated at 37°C for 5 min. After that 100 µL of enzyme was added and the reaction mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 1.4 mL of sodium carbonate solution (0.2 M). The resulting yellow colour was measured at 420 nm by a spectrophotometer using *p*-nitrophenol as standard.

Assay of β-galactosidase

The substrate (*p*-NP-β-D-galactopyranoside) was prepared in sodium acetate buffer (pH-4.0). To 0.25 mL of the substrate, 0.25 mL of enzyme solution was added and incubated it for 15 min at

32°C. The reaction was stopped by adding 0.1 M sodium hydroxide solution and the yellow colour was read at 410 nm by a spectrophotometer using *p*-nitrophenol as standard.

Sampling for Activities at Different Plant Parts

Samples of harvestable shoot, twig, bark and feeder roots were collected from clone UPASI-9 from the same field. The harvestable shoots were separated into bud, 1st, 2nd, 3rd leaf and 1st internode (stalk between 1st and 2nd leaf) and 2nd internode (stalk between 2nd and 3rd leaves). The activity of enzymes was assayed using the procedure mentioned above. The same procedure was repeated for five times during the year (2005) at regular intervals.

Sampling for Activity in Various Stages of Manufacturing

Fresh tea leaves comprising three leaves and a bud were collected from clone UPASI-9 and processed into different stages of manufacturing as outlined in Fig. 1. The fresh leaves were spread on a net indoors and allowed to wither naturally for 18 h at ambient temperature. At the end of this process a small portion of sample was drawn and assayed for enzyme activity. The remaining sample was processed through cutting operations, where the samples were cut four times using CTC (Crush, Tear, Curl) rollers. At the end of this process samples were drawn for assaying enzyme activities. The remaining cut leaves were processed through fermentation at 27°C for 60 min. At this stage also samples were drawn and enzyme activity was assayed. The fermented tea was fired further at 100°C to make black tea from which the samples were collected for enzyme assays.

Extraction and Analysis of Volatile Flavour Compounds (VFC)

The samples drawn as green leaf, withered leaf, cut dhool, fermented dhool, made tea and brew at various stages of manufacturing were extracted with dichloromethane using simultaneous distillation extraction (SDE) apparatus (Yamanishi *et al*., 1989). About 25 g of the above samples were placed in single neck round bottom flask (1000 mL) and covered with distilled water (250 mL) at 70°C. This was placed in heated mantle (100°C) and linked to one arm of the SDE apparatus, whilst a small flask (250 mL) which contained 50 mL dichloromethane at 40°C (in a water bath) was simultaneously connected to the other arm of the apparatus. Along with the above connections a small volume of dichloromethane (10 mL) was added into the U-tube located in the middle of the apparatus. Distillation was limited to 1 h. The condensate of dichloromethane was dried with anhydrous sodium sulphate and a small aliquot of this concentrate was used for VFC analysis.

The analysis was carried out using a Gas Chromatograph (Perkin Elmer Autosystem XL) using ethyl caproate as internal standard. About 1 μL of the extract was injected and Flame Ionization

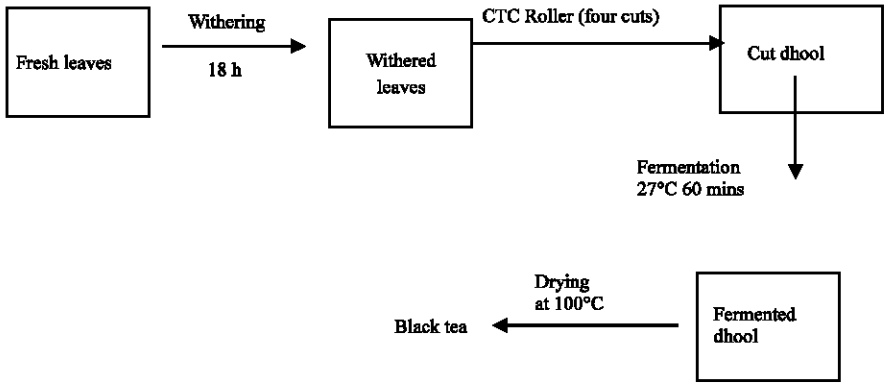

Fig. 1: Schematic diagram of CTC black tea manufacture

Detector (FID) was used for detection. The temperature of injector and FID were 200 and 250°C, respectively. A 60 m X 0.25 mm i.d capillary column was used. The initial temperature of the column was 50°C for 2 min and increased at the rate of 5°C per min to 180°C, thereafter maintained at the same temperature (180°C) for 15 min. Nitrogen was employed as the carrier gas with a flow rate of 1 mL per min. The compounds of the sample were identified in comparison to their GC retention times with those of authentic chemicals (Sigma). The identified flavours were expressed in terms of percentage of their relative distribution with respect to ethyl caproate.

RESULTS AND DISCUSSION

Activity in Different Cultivars

The β-D-glucosidase activity varied between 1.91 and 0.06 units (the cultivar UPASI-7 and UPASI-22, respectively), among the cultivars studied (Table 1). The clones UPASI-17, UPASI-6 and UPASI-1 bagged the first three ranks of β-D-glucosidase activity while it was UPASI-23, UPASI-21 and UPASI-22 which showed the minimum activity (last three ranks). A correlation between the mean annual productivity recorded during the past four years (Table 1) and the β-D-glucosidase activity resulted in insignificant correlation coefficient ($r = 0.241$, not significant, degree of freedom (n) = 19). The correlation was done to find out whether the β-D-glucosidase activity can be regarded as the productivity index of tea. The insignificant correlation coefficient suggests that it cannot be regarded as productivity index of tea, under south Indian conditions.

The tea cultivars showed a significative variability for β-galactosidase activity. The cultivars UPASI-14, UPASI-2 and UPASI-10 present the highest enzyme activity while the cultivars UPASI-21, UPASI-3 and the Assam seedling had the lowest activity (Table 1). Among the cultivars, a range of variability of 0.63 to 5.45 units of enzyme activity (cultivars UPASI-14 and Assam seedling, respectively) was observed. Activity of β-galactosidase also gave insignificant correlation coefficient with productivity levels ($r = -0.230$, not significant, $n = 19$).

Table 1: β-D-glucosidase and β-galactosidase activities (μ moles min^{-1} g^{-1} acetone powder) in harvestable tea shoots of a few tea cultivars

			β-D-glucosidase		β-galactosidase	
Clones	Variety	Mean made tea yield	Activity in (μ moles min^{-1} g^{-1} acetone powder)	Rank	Activity in (μ moles min^{-1} g^{-1} acetone powder)	Rank
Assam seedling	Assam	3000	1.28±0.030	11	0.63±0.026	19
UPASI-1	Assam	2000	1.70±0.021	3	2.97±0.029	8
UPASI-2	Assam	3000	1.25±0.040	12	5.37±0.025	2
UPASI-3	Assam	4600	1.67±0.012	4	0.95±0.035	18
UPASI-6	Assam	4748	1.63±0.051	6	1.24±0.012	17
UPASI-7	Assam	2800	1.35±0.040	10	1.81±0.045	12
UPASI-8	China	2200	0.69±0.056	14	3.63±0.035	5
UPASI-10	China	3553	1.64±0.044	5	5.20±0.012	3
UPASI-12	Cambod	2400	0.52±0.036	15	2.25±0.010	11
UPASI-13	Assam	2600	1.50±0.036	9	4.30±0.015	4
UPASI-14	Cambod	2000	1.53±0.015	8	5.45±0.020	1
UPASI-15	China	2800	1.24±0.035	13	1.26±0. 021	16
UPASI-16	Assam	3000	1.71±0.031	2	2.30±0.006	10
UPASI-17	Cambod	3300	1.91±0.035	1	1.62±0.015	13
UPASI-20	China	3000	1.57±0.029	7	1.30±0.026	15
UPASI-21	Assam	3000	0.19±0.036	18	1.24±0.026	17
UPASI-22	Assam	2200	0.06±0.025	19	1.31±0.010	14
UPASI-23	Assam	3000	0.27±0.040	17	2.32±0.012	9
CR 6017	Cambod	2500	0.30±0.010	16	3.29±0.010	7
TRI 2025	Assam	4733	0.69±0.049	14	3.56±0.000	6

Values followed by ±are the relative standard deviation of five replications

Table 2: β-D-glucosidase and β-galactosidase activities (μ moles min^{-1} g^{-1} acetone powder) in various plant parts of tea cultivar (UPASI-9)

	β-D-glucosidase		β-galactosidase	
Plant parts	Activity in (μ moles min^{-1} g^{-1} acetone powder)	% activity in comparison to bud	Activity in (μ moles min^{-1} g^{-1} acetone powder)	% activity in comparison to bud
Bud	2.37±0.015		2.85±0.06	
First leaf	2.19±0.015	92.41	2.52±0.095	88.42
Second leaf	1.21±0.012	51.05	2.29±0.015	80.35
Third leaf	0.41±0.021	17.30	0.90±0.015	31.58
I Internode	1.09±0.006	45.99	2.12±0.025	74.39
II Internode	0.64±0.021	27.00	0.77±0.015	27.02
III Internode	0.39±0.025	16.46	0.56±0.006	19.65
Top most mature leaf	0.88±0.02	37.14	1.27±0.01	44.66
Bottom most mature leaf (bottom)	0.61±0.02	25.74	0.90±0.02	31.58
Twig	0.12±0.01	4.92	0.13±0.01	4.71
Bark	0.05±0.02	1.97	0.02±0.00	0.62
Feeder root	0.93±0.04	39.24	0.44±0.01	15.46

Values followed by ±are the relative standard deviation of five replications

Since all twenty cultivars are located in a single area of the UPASI Experimental Farm and since the samples were collected at the same time and the analyses were carried out simultaneously, the possibility of an influence of environmental conditions on the β-D-glucosidase and β-galactosidase activity is eliminated. Hence the variation existing in the activity of these two enzyme could be due to genetic factors. Carro-Panizzi and Kitamura (1995) and Carrao-Panizzi and Bordingnon (1999) have observed similar kind of genetic variations in glucosidase activity of soyabean. Since both β-D-glucosidase and β-galactosidase are involved in carbohydrate metabolism, an attempt was made to correlate the relationship between them. The correlation coefficient was found to be insignificant ($r = 0.091$, not significant, $n = 19$) and hence it is concluded that they are not dependent on each other.

Glycosidase Activity vs Various Plant Parts

The β-D-glucosidase activity was in the order of bud > first leaf > second leaf > first internode > feeder root > top most mature leaf > second internode > bottom most mature leaf > third leaf > third internode > twig > bark. The β-galactosidase activity was in the order of bud > first leaf > second leaf > first internode > top most mature leaf > third leaf = bottom most mature leaf > second internode > third internode > feeder root > twig > bark (Table 2). The study carried out by Yeoh (1989) in Cassava has also shown similar kind of observation. According to him the activity was maximum in leaf followed by peel and tuber cortex. Activity of both β-D-glucosidase and β-galactosidase was highest in bud. It is probable that the activity/carbohydrate metabolism was taking place rapidly in actively growing tea shoots. It is interesting to note that measurable quantity of activity was existing even in the bark of the tea plant (1.97 and 0.62% of activity in comparison to bud). Since the enzymes are present all over the system it could be called as ubiquitous.

Activity in Various Stages of Manufacturing

Withering is a process of removal of moisture where break down of proteins and amino acids are taking place (Fig. 2). The β-D-glucosidase activity was found maximum in the withered leaves followed by the fresh leaves. The organic compounds like linalool, geraniol, phenyl acetaldehyde, nerol and methylsalicylate were also found maximum in withered leaves (Table 3), which are believed to be present as their β-D-glucopyranoside (Sarry and Gunata, 2004). This result showed the strong likelihood that the glucosides are hydrolyzed during withering process (Wang *et al*., 2001). The activity was almost standstill in cut dhool, fermented dhool and made black tea.

Table 3: Percentage relative distributions of individual volatile flavour compounds observed during various stages of manufacture

Compound	GL	WL	CD	FD	MT	Brew	PT%
α-pinene	0.52	1.57	0.86	0.78	1.86	0.71	38.17
Hexenal	0.49	3.72	5.69	1.80	0.82	3.54	431.70
1-penten-3-ol	0.80	4.06	6.68	1.87	0.75	5.45	727.67
2-hexanol	1.13	0.74	2.90	3.38	3.04	1.95	64.14
trans-2-hexenal	6.86	72.93	67.90	39.08	6.15	4.94	80.33
Ethyl caproate	100.00	100.00	100.00	100.00	100.00	100.00	-----
1-pentanol	0.81	11.77	5.76	4.40	1.26	0.93	73.81
6-methyl-5-hepten-2-one	0.19	12.55	0.61	0.32	0.10	0.25	250.00
cis-3-hexenol	10.47	44.88	33.84	17.61	6.42	4.98	77.57
1-octen-3-ol	1.27	2.11	1.12	0.71	0.49	0.00	0.00
Linalool Oxide I	0.68	2.38	1.47	1.39	0.73	1.59	217.81
Linalool Oxide II	0.75	5.55	4.21	3.50	2.82	6.98	247.52
citronellal	0.25	0.54	0.45	0.41	0.40	0.21	52.50
Benzaldehyde	0.15	0.37	0.21	0.47	0.39	0.12	30.77
Linalool	6.90	33.42	30.80	23.78	3.79	1.41	37.20
Phenyl acetaldehyde	0.77	31.82	29.45	20.37	2.93	3.36	114.68
Geranyl acetate	0.04	0.33	0.40	0.41	0.44	0.06	13.64
Methyl salicylate	1.12	13.18	4.77	3.37	2.56	6.19	241.80
Nerol	0.24	5.79	3.05	2.56	0.72	0.88	122.22
Geraniol	2.46	13.09	11.00	8.65	2.82	1.11	39.36
α-ionone	0.39	1.09	0.97	0.75	1.03	0.87	84.47
Benzyl alcohol	0.30	1.34	0.41	0.69	1.10	1.63	148.18
Phenyl ethanol	0.32	1.67	0.90	1.26	1.24	2.32	187.10
β-ionone	0.19	2.21	0.51	0.55	1.55	1.23	79.35
cis-nerolidol	0.24	0.87	0.03	0.12	0.15	0.50	333.33
methyl jasmonate	0.00	0.06	0.12	0.52	1.01	0.98	97.03
VFC I	22.02	152.75	124.50	69.16	19.03	22.04	
VFC II	15.32	115.28	89.59	76.25	25.54	30.16	
Total Flavour	37.33	268.03	214.10	145.40	44.57	52.20	

GL = Green Leaf, WL = Withered Leaf, CD = Cut Dhool, FD = fermented dhool, MT = made tea, PT = Percent Transfer from black tea to tea brew

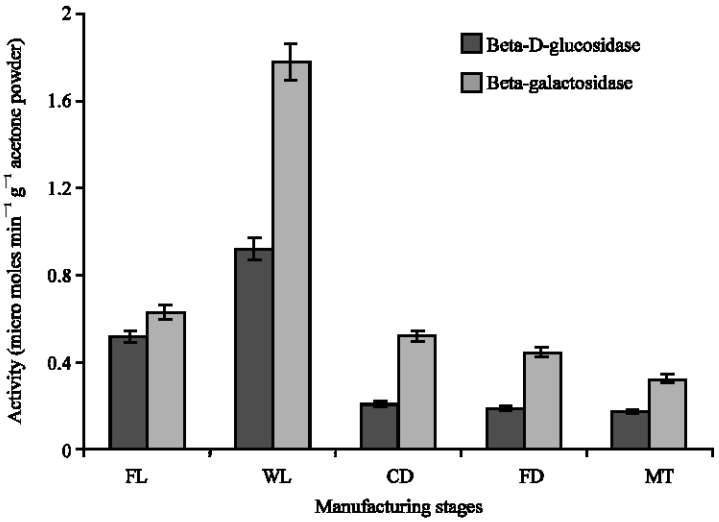

Fig. 2: Changes in β-D-glucosidase and β-galactosidase activities (μ moles min^{-1} g^{-1} acetone powder) during various stages of black tea manufacture (UPASI-9); Error bars represent the standard error of the mean of five replicates; FL = Fresh Leaf, WL = Withered Leaf, CD = Cut Dhool, FD = Fermented Dhool, MT = Made Tea

Similar to β-D-glucosidase activity, it started declining significantly from withered leaves to made tea which means that the enzymatic cleavage of at least a few flavour compounds of β-D-galactopyranoside is existing till the made tea preparation was completed. (Fig. 2). At any given stage of black tea manufacture the activity of β-galactosidase was higher than that of β-D-glucosidase. However in both the cases the activity maximum was seen in withered leaves. During the process of making made tea from fermented dhool, it is subjected to heating (indirect firing) at 100-110°C. Even then the enzymes were active, which is little surprising. It is probable that they are resistant to heat to an extent.

Changes in Flavour Profile During Manufacture

The glycosides of volatile compounds like geraniol, linalool, methylsalicylate, nerol, benzyl alcohol and phenyl ethanol had remained higher in withered leaves where the activity of β-D-glucosidase and β-galactosidase were also maximum (Table 3). It is predicted that the above mentioned flavours must have formed from their glucosides as evidenced by the higher β-D-glucosidase and β-galactosidase activities (Sarry and Gunata, 2004; Wang *et al*., 2001). In general out of twenty six volatile compounds that were studied, twenty five compounds have shown increase except 2-hexanol during withering stage. Since all biochemical operations are expected to take place during cutting operation, the concentration of the majority of the compounds have gone down probably due to formation of new compounds except the compounds like hexenal, 1-penten-3-ol, 2-hexenol and geranyl acetate where an increase was noted. Further reduction in the quantum of flavour compounds was noted due to fermenting operation except in a few compounds like 2-hexenol, benzyl alcohol and phenyl ethanol, where a decrease was observed. Similar kind of trend was observed in the made tea also. Made tea brew was prepared and analyzed for volatile flavour compounds. It was interesting to note that 1-octen-3-ol which is an undesirable compound is completely absent in the brew while a significant quantity is present in made tea. The percent transfer of compounds from made tea to brew was also worked out. The percentage transfer was maximum in the case of 1-penten-3-ol followed by hexenal and minimum in 1-octen-3-ol followed by geranyl acetate. The higher per cent transfer observed in many compounds could be due their formation from other compounds during the boiling process of brewing operation.

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