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PROPERTIES OF TEA POLYPHENOL-OXIDASE

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The properties of tea polyphenoloxidase in relation to tea manufacture are described. The enzyme is separable to six isozymes on electrophoresis, and shows optimal activity in the range pH 6.2 to 6.6 The molarity of the reaction affects polyphenoloxidase activity, and is optimal at 0.05. The period of withering has no effect on enzyme activity, but there is a progressive fall as the period of fermentation is extended. This latter effect is due to inhibition of the enzyme by the products of oxidation, aided by the decrease in pH. The products of oxidation of individual and mixed flavanols are described, and addition of amino-acids have a profound effect on the formation of theaflavins and thearubigins. Studies of the effect of substrate concentration showed that there was an optimal level for theaflavin production. Tannins had the effect of causing a reduction in theaflavin production, but an increase in thearubigin content.

Polyphenoloxidase (PPO) plays an important role in the manufacture of black tea, because it is the enzyme which is responsible for the oxidation of polyphenols and the formation of theaflavins, compounds which are responsible for the brightness and much of the quality of tea liquors. The enzyme was first identified as a phenol oxidase by Sreerangachar (1943), and its properties studied by Roberts and Wood (1950, 1951). The enzyme was purified by Gregory and Bendall (1966), who discribed its kinetic and other properties. Both groups of workers used acetone powders of tea leaf as the source of their enzyme. Sanderson (1965) devised a neat method for the extraction of polyphenoloxidase directly from the tea leaf. This method was based on the inclusion of an insoluble adsorbent (Loomis & Battaile 1966) for polyphenols in the extraction medium. The properties of the soluble polyphenoloxidase prepared by this method were studied in this investigation. Enzyme activity was measured by theaflavin (TF) production, because this was considered to be the method of practical significance. The experimental findings were, in the majority of cases, compared for their effect on actual tea manufacture, carried out on a miniature scale.

METHODS

Preparation of enzyme

Fresh shoot tips (20 g), of clone TRI 2023, growing at the Low-Country Station of the Tea Research Institute of Ceylon, Ratnapura, comprising the bud, two adjacent leaves and included stem, were frozen at -20°C for 18 hr. The frozen shoots were ground with Polyclar AT (10g), acid-washed sand (4g), and 60 ml of 0.05 M phosphate buffer, pH 5.8. The homogenate was sieved through muslin cloth, and additional Polyclar AT (1g) added to the filtrate to remove any residual polyphenols.

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This was then centrifuged at 3000 r.p.m for 10 minutes in a Servall refrigerated centrifuge at -5°C. All of the above operations were carried out in a cold room (4°C) using cold reagents and equipment. The supernatant solution (which still contained traces of chlorogenic acid and p-coumaryl-quinic acid), was dialysed in the cold room against 40 L of distilled water.

Fluorescent enzyme for electrophoresis was prepared by conjugation with fluorescein isothiocyanate according to Nairn (1964).

Starch gel electrophoresis

Starch gal electrophoresis was effected using vertical migration. Hydrolysed starch (15g), purchased from Connaught Laboratories, was mixed with tris-citric acid buffer, pH 8, (100 ml). Electrophoresis was carried out at 275 volts for 18 hours at 4°C, and the polyphenol oxidase detected by placing a No. 3 MM Whatman filter paper, dipped in a solution of 3% D-catechin and 0.5% $\propto$ - alanine over the developed electrophoregram, and noting the appearance of the yellowish-red colour. When fluorescent enzyme was used, the bands were visualized under the ultra-violet lamp.

Preparation of flavanols

1—Mixture of flavanols :

Freshly plucked flush (100g) was extracted in a Waring Blender for 5 min. with 80% (v/v) ethanol (400 ml), and the extract passed through Polyclar AT. The column was eluted with acetone, and the eluate evaporated do dryness under reduced pressure. The light tan product was then washed with diethyl ether to yield a white powder containing all the flavanols, as determined by paper and thin layer chromatography.

2—Individual flavanols :

Individual flavanols were obtained by separation on two dimensional paper chromatograms, developed in butanol-acetic acid-water (6:1:2) and 6% acetic acid, followed by visualization under the ultra-violet lamp. The relevant 'spots' were then cut off, and eluted with warm water. The eluates were concentrated under reduced pressure, and stored at - 20°C in sealed vials.

Preparation of mixture of tea amino acids and sugars

These were separated from each other by ion exchange column chromatography, and the amino acids recovered from the column with 2N ammonia. The ammoniacal extract of amino acids was evaporated to dryness under reduced pressure, and the residue taken up in distilled water.

Theaflavins and thearubigins

Theaflavins (TF) and thearubigins (TR) were estimated spectrophotometrically according to Roberts and Smith (1961).

Vanillin-reacting polyphenols

Vanillin-reacting polyphenols were estimated colorimetrically according to the method of Swain and Hillis (1959).

RESULTS AND DISCUSSION

The starch gel electrophoregram (Fig. 1), of the dialysed enzyme showed the presence of seven distinct bands which gave a yellowish-red colour with the D-catechin - α- alanine reagent. Six of these bands migrated from cathode to anode, whilst the other moved in the opposite direction. Starch gel electrophoresis (Fig. 2) of the enzyme conjugated with fluorescein isothiocyanate showed six bands when viewed under the ultra-violet lamp (2537 A), all of which migrated towards the anode at pH 8.0, but conjugation resulted in a loss of enzyme activity, and none of the six bands gave any colouration with the D-catechin - α-alanine reagent. The multiplicity of forms of tea polyphenol oxidase may be compared with the findings of other workers on polyphenol oxidases, *eg* Robb,Mapson and Swain (1965) reported on the heterogeneity of the tyrosinase of broad bean, Constantinides and Bedford (1967) described multiple forms of polyphenoloxidase from mushrooms, apples and potatoes, whilst Dizik and Knapp (1970) separated Avocado polyphenoloxidase to several components. However, the purified tea polyphenoloxidase obtained from an acetone powder of tea leaves by Gregory and Bendall (1966) was electrophoretically homogeneous.

Effect of pH on theaflavin production

Incubation of the dialysed enzyme with a mixture of tea flavanols produced the highest level of theaflavins at pH values between 6.2 and 6.6 (Fig. 3). This broad range may be due to the use of a mixture of isozymes and a mixture of flavanols, and is in agreement with the observation of Gregory and Bendall (1966) that pH 5.7 and pH 5.0 were the optimal pH values for the oxidation of pyrogallol and 4-methyl catechol respectively by their homogenous enzyme preparation. The effect of pH on actual black tea manufacture was made by spraying unwithered leaf with buffers of different pH values. The results (Table 1) showed that the optimal pH of TF production was here the same as that obtained with the partially purified enzyme. In the case of TR production, there was a steady rise with increasing pH.

The increased TF production at pH 6.4 may be due to the prevention of inhibition of PPO by its oxidation products, as the H-bonding of quionones to proteins decreases as the pH is raised. When the pH is raised to 7.0, conditions are unfavourable for PPO activity, and this results in a reduction in TF production; however, the higher pH values promote the occurrence of non-enzymic oxidation and non-enzymic browning reactions, and this leads to an increase in TR formation.

TABLE 1—*Effect of pH of the leaf on the production of theaflavins and thearubigins*

pH	*% TF*	*% TR*
3.0	0.80	10.2
4.5	0.96	8.0
5.8	0.96	14.6
6.4	1.04	16.2
7.0	0.76	16.0
8.0	0.34	18.0

Effect of molarity of buffer on TF production

The results of incubating the dialysed PPO with the mixture of flavanols in the presence of phosphate buffers (pH 5.8) of different molarities, and the effect of manufacture of spraying tea leaves, prior to withering, with these buffers are shown in Fig. 4 and Table 2 respectively. It is seen that TF production is maximal with 0.05

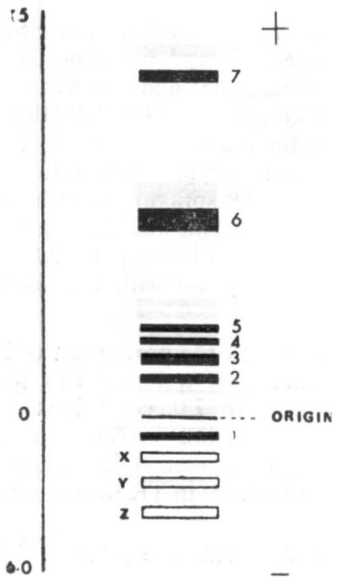

FIG. 1—*Starch-Gel electrophoregram of the solubilized polyphenol oxidase. Bands revealed with P. Catechin: ∝ - alanine reagent; Voltage of electrophoresis* 275 *Volts; Period of migration* 18 *hrs. at pH* 8.

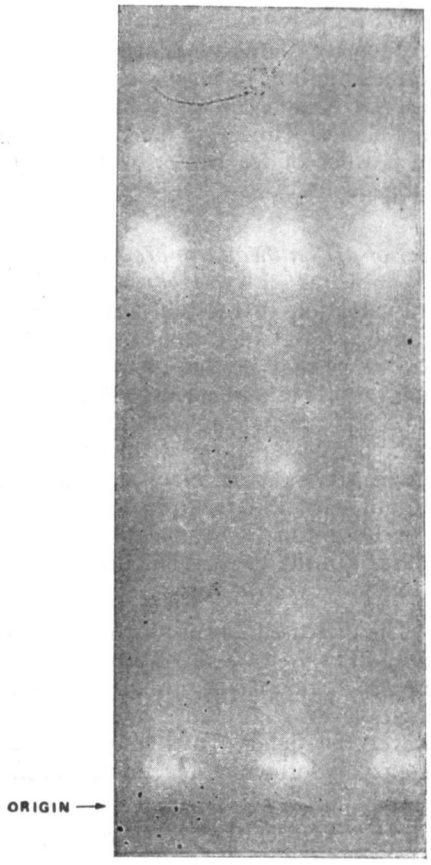

FIG. 2—*Starch-Gel electrophoregram of the solubilized polyphenol oxidase conjugated with fluorescein isothiocyanate. Bands were visualized under ultra-violet lamp.*

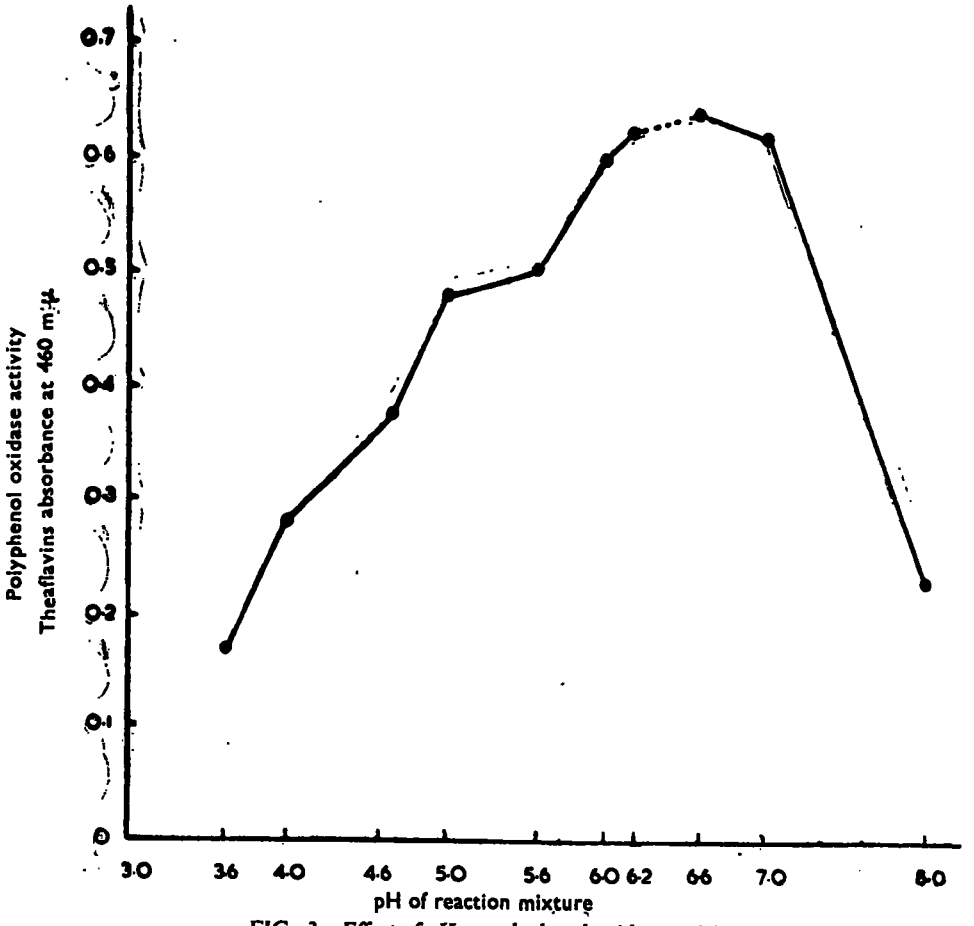

FIG. 3—*Effect of pH on polyphenol oxidase activity*

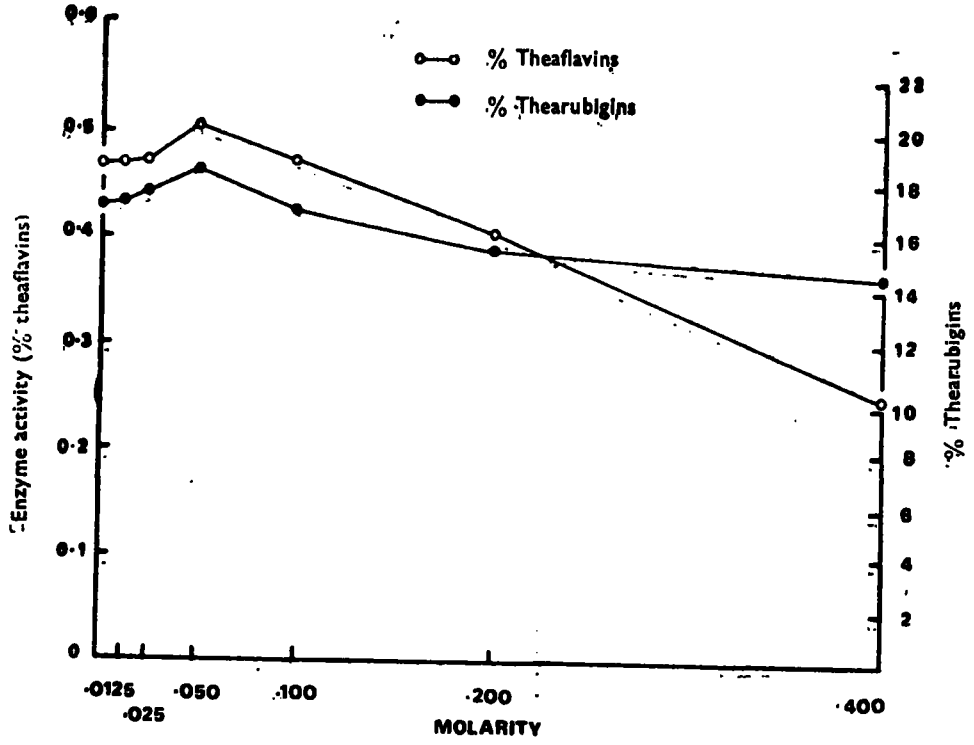

FIG. 4—*Effect of phosphate buffer (pH 5.8) molarity on polyphenol oxidase activity*

to 0.01 M buffers, and that TR levels are hardly effected by the buffer molarity. This effect of molarity on TF production may be a contributory factor to the effect of rain on tea quality, as wet weather would tend to cause a reduction in the molarity of the cell contents.

TABLE 2—*Effect of the molarity of the buffer on the production of theaflavins and thearubigins*

Molarity of the phosphate buffer pH 5.8	*% TF*	*% RT*
0.0125	0.82	14.3
0.0250	0.80	14.2
0.0500	0.86	14.6
0.1000	0.86	14.6
0.2000	0.81	14.8
0.3000	0.75	14.6
0.4000	0.62	14.9

Effect of different periods of wither and of fermentation on polyphenol oxidase activity

The effect on TF production by PPO, of different periods of withering and of fermentation are shown in Fig. 5. It is seen that there were only slight variations in the quantities of TF produced after different periods of wither, but that increasing the time of fermentation led to a steep fall in the level of TF produced by the enzyme. It is noteworthy that extracts of the fired tea showed some PPO activity which, however, completely disappeared on keeping the tea for about seven days.

The finding that the period of withering has no effect on TF production by enzyme extracts of the leaf is in contrast to that of Sanderson (1964), who measured enzyme activity in acetone powders of the leaf by a modification of the ascorbic acid method described by Sreerangachar (1943). The decrease in enzyme activity on fermentation is probably due to inhibition (by precipitation or denaturation) of the enzyme by the oxidation and condensation products of its substrates. This possibility is supported by the marked decline in the level of vanillin reacting polyphenols during fermentation (Table 3), whereas the period of wither had no effect on this group of compounds. The decline during fermentation is probably due to the formation of insoluble complexes between the enzyme protein, and the oxidized and condensed polyphenols, and the occurrence of such complex formation would be favoured by the observed decrease in pH.

TABLE 3—*The pH values and the vanillin-reacting polyphenols during different periods of wither and of fermentation.*

	pH values	*Vanillin-reacting polyphenol (Abs. MeOH extract) (Mg/g dry wt)*
Fresh leaf	5.7	236.2
Withered leaf		
6 hr	5.7	238.0
12 hr	5.5	240.8
18 hr	5.7	237.6
24 hr	5.6	240.1
Fermented Dhool		
30 mns	5.2	180.6
60 mns	5.1	162.0
120 mns	5.0	108.0
240 mns	4.8	102.0

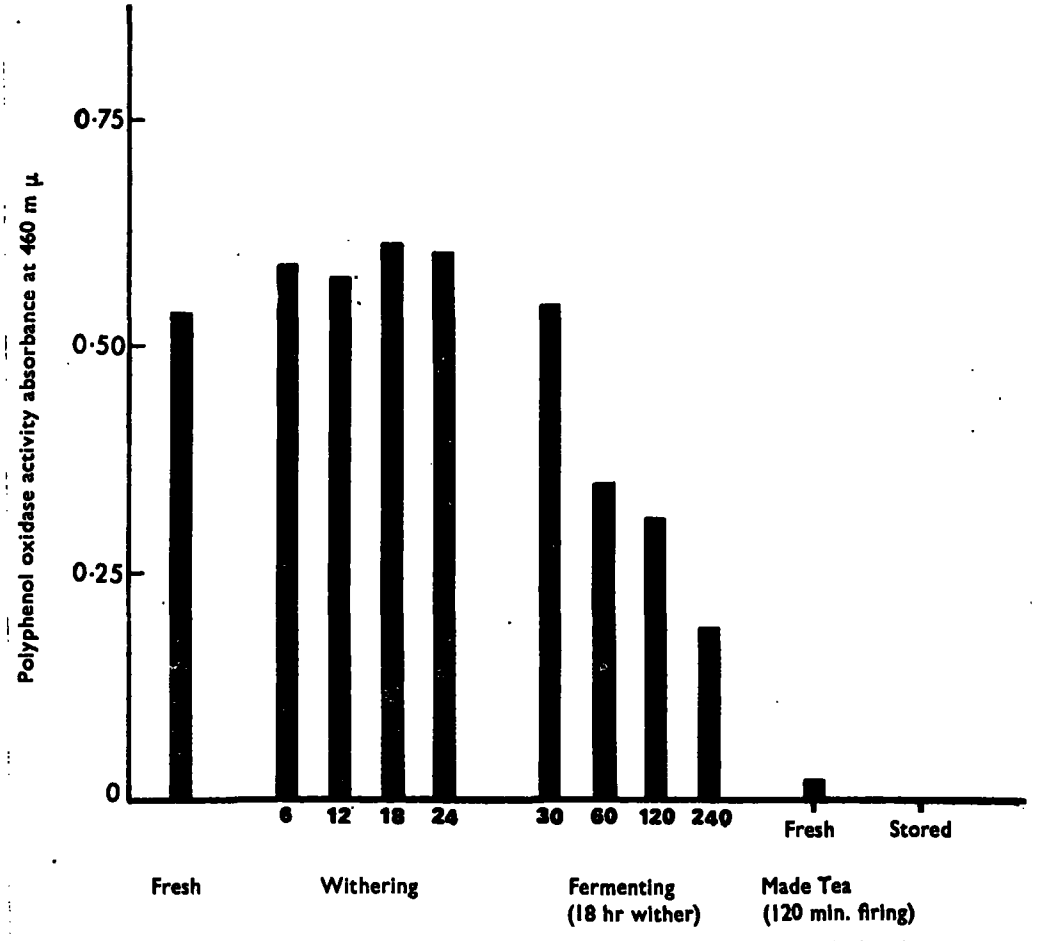

FIG. 5—*Effect of different periods of withering and of fermentation on polyphenol oxidase activity.*

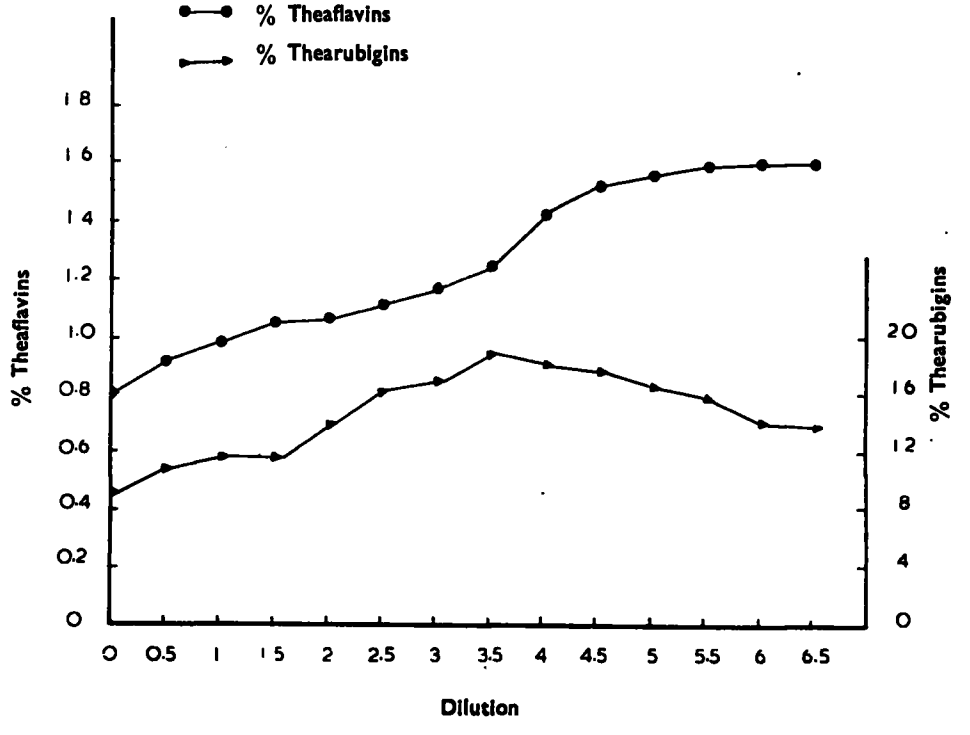

FIG. 6—*Effect of Substrate dilution on enzyme activity*

Substrates of polyphenoloxidase

(a) *Preliminary study*—The individual flavanols were separated on paper, after which the two-dimensional chromatograms were dipped in fresh enzyme extract. Six spots were seen to develop various colours (Table 4) and these coloured spots were cut, eluted with warm water, and the eluates analysed by two-dimensional paper chromatography. The results, incorporated in Table 4 indicate the identity of the six individual compounds which developed colours in these conditions, and also that in the conditions used theaflavin gallate appeared as an oxidation product of EGCG alone. Triacetidinin was seen to form on aerial oxidation of EGCG, even in the absence of enzyme. The coloured compounds of RfO are probably complexes of the oxidized polyphenol and enzyme protein.

(b) *Oxidation of mixtures of substrates*

(i) *Mixed flavanols*—The individual flavanols, eluted from paper chromatograms, were mixed in different combinations, and incubated, with PPO. The results (Table 4) indicated that the brightest colour was produced by the EGCG-EGC mixture, and the deepest colour by the mixture of the four flavanols. The pink spot corresponding the epitheaflavic acid (Berkowitz, Coggon & Sanderson 1971), is seen to occur only when ECG & EGC are present together, whereas TF and TFG are formed from all mixtures.

TABLE 4—*Substrates and the detected colour products*

Substrate	*Coloured Oxidized product*	*Visible Colour*
EGC	Polyphenol-protein complex (TR)	Orange yellow
EGCG	Theaflavin gallate, polyphenol-protein complex (TR) Triacetinidin (pink)	Orange yellow
ECG	Polyphenol-protein complex (TR)	Bright yellow
EC	Polyphenol-protein complex (TR)	Greenish
Cat.	Polyphenol-protein complex (TR)	Orange yellow
Chlorogenic acid	Polyphenol-protein complex (TR)	Brown
ECG + EGCG	Theaflavin Theaflavin gallate, Tricetinidin, polyphenol-protein complex (TR)	Bright Orange red
EGC + ECG	Theaflavin, theaflavin gallate pink spots similar to tricetinidin, polyphenol-protein complex (TR)	Orange
EGCG + ECG	Theaflavin, theaflavin gallate, tricetinidin, polyphenol-protein complex (TR)	Orange red
EGC + EC	Theaflavin, theaflavin gallate, polyphenol-protein complex (TR)	Yellow
All flavanol	Theaflavin, theaflavin gallate, tricetinidin, pink spot, polyphenol-protein complex (TR)	Deep orange yellow

ECG : (—) Epigallocatechin
EGCG : (—) Epigallocatechin gallate
ECG : (—) Epicatechin gallate
EC : (—) Epicatechin
Cat. : (+) Catechin

(ii) *Effect of addition of amino acids to TF & TR production from mixed flavanols*—The addition of increasing quantities of a mixture of tea amino acids to the reaction system consisting of mixed flavanols and dialysed enzyme led to a reduction of TF, but an increase of colour. This increase in colour was due to an increase in the quantities of TR (Table 5). This indicates the importance of amino acids in determining the colour of tea liquors, which probably is partly a result of non enzymic browning reactions.

TABLE 5—*Effect of addition of amino acids to the reaction mixture containing the flavanols and the purified enzyme*

Incubation Mixture	*TF*	*TR*
	(Absorbance at 460 m u)	
Flavanols + enzyme (F+E) (standard)	0.36	0.92
F+E+0.1mg to amino acid mixture (AM)	0.37	0.94
F+E+0.2mg AM	0.36	0.94
F+E+0.4mg AM	0.36	0.98
F+E+0.8mg AM	0.34	1.20
F+E+1.0mg AM	0.31	1.25
F+E+2.0mg AM	0.28	1.25
F+E+4.0mg AM	0.24	1.30

The effect of addition of the individual amino acids occurring in tea, on the mixed flavanol-PPO system is shown in Table 6. It was observed that the general effect was a reduction in TF and increase in TR on addition of most of the amino acids; cysteic acid and glutamic acid were the two exceptions, which showed the reverse effect. The highest increase in TR and most striking decrease in TF occurred on the addition of arginine.

TABLE 6—*Effect of individual amino acids (unbuffered) on the theaflavins and thearubigins*

AMINO ACID (0.1M concentration)	*TF* (Absorbance at 460 mu)	*TR* (Absorbance at 460 mu)
1—Control (water)	0.41	0.62
2—L-Leucine	0.28	0.84
3—L-Tyrosine	0.21	0.86
4—Hydroxy-L-Proline	0.24	0.80
5—L-Cysteine	0.23	0.88
6—Amino butyric acid	0.22	0.90
7—L-Proline	0.23	0.86
8—L-Asparagine	0.15	0.98
9—B-Alanine	0.22	0.98
10—Cysteic acid	0.52	0.62
11—Glycine	0.21	0.88
12—D-Amino-n-butyric acid	0.12	0.99
13—L-Aspartic acid	0.42	0.68
14—Arginine	0.03	1.05
15—Glutamine	0.22	0.92
16—Theanine	0.32	0.88
17—DL-Nor-Valine	0.23	0.88
18—Glutamic acid	0.54	0.48
19—L-Valine	0.32	0.60
20—Alanine	0.21	0.85
21—DL-Threonine	0.22	0.85
22—Serine	0.22	0.85
23—Methionine	0.21	0.80
24—D-Threonine	0.32	0.74

Effect of substrate concentration on enzyme activity

The effect of substrate concentration on enzyme activity is shown in Fig. 6. The activity of enzyme was inhibited by high concentrations of substrate and there was an increase in activity with dilution, as shown by increased TF production. TR too showed an initial increasing trend which, however fell off when the flavanols were diluted. A similar effect of dilution on PPO activity was observed by Roberts (1940) and attributed (Gregory & Bendall 1966) in inhibition of enzyme activity by the catechins. It had also been suggested (Wickremasinghe & Swain 1965), that there is an optimal level of catechins for maximal TF production during tea manufacture. The early increase in TR may be explained as being due to the increased availability of TF, which are known to be part of the TR complex (Roberts 1962). The subsequent decrease may be due to the dilution of other constituents which are necessary for the formation of the TR complexes, *eg* caffeine (Roberts 1962) and theanine (Wickremasinghe & Perera 1971). It is noteworthy that TF production increases, markedly at the beginning of this stage. The subsequent flattening of the curve for TF production is due to the limitation of substrate availability.

Effect of tannins on TF and TR production

The slow-fermenting clones TRI 9 and TK 48 were found to contain high levels of tannins (Wickremasinghe 1965) which could tan PPO and so inhibit fermentation. These same tannins were found to occur in tea bark as well as in myrobalans (*Terminalia catappa*), and experiments were carried out to determine whether extracts of these tanniferous materials would interfere with TF production during tea manufacture. Analysis of the black tea (Table 7) showed that the addition of these tanniferous materials did, indeed, lead to a reduction in TF production. The liquors of these teas were, however, coloury, due to an increase in the levels of TR, a situation reminiscent of the nature of low-grown tea. The analyses of low-grown tea conformed with this observation and were found to contain higher levels of tannins than high-grown teas.

TABLE 7—*Inhibitory effect of various tannins on tea fermentation*

Sources of tannin	*% TF*	*% TR*
Control	0.96	12.6
Tannins from clone TRI 9	0.88	12.9
Tannins from clone TK 48	0.92	13.6
Tannins from tea bark	0.84	14.2
Tannins from *T. catappa*	0.73	16.2

SUMMARY

1—Polyphenoloxidase is not a homogeneous enzyme, but consists of at least six isozymes.

2—The optimal pH for polyphenoloxidase activity is 6.2 to 6.6

3—The optimal molarity for polyphenoloxidase activity is 0.05 to 0.1

4—Period of wither does not affect the activity of the enzymes, as far as theaflavin production is concerned, but the activity falls as fermentation proceeds.

5—Oxidation of individual flavanols, mixtures of flavanols, and mixtures of flavanols with amino acids are described.

6—There is an optimal substrate concentration for polyphenoloxidase activity.

7—Tannins inhibit polyphenoloxidase activity.

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