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SOME OBSERVATIONS ON THE NATURE OF CATECHOL OXIDASE IN TEA LEAVES

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The catechol oxidase enzyme has been investigated in some detail. Enzyme activity can be solubilized from acetone powders it a sufficient quantity of polyclar AT is added to the preparation. Treatments with lipid solvents and detergents are used to show that catechol oxidase is not lipid bound or latent.

The catechol oxidase enzyme system is recognized to play a vital role in the oxidation reactions taking place during the fermentation step of tea manufacture (Sanderson 1965). The enzyme was assumed to be insoluble or particle bound until Sanderson (1964a) found that catechol oxidase was, in reality, a soluble enzyme that was readily precipitated by the high concentration of polyphenols found in tea shoots. The basic concept used for the solubilization of the enzyme was to bind the polyphenols onto an insoluble polyamide adsorbent during the extraction process, thereby preventing them from interacting with the enzyme protein. This study extends these ideas to show that, contrary to earlier conclusions (Sanderson & Roberts 1964), the enzyme can be readily solubilized even from acetone powders. It is further shown that the enzyme is readily available for fermentation reaction and not latent or lipid bound.

EXPERIMENTAL

The tea shoots used in these investigations were collected from vegetatively-propagated tea plants at St Joachim Estate, Ratnapura (elevation 75 m amsl). Clone TRI 2025 was used in most experiments.

Acetone powders—Acetone powders were prepared according to the method described by Sanderson (1964b). In some experiments polyclar AT powder 1966) was mixed with the leaves (Sanderson & Perera prior to homogenization with acetone. Attempts at solubilizing the enzyme activity present in acetone powders were carried out by shaking the acetone powder (0.1g) with Mc. illvain Buffer pH 7 (10 mls) for 1 hr at 4° C. The resulting mixture was centrifuged at 2000 x g for 5 min. and the supernatant used for the experiments. Longer periods of agitation resulted in lower activity. *Soluble enzyme preparation*—The soluble enzyme was prepared according to the method described by Sanderson (1964a). Portions of the extract were used without centrifugation. The details of the enzyme assay procedure have been described earlier (Sanderson 1964b).

Nitrogen determination—Weighed quantities of acetone powder were used for nitrogen determination by the standard microkjeldahl procedure. Protein in the soluble enzyme preparation was separated by mixing a small volume of extract (usually 2 ml) with an equal volume of trichloroacetic acid (20%). The precipitate was separated by centrifugation and used for the determination of nitrogen by the Kjeldahl procedure.

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RESULTS

The values for enzyme activity obtained from acetone powders as compared with the soluble enzyme preparation may have appeared to be divergent in earlier reports because of the variety of units used. The activity is almost identical when expressed on a protein nitrogen basis (see Table 1 (b)).

TABLE 1—*Enzyme activity of acetone powders and soluble enzyme preparations*

Acetone powder	*Expt 1*	*Expt 2*
(a) μ moles catechol/min/mg acetone powder	20.02	9.25
(b) μ moles catechol/min/mg protein N	0.346	0.191
Soluble enzyme preparation		
(a) μ mole catechol/min/g fresh weight tissue	2.75	1.85
(b) μ mole catechol/min/mg protein N	0.350	0.215

The quantities of enzyme activity that can be solubilized from acetone powders prepared by homogenizing the tissue with acetone in the presence of increasing quantities of polyclar AT powder are shown in Table 2.

TABLE 2—*The effect of polyclar AT on the solubilization of enzyme activity from acetone powders*

Quantity of Polyclar AT powder in homogenate (g/g fresh wt tissue)	Activity of acetone powder (μ moles catechol/min/g/powder)		Percent activity solubilized
	Total	Soluble	
0	41.05	12.41	31
0.25 g	60.50	26.00	43
1.0 g	17.60	13.40	76
1.0 g	18.20	14.73	81

It is evident that an increase in the polyclar content to 1:1 with respect to tissue weight results in solubilization of 75-80% of the enzyme activity in the acetone powders. Considerable variation in the absolute values for enzyme activity with different samples has already been reported to occur (Sanderson 1964c).

Tea shoots were further examined to determine whether part of the catechol oxidase activity is latent or lipid bound and subsequently released on treatment with lipid solvents or detergents. The results of the experiment are shown in Table 3, the details of the treatments being listed below. None of the treatments produced any significant increase in enzyme activity, there is evidence for the slight depression of activity by some treatments.

TABLE 3—*The effect of pre-treatment of leaf on catechol oxidase activity*

Treatment (see below)	μ m catechol/min/g fresh weight Activity after treatment*	Activity of control
1—Tissue wilted	(a) 4.65	4.75
	(b) 3.85	4.75
2—Exposed to chloroform	4.65	4.70
3—Homogenized with chloroform	2.80	3.50
4—Homogenized with ether	3.20	4.75
5—Homogenized with hexane	3.10	(3.75)
6—Teepol — 0.1%	5.50	5.20
0.2%	5.20	5.20
7—Borate buffer	5.20	5.20

Treatment

1—Tissue was allowed to wilt for 18 hr at room temperature.
2—Tissue was exposed to chloroform for 20 min. until browning begins and then extracted by the standard procedure (compare with Sanderson 1964).
3—Tissue was homogenized with chloroform for 1 min. in an atomix blender running at full speed, filtered on a Buchner funnel to remove the solvent and then the enzyme was extracted and assayed by the standard procedure.
4—Tissue was treated as in (3) using ether instead of chloroform.
5—Tissue was treated as in (3) using hexane instead of chloroform.
6—Teepol (Shell Chemicals Ltd) was added at concentrations of 0.1 and 0.2% v/v/ to the extraction buffer.
7—Borate buffer pH 7 0.025 M was used instead of Mc illvain buffer in the extraction media.

A portion (15 ml) of the soluble enzyme preparation was fractionated on columns of Sephadex G 75 (10 cm x 2 cm). The columns were eluted with Mc illvan buffer pH 7 and 4 ml fractions collected on a automatic fraction collector. Portions of the eluate were used for the determination of protein nitrogen. The results are shown in Table 4. It is evident that fraction 3 has the highest specific activity but not markedly higher than that of the original extract.

TABLE 4—*The fractionation of soluble enzyme preparation on Sephadex G 75*

Fraction	*mg Protein* (N/ml)	*Enzyme activity* (μ moles catechol/min/mg N)
Original extract	0.930	2.33
Fraction 1	0.575	0.191
2	0.266	0.89
3	0.523	2.51
4	0.302	1.51
5	0.129	1.29
6	0.109	1.08

DISCUSSION

The results described above show that the enzyme activity extracted from the older acetone powder method is substantially the same as that obtained by Sanderson's procedure (1964a) for the preparation of a soluble enzyme. The inability of earlier workers (Sanderson & Roberts 1964; Gregory & Bendall 1966) to quantitatively solubilize the catechol oxidase enzyme from the acetone powders was due to the irreversible precipitation of proteins by the polyphenols and not due to the denaturing of the protein by acetone as was concluded earlier (Sanderson & Roberts 1964).

This is supported by the finding that up to 81% of activity in acetone powders is soluble when sufficient polyclar AT powder is incorporated to remove the polyphenols at an early stage (Table 2). The enzyme activity may be concluded to be totally soluble if it is assumed that there could be a partial loss in enzyme activity during extraction of the acetone powder with buffer. This assumption is substantiated by the observation that longer extraction periods generally led to lower enzyme activity in the soluble fraction.

The failure of the seven additional treatments described in Table 3 to produce an increase in catechol oxidase activity leads to the conclusion that the catechol oxidase enzyme is not lipid bound or latent in shoots of the tea plant. A latent polyphenol oxidase has however been described in other plants (Kenton 1958).

The absence of low molecular weight inhibitors in the enzyme extract is suggested by the finding that fractionation of the enzyme on different grades of Sephadex does not produce any fraction with higher specific activity than the crude soluble enzyme preparation. A higher specific activity would be expected if an inhibitor were removed from the enzyme site by the Sephadex. The tea catechol oxidase enzyme system has however been previously separated into six fractions by starch gel electrophoresis (Perera 1972).

The conclusions arrived at in this study confirm and further extend Sanderson's findings (1964a) that tea shoots contain a readily available catechol oxidase enzyme system. The very rapid precipitation of the enzyme with polyphenols even in the presence of a solvent like acetone indicates that the enzyme would likewise be very rapidly precipitated when the cells are broken up during tea manufacture. The enzyme will, however, remain active in the insoluble state and can carry out the oxidation reactions in situ to provide the primary oxidized substracts for the formation of coloured complexes like the theaflavins and thearubigins during the fermentation of tea.

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