Studies on the 'Fermentation' of Ceylon Tea

5. COMPARATIVE RATES OF OXIDATION OF DIFFERENT POLYPHENOLIC SUBSTRATES BY TEA-OXIDIZING ENZYMES

By H. B. SREERANGACHAR, *Tea Research Institute of Ceylon, Talawakelle*

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The oxidizing-enzyme preparation employed in previous work was obtained from tea by grinding fresh leaf under acetone until the residue was almost completely free of all colouring constituents of the leaf.

Among the differences that exist between the *in vitro* action of this acetone-prepared tea enzyme and the *in vivo* reaction in the minced leaf are the rates of oxidation of different polyphenolic substrates. Thus, acetone-prepared enzyme oxidizes catechol about two or three times as rapidly as it does tea tannin [Lamb & Sreerangachar, 1940], whereas in the fermenting leaf-mince to which the polyphenolic substrates are introduced after completion of its initial fermentation, tea tannin is oxidized at about three times the rate of catechol oxidation [Harrison & Roberts, 1939].

There is obviously one main difference between the conditions that exist in the minced-leaf system prior to the addition of the polyphenolic substrates, and those obtaining in the acetone-prepared enzyme reaction system. In the former, the leaf polyphenols are, as a result of initial reactions, in various stages of oxidation and condensation, whereas in the latter such oxidized products of tea tannin are absent at the commencement of the reaction. The object of the present investigation is to find out the exact influence of these oxidized polyphenols on subsequent oxidation reactions, and to arrive at a clear distinction between the direct reactions of the enzyme and the possible indirect action of other substances present.

EXPERIMENTAL

Methods. The rates of oxidation of different polyphenols by tea enzymes, as shown by their oxygen uptake, were measured at 25° with Barcroft's apparatus [cf. Sreerangachar, 1941]. Observations included reactions in the fresh-leaf mince and also *in vitro* reactions of prepared enzyme and substrate. In the latter type of reaction, the enzyme powder was initially kept floating on the substrate solution in small 'pliofilm' boats which could be upset whenever desired by shaking the apparatus, thus bringing the enzyme and substrate into contact with each

other. Any complication due to respiration changes was avoided by not introducing KOH into the central tube. Such a contingency arose only when fresh-leaf mince was used; for in the case of *in vitro* reaction mixtures the system was entirely free from living tissues. The polyphenolic substrate solutions employed were all of M/10 concentration. The amorphous tea tannin was prepared by the Tocklai modification of Shaw's method [1935], and all gallic acid was removed from it by extraction with dry ether.

Substrate specificity. Attention was first directed towards finding out the relative rates of oxidation

Fig. 1. Oxidation by acetone-prepared tea enzyme. I, catechol; II, tea tannin.

of catechol and tea tannin by the use of the same enzyme preparation. Acetone-prepared enzyme powder (50 mg.) was allowed to react on 2 ml. substrate solution to which 2 ml. McIlvaine's buffer solution at pH 5.0 were added. Fig. 1 represents the course of oxygen uptake by two such systems, and it will be seen that the rate of oxidation of catechol is higher than that of tea tannin, although the difference is not as great as that obtained previously with different enzyme preparations. This has been confirmed in several experiments.

The rate of oxidation of a few other substrates available here, namely, pyrogallol, ascorbic acid and quinol, were then studied in comparison with catechol and tea tannin (Fig. 2). The enzyme oxidizes catechol, tea tannin and pyrogallol, members of a class of substrates to which the enzyme is specific, more rapidly than it does ascorbic acid

and quinol, which do not belong to this specific class. The slight oxidizing action observed on ascorbic acid and quinol is due to the indirect influence of minute quantities of original polyphenols in the enzyme powder which resist complete extraction with acetone.

Fig. 2. Oxidation of polyphenols by acetone-prepared enzyme. I, catechol; II, tea tannin; III, pyrogallol; IV, ascorbic acid; V, quinol.

The oxygen uptake of the leaf-mince suspension came almost to a standstill after about 90–120 min. and the relative rates of oxidation of polyphenolic substrates were then measured by introducing them at this stage to the leaf-mince system. It was observed that tea tannin was oxidized more rapidly than catechol as shown in Fig. 3, I. Harrison & Roberts [1939] in Assam also found that tea tannin is oxidized about three times as rapidly as catechol with minced leaf.

Influence of oxidation products. A study of these results suggested that the tea polyphenols existing in different stages of oxidation and condensation as a result of the initial fermentation reaction in the leaf mince might probably be the chief cause of the difference between the results with acetone-prepared enzyme and those obtained with leaf mince. The interference of the oxidized products of tea polyphenols with the oxidation rates of polyphenols subsequently added might have been so pronounced as even to reverse the relative rates of oxidation of tea tannin and catechol. In order to test this hypothesis, fresh-leaf mince was repeatedly washed with water after its initial reaction, to remove the accumulated oxidation products, and the residue allowed to react on catechol and tea tannin. It was then found that the oxidation of tea tannin not only ceased to be faster than that of catechol but its rate was either the same or even lower than that of catechol as shown in Fig. 3, II.

The use of acetone, to remove the oxidation products from the leaf mince after the initial reaction, yields still better results, due to a greater extraction of these materials, though perhaps not so completely as in the preparation of enzyme from

Fig. 3. O_2 uptake of leaf-mince suspensions with added substrates. I, reactions in presence of tannin oxidation products; II, ditto after removal of these by washing the tissues; III, ditto after removal by acetone extraction.

fresh leaf. Comparison with the results of water extraction reveals that acetone treatment does not appreciably inhibit the enzyme with respect to its rate of polyphenol oxidation. The rates of oxidation of catechol and tea tannin by the acetone-extracted residue, from leaf mince which had undergone the initial fermentation reaction, are in the same order as those observed in the case of acetone-prepared enzyme, although in the former case the difference between the oxidation rates of catechol and tea tannin is not as great as in the latter. Fig. 3, III shows that catechol is being oxidized slightly more rapidly than tea tannin by the acetone-extracted residue of leaf mince which had previously been fermented.

Further confirmation of the influence of the tea-tannin oxidation products on subsequent oxidations of catechol and of tea tannin was obtained when these products, from another experiment, were

added to the reaction mixtures of the acetone-prepared enzyme on the one hand and catechol and tea tannin on the other. The relative rates of reaction at once changed, tea tannin being then oxidized more rapidly than catechol.

When this evidence concerning the influence of tea-tannin oxidation products on the subsequent oxidations of catechol and tea tannin had been

mentally the oxidized product of quinol, as pure *p*-quinone was available. A M/20 solution of *p*-quinone was used. Solutions (2 ml.) of these three oxidized polyphenols were added to each of the polyphenol-enzyme mixtures and the oxygen uptake measured by manometric methods. Fig. 4 represents the results obtained. The results shown in dotted lines (Fig. 4, A, D) were obtained with a

Fig. 4. A, oxidation of polyphenols by acetone-prepared enzyme from tea; B, reactions with added catechol oxidation products; C, ditto with added *p*-quinone; D, ditto with added tea-tannin oxidation products. Dotted lines in A and D represent reactions with a different enzyme.

gathered it was of interest to find out whether such behaviour was peculiar only to the tea-tannin oxidation products or whether it was generally true of the oxidized products of other polyphenols as well. The general scheme of the experiment was that the oxidized products of three phenolic bodies, catechol, quinol and tea tannin, should be added at the commencement of the reaction to systems each containing one of these polyphenols and the acetone-prepared enzyme. Comparisons of oxygen uptake results of two otherwise corresponding systems, with and without the addition of oxidation products, would reveal the effect, if any, of such additions. The oxidized products of catechol and tea tannin were obtained by oxidizing M/20 solutions of these polyphenols by acetone-prepared enzyme. It was not necessary to prepare experi-

separate sample of enzyme, as the sample used in the case of all other reactions became exhausted, a circumstance which cannot, however, affect the conclusions. These results are summarized in Table 1.

Table 1. Oxygen uptake during various polyphenol oxidations

Product added	Polyphenol oxidation, $\mu\text{l. O}_2/\text{hr./mg. enzyme}$			
	Catechol	Tea tannin	Quinol	
Nil	5.5	4.6	1.2	0.4*
Catechol oxidation products	7.4	5.6	5.3	—
<i>p</i> -Quinone	6.8	4.4	0.6	—
Tea tannin oxidation products	2.6	4.3	—	1.2*

* A different sample of enzyme.

Catechol oxidation products accelerate oxidations of catechol and tea tannin almost to the same extent, while quinol oxidation is increased four to five times. *p*-Quinone accelerates catechol oxidation and does not much affect tea-tannin oxidation; but it markedly inhibits quinol oxidation. Tea-tannin oxidation products increase the rate of quinol oxidation nearly three times but inhibit catechol oxidation to less than half its original activity. Oxidation of tea tannin also suffers a slight inhibition. These results point to the general conclusion that the oxidation of polyphenols is markedly affected by the presence, at the beginning of the reaction, of certain polyphenol oxidation products. There appears to be, however, no correspondence between the polyphenol reaction affected and the polyphenol-oxidation products which influence the reaction. In particular, the reversal of the rates of oxidation of catechol and tea tannin in presence of oxidation products of the latter appears to be due to a marked depression of catechol oxidation, but not to an acceleration of the tea-tannin oxidation.

Corroboration of this point is noticeable in the results of fresh leaf-mince reactions and those with washed tissues. The oxygen uptakes during tea-tannin oxidation of 200 mg. leaf mince in 1 hr. (Fig. 3) are 135 and 150 μ l. for unwashed and washed tissues respectively, while the corresponding figures for catechol oxidation are 98 and 163 μ l. for the same unwashed and washed tissues. The marked inhibition of catechol oxidation by tea-tannin oxidation products is thus indicated.

When tea tannin is oxidized either in the fermenting leaf or *in vitro* by the prepared enzyme, the product consists of the oxidized tannins in a large range of condensations. In the present state of our knowledge about the chemistry of tea it is not possible to distinguish between the simple tea tannin-*o*-quinone and its various condensation products, or to ascertain their relative amounts in a solution of oxidized tea tannin. For this reason, the effect of tea-tannin oxidation products on subsequent polyphenol oxidations cannot be attributed singly either to the one or the other of these constituents. The following experiment, however, affords some evidence on this point. Condensation in a solution of oxidized tannin can be increased by rendering the solution alkaline, and it was desired to find out whether such an increase in the condensation products would materially affect the results. Five ml. of an oxidized tea tannin solution (M/20) were made alkaline to pH 9.5–10.0, boiled and cooled, and the pH readjusted to 5.0. This solution and 5 ml. of original solution were both made up to 10 ml., and 2 ml. of each were added to the oxidation-reaction systems of catechol and tea tannin.

It can be concluded from the results (Fig. 5) that increasing the condensation products has no significant effect on the rate of tannin oxidation, while it slightly enhances the inhibition of catechol oxidation. Therefore, the inhibition effect observed in catechol oxidation by addition of tannin-oxidation products is probably to be associated more with the

Fig. 5. Oxidations of catechol and tea tannin in presence of tea-tannin oxidation products. I, tea-tannin oxidation, lower condensed products; II, ditto, higher condensed products; III, catechol oxidation, lower condensed products; IV, ditto, higher condensed products.

higher condensation products of tannin-*o*-quinone than with the simpler tannin-*o*-quinone itself. The difference between the rates of catechol oxidation with the two concentrations of condensed products is, however, small, but it may probably be due to the fact that the original solution of oxidized tea tannin which was standing for 24 hr. already contained an effective amount of condensed tannin-oxidation products, which might have been increased only slightly on the solution being rendered alkaline.

Reaction in a mixture of substrates. When a single enzyme oxidizes two substrates, the rate of oxidation of their mixture generally falls between the two individual values owing to the competition of substrates for the enzyme. An investigation of the action of the acetone-prepared enzyme on a mixed substrate of catechol and tea tannin showed (Fig. 6) that the initial rate of oxidation of the mixture was intermediate between the rates of individual oxidations of catechol and tea tannin. This is in accordance with the above general rule and indicates that a single enzyme is concerned in the reaction. Towards the end of the reaction, however, the accumulation of the oxidation products begins to exert its influence and tends to depress the rate of reaction.

Addition of catechol to the fermenting leaf leads to conditions in which the same tea enzyme is acting simultaneously both on catechol and tea tannin. The rate of oxidation would then be intermediate between the oxidation rates of the two

observed at Tocklai and St Coombs is due to the presence of two different enzymes in the materials derived from the two different sources. In the literature there is, no doubt, some evidence for the presence of more than one oxidase system in the same plant tissue [Boswell & Whiting, 1938; Marsh & Goddard, 1939]. But in this instance such a suggestion seems unnecessary, since this particular disagreement between St Coombs and Tocklai is due more to a difference in technique than to the nature of the materials. The reaction in the minced leaf as employed by Roberts is complicated by the accumulated tannin-oxidation products, which exert some influence on the course of the subsequent reactions.

Fig. 6. Oxidation of catechol, tea tannin and their mixture by acetone-prepared enzyme.

individual substrates. Harrison & Roberts [1939], however, found in a similar experiment that the rate of oxidation of the mixture was higher than that of the individual substrates. In arriving at this conclusion they made use of their previous result that catechol is oxidized at about one-third the rate of tea tannin, a result which was obtained without taking into consideration the presence and, therefore, the influence, of tannin-oxidation products. But, in view of the facts shown in the preceding section, the validity of this conclusion is open to doubt. When the individual rates of oxidation of catechol and tea tannin are investigated under conditions free from any extraneous interference, it is possible that the rate of oxidation of the mixture of substrates by the fermenting leaf system is actually intermediate between the two individual values.

DISCUSSION

Roberts [1941] has suggested that the difference in the rates of oxidation of catechol and tea tannin

SUMMARY

1. The relative rates of oxidation of catechol, tea tannin, pyrogallol, quinol and ascorbic acid by the tea enzyme prepared with acetone have been studied by manometric methods.

2. The discrepancy in the comparative rates of oxidation of catechol and tea tannin between our results and those of Assam workers has been shown to be due to an inhibitory effect, under the conditions of Assam experiments, of the condensation products of oxidized tea tannin on the oxidation of catechol. In view of this, the supposition of two different enzymes in Assam and Ceylon seems unnecessary.

3. It is shown that the presence of oxidized products of polyphenols at the commencement of a subsequent polyphenol oxidation reaction affects, in general, the course of such reaction.

4. The action of the enzyme prepared with acetone in a mixture of catechol and tea-tannin substrates has yielded results consistent with the view that both these substrates are oxidized by a common enzyme, namely, the tea polyphenol oxidase.

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