Studies on the 'Fermentation' of Ceylon Tea

4. ESTIMATION OF THE OXIDIZING ENZYME ACTIVITY

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(*Received* 8 *July* 1943)

In our previous studies [Lamb & Sreerangachar, 1940*a*, *b*] the oxidizing activity of the tea-leaf enzyme was measured by following the changes in the tannin content of an enzyme-substrate reaction mixture by the iodine titration method. In stressing the limitations of this method it was stated that it did not afford any indication of complex changes such as condensation, and there existed a possibility that the results were subject to an error due to these causes.

The oxidizing enzyme in tea leaf is a polyphenol oxidase and ascorbic acid is only indirectly oxidized in the presence of a suitable polyphenol. The function of the polyphenol in this reaction is that of an oxygen carrier, and its permanent oxidation becomes noticeable only after the complete oxidation of all ascorbic acid. The *o*-quinones are continually removed from the sphere of reaction and therefore their condensation cannot occur as long as there is an excess of ascorbic acid in the system. The ascorbic acid reaction thus provides the basis of a more reliable method for measuring enzyme activity in the tea-leaf enzyme preparation. Ezell & Gerhardt [1940] have likewise employed ascorbic acid as a substrate in the determination of oxidase activity in fruits and vegetables.

EXPERIMENTAL

Enzyme preparation and properties. The enzyme preparation employed in this investigation was obtained by grinding tea leaf with sand under acetone, washing until free of all colouring constituents and rapidly drying under vacuum. This acetone-prepared leaf powder was all but polyphenol-free and exerted only a very weak oxidizing action on pure *l*-ascorbic acid. Gradual removal of the associated polyphenols from this tissue powder rendered the enzyme progressively inactive towards ascorbic acid. Thus on extraction with water and exhaustive washing, a large fraction of the residual tannin was removed and the ascorbic acid oxidation activity fell markedly. Aeration for 2–3 hr. of a suspension in water of the extracted enzyme powder did not remove any further quantities of polyphenol; grinding the extracted powder with sand and water, filtering and washing resulted in the material still giving a positive ferric chloride reaction for tannin substrates. However, when the ground mass was treated with an excess of a dilute solution of ascorbic acid it was found possible to extract completely the last traces of polyphenol. The ascorbic acid oxidation activity of this final preparation is almost negligible. Table 1 gives results showing both the polyphenol oxidase activity on a prepared tea-tannin substrate solution measured iodometrically and the ascorbic acid oxidation activity estimated by the use of the 2:6-dichlorophenol-indophenol method at the above different stages of extraction. The reactions were all carried out in presence of McIlvaine's buffer at pH 5·4.

Table 1. *Effect of polyphenol removal on enzyme activity*

Extraction stage	Activity/0·1 g. enzyme powder: Polyphenol oxidase activity (4 hr.) (ml. N/20 $Na_2S_2O_3$)	Activity/0·1 g. enzyme powder: Ascorbic acid oxidation (5 min.) (mg. ascorbic acid)
1. Unextracted powder	10·5	0·29
2. After extraction with McIlvaine's buffer at pH 7·0 and complete washing	12·8	0·09
3. After aeration	12·0	0·07
4. After grinding with sand and water	—	0·06
5. After treatment with ascorbic acid	18·4	0·002

These results indicate that the enzyme in question is not ascorbic acid oxidase but that it is polyphenol oxidase, of which the activity increases on partial purification. Since the complete removal of all tissue-bound polyphenols renders the enzyme practically inactive towards ascorbic acid there can be no reasonable doubt that the ascorbic acid oxidation activity of the unextracted powder was due to an indirect action of a polyphenol-polyphenol oxidase system.

The ascorbic acid oxidation activity exhibited by the enzyme in the presence of polyphenol was, however, found to vary according to the amount of polyphenol present. It became, therefore, necessary to find out the optimum quantity of polyphenol that would give the maximum enzyme activity. Two polyphenols, catechol and amorphous tea tannin, were so studied and Table 2 shows the effect of their addition on ascorbic acid oxidation by the enzyme. The reaction mixture consisted of 5 ml. 0·1% ascorbic acid solution, 4 ml. distilled water, 1 ml. catechol or tea-tannin solution containing various amounts of polyphenol, and 20 mg. acetone-prepared enzyme powder. The mixture was aerated for 1 hr., after which it was filtered and 5 ml. titrated with a standard solution of 2:6-dichlorophenol-indophenol. Neither 100 mg. catechol nor 20 mg. tea tannin, the maximum amounts of polyphenols employed in this experiment, gave any blank 2:6-dye titre when dissolved in 5 ml. A control experiment in which the polyphenol

solution was replaced by 20% solution of trichloroacetic acid was also run at the same time, and the enzyme activity obtained by the difference between the control and experimental values.

Table 2. *Effect of addition of polyphenols on ascorbic acid oxidation activity*

Catechol			Tea tannin		
	Enzyme activity (mg. ascorbic acid)			Enzyme activity (mg. ascorbic acid)	
mg. added	(*a*)*	(*b*)*	mg. added	(*a*)*	(*b*)*
0	0·27	0·98	0	0·80	0·98
0·5	0·82	—	0·5	1·16	—
1·0	0·96	—	1·0	1·20	—
2·0	0·92	—	2·0	1·59	—
3·0	0·98	—	4·0	1·63	—
4·0	1·06	—	5·0	—	3·77
5·0	1·33	—	10·0	—	4·06
10·0	1·53	—	20·0	—	5·02
15·0	1·94	—	40·0	—	4·53
20·0	2·10	—	—	—	—
25·0	2·22	2·90	—	—	—
50·0	—	2·04	—	—	—
100·0	—	1·22	—	—	—

* (*a*) and (*b*) represent two separate enzyme preparations.

The results (Table 2) show that in a total volume (10 ml.) of the reaction mixture either 25 mg. catechol or 20 mg. tea tannin gave optimum values for ascorbic acid oxidation activity, which is higher in the case of tea tannin than catechol. But as the preparation of tea tannin in pure state is tedious and its identity still uncertain it was thought desirable to employ only the more definite compound, catechol. Instead of using catechol and ascorbic acid solutions separately it is convenient to prepare a mixed substrate solution containing 0·1% ascorbic acid and 0·5% catechol. 5 ml. of this solution, containing 5 mg. ascorbic acid and 25 mg. catechol, are usually employed in each reaction.

Optimum pH *and temperature.* The optimum conditions for ascorbic acid oxidation by the tea-leaf enzyme system are shown in Table 3. McIlvaine's buffer at various pH values was employed and the reactions for determination of optimum temperature were carried out in a thermostat at pH 5·4. There was slight autoxidation of ascorbic acid at pH > 6·0 and the values were corrected for such autoxidation errors.

Table 3. *Optimum* pH *and temperature*

pH	Enzyme activity (mg. ascorbic acid) Mixed enzyme	Insoluble enzyme	Temp. (°C.)	Enzyme activity (mg. ascorbic acid) Mixed enzyme	Insoluble enzyme
4·0	2·34	2·00	16	1·14	1·22
4·6	2·48	1·80	21	1·59	1·47
5·0	2·85	—	27	1·79	1·92
5·4	2·73	2·53	32	1·26	1·71
6·0	1·82	1·84	38	1·06	1·29
7·0	1·49	1·13			
8·0	1·36	1·18			

The mixed and insoluble enzymes were derived from different samples of leaf. The optimum pH for the mixed and the insoluble enzymes is 5·0 and 5·4 respectively, while both enzymes have the same optimum temperature, 27°.

Relation between soluble, insoluble and mixed enzymes. In earlier investigations it was shown that the activity of the unextracted powder was not equal to but always less than the sum of the activities of the extract and the residue. When this question was reinvestigated by the ascorbic acid method, it was observed that the activity of the unextracted powder consisted of those due to the soluble and insoluble enzymes. To a mixture of 5 ml. of ascorbic acid-catechol substrate and 5 ml. McIlvaine's buffer at pH 5·4 the enzyme was added in the following forms: (1) unextracted powder (20 mg.), (2) insoluble enzyme from 20 mg. unextracted powder, and (3) enzyme extract (2 ml.) derived from 20 mg. unextracted powder. The reactions were carried out separately. At the end of 1 hr., the enzyme activities were estimated by titration of 5 ml. filtrates in the case of solid enzymes and 6 ml. in the case of the enzyme extract. The results are shown in Table 4.

Table 4. *Activities of soluble, insoluble and mixed enzymes*

	mg. ascorbic acid oxidized		
	I	II	III
Soluble enzyme	0·82	0·41	0·28
Insoluble enzyme	3·56	1·41	0·90
Mixed enzyme	4·32	1·80	1·15

It can be concluded that the activity of the mixed enzyme represents the total of the soluble and insoluble enzyme activities, and therefore the acetone-prepared enzyme can be employed in any comparative study of the enzyme contents of tea-leaf samples. Condensation is eliminated in the present method, and it is possible that the explanation of the discrepancy between these results and the previous ones is in some way connected with this condensation phenomenon.

Effect of concentration of enzyme. That the activity is proportional to the amount of the reacting enzyme is shown (Table 5) by an experiment in which the quantities of the

Table 5. *Effect of enzyme concentration*

Quantity of enzyme powder (mg.)	Activity (mg. ascorbic acid)
5	0·3
10	0·58
20	1·17
40	2·45
60	3·57

enzyme were varied, keeping the other conditions the same. The period of reaction was, however, reduced to 30 min. in order to compare the activities while the reaction is proceeding briskly.

The ascorbic acid method can therefore be employed in tea-fermentation studies, especially in cases where it is required to assess or compare the enzyme activities. Such an application of the method has already been made in the study of enzyme activity in individual bushes.

Description of the proposed method and its advantages

Acetone-prepared enzyme powder (20 mg.) is weighed into a 50 ml. test-tube containing 5 ml. McIlvaine's buffer at pH 5·0 and 5 ml. catechol-

ascorbic acid substrate solution. The mixture is aerated for 1 hr., and 5 ml. filtrate are titrated in presence of 1 ml. 20 % trichloroacetic acid with 2:6-dichlorophenol-indophenol solution standardized according to the method of Menaker & Guerrant [1939] for its ascorbic acid equivalent.

A control with the same quantities of enzyme and substrate but with 5 ml. 10 % trichloroacetic acid solution instead of buffer is also run at the same time. The difference between the control value, which provides the initial amount of ascorbic acid, and the experimental result gives the amount of ascorbic acid oxidized during the reaction or, in other words, the enzyme activity. It has been established by a separate control that there are no autoxidation errors in the reaction at pH 5·0. No difficulty has been encountered in the dye titration of ascorbic acid due to the normal colour of either the enzyme extracts from tea leaf or its juice.

The iodometric method of measuring enzyme activity depends upon estimations of tannin by iodine titration, the limitations of which have been already stressed. The present method, on the other hand, has several advantages: (1) Both the chemicals used in substrate solution are of well-defined constitution and can be obtained in a high degree of purity. (2) The method is fairly rapid and does not involve the necessity of observing a standard time of reaction during titrations. (3) Greater accuracy is obtained not only in titrations but also from the fact that condensation errors are eliminated. (4) In iodometric titrations it was either necessary to obtain a completely tannin-free enzyme preparation or a control had to be run to determine the amount of buffer-extractable polyphenols. In the ascorbic acid method this is obviously unnecessary, as polyphenols are in fact added to the reaction mixture.

DISCUSSION

Condensation changes which follow the accumulation of *o*-quinones in a reaction mixture can take place between two or more molecules of *o*-quinones themselves or between one molecule of *o*-quinone and one or more molecules of unoxidized polyphenol. Such condensed products are oxidized by iodine relatively more slowly than the original polyphenols, and this fact is reflected in an apparent fall in their iodine titre. Some indication of this is afforded by our results on catechol oxidation by oxidase and peroxidase [Lamb & Sreerangachar, 1940*b*]. Oxidase activity produced very little change in iodine titre, whereas peroxidase produced a marked fall in titre, and it appeared that peroxidase activity resulted in the formation of more condensation products than does oxidase activity.

The criticism of Shaw's iodometric method for the volumetric estimation of tea tannin by Barua & Roberts [1940] is based on this question of condensation of tannin bodies after oxidation and the errors that may be introduced thereby. Reference to Table 3 shows that the conditions of optimum pH and temperature for the action of the tea-insoluble enzyme are almost the same when determined by the iodometric method previously used and by the present ascorbic acid method, which is independent of any interference due to condensation. Our previous conclusions, which were mostly based on the iodometric method, may not then be substantially affected by errors due to condensation.

An explanation of this agreement may perhaps be suggested. As condensation is a chemical reaction, it is reasonable to assume that the rate and amount of condensation of oxidized tannins will bear a definite relation to the amount of oxidized tannins present, which in its turn will be related to the activity of the enzyme. The fall in iodine titre due to the condensation factor would, therefore, vary in proportion to the enzyme activity itself, so that the method can still be employed in any comparative study of enzyme activities.

Further investigation is needed to amplify our present insufficient knowledge about this condensation process of oxidized tea tannin bodies. For example, it is not known whether or not the condensation phenomena are attended by any further oxygen uptake or even by simple dilatometric changes, in which case the manometric results on the fermentation of tea may be regarded as subject to an error.

SUMMARY

1. A new method of estimating oxidase activity in tea, by the use of ascorbic acid as substrate, has been described. The method is simple, accurate and avoids errors due to condensation of oxidized polyphenols, which was the chief drawback of the iodine titration method.

2. The values for the optimum pH and temperature of the insoluble enzyme are confirmed by this new method.

REFERENCES

Barua, D. N. & Roberts, E. A. H. [1940]. *Biochem. J.* **34**, 1524.

Ezell, B. D. & Gerhardt, F. [1940]. *J. agric. Res.* **60**, 89.

Lamb, J. & Sreerangachar, H. B. [1940*a*]. *Biochem. J.* **34**, 1472.

Lamb, J. & Sreerangachar, H. B. [1940*b*]. *Biochem. J.* **34**, 1485.

Menaker, M. H. & Guerrant, N. R. [1939]. *Industr. Engng Chem.* (Anal. ed.), **10**, 25.