CCXCII. ASCORBIC ACID OXIDASE FROM DRUMSTICK, *MORINGA PTERYGOSPERMA*.¹

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THE high content in ascorbic acid of the pods of the drumstick tree, *Moringa pterygosperma*, was demonstrated by Damodaran & Srinivasan [1935], using the Tillmans-Harris method. The starting point of the present investigation was the observation that the press juice from this material, in contrast to its trichloroacetic acid extract, exercised no reducing action on 2:6-dibromophenol-indophenol. That this anomalous behaviour was due to a vigorous enzymic oxidation of ascorbic acid in the press juice became evident from further experiments which showed, in the first place, that the juice when added to other normally stable sources of ascorbic acid such as orange juice or the press juice of *Phyllanthus emblica* caused a rapid diminution in their ascorbic acid content and secondly that if the drumstick pods in the intact condition were first immersed for a short time in boiling water, the juice obtained on pressing had a Tillmans titre practically equivalent to, or slightly higher than, that of a trichloroacetic acid extract.

Studies on the enzymic oxidation of ascorbic acid were begun by Szent-Györgyi [1928] who concluded that the oxidation of ascorbic acid by plant tissues was due to peroxidase acting through the intermediary of a phenolic compound, the phenol being first oxidized by peroxidase to the quinone which in turn oxidized the ascorbic acid, presumably without the intervention of a second enzyme. In later communications [1930; 1931] he modified this view and postulated the existence in cabbage juice of a specific "hexoxidase" which was responsible for the oxidation of ascorbic acid. From a study of the kinetics of the reaction which showed that the rate of oxidation increased with increasing concentrations of substrate, the conclusion was drawn that this oxidizing enzyme was of a complex nature and involved the intervention of an intermediate substance "x".

Zilva [1934] also described experiments to show that Bramley's seedling apples contained an enzyme, distinct from and unconnected with peroxidase, which was capable of reversibly oxidizing vitamin C and which he considered to be similar in nature to Szent-Györgyi's "hexoxidase".

During the course of the present work Tauber *et al.* [1935] have demonstrated the presence in the pericarp of *Cucurbita maxima* of a specific oxidase for ascorbic acid, which differs in many respects from Szent-Györgyi's "hexoxidase".

The enzyme from *M. pterygosperma* now described presents few analogies with Szent-Györgyi's "hexoxidase", but appears to be similar in most respects to that obtained by Tauber *et al.* from *C. maxima*. In the presence of molecular oxygen ascorbic acid is oxidized by it directly and specifically to its reversible first oxidation product; there is no evidence of the intervention of an intermediate carrier. A very active concentrate capable of quantitatively oxidizing large amounts of ascorbic acid in an extremely short time is readily obtained by

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fractional precipitation of the press juice with ammonium sulphate. Such a preparation has no action upon guaiacum, benzidine, catechol, pyrogallol or *p*-phenylenediamine, showing the absence of a polyphenol or an indophenol oxidase. On the other hand, it always shows peroxidase activity, blueing guaiacum in the presence of H_2O_2 . The differential action of inhibiting agents, however, leaves no doubt of the fact that the oxidation of ascorbic acid is entirely independent of the presence of peroxidase. Thus, after treatment with acetone or alcohol the preparation has little oxidizing action on ascorbic acid, though the peroxidase remains unimpaired (Table II). On the other hand, 10^{-4} *M* cyanide completely inhibits the reaction with guaiacum and H_2O_2 , while 65% of the original capacity to oxidize ascorbic acid is still retained (Table III and Fig. 2). Further, in the press juice of the radish, *Raphanus sativus*, which contains both ascorbic acid and a peroxidase, the latter is without action on the former. Szent-Györgyi also showed [1928] that a purified peroxidase preparation is without action on ascorbic acid. In the present instance the usual colour reactions gave no evidence of the presence of a phenol or a quinone. No mention is made by Tauber *et al.* [1935] as to the presence or absence of peroxidase in their ascorbic acid oxidase preparation from *C. maxima*.

A study of the course of the oxidation of ascorbic acid by the enzyme from drumstick reveals that it is unimolecular (Table IV). The rate of oxidation is proportional to enzyme concentration (Table VI) and is unaffected by increasing quantities of substrate.

In one important respect, viz., in its sensitiveness to cyanide, ascorbic acid oxidase from drumstick differs from the corresponding enzyme in *C. maxima*, as well as from "hexoxidase". While both the latter enzymes have been shown to be affected only by high concentrations of cyanide (0.01%), the activity of the enzyme from drumstick is greatly inhibited by 10^{-3} *M* cyanide or H_2S (Table III and Fig. 2). Even 10^{-5} *M* cyanide has a definite inhibitory effect.

EXPERIMENTAL.

Preparation of the enzyme. From preliminary experiments it was found that by treating the pressed juice of drumstick with solid $(\text{NH}_4)_2\text{SO}_4$ to 1/9 saturation (5%), an inert precipitate was thrown down, while the resulting filtrate contained almost the whole of the original enzyme activity; further saturation of the active filtrate with $(\text{NH}_4)_2\text{SO}_4$ to 3/4 saturation (35% final concentration) precipitated most of the enzyme, the filtrate being practically inactive. The following procedure was therefore adopted in preparing the enzyme.

About 15–20 drumsticks, weighing nearly 700 g. with the outer skin scraped off, were passed through a mincer, the juice pressed out and centrifuged for 20 min. The greenish yellow supernatant liquid (about 400 ml.) was cooled in ice and $(\text{NH}_4)_2\text{SO}_4$ added to a concentration of 5%. The heavy precipitate settling down on standing in the ice-chest overnight was filtered off and the resulting brownish yellow filtrate further treated with $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 35%. The fine precipitate which appeared was separated by centrifuging (3000 r.p.m.) for half an hour, dispersed in about 50 ml. of water and filtered. The clear yellow filtrate thus obtained possessed powerful enzyme activity and was the material used for all experiments described here. Kept under toluene in the refrigerator, such a preparation retained its activity undiminished for over a month.

Methods.

Water, redistilled four times in all-glass (pyrex) apparatus was used for making up all the experimental solutions. A solution of ascorbic acid (B.D.H.) prepared in glass-distilled water and brought to pH 5.3 (*v. infra*) with Na₂CO₃ served as substrate, the exact strength of this solution being determined by titration against standard iodine (*N*/200).

Table I. Oxidation of ascorbic acid at different pH values.

McIlvaine's citric acid-phosphate buffer		Sørensen's phosphate buffer	
pH	Ascorbic acid oxidized %	pH	Ascorbic acid oxidized %
3.0	27.94	5.3	100.00
3.4	55.89	5.6	100.00
3.8	76.47	5.9	96.16
4.2	85.31	6.5	84.63
4.6-5.6	89.71	6.8	67.32
5.8	82.75	7.2	42.32
6.0	81.04	8.0	23.08
6.4	68.96	—	—
6.8	44.82	—	—
7.2	34.48	—	—
7.6	24.14	—	—

Fig. 1. Activity-pH curves for ascorbic acid oxidase.

The reaction mixture was contained in 50 ml. beakers held in a thermostat at 37°. After the addition of enzyme and during its action, the contents of the beaker were kept well stirred. Enzyme action was arrested, when desired, by adding acetic acid to the reaction mixture, in which unoxidized ascorbic acid was determined at any time by titrating against a standard solution of 2:6-dibromophenolindophenol.

Control experiments were run under identical conditions, but with the boiled enzyme. Though stock solutions of ascorbic acid prepared as described suffered no oxidation on standing for two or three days in stoppered bottles, the control experiments, conducted as they were in open beakers and with the probable metallic contamination from the boiled enzyme showed small decreases in the Tillmans titres. Such decreases were extremely minute compared with those caused by the enzyme; the corresponding corrections were, however, made in the experimental values.

The optimum pH for enzyme action.

McIlvaine's phosphate-citric acid and Sørensen's phosphate buffers were used for obtaining solutions of various reactions. The oxidation taking place in 3 min. at 37° in 5 ml. of a reaction mixture composed of 0.4 mg. ascorbic acid, 0.1 ml. enzyme and 1 ml. of buffer solution of the desired pH was determined by titration of the unoxidized ascorbic acid.

From Table I and Fig. 1 it will be seen that the optimum reaction for this enzyme lies over a range pH 4.6–5.6 in citric acid-phosphate buffer and 5.3–5.6 in phosphate buffer, the natural pH of the press juice of drumstick being 5.6–5.8. Further, the extent of oxidation of ascorbic acid by the enzyme is greater in the latter than in the former buffer. In all subsequent experiments phosphate buffer of pH 5.3 (9.75 ml. $M/15$ NaH_2PO_4 + 0.25 ml. $M/15$ Na_2HPO_4 mixture according to Sørensen) was therefore used.

Nature and extent of oxidation of ascorbic acid by the enzyme.

The reaction does not proceed in the absence of oxygen, no oxidation taking place in evacuated Thunberg tubes.

The enzyme oxidizes ascorbic acid to its reversible oxidation product as is evident from the following experiment: 0.5398 mg. ascorbic acid was completely oxidized (as judged by a zero Tillmans titre in a duplicate experiment) by 0.1 ml. enzyme at pH 5.3 at 37°. Through the oxidized solution H_2S was bubbled for 10 min. and the solution kept overnight in a stoppered flask. H_2S was then completely removed by a current of carbon dioxide; the solution then had an ascorbic acid content of 0.5202 mg.

Action of the enzyme on other compounds.

Guaiacum, benzidine, catechol, pyrogallol and *p*-phenylenediamine were not affected by the enzyme preparation, suggesting that the enzyme, which oxidizes ascorbic acid, is not a polyphenol or an indophenol oxidase.

All these substances were oxidized by the enzyme preparation in the presence of H_2O_2 , indicating the presence of peroxidase, but the following experiments show that the peroxidase action was totally unconnected with the oxidation of ascorbic acid.

Effect of alcohol or acetone on ascorbic acid oxidase and peroxidase.

2 ml. enzyme solution were treated in a 15 ml. centrifuge-tube with 4 vol. of 95 % ethyl alcohol, methyl alcohol or acetone. The precipitate formed in each

case was separated by centrifuging and redispersed in 2 ml. water. The supernatant liquid was evaporated at a low temperature *in vacuo* and the resulting residue also taken up in 2 ml. water. The enzymic activities of these two fractions were compared with that of the untreated enzyme preparation. The results are given in Table II.

Table II. *Effect of alcohol or acetone on ascorbic acid oxidase.*

Reaction mixture: 0.5 mg. ascorbic acid, 0.1 ml. enzyme in a total volume of 5 ml. at pH 5.3 at 37°.

	Ascorbic acid oxidized in 2 min. %
1. Untreated enzyme	77.52
2. Enzyme treated with ethyl alcohol	8.90
3. Enzyme treated with methyl alcohol	2.60
4. Enzyme treated with acetone	26.97
5. Filtrates from 2, 3 or 4	0.0

The enzyme is thus seen to be highly sensitive to ethyl or methyl alcohol and to a less extent to acetone. The peroxidase activity of the preparation as studied qualitatively by the colour developed with a solution of pyrogallol in the presence of H_2O_2 , remained unimpaired by such treatment.

Effect of cyanide or H_2S on ascorbic acid oxidase and peroxidase.

The action of the enzyme on ascorbic acid under standard conditions was studied with and without the addition of cyanide or H_2S . Simultaneously, peroxidase activity was also studied qualitatively as before.

Table III. *Effect of cyanide or H_2S on ascorbic acid oxidase and peroxidase.*

Reaction mixture: 0.3 mg. ascorbic acid, 0.1 ml. enzyme in a total volume of 5 ml. at pH 5.3 at 37°.

Time in min.	Ascorbic acid oxidized (%)					
	Enzyme	Enzyme + $10^{-5} M$ KCN	Enzyme + $5 \times 10^{-5} M$ KCN	Enzyme + $10^{-4} M$ KCN	Enzyme + $10^{-3} M$ KCN	Enzyme + $10^{-3} M$ H_2S
1	33.88	29.03	25.81	22.58	6.45	6.34
2	56.45	53.23	46.77	43.55	9.68	11.11
3	75.82	70.98	67.75	64.52	12.9	14.29
4	90.33	88.72	85.49	82.26	14.52	—
Peroxidase activity	+	±	—	—	—	—

From the above table and the graphs in Fig. 2, the inhibitory influence of cyanide or H_2S at low concentration is evident. Slight though definite inhibition of the oxidation of ascorbic acid takes place even at a cyanide concentration of $10^{-5} M$. But the effect is marked only above a concentration of $10^{-4} M$. Peroxidase activity, on the other hand, ceases completely long before such a concentration of cyanide is reached.

Enzyme kinetics.

Order of the reaction. The oxidation of ascorbic acid by the enzyme is a reaction of the first order, as is seen from the calculated values for the velocity constant

given in the last column of Table IV. For this particular experiment a very dilute solution of 2:6-dibromophenolindophenol, equivalent to 0.05 mg. ascorbic acid per ml. was used to ensure greater accuracy.

Fig. 2. Effect of KCN or H_2S on ascorbic acid oxidase.

Table IV. *Rate of oxidation of ascorbic acid by the enzyme.*

Reaction mixture: 0.41 mg. ascorbic acid, 0.1 ml. enzyme in a total volume of 5 ml. at pH 5.3 at 37°.

Time in min.	Ascorbic acid oxidized %	$k = \frac{1}{t} \log_{10} \frac{a}{a-x}$
1	22.67	0.11160
2	38.68	0.10650
3	52.00	0.10633
4	62.67	0.10697
5	70.68	0.10652
7	82.00	0.10639

Relation between concentration of enzyme and its action. Mixtures of 1 ml. ascorbic acid solution containing 0.41 mg. of acid, 1 ml. phosphate buffer pH 5.3 and varying quantities of glass-distilled water to make the final volume

after the addition of enzyme 5 ml., were incubated with different quantities of enzyme at 37° and the rate of oxidation determined by titration. The results are given in Table V.

Table V. Influence of the concentration of enzyme on the rate of oxidation of ascorbic acid.

Time in min.	mg. ascorbic acid oxidized by			
	0.3 ml. enzyme	0.2 ml. enzyme	0.1 ml. enzyme	0.05 ml. enzyme
1	0.2033	0.1621	0.09344	0.04397
2	0.3243	0.2583	0.1597	0.08245
3	—	0.3243	0.2061	0.1099
4	—	—	0.2583	—
5	—	—	0.2913	0.1759
7	—	—	0.3380	0.2199
10	—	—	—	0.2859

Fig. 3. Enzyme concentration and ascorbic acid oxidation.

The above results are plotted graphically in Fig. 3. From these graphs the results given in Table VI were obtained.

From Table VI, it is clear that the rate of oxidation of ascorbic acid is directly proportional to enzyme concentration.

Substrate concentration and enzyme action. 5 ml. reaction mixture composed of 1 ml. phosphate buffer pH 5.3, 0.2 ml. enzyme and different quantities of ascorbic acid ranging from 0.5633 to 4.893 mg. were incubated for 3 min. at 37°. The amount of ascorbic acid oxidized at the end of the interval was determined in each case. 0.4074 mg. ascorbic acid was uniformly oxidized, showing that under these conditions the rate of oxidation is unaffected by increasing quantities of substrate.

Table VI. *Enzyme concentration and ascorbic acid oxidation.*

Enzyme quantity ml. (E)	Time in min. (T)	$E \times T$	Ascorbic acid oxidized mg.
0.30	1.0	0.3	0.2150
0.30	2.0	0.6	0.3125
0.20	1.5	0.3	0.2175
0.20	3.0	0.6	0.3200
0.10	3.0	0.3	0.2150
0.10	6.0	0.6	0.3150
0.05	6.0	0.3	0.2025
0.05	12.0	0.6	0.3200*

* Obtained by extrapolation.

SUMMARY.

1. From the press juice of the drumstick, *Moringa pterygosperma* an enzyme preparation has been obtained which, in presence of oxygen, oxidizes ascorbic acid to its reversible oxidation product.

2. The enzyme does not oxidize guaiacum, benzidine, catechol, pyrogallol or *p*-phenylenediamine.

3. There is present in the preparation a peroxidase which, however, has been shown to have no role in the oxidation of ascorbic acid.

4. The oxidation of ascorbic acid by the enzyme is a reaction of the first order. The rate of oxidation is directly proportional to enzyme concentration and above a certain limit is unaffected by increasing quantities of substrate.

5. From the above findings, the ascorbic acid-oxidizing action of the preparation is to be attributed to an oxidase therein specific for ascorbic acid.

6. Low concentrations of cyanide or H_2S inhibit the activity of the enzymes. It is destroyed almost completely by alcohol and partially by acetone.

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