THE ENZYME-CATALYSED OXIDATION OF ASCORBIC ACID IN FRUIT AND VEGETABLE SUSPENSIONS

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Summary

Enzymic oxidation of ascorbic acid is particularly rapid in orange rind, parsley, french bean, and cabbage suspensions. It is only slight in onion and swede turnip, and negligible in orange juice, rose hip, and tomato suspensions. Apple suspensions give very variable results.

In many suspensions the oxidase activity is far greater than could be due to the copper concentration, if present in ionized form.

The ascorbic acid oxidase of cabbage has an optimum pH of approximately 6. In suspensions of cabbage in water the activity is mainly in the insoluble particles, but the solubility is increased in the presence of neutral salts. The enzyme is precipitated by saturation with ammonium sulphate.

Evidence has been obtained that the mechanism of oxidation in apple suspensions is largely direct.

I. Introduction

In a previous paper (Huelin and Stephens 1948), the authors described studies of the copper-catalysed oxidation of ascorbic acid in fruit and vegetable suspensions. In the work on copper catalysis, the suspensions were boiled for two minutes to inactivate the enzymes. The relative importance of enzymic oxidation in unboiled suspensions is discussed in the present paper.

Enzymes which oxidize ascorbic acid have been found in a number of tissues. Johnson and Zilva (1937) found that cabbage, cauliflower, cucumber, and marrow contain enzymes which oxidize ascorbic acid directly. They showed that apple and potato tissue did not oxidize ascorbic acid directly but contained phenolases which oxidized ascorbic acid in the presence of catechol or juice. However, Hackney (1946) has obtained evidence of a true ascorbic acid oxidase (which can oxidize ascorbic acid directly) in apple juice.

Highly active preparations of ascorbic acid oxidase have been obtained by various workers. Tauber, Kleiner, and Mishkind (1935) obtained the enzyme from an extract of Hubbard squash by precipitation with acetone. Highly purified preparations have been obtained by Lovett-Janison and Nelson (1940) and by Powers, Lewis, and Dawson (1941). The purified preparations were found to contain 0.15-0.24 per cent. of copper. Tadokoro and Takasugi (1939) obtained the enzyme in crystalline form.

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Szent-Gyorgyi (1931) precipitated an ascorbic acid oxidase from cabbage extract by saturation with ammonium sulphate. This enzyme oxidized ascorbic acid directly and was devoid of polyphenolase activity.

As the most highly purified preparations of ascorbic acid oxidase contain definite amounts of copper, this enzyme is regarded by some workers as a copper protein complex. Meiklejohn and Stewart (1941) found that the ascorbic acid oxidase activity of cucumber juice was proportional to the non-dialysable copper but greater than the catalytic activity of the copper alone. Other workers, however, have doubted the existence of a specific oxidase and claimed that the activity is simply due to copper either alone or in non-specific association with protein. Stotz, Harrer, and King (1937) found copper-gelatin or copper-albumen mixtures to simulate most of the properties of ascorbic acid oxidase. The catalytic activity of such mixtures was, however, less than that of the copper alone.

As the work described in this paper is primarily a study of enzymic oxidation such as would occur during fruit and vegetable processing, i.e. in the preparation of pulps and juices, and in the partial disintegration of tissue for freezing, drying, or canning, it follows that only enzymes which are active in the disintegrated tissue are considered. It is possible for enzymes to be inactive at the pH of the minced tissue or tissue extract but highly active in regions of different pH which may occur in the intact cells.

II. Experimental Procedure

The fruit and vegetable suspensions were prepared in the Waring Blendor from one part by weight of tissue and four parts of glass distilled water. However, the rose hip suspension was found too viscous to handle, and had to be diluted to half this concentration before use.

The measurements of oxidation were carried out at 40°C. as previously described (Huelin and Stephens 1948). After adding 2 ml. of 0.5 per cent. ascorbic acid to 50 ml. of suspension, the usual procedure was to pipette aliquots of the suspension into 3 per cent. metaphosphoric acid at definite intervals and titrate with 2.6 dichlorophenolindophenol. Some suspensions, however, were difficult to pipette and titrate, and a modified procedure was necessary. This consisted of adding metaphosphoric acid solution to the whole of the suspension after a definite time interval and making up to volume with metaphosphoric acid. An aliquot of the centrifuged liquor was taken for titration. To determine the ascorbic acid present at zero time, metaphosphoric acid was first added to the suspension followed by 2 ml. of 0.5 per cent. ascorbic acid and a similar procedure followed. (It is essential to study the oxidation in whole suspensions, as most of the enzyme activity may be in the suspended particles).

The results for unboiled suspensions were expressed as per cent. oxidation of ascorbic acid in five minutes. This was preferred to a velocity constant, as the reaction kinetics were not similar in all cases. The per cent. oxidation in suspensions boiled for two minutes, which is due to traces of copper or other

thermostable catalysts, was subtracted to obtain the true enzymic oxidation. It was assumed that the enzymic and non-enzymic catalysis were independent.

III. Oxidation in Different Tissue Suspensions

The enzymic oxidation of ascorbic acid in various fruit and vegetable suspensions is given in Table 1.

Table 1

Enzymic Oxidation in Fruit and Vegetable Suspensions

Suspension	pH of Unboiled Suspension	Copper Concentration (p.p.m.)	Per Cent. Oxidation in 5 Minutes
Apple	3.2	0.2	88.5
Orange juice	3.6	0.1	0
Rose hip	3.6	0.2	0
Tomato	4.3	0.2	0
Orange rind	4.9	0.2	59.7
Onion	5.4	0.1	1.0
Parsley	5.7	—	88.6
French bean	6.1	0.2	71.0
Potato	5.9	0.2	13.1
Cabbage	5.9	0.2	93.8
Asparagus	6.2	0.3	20.1
Swede Turnip	6.3	0.1	1.9
Silver beet	6.7	0.4	39.1

These data indicate very high enzyme activity in apple, orange rind, parsley, french bean, and cabbage suspensions; only slight activity in onion and swede turnip suspensions; and negligible activity in orange juice, rose hip, and tomato suspensions.

In the initial experiments, when single samples of a number of different fruits and vegetables were compared, the apple suspension gave a very high enzyme activity. On further investigation of a number of apple samples considerable variation was experienced. The per cent. oxidation in five minutes for thirteen apple suspensions of pH 3.2-3.7 was found to vary from 3 to 91, with a mean of 24. It is of considerable interest to find a definite oxidase activity in this range of pH, as neither ascorbic acid nor polyphenol oxidase is usually found active below pH 4.

In many of the suspensions the oxidase activity is far greater than would be expected if all the copper were in the free ionic form. This is particularly noticeable in the apple, french bean, and cabbage suspensions (Table 1), which all had a copper content of 0.2 part per million. If the enzyme is inactivated by boiling, the addition of even ten parts of copper per million fails to restore the original level of oxidation. The results are shown in Table 2.

TABLE 2

COMPARISON OF ENZYMIC AND COPPER CATALYSIS

Suspension	Per Cent. Oxidation in 5 Minutes		
	Enzyme	Copper (1 p.p.m.)	Copper (10 p.p.m.)
Apple	88.5	9.0	34.5
French bean	71.0	3.8	42.3
Cabbage	93.8	4.3	47.8

If the oxidation is due to copper enzymes, they must hold the copper in a special type of combination, which considerably enhances its catalytic effect. Considerably more than non-specific association with protein is involved as the addition of gelatin or albumen can actually reduce the catalytic effect of copper (Stotz, Harrer, and King 1937; Huelin and Stephens 1948).

IV. THE ASCORBIC ACID OXIDASE OF CABBAGE

The per cent. oxidation in five minutes in four cabbage suspensions of pH 5.9-6.4 varied from 45 to 94. Oxidation in the centrifuged liquor was only a fraction of that in the whole suspension and varied from 0 to 10 per cent. Most of the enzyme activity appears to be in the insoluble particles. The enzyme in the original suspension was found to be completely inactivated in 30 seconds at 100°C.

The effect of pH on the activity of the insoluble enzymes was determined very readily. The suspension was centrifuged at 2,000 r.p.m. and the insoluble portion washed twice with glass distilled water. It was then made up to the same volume as the original suspension with citrate-phosphate buffer of the appropriate pH. Studies of oxidation gave the following results (Table 3).

TABLE 3

EFFECT OF pH ON CABBAGE OXIDASE ACTIVITY

pH	Per Cent. Oxidation in 5 Minutes
3	2.3
4	0.8
5	50.2
6	65.7
7	45.4
8	22.4

Maximum activity was obtained at pH 6, which was approximately the pH of the original cabbage suspension. The relation is similar to that obtained with ascorbic acid oxidase of Hubbard squash (Tauber, Kleiner, and Mishkind 1935).

In suspensions of cabbage in water, not more than one-tenth of the enzyme activity was in the soluble portion. The solubility of the enzyme was considerably increased in the presence of neutral salts. The addition of 0.5M sodium chloride,

potassium chloride, or ammonium sulphate to the suspension increased the proportion of soluble enzyme to about half. These salts reduced the pH by about 0.2. After adding the salt, the suspension was allowed to stand at 1°C. for two hours before centrifuging. The effect of various concentrations of sodium chloride is shown in Table 4.

TABLE 4

EFFECT OF SODIUM CHLORIDE ON SOLUBILITY OF CABBAGE OXIDASE

Sodium Chloride (Concentration)	Per Cent. Oxidation in 5 Minutes in Suspension (S)	Centrifuged Liquor (L)	Proportion of Soluble Enzyme (L/S)
Nil	67.9	7.2	0.11
0.1M	91.4	17.2	0.19
0.2M	94.6	30.5	0.32
0.3M	86.4	40.9	0.47
0.4M	89.9	51.7	0.57
0.5M	70.5	36.7	0.52
0.6M	70.9	28.0	0.39
0.8M	62.9	26.4	0.42
1.0M	57.4	20.8	0.36

Maximum solubility was obtained in 0.4M sodium chloride.

Sodium chloride also brings about a gradual inactivation of the enzyme, and the suspensions and liquors should be held at a low temperature except when measurements of oxidation are actually being made. Concentrations of sodium chloride up to 1.0M gave negligible loss of activity in six hours at 1°C, but considerable loss at 40°C. The loss at 40°C. is shown in Table 5.

TABLE 5

INACTIVATION OF CABBAGE OXIDASE BY SODIUM CHLORIDE AT 40°C.

Hours at 40°C.	Per Cent. Oxidation in 5 Minutes in Suspensions in		
	Water	0.4M NaCl	1.0M NaCl
0	67.1	79.0	56.3
3	61.0	40.6	32.1
6	56.4	19.5	14.5

The solution of enzyme in 0.4M sodium chloride was purified with barium acetate and the enzyme was precipitated by saturation with ammonium sulphate. This follows the procedure of Szent-Gyorgyi (1931). The precipitated enzyme was redissolved in 0.4M sodium chloride buffered to pH 6.0 and showed negligible loss of activity.

V. THE OXIDIZING SYSTEM OF APPLE

Further work on the oxidizing system of apple has been limited by the difficulty of obtaining material of uniformly high activity. However, the investigations have generally confirmed the conclusion of Johnson and Zilva (1937) that the mechanism of oxidation is largely indirect.

An enzyme preparation was obtained from one suspension by precipitation with acetone and was dispersed in the same volume of citrate-phosphate buffer.

A further quantity of enzyme was dispersed in the acetone filtrate (from which the acetone had been boiled off), and diluted to the same volume as the original suspension. Measurements of oxidation (per cent. in five minutes) gave the following results:

Original suspension (pH 3.2) - - - -	13.9
Enzyme in citrate-phosphate buffer (pH 3.2) -	0.9
Enzyme in acetone filtrate - - - - -	14.8

For all except a small proportion of the oxidation, the presence of the thermostable acetone-soluble material was required as well as the enzyme. Presumably oxidation products are first formed from the soluble material, and these oxidize the ascorbic acid. The oxidizable material in the acetone filtrate may be phenolic in nature but it is doubtful if the oxidation involves a polyphenol oxidase of the usual type, which has a maximum activity at pH 6-8. The activity of the enzyme in citrate phosphate buffer of pH 3.2 was not affected by the addition of 0.1 per cent. pyrogallol.

Typical polyphenol oxidases appear to be still present, if inactive in the original suspension. When the enzyme was dispersed in citrate phosphate buffer of pH 7, the addition of 0.1 per cent. pyrogallol definitely promoted the oxidation of ascorbic acid. The results are given as per cent. oxidation in five minutes.

Enzyme in citrate-phosphate buffer (pH 7) -	1.3
Enzyme + 0.1 per cent. pyrogallol - - -	17.7

VI. Discussion

The data which have been obtained both in this and the previous paper (Huelin and Stephens 1948) give some indication of the factors responsible for the varying stability of ascorbic acid in different fruits and vegetables. Ascorbic acid has been found particularly stable in onion and rose hip tissue, and in orange juice. Onion tissue has only slight oxidase activity and also contains "protective" substances which reduce copper catalysis to a very low level. Rose hip tissue has negligible oxidase activity and also contains "protective" substances. In this tissue the low pH and very high concentration of ascorbic acid (about 500-1,000mg./100g.) also tend to reduce copper catalysis. The stability of ascorbic acid in orange juice is due to similar factors. Orange juice has a comparatively high level of ascorbic acid (50-80mg./100ml.), but not as high as rose hip.

Pulped tomato tissue appears to have little oxidase activity, but offers little protection against copper catalysis. The avoidance of copper contamination in tomato products is particularly desirable.

Retention of ascorbic acid in apple products is usually very poor. Oxidase activity is very variable, but generally quite appreciable. Pressed apple juice is often completely devoid of ascorbic acid. Copper catalysis is of less importance, due to the low pH.

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