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ASCORBIC ACID OXIDASE IN CITRUS

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Enzymes are in the class of those substances known as "catalysts," which cause chemical reactions to proceed at higher rates than would be the case in the absence of the catalyst. In many important cases the rate in the absence of the enzyme is to all intents and purposes equal to zero. Enzymes are distinguished from catalysts by the fact that they occur only within or as secretions of living organisms. All enzymes about which sufficient information is available are known to be proteins. They consequently share all the special properties of proteins, and their physical state and their catalytic function depend markedly upon factors such as pH, temperature and ionic strength.

This is a study of the copper containing enzyme, ascorbic acid oxidase, in citrus fruits. It is responsible for the conversion of ascorbic acid (Vitamin C) to dehydroascorbic acid and is therefore of interest to the citrus industry. Recently (2, 3) evidence has been presented that this enzyme is present in young citrus fruits. The enzyme activity has been followed in developing whole fruits of a number of species and varieties of citrus and in the various fruit parts.

MATERIALS AND METHODS

Six different varieties of citrus were selected according to their size, apparent age and availability—Villafranca lemons, Persian limes, Valencia oranges, Pineapple oranges, Thompson Pink grapefruit and Marsh Seedless grapefruit. Fruits of each variety, picked at the same date, were separated into eight sizes in order to check the ascorbic acid oxidase activity at various sizes of growth.

Enzyme Preparation.—The samples were washed clean of residues, and the fruit was sliced and ground in a Mouli Persil mincer. Larger fruit was separated into flavedo, albedo and juice with a potato peeler or by hand, and the component parts were weighed. Aliquants of the minced tissue and 0.1 M phosphate buffer at pH 9.0 (1:5 W/V) were ground in a cold Potter homogerizer. Older tissue, especially flavedo and albedo, required mechanical grinding to break the cells, as did the outer seed coat and cotyledons. Care was taken to keep the mortar in the ice bath at all times.

The homogenate was centrifuged at 0° C. in a Servall refrigerated centrifuge at 5,000 X G for 10 minutes; the supernatant was decanted and retained for enzyme assay. Previous results showed that the enzyme remained in the supernatant at centrifugal speeds up to 30,000 X G for 10 minutes.

pH Level of Extracting Medium.—In order to avoid gel formation by older fruit during homogenation, a pH change from 6.5 used in other tissue extractions (1) was necessary. The extracting (0.1 M phosphate) buffer solution was adjusted in pH units from 6 to 10. The resulting enzyme preparations were assayed for activity.

Spectrophotometric Assay.—A Beckman DK2 twin beam ratio recording spectrophotometer with time drive was used for enzyme assay. Ascorbic acid has an absorption peak at 265 $m\mu$ (Figure 1). The rate of decrease in absorption was measured while ascorbic acid was enzymatically oxidized to dehydroascorbic acid. Phosphate buffer with ascorbic acid was used as the blank. All measurements were based on the change in absorbance at 25° C. during the first minute after the reaction was started.

The reaction mixture contained 0.5 μmoles of ascorbic acid as substrate and 1 to 5 μl of enzyme preparation, and was made to 3 ml with 0.1 M phosphate buffer at pH 5.6.

Manometric Assay.—Standard methods (4) using the Warburg respirometer were employed in all oxygen uptake measurements. The reaction

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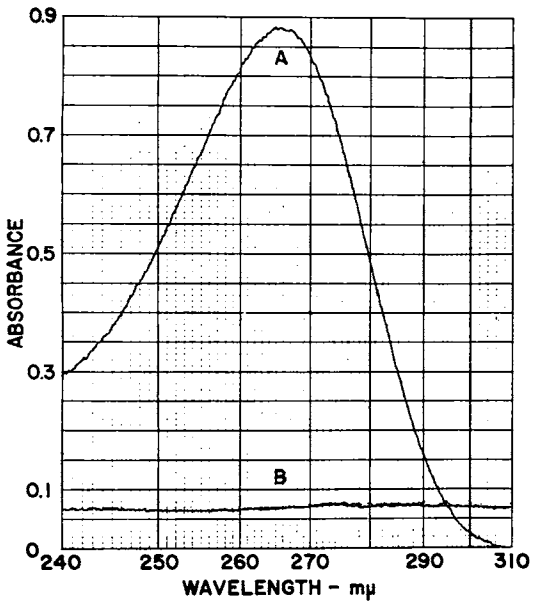


Figure 1.—Absorption spectra of ascorbic acid before and after enzymatic oxidation. Reference cell contained 3 ml of 0.1 M phosphate buffer at pH 5.6. Reaction mixture contained 0.2 μmoles of ascorbic acid in 3 ml of 0.1 phosphate buffer at pH 5.6 (curve A). Curve B shows spectrum 5 minutes after 2 μl of the enzyme preparation was added.

mixture contained 30 μmoles of ascorbic acid and 0.1 ml of enzyme preparation in a total volume of 3 ml of 0.1 M phosphate buffer at pH 5.6. All measurements were made at 25° C.

Results

Extraction.—When extractions were carried out at pH 6.5, there was a gel formation which became increasingly troublesome as the fruit matured. A change to a pH of 9.0 completely eliminated or drastically reduced this problem, thereby allowing good enzyme extraction. These data are presented in Table 1. The optimum pH of 9.0 for extraction is not to be confused with the optimum pH for enzyme activity discussed later.

Manometric.—Established enzyme units are on the basis of oxygen uptake (1 E.U. equal to 10 μl O_2 uptake per minute) rather than on absorbance change induced enzymatically. Figure 2 shows that the oxygen uptake measurements were linear for the first 10 minutes. Data beyond 10 minutes is inaccurate because of possible substrate depletion. From a number of these studies it was apparent that the enzyme concentration varied with the fruit size and with the variety. Previous reports (2) have shown that 10 μl of oxygen at 25° C. is equivalent to a change of 3.85 absorbance units at 265mμ using the substrate ascorbic acid. Because of the low enzyme requirement, speed and accuracy of the spectrophotometric assay technique, this method was used in the studies reported here.

pH.—The enzyme activity was measured spectrophotometrically at pH 5.6, which has previously been reported as the optimum by Dawson and

Table 1. Ascorbic acid oxidase activity. Effect of pH of extracting medium on gelation and efficiency of enzyme extraction.

pH of Solution		Ascorbic Acid Oxidase Activity		
Original Buffer	After Extraction	When Assayed at pH of Resulting Supernatant	When Supernatant Adjusted to Original Buffer pH	Gel Formation
		Δ Absorbance/min/μl EP*		
6.0	5.5	0.140	0.130	Extreme
7.0	5.7	0.157	0.122	Moderate
8.0	6.5	0.152	0.133	Slight
9.0	7.5	0.230	0.230	None
10.0	7.5	0.205	0.197	None

*All assays measured at pH 5.6 and 25° C.

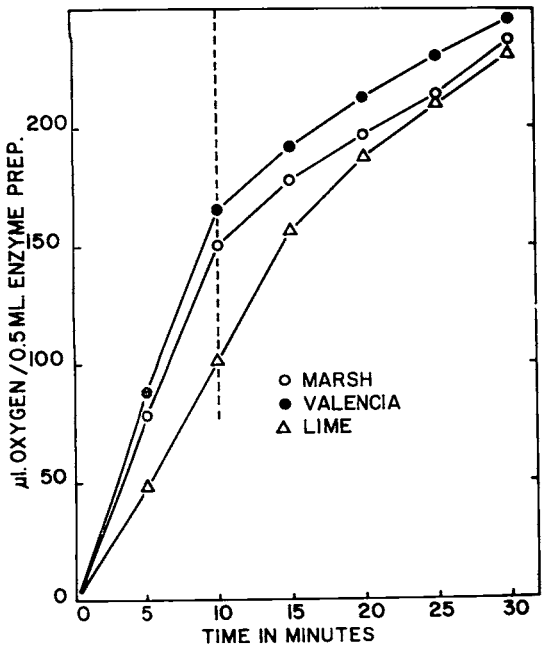


Figure 2.—Oxygen uptake of enzyme preparations from whole fruit of Marsh Seedless grapefruit, Valencia orange and lime. Reaction vessels contained 30 µmoles of ascorbic acid and 0.2 ml of enzyme preparation in a total of 3 ml of 0.1 M phosphate buffer at pH 5.6.

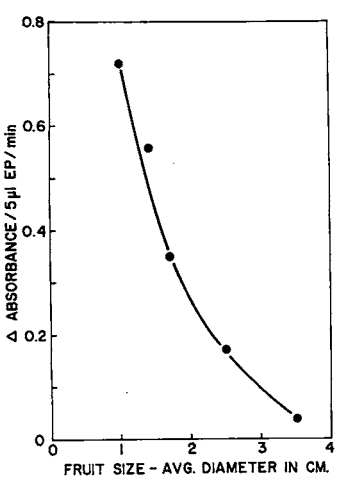


Figure 3. Ascorbic acid oxidase. A comparison of enzyme activity between orange fruits ranging in diameter from 1 cm to 3.5 cm. Enzyme activity is expressed as the rate of change in absorbance at 265 mµ per minute. Reaction vessel contained 0.5 µmoles ascorbic acid and 1, 5, 10, or 20 µl of enzyme preparation depending on the activity in a total of 3 ml of 0.1 M phosphate buffer at pH 5.6.

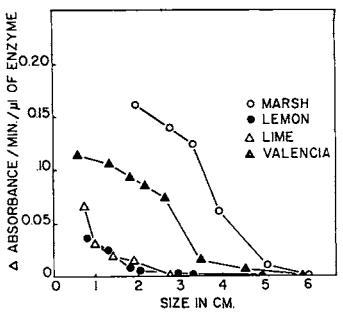


Figure 4. Ascorbic acid oxidase. A comparison of enzyme activity between different citrus fruits ranging in diameter from less than 1 cm to 6 cm. Enzyme activity is expressed as the rate of change in absorbance per minute at 265 mµ per µl with ascorbic acid as the substrate.

Magee (1) and Vines and Oberbacher (3). The agreement in the optimum pH is further evidence that the enzyme is ascorbic acid oxidase.

Comparison Between Varieties and Species.—Figure 3 shows data on enzyme preparations from Pineapple orange at different sizes. These are the averages of a number of analyses on fruit from different locations and are plotted as change in absorbance per 5 µl of enzyme preparation. The decrease in enzyme activity was consistent with the increase in fruit size.

A comparison of ascorbic acid oxidase activity in grapefruit, lemon, lime, and oranges at different sizes is shown in Figure 4. Similar decrease in the peroxidase activity in the same fruit is shown in Figure 5. The only difference noted was in lemon and lime, which had low

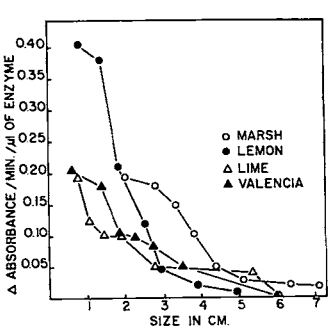


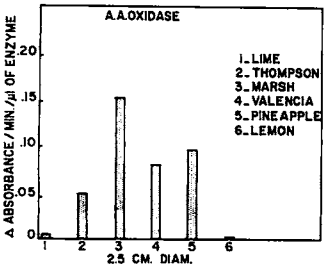
Figure 5. Peroxidase. A comparison of the enzyme activity between different citrus fruits ranging in diameter from less than 1 cm to 6 cm. Enzyme activity is expressed as the rate of change in absorbance per minute at 470 mµ per µl with hydrogen peroxide as the substrate and o-phenylenediamine as indicator.

Table 2. Lemon ascorbic acid oxidase.

Fruit Size Diam-cm	No. of Fruit/Sample	Weight/Fruit				Total Ascorbic Acid Oxidase Activity/Fruit				Total Ascorbic Acid Oxidase Activity/Fruit			
		Total	Flav.	Alb.	Juice	Flav.	Alb.	Juice	Whole	Flav.	Alb.	Juice	Whole
		gms.				Δ Absorbance/min.				Enzyme Units*			
0.6	6	0.167	-	-	-	-	-	-	4,917	-	-	-	1,277
0.8	2	0.505	-	-	-	-	-	-	10,000	-	-	-	2,597
1.95	1	4.992	1.8	1.6	1.6	13,500	12,000	12,000	37,500	3564	3117	3117	9,740
3.70	1	33.40	4.0	4.4	24.3	14,000	4,400	0	18,400	3636	1143	0	4,779
5.46	1	91.00	8.4	10.7	70.0	16,800	0	0	16,800	4364	0	0	4,364

*1 emzyme unit = 3.85 absorbance units change per minute.

ascorbic acid oxidase but high peroxidase activity. This can be better seen in the bar graph for the fruit size of 2.5 cm in diameter (Figure 6 and 7). Marsh Seedless grapefruit had the highest enzyme activity for both enzymes at this size.



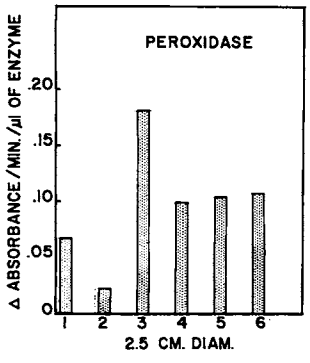


Figure 6. (upper) Ascorbic acid oxidase.

Figure 7. (lower) Peroxidase. Enzyme preparation from whole fruits of 1) lime, 2) Thompson grapefruit, 3) Marsh grapefruit, 4) Valencia orange, 5) Pineapple, and 6) lemon which were 2.5 cm in diameter. Enzyme activity expressed as the rate of change in absorbance per minute per μl of enzyme preparation as described in the text.

Enzyme in the Total Fruit.—Since analyses showed a decrease in enzyme concentration on a fresh weight basis as the fruit size increased, it was questioned whether this was a dilution rather than a decrease in total enzyme. Table 2 shows total ascorbic acid oxidase of component parts and fruit of various sized lemons. The total enzyme in flavedo increased in amount as the size increased; however, the enzyme units per fruit reached a peak in the fruit having a size of 1.95 cm diameter, and decreased thereafter.

SUMMARY

Marsh Seedless grapefruit had the highest enzyme activity, while lemons and limes had the lowest. On the basis of fresh weight, ascorbic acid oxidase was highest in the young citrus fruit and decreased as the fruit increased in size. On a whole fruit basis, this appeared to be a dilution factor, because the total enzyme activity per fruit actually increased during the period of enlargement. The enzyme was concentrated in the flavedo and disappeared from the albedo and juice.

Peroxidase activity followed the same pattern, although lemon and lime had a respectable amount.

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