Citrus Fruit Enzymes. I. Ascorbic Acid Oxidase in Oranges

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Introduction

Ascorbic acid oxidase was first described by Szent-Györgyi (14) in 1931 from cabbage. Frankenthal postulated its presence in citrus peel on the basis of indirect evidence in 1939 (6). In a survey of different tissues, Huelin and Stephen (8) reported the presence of ascorbic acid oxidase in citrus peel but found negligible amounts in orange juice. These reports, indicating the presence of this enzyme, were based on the ability of the enzyme preparation to oxidize ascorbic acid; however, many compounds are capable of oxidizing ascorbic acid in the absence of ascorbic acid oxidase. This criterion alone is not sufficient evidence for determining the presence of ascorbic acid oxidase (1).

The purpose of this paper is to establish the fact that ascorbic acid oxidase is present in citrus. The citrus enzyme characteristics are compared with ascorbic acid oxidase from other sources (2, 3, 6, 8, 14) on the basis of spectrophotometric and manometric studies, inhibition by potassium cyanide and diethyl-dithiocarbamate, optimum pH, copper content, thermodlubility, dependence on oxygen, and ammonium sulfate fractionation studies.

Materials & Methods

Enzyme Preparation. The material used throughout these studies was orange fruits (Citrus sinensis) var. Pineapple. Whole fruit (ca. 2.5 cm in diameter) were ground in the cold (0-3°) with a hand mincer and then homogenized in a Potter homogenizer with five parts 0.1 m potassium phosphate buffer pH 6.5. In order to break the cells of citrus flavedo and albedo which are tough and elastic, 300-mesh glass beads were glued with epoxy resin to both the pestle tip and the mortar. The homogenate was strained through two layers of cheesecloth to remove the unbroken cells. The soluble fraction was obtained as a supernatant solution following centrifugation at 3000 × g for 15 minutes at 0°. This fraction, prepared daily, was used for inhibition studies, ammonium sulfate precipitation studies and enzyme assay. Nitrogen and copper analyses were also made on this fraction.

Some of the enzyme remains adsorbed to the cell wall or possibly as an integral part of it (9). Although this was of secondary interest, the enzyme was separated into the apparently soluble and insoluble or cell wall fraction by repeated washing. When no additional enzyme activity could be removed, the cell wall fraction was suspended in the same volume as above and assayed for enzyme activity.

Spectrophotometric Measurements. The ultraviolet properties of ascorbic acid absorption at 265 μm was employed for ascorbic acid oxidase assay by following the diminution of the absorption peak as ascorbic acid was enzymatically oxidized. The Beckman DK2 recording spectrophotometer with a time drive was employed. Paired silica cuvettes with a volume of 3 ml with a 1 cm light path were used. By reversing the reference and sample cuvettes, a positive absorbance change was obtained for a decrease in ascorbic acid. This method has been described in detail (12).

The assay mixture of 3 ml contained 0.5 μmole of ascorbic acid, enzyme preparation containing 4.56 μg N and 33 mmoles of potassium phosphate at a pH of 5.6. This amount of substrate and enzyme was sufficient to give linear response over a 5-minute period. The ascorbic acid substrate was prepared fresh (not in excess of 2 hr).

The autoxidation of 0.5 μmole of ascorbic acid gave an absorbance change of 0.03 per minute at 25°. Since ascorbic acid was included in both cuvettes during assay, autoxidation was compensated for automatically. All solutions except the enzyme preparation were brought to 25° and all measurements were made at this temperature.

Manometric Measurements. Standard Warburg respirometer procedures (16) were followed in measuring O₂ uptake at 25°. If not specified otherwise, the reaction mixture contained 30 μmoles of ascorbic acid, 33 mmoles of potassium phosphate at a pH of 5.6, an enzyme preparation containing 228 μg N in a total volume of 3 ml. This amount of substrate and enzyme was sufficient to give linear response for a 30-minute period (figs 4 & 5). Enzyme calculations were based on the first 10 minutes. Companion flasks to measure autoxidation and endogenous rates were employed in all experiments. Corrections were made for autoxidation which did not exceed 10 μl O₂/30 minutes in any experiment.
endogenous oxidation or change in pH was noted in any experiment.

Comparison of Manometric and Spectrophotometric Assay. Absorbance data obtained spectrophotometrically were converted to enzyme units obtained manometrically (the amount of enzyme that will cause an oxygen uptake of 10 ml per minute when catalyzing the oxidation of 30 mmol of ascorbic acid at 25° in 3 ml of potassium phosphate buffer at a pH of 5.6) (5). At 25° one enzyme unit is equivalent to 0.405 mmol of O₂ per minute. Since ascorbic acid requires ½ O₂ per molecule oxidized, then 0.405 mmol of O₂ would oxidize 0.81 mmol of ascorbic acid. According to the data of Daglich (4) ascorbic acid has an E₅₀ cm of 760 at 265 mμ or an absorbance of 4.4 per mmol in a 3 ml volume. Therefore, one unit of enzyme would be equivalent to an absorbance change of 3.58 minute (12).

Manometric and spectrophotometric assay of enzyme units of the same enzyme preparation gave 24 and 26 units per mg N respectively.

Fractionation. Fractionation was carried out as follows: Ammonium sulfate necessary for the increase in saturation of the supernatant solution from 0 to 100% in 10% increments was weighed. The first increment of ammonium sulfate was added slowly to the enzyme preparation while stirring in an ice bath. The precipitate was sedimented in a refrigerated centrifuge at a speed of 8000 × g for 10 minutes and resuspended in cold 0.1 M potassium phosphate buffer at pH 5.6. Additional increments of ammonium sulfate were added progressively to the same supernatant solution and the above operation repeated. The pH change after each fractionation was minimal, varying from 5.5 to 5.7. The enzyme activity for each increment was assayed spectrophotometrically at 265 mμ as described earlier.

Nitrogen Analysis. The method of Hiller et al. (7) as revised by Thompson et al. (15) was used in nitrogen determinations of the enzyme preparation.

Copper Analysis. Copper was analyzed according to the manometric method of Warburg (18). This method employs the principle that cysteine oxidizes only in the presence of heavy metals, therefore the oxidation of cysteine solution can be utilized in order to trace copper and determine it quantitatively in micro quantities.

Results

Polyphenol Oxidase Assay. A browning discoloration during and immediately following the preparation of the enzyme solution first led to the postulation that polyphenol oxidase was present. If so, this could explain the disappearance of ascorbic acid due to reduction of oxidized phenols (10, 13, 17). Wachholder (17) has reported a positive test of polyphenol oxidase in Citrus limon.

To determine whether or not polyphenol oxidase was present, manometric measurements were made with the addition of substrate quantities of catechol which has been reported as a good substrate for this enzyme (3, 13). Other phenols tried in substrate quantities were chlorogenic acid, gallic acid, tyrosine,
dihydroxyphenylalanine, and resorcinol. The results showed no increase in oxygen uptake over the endogenous rate with any of these compounds.

Negative results were also obtained using a spectrophotometric method of assay for catecholase with chlorogenic acid.

Catecholase activity was thoroughly investigated using the procedure outlined by Dawson and Magee (3) and none could be detected in the supernatant solution or in additionally purified enzyme preparations.

Optimum pH. Monobasic and dibasic phosphate solutions were made in 0.1 M concentrations and combined in differential quantities to prepare a pH series from 3.5 to 6.5 in 0.5 pH increments. Orthophosphoric acid was used when necessary for adjustments of pH. The addition of enzyme preparation containing 4.6 µg N and 0.5 µmole of ascorbic acid were found to have no effect on the pH at any level.

The results in figure 1 show an optimum pH of 5.6 for the enzyme. This agrees with the results of ascorbic acid oxidase from yellow squash (3) and from fungus spores (10).

Stoichiometry of Manometric Assay. The manometric data in figure 2 show two important points. First it can be seen that as the enzyme concentration is increased, the rate of oxygen uptake is increased; second that 30 µmoles of ascorbic acid required 13 to 14 µmoles of O₂ for oxidation. This agrees relatively close with the proposed stoichiometry of \( \frac{1}{2} \) O₂ per ascorbic acid molecule oxidized and indicates that there was no recycling of ascorbic acid.

Cell Wall Fraction. After 10 washings 7.5 units (31.5%) of the enzyme activity remained in the cell wall fraction and 16.5 units (68.5%) was in the soluble fraction. This agrees with findings of Kivi-Ilaan, et al. (9) in their studies on enzymes associated with the cell wall.

Fig. 3 (top). The effect of potassium cyanide on ascorbic acid oxidase activity. Reaction vessels contained 0.5 µmole of ascorbic acid. 33 mmoles of potassium phosphate, enzyme preparation containing 2.3 µg N at a pH of 5.6. The reactions were started with the addition of the enzyme preparation. After one minute cyanide sufficient to make final concentrations of 6.66 \( \times \) 10⁻⁴ M, 3.33 \( \times \) 10⁻⁴ M, 10⁻³ M, and water to the control was added to make a final volume of 3 ml. The assay is described in detail in the text.

Fig. 4 (bottom). The effect of potassium cyanide on ascorbic acid oxidase activity. Reaction vessel contained 40 µmoles of ascorbic acid, 33 mmoles of potassium phosphate, enzyme preparation containing 228 µg N at a pH of 5.6. The reaction was started with the tipping of the enzyme preparation. After 10 minutes cyanide sufficient to make final concentrations of 3.3 \( \times \) 10⁻⁴ M, 6.6 \( \times \) 10⁻⁴ M, 10⁻³ M and water to the control was added to make a final volume of 3 ml. The assay is described in detail in the text.
Inhibition. Cyanide and diethylthiocarbamate have been reported as inhibitors of ascorbic acid oxidase (1, 2, 10, 11); therefore, the effect of these substances in the enzyme preparation was tested.

Figure 3 shows inhibition of enzyme activity with cyanide in concentrations ranging from $10^{-2}$ M to $6.67 \times 10^{-4}$ M. Manometric measurements (fig 4) show complete inhibition of oxygen uptake by cyanide at a final concentration of $3.3 \times 10^{-4}$ M.

Diethylthiocarbamate in final concentrations of $10^{-2}$ M and $6.67 \times 10^{-4}$ M was found to inhibit ascorbic acid oxidase 50 and 60% respectively as indicated by spectrophotometric assay.

Fractionation. Dawson and Magee (3) reported fractionation of ascorbic acid oxidase from yellow squash with ammonium sulfate between 30 and 60%. Similar results were obtained with the citrus enzyme. It can be seen in figure 5 that one-half the enzyme activity appears in the 60% saturation fraction. The original E. P. had 24 units per mg X and this amount was used for fractionation with the percentage enzyme activity recovery shown in figure 5.

Dependency on Oxygen. The oxygen requirement was verified by measuring the rate of enzymatic oxidation of ascorbic acid in the presence and absence of oxygen.

Thermolability. Boiling the enzyme preparation for 2 minutes completely destroyed the activity as measured manometrically. This indicates that the O$_2$ uptake is catalyzed by an enzyme which is likely proteinaceous, and not by the copper alone.

Discussion & Conclusions

The data here show that ascorbic acid oxidase is present in young orange fruits. After fractionation the soluble fraction was found to contain approximately 70% of the total ascorbic acid oxidase in small whole oranges and 30% bound to the cell wall fraction. This fraction contained 26 units per mg nitrogen or 30 units per µg copper.

The enzyme was found to have the following properties:

I. Ascorbic acid is a substrate for the enzyme as indicated by oxygen uptake data. Oxygen uptake measurements which were carried to completion verified the accepted over-all reaction (1/2 O$_2$/mole ascorbic acid). Ascorbic acid disappearance was also followed spectrophotometrically as it was enzymatically oxidized. A comparison of enzyme units per mg X assayed by oxygen uptake methods with those assayed spectrophotometrically agreed (24 & 26 respectively).

The ratio of oxygen uptake to ascorbic acid oxidized was approximately 1:2, which indicates that the ascorbic acid was almost completely oxidized and no recycling occurred.

II. The optimum pH of 5.6 determined in these studies corresponds to previous findings of pH optimum for ascorbic acid oxidase from other sources.

III. The orange enzyme was inhibited by cyanide concentrations of from $10^{-2}$ to $10^{-4}$ M as indicated by oxygen uptake measurements and ascorbic acid disappearance. Diethylthiocarbamate at concentrations of $10^{-5}$ to $6.67 \times 10^{-4}$ M also inhibited enzyme activity about 50%. These inhibitors are known to form stable compounds with copper and have been previously reported as inhibitors of ascorbic acid oxidase.

IV. The enzyme activity was oxygen-dependent.

Summary

Evidence for the occurrence of ascorbic acid oxidase in immature oranges is presented. This evidence is based on the findings that the enzyme was: A. specific for ascorbic acid and not for various phenolic compounds tested, B. was stoichiometric with respect to substrate and O$_2$ uptake, C. was inhibited by cyanide and diethylthiocarbamate, D. was oxygen-dependent. The pH optimum was 5.6. Approximately two-thirds of the enzyme was soluble in 0.1 M potassium phosphate buffer at pH 5.6 and the balance remained with the cell wall fraction. Assay conducted by both manometric and spectrophotometric methods gave comparable results with the latter technique having certain advantages.

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Literature Cited