Prospect of a Specific Enzymatic Assay for Ascorbic Acid (Vitamin C)

Ascorbate peroxidase, an enzyme recently detected in plant leaves, has been utilized for the enzymatic estimation of ascorbic acid in extracts of fruits and vegetables. Values obtained agreed with those produced by titration with 2,6-dichloroindophenol. Use of ascorbate peroxidase permits rapid estimations on small (10–100 mg) samples of tissue.

An acceptable enzymatic procedure for estimating ascorbic acid is not yet available. The enzyme ascorbate oxidase has been used (Fujita and Sakamoto, 1938; Hewitt and Dickes, 1961) but has the drawbacks that purified enzyme is expensive and that the reaction can only be initiated by the addition of enzyme since the second substrate (oxygen) is normally present in most solutions. An alternative enzymatic assay, based on ascorbate peroxidase which catalyzes the reaction

$$\text{ascorbate} + H_2O_2 \rightarrow \text{dehydroascorbate} + 2H_2O$$

has been developed recently in this laboratory following the detection of high levels of a soluble form of this enzyme in plant leaves (Kelly and Latzko, 1979). Ascorbate peroxidase has the advantage that reactions for the estimation of ascorbic acid can be initiated by addition of either enzyme or the second substrate (H_2O_2). Although ascorbate peroxidase is not yet available in purified form, this communication describes how partially purified enzyme was already found to be suitable for estimating the ascorbic acid contents of a wide variety of fruits and vegetables. In almost all cases the values observed agreed well with those obtained by the visual titration procedure (Association of Official Analytical Chemists, 1975). An improved method for obtaining partially purified enzyme is also included. With the ascorbate peroxidase procedure, it is possible to estimate ascorbic acid concentrations in tissue samples too small to be dealt with by the visual titration technique.

MATERIALS AND METHODS

Pea seeds were germinated in vermiculite. All fruits and vegetables were purchased from the local market, except parsley and spinach leaves and carrot roots were freshly harvested.

Partial Purification of Pea Shoot Ascorbate Peroxidase. A 55–80% ammonium sulfate fraction was obtained from young pea shoots as described previously (Kelly and Latzko, 1979) and divided into several 0.5-mL portions which were stored frozen. Before use, each portion was thawed and diluted to 5 mL with extraction buffer (10 mM sodium phosphate, pH 7.0, containing 5 mM $MgCl_2$ and 1 mM EDTA), and then 0.5 g of calcium phosphate gel was added. The mixture was swirled intermittently for 5 min and centrifuged at 12000*g* for 5 min, and the supernatant was collected as the partially purified preparation. All operations were at 2 °C.

Extraction of Tissues for Ascorbic Acid Determination. Between 0.1 and 1.0 g of tissue was added to 4 mL of cold 2% HPO_3 (containing 2 mM EDTA) and ground manually by using a glass Ten Broeck hand homogenizer. After the pH was adjusted to approximately 5 by addition of 2 mL of 10% sodium citrate (containing 2 mM EDTA), the mixture was centrifuged at 4000*g* for 7 min and the supernatant collected for assay.

Estimation of Ascorbic Acid. Determination of ascorbic acid by titration against 2,6-dichloroindophenol was performed on 2–5-mL aliquots of extract essentially as outlined by the Association of Official Analytical Chemists (1975).

Table I. Comparison of the Enzymatic Procedure and the Visual Titration Method for the Estimation of Ascorbic Acid in Fruits and Vegetables

	ascorbate, mg/100 g fresh wt	
tissue	ascorbate peroxidase	2,6-dichloro-indophenol
bean (French) pod	8.4	9.6
blackberry fruit	8.4	9.2
Brussels sprout	104	99
carrot root	2.7	2.6
cauliflower inflorescence	53	52
chive leaf	45	46
horseradish root	44	46
kiwi fruit	86	82
onion bulb	9.1	8.9
orange juice	51	50
parsley leaf	266	272
pepper (sweet) fruit	118	123
potato tuber	12.6	9.5
spinach leaf	81	86
strawberry fruit	67	65
tomato fruit	7.7	8.8
zucchini fruit	10.4	10.6

For estimations using ascorbate peroxidase, the following were added to a final volume of 1 mL in a quartz spectrophotometer cuvette: 50 μmol of sodium phosphate buffer (pH 7.0), 2 μmol of EDTA, 0.5 μmol of β-mercaptoethanol, 50 μL (containing approximately 35 μg of protein) of partially purified ascorbate peroxidase, and sufficient tissue extract (usually 10–100 μL) to give an absorbance at 265 nm of between 0.5 and 1.0. This initial absorbance was recorded, and then the reaction was initiated by addition of 5 μL of 50 mM H_2O_2. The absorbance decreased (due to the oxidation of ascorbate to dehydroascorbate) until a final value could be recorded (Figure 1). The ascorbic acid content was calculated from the difference between the initial and final absorbance readings and the millimolar extinction coefficient for ascorbate at pH 7.0 determined with a standard solution of ascorbic acid (in these experiments this value was 14.1 mM^{-1} cm^{-1}). Reactions were run at 22 °C.

RESULTS AND DISCUSSION

Typical assays of ascorbic acid using ascorbate peroxidase are shown in Figure 1. The observed decrease in absorbance was dependent on the presence of both ascorbate peroxidase and H_2O_2, and the observed final absorbance was independent of the order of addition of these two components (cf. h and j, Figure 1). However, due to the absorbance of the partially purified enzyme preparation itself, it is more straightforward to carry out estimations by starting reactions with H_2O_2. Control experiments verified that the amount of ascorbate estimated was directly proportional to the amount of extract added to the

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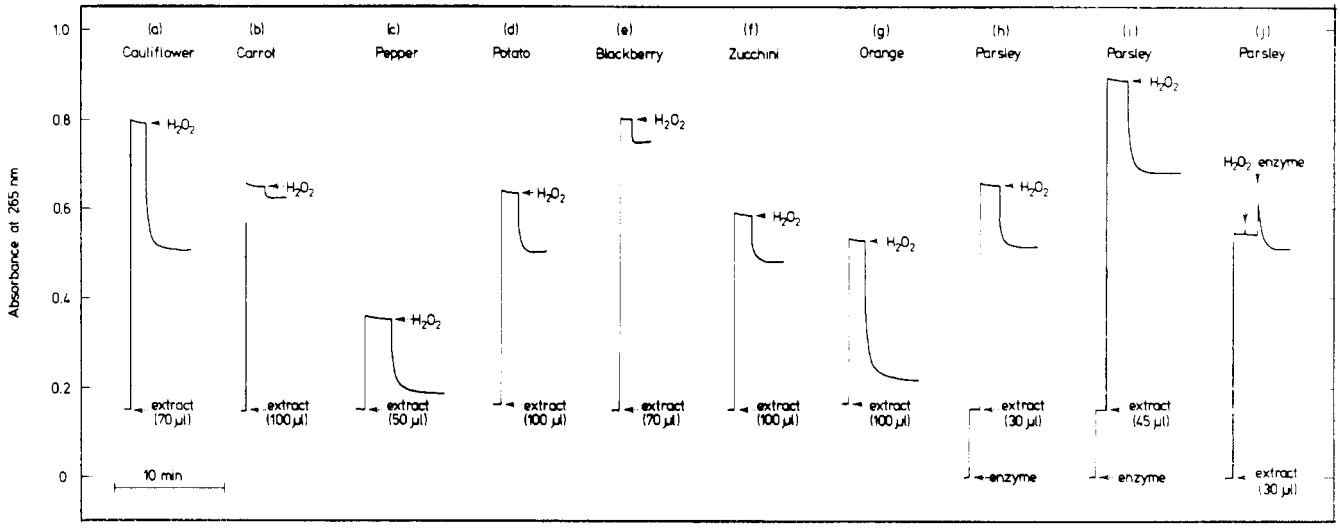

Figure 1. Estimation of ascorbic acid using ascorbate peroxidase. Extraction and assay were as described under Materials and Methods, except in (j) the reaction was initiated by addition of enzyme. Plant parts were as listed in Table I; amounts extracted were (a) 648, (b) 785, (c) 227, (d) 908, (e) 777, (f) 890, (g) 500, and (h–j) 134 mg.

reaction mixture (cf. h and i, Figure 1).

The pH of tissue extracts was adjusted to 5 (see Materials and Methods) so that the pH of the reaction mixture was not appreciably affected upon addition of reasonable volumes of the extract. Control experiments in which plant tissue was ground in the presence of an additional known amount of ascorbic acid confirmed that this pH adjustment did not result in any detectable oxidation of ascorbic acid in the extracts.

A variety of fruits and vegetables were selected and their contents of ascorbic acid determined by both the 2,6-dichloroindophenol method and the ascorbate peroxidase procedure. Within the limits of experimental error, values obtained by the two methods agreed in practically all cases (Table I), the one possible exception being potato tuber. In no case was a fruit or vegetable extract found which gave any indication of interfering with the ascorbate peroxidase test. These results indicate that, as far as tested, the use of this enzyme for ascorbic acid estimation is as reliable a method as that employing 2,6-dichloroindophenol. Nevertheless, it is hoped that more purified enzyme will be available in the future, thus further increasing the effectiveness and reliability of the method. Preliminary tests suggest that the enzyme has a high specificity for ascorbate; even with isoascorbate the activity was only 30% of that with ascorbate. Neither ferric nor stannous ions (5 µM) interfered with the assay, but 5 µM ferrous ions caused slow ascorbate oxidation in the absence of H_2O_2. Interference by reductones (triose reductone and reductic acid) was not examined, but Shigeoka et al. (1980) have recently reported that soluble ascorbate peroxidase from *Euglena* is only weakly active with reductones.

The accuracies of the enzymatic and visual titration methods are similar, but the enzymatic method is more sensitive. Hence, when the extraction is scaled down, it represents a simple procedure for the rapid estimation of ascorbic acid in small samples of 10–100 mg of tissue. For example, in this laboratory it is being used to estimate ascorbic acid in individual wheat grains at various stages of maturity.

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