CHUGOKUGAKUEN J. 2008
Vol. 7, pp. 7-10

CHUGOKUGAKUEN Journal
http://www.cjc.ac.jp/

Original Article

Ascorbic Acid and Ascorbic Acid Oxidase in Vegetables

Yoshihiro Shimada* and Sanae Ko

Department of Human Nutrition, Faculty of Contemporary Life Science, Chugokugakuen University, Okayama 701-0197, Japan

Ascorbic acid, dehydroascorbic acid and total ascorbic acid content in fruits and vegetables and the amounts of remaining after 24 hours were measured and compared by high-performance liquid chromatography. Activity of ascorbic acid oxidase was determined in nine kinds of vegetables. The relationship between the activity of ascorbic acid oxidase and the amount of ascorbic acid, dehydroascorbic acid and total ascorbic acid remaining is discussed. The residual ratio of dehydroascorbic acid increased and the amount of total ascorbic acid decreased in the vegetables with high enzymic activity. The distribution of ascorbic acid oxidase activity in stem and leaf of tingentsai and taatsai was examined. Ascorbic acid oxidase activity in the leaf was twice as high as that in the stem for both vegetables.

Key Words: ascorbic acid, dehydroascorbic acid, ascorbic acid oxidase, high-performance liquid chromatography, vegetables

Introduction

Ascorbic acid is one of the most important components in both plants and animals. Ascorbic acid has important antioxidant and metabolic functions in plants and animals in the form of vitamin C, but humans, and a few other animal species, have lost the capacity to synthesize it [1]. Plant derived ascorbic acid is thus the major source of vitamin C in the human diet. Although the biosynthetic pathway of L-ascorbic acid in animals is well understood, the plant pathway has remained little understood [2].

Ascorbic acid and dehydroascorbic acid are equally biologically active forms of vitamin C in humans [3]. Therefore, ascorbic acid and dehydroascorbic acid levels should be determined in order to know the total amount of vitamin C in vegetables.

There have been several investigations on ascorbic acid metabolism and its function in plants which provide the major source of dietary vitamin C for humans [4]. Ascorbic acid is oxidized by enzymatic or non-enzymatic reactions.

Ascorbic acid oxidase is an enzyme that catalyses the oxidation of ascorbic acid as follows [5]:

$$\text{Ascorbic acid} + 1/2O_2 \longrightarrow \text{Dehydroascorbic acid} + H_2O$$

Ascorbic acid oxidase which catalyzes the oxidation of ascorbic acid to yield dehydroascorbic acid, occurs in various higher plants [6, 7]. The physicochemical properties of ascorbate oxidase from zucchini squash have been studied in detail because of the success in X-ray crystallographic analysis of the enzyme [8, 9]. The occurrence of ascorbic acid oxidases in microorganisms (*Myrothecium verrucaria* and *Aerobacter aerogenes*) has also been reported [10, 11].

*Corresponding author.
Yoshihiro Shimada
Department of Human Nutrition, Faculty of Contemporary Life Science, Chugokugakuen University, 83, Niwase, Okayama 701-0197, Japan

In the present report, the stability of vitamin C (ascorbic acid and dehydroascorbic acid) in fruits and vegetables was measured, and the relation between it and ascorbic acid oxidase is discussed.

Materials and Methods

1. Reagents

L(+)-ascorbic acid, tetra-n-butylammonium bromide and other chemicals were obtained from the Wako Pure Chemical Ind. Ltd., Osaka, Japan. DL-homocysteine was obtained from Sigma-Aldrich Inc., St. Louis, USA. All the reagents used were reagent grade.

2. Samples

Fresh vegetables were obtained from local supermarkets, and analyzed on the day of purchase.

3. High-performance liquid chromatography (HPLC)

Separation of ascorbic acid was achieved with a Yanaco L-5000 Liquid chromatographic apparatus equipped with a Rheodyne Model 7125 injector. Column effluents were monitored at 265nm with a Yanaco M-515 variable-wavelength detector. Peak areas were determined using an SIC Chromatocorder 12. A Shodex RSpak DE-613 column (150mm×6mm inside diameter) was used. The mobile phase was composed of 8mM phosphate buffer, pH6.8, containing 3mM tetra-n-butylammonium bromide. The flow rate was 1.0ml/min.

4. Preparation of sample

Samples of tahtsai, spinach, and chingentsai were divided into leaf and stem. All vegetables were then chopped into small sections with a kitchen knife.

Ten g of these small sections were homogenized with 10ml of 10% metaphosphoric acid and sea sand in a mortar. The slurry obtained was transferred to a centrifuge tube with 20ml of 5% metaphosphoric acid and centrifuged for 20min at 3,000rpm. The supernatant was diluted 20 fold with distilled water.

5. Ascorbic acid assay

Ten ml of diluted supernatant was diluted with 0.13ml of 2.5M K_2HPO_4 to give a final pH of 7.0. A 20-μl aliquot of this solution was injected into the HPLC system.

6. Total ascorbic acid assay

Total ascorbic acid was assayed by adding 0.015g of homocysteine to 5ml of the neutralized sample for ascorbic acid assay. After 30min at 25℃, 20-μl aliquot of this solution was injected into the HPLC system. The concentration of dehydroascorbic acid was calculated by subtracting the amount of assayed ascorbic acid from that of total ascorbic acid.

7. Measurement of ascorbic acid oxidase activity

(1) Preparation of crude enzyme

Ten g of small sections of samples were homogenized with 10ml of water and sea sand in a mortar. The slurry obtained was transferred to a centrifuge tube and centrifuged for 5min at 3,000rpm. The supernatant was used as crude enzyme.

(2) Assay of ascorbic acid oxidase activity

Prior to diluting the crude enzyme, the reaction mixtures were prepared by adding 0.5ml of ascorbic acid solution (1mg/ml in 01M acetate buffer) and 0.5ml of 01M acetate buffer (pH5.6) to each flask. Then 1.5ml of the crude enzyme was added immediately to the flasks, and the flasks were incubated at 30℃ for 10minutes of slow shaking. The flasks were centrifuged for 20min at 3,000rpm after adding 2.5ml of 10% metaphosphoric acid. AA was measured in the supernatant.

(3) Definition of Unit and Specific Activity

One unit of ascorbic acid oxidize activity is defined as that amount of enzyme which oxidizes 1μM of ascorbic acid per one minute. Specific activity is expressed as units per 100g of samples.

Results

1. Stability of vitamin C in fruits and vegetables

The amounts of ascorbic acid, dehydroascorbic acid and total ascorbic acid remaining in fruits and vegetables after 24 hours were compared (Table 1). The residual ratio of total ascorbic acid was high, above 60%, in lemons, kiwi fruit, radishes and strawberries and was low, under 60%, in broccoli, pumpkins, spinach, cabbage, tingentsai, carrots, taatsai, stalk garlic. Conversely, the residual ratio of dehydroscorbic acid was high in broccoli, pumpkins, spinach, cabbage, tingentsai, carrots, taatsai, and stalk garlic.

2. Activity of ascorbic acid oxidase

The results of measurements of ascorbic acid oxidase activity in several kinds in vegetables is shown

Table 1 Stability of vitamin C in fruits and vegetables.

	0 hour			after 24 hours			
	Total(mg/100 g)	AA(mg/100 g)	DAA(mg/100 g)	Total(mg/100 g)	AA(mg/100 g)	DAA(mg/100 g)	residual ratio(%)
Lemon	50.0	47.0 (94.0)	3.0 (6.0)	37.0	33.30 (90.0)	3.70 (10.0)	74.0
Kiwi fruit	75.0	70.0 (93.3)	5.0 (6.7)	55.1	51.20 (92.9)	3.93 (7.1)	73.5
Radish	12.3	11.6 (94.3)	0.7 (5.7)	9.2	9.10 (98.6)	0.70 (1.4)	75.0
Strawberry	60.0	55.0 (91.7)	5.0 (8.3)	37.8	33.60 (88.9)	4.20 (11.1)	63.0
Broccoli	74.0	65.8 (88.9)	8.3 (11.1)	33.5	2.00 (6.0)	8.30 (94.0)	45.3
Pumpkin	35.5	25.1 (70.0)	10.4 (29.3)	18.2	10.00 (54.9)	10.40 (45.1)	51.3
Spinach	33.1	30.8 (93.1)	2.3 (6.9)	16.7	8.90 (53.2)	2.30 (46.8)	50.5
Cabbage	34.3	30.8 (89.8)	3.5 (10.2)	16.6	6.00 (36.1)	3.50 (63.9)	48.4
Tingentsai	42.7	35.0 (82.0)	7.7 (18.0)	22.0	8.40 (38.2)	7.70 (61.8)	51.5
Carrot	4.0	0.7 (17.5)	3.3 (82.5)	0.0	0.00 (0.0)	0.00 (0.0)	0.0
Taatsai	55.4	35.3 (63.7)	20.0 (36.3)	31.9	14.40 (45.2)	20.00 (54.8)	57.5
Stalk garlic	39.7	31.8 (80.1)	0.8 (19.9)	23.0	10.80 (46.9)	0.80 (53.1)	58.0

Total: total ascorbic acid, AA: ascorbic acid, DAA: dehydroascobic acid
Numbers in parentheses indicate the ratio to total ascobic acid.

in Table 2. The enzymic activity was highest in broccoli. Pumpkin, spinach, tingentsai and cabbage followed in the level of enzymic activity. The enzymic activity was the lowest in radishes.

3. Distribution in stem and leaf of ascorbic acid oxidase activity

The distribution of ascorbic acid oxidase activity in the stem and leaf of tingentsai and taatsai is shown in Table 3. In both, ascorbic acid oxidase activity of the leaf was the twice as high as that of the stem.

Table 2 Activity of ascorbic acid oxidase (units/100 g).

	activity
Broccoli	11.88
Pumpkin	8.45
Spinach	7.30
Tingentsai	5.12
Cabbage	4.96
Carrot	4.22
Taatsai	3.42
Stalk garlic	3.27
Radish	0.14

Table 3 Distribution in stem and leaf of ascorbic acid oxidase activity (units/100 g).

	Leaf	Stem	Edible portion
Tingentsai	7.98	4.32	5.12
Taatsai	4.87	2.82	3.42

Discussion

Many methods have been developed for the determination of amounts of vitamin C (L-ascorbic acid), however, most of them have been based on its reducing properties [12–15]. These methods lack specificity, and are prone to interference by other reducing agents present in the sample. High-performance liquid chromatography is the proper method for analysis in complex matrices such as food samples.

From the result of the residual ratio of ascorbic acid and activity of ascorbic acid oxidase (Tables 1 and 2), the following conclusions were derived. The residual ratio of dehydroascorbic acid increased and that of total ascorbic acid decreased in the vegetable with high enzymic activity. This evidence shows that ascorbic acid oxidase catalyzes the oxidation of ascorbic acid to yield dehydroascorbic acid, followed by the decomposition of dehydroascorbic acid.

Ascorbic acid content was measured in four parts of broccoli by Nishikawa et al. [16]. The amount of total ascorbic acid in the florets was the highest of all the portions. It stayed at a relatively high level for about 12 hours, followed by a rapid decline to a low level during senescence. In the stem tissue, the levels of ascorbic acid remained almost unchanged or increased slightly as time progressed. It is estimated that this result depends on the difference of the enzymic activity in the different parts of the vegetable just as ascorbic acid oxidase activity of the leaf was

the twice as high as that of the stem in both tingentsai and taatsai.

References

1. Nishikimi M, Fukuyama R, Minoshima S, Simizu N and Yagi K: Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-γ-lactone oxidase, the enzyme for L-ascorbic and biosynthesis missing in man. J Biol Chem (1994) **269**, 13685–13688.
2. Smirnoff N: The function and metabolism of ascorbic acid in plants. Ann Bot (1996) **78**, 661–669.
3. Sabry JM, Fisher KH and Dodds ML: Human utilization of dehydroascorbic acid. J Nutr (1956) **64**, 457–466.
4. Noctor G and Foyer CH: Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol (1998) **49**, 249–279.
5. Greenway GM and Ongomo P: Determination of L-ascorbic acid in fruit and vegetable juices by flow injection with immobilised ascorbate oxidase. Analyst (1990) **115**, 1297–1299.
6. Lee MH and Dawson CR: Ascorbate oxidase. Further studies on the purification of the enzyme. J Biol Chem (1973) **248**, 6596–6602.
7. Nakamura T, Makino N and Ogura Y: Purification and properties of ascorbate oxidase from cucumber. J Biochem (1968). **64**, 189–195.
8. Messerschmit A and Huber R: The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. Eur J Biochem (1990). **187**, 341–352.
9. Messerschmit A, Rossi A, Laudenstein R, Huber R, Bolognesi M, Gatti G, Marchesini A, Petruzzelli R and Finazzi-Argo A: X-ray crystal structure of the blue oxidase ascorbate oxidase from zucchini. Analysis of the polypeptide fold and a model of the copper sites and ligands. J Mol Biol (1989) **206**, 513–529.
10. Fahraeus G and Rainhammar B: Large scale production and purification of laccase from cultures of the fungus Polyporus versicolor and some properties of laccase A. Acta Chim Scand (1967) **21**, 2367–2378.
11. Froehner SC and Eriksson KE: Purification and properties of Neurospora crassa laccase. J Bacteriol (1974) **120**, 458–465.
12. Pachla LA, Rcynolds DC and Kissenger PT: Analytical methods for measuring uric acid in biological samples and food products. J Assoc Off Anal Chem (1985) **68**, 1–14.
13. Dabrowski K and Hinterleitner S: Applications of a simultaneous assay of ascorbic acid, dehydroascorbic acid and ascorbic sulphate in biological materials. Analyst (1989) **114**. 83–87.
14. Salinas F and Galeano Diaz T: Spectrophotometric determination of L-ascorbic acid in pharmaceutical preparations, foods and urine by formation of a 2-oximinocyclohexanone thiosemicarbazone-iron (II) complex. Analyst (1988) **113**, 1657–1659.
15. Lau OW, Luk SF and Wong KS: Background correction method for the determination of ascorbic acid in soft drinks, fruit juices and cordials using direct ultraviolet spectrophotometry. Analyst (1986) **111**, 665–670.
16. Nishikawa F, Kato M, Hyodo H, Ikoma Y, Sugiura M and Yano M: Ascorbate metabolism in harvested broccoli. J Exp Bot (2003) **54**, 2439–2448.

Accepted March 30, 2008.