J Life Sci Res 2021; **18**: 15-19

Journal of Life Science Research
Online ISSN 2186-5809

Rapid paper

Irreversible degradation of vitamin C in broccoli during cryopreservation

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Received 5 November 2021; accepted 15 November 2021

Key words: ascorbic acid; dehydroascorbic acid; diketogulonic acid; food storage

1 Introduction

Ascorbic acid (ASC) or vitamin C is a water-soluble vitamin with antioxidant properties, and its physiological effects have long been recognized as antiscorbutic.[1, 2] ASC possesses many physiological effects, including its function as a cofactor for enzymes that polymerize collagen and synthesize catecholamines, and its clinical use based on pharmacological effects for cancer therapy.[3, 4]

Vitamin C is typically present in the anionic state in the cell and produces monodehydroascorbic acid (MDA) during oxidation. MDA is either reduced back to ASC by reductase or broken down to produce one molecule of ASC and one molecule of dehydroascorbic acid (DHA). DHA is then reduced to ASC by reductase or thiol compounds such as glutathione, non-enzymatically (Fig. 1). [5-8] The redox system is known as the glutathione/ascorbate cycle and is involved in the decomposition of hydrogen peroxide and various hydroxylation reactions. Both ASC and DHA are known to act as vitamin C *in vivo*, and the total amount of ASC is described as vitamin C in the standard tables of food composition in Japan. However, it has recently been shown that some DHA is metabolized to 2,3-diketogulonic acid (DKG) via irreversible hydrolysis. DKG is not as active as vitamin C and is further degraded to threonate and erythrulose under oxidative stress.[9] The degradation of DHA in the metabolic pathway of ASC has not been considered, and little is known about the synthesis and degradation of DKG. However, there have been several reports on the toxicity of DKG due to its high reactivity with proteins.[23-25]

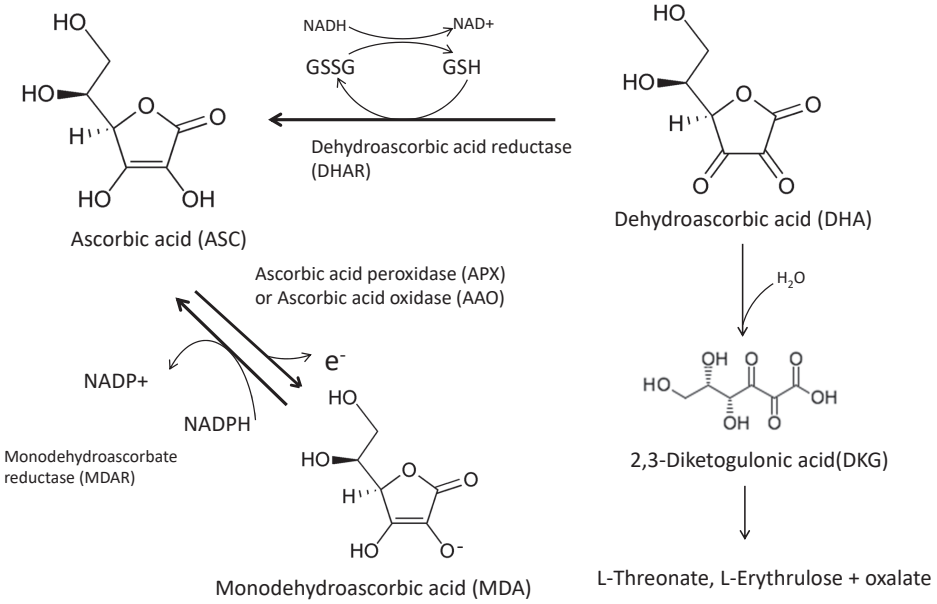

Fig. 1 Ascorbic acid metabolic pathway.

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In this study, we focused on the degradation pathway of ascorbic acid in vegetables for clarifying the changes in the dynamics of ascorbic acid under various storage conditions. The present results will provide the important knowledge on food preservation and cooking.

2 Materials and Methods

Chemicals

All reagents were of the highest available quality. Ascorbic acid, DHA, 2,6-dichlorophenolindophenol (DCIP), tris [2-carboxyethyl] phosphine hydrochloride (TCEP), and dinitrophenylhydrazine (DNPH) were purchased from Nacalai Tesque (Kyoto, Japan). Meta-phosphate (MPA) was purchased from Santa Cruz Biotechnology (TX, USA).

Plant materials and thermal treatments

Fresh broccoli (*Brassica oleracea* L. var. *Italica*) was purchased from a local market. Briefly, 100 mg of florets from a broccoli head were washed with distilled water and then packed in a plastic bag. For thermal treatments, samples were stored at 4°C or -20°C for 0, 6, or 24 h. After the treatments, the samples were frozen in liquid N_2 and homogenized with a mortar and pestle. The fine powders of the florets were thawed in 1 ml of 5% MPA, transferred into microtubes, and then centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were collected and used as assay samples for ASC analysis, as described below.

Cooking

For steaming, 50 g of florets were suspended in a domestic steamer with 500 ml boiling water for 2 min. The surface temperature of the broccoli was approximately 70°C with this heat treatment.

For boiling, 250 ml of water in a 500 ml beaker was heated to 100°C. Florets (50 g) were added to the boiling water for 2 min. The samples were drained and transferred into plastic bags.

For microwave cooking, 50 g of broccoli florets were immersed in water and cooked in a microwave oven (Panasonic, Osaka, Japan) at a middle power level (500 W) for 2 min. The surface temperature of the broccoli was approximately 65°C using this heat treatment. After cooking, the samples were frozen in liquid nitrogen prior to extraction.

Determination of ASC, DHA, and DKG with DNPH derivatization method

DNPH derivatization and ASC determination were performed according to the method of Omaye et al.[10] and Kishida et al.[11], respectively.

For the total ascorbic acid determination, the samples (500 µL) were added to 5 µL of 0.2% DCIP, mixed well, 2% thiourea (500 µL) and 2% DNPH in 4.5 M H_2SO_4 (125 µL) was added, and then incubated for 40 min at 60°C in closed glass test tubes. After incubation, 500 µL of ethyl acetate was added to each tube, and the tubes were shaken at 200 rpm for 5 min and centrifuged at 12,000 × g for 2 min. The ethyl acetate layer and the aqueous layer were separated, and 200 µL of the upper ethyl acetate layer was transferred to a 1.5 mL Eppendorf tube containing 100 µg of sodium sulfate. The tube was centrifuged at 12,000 × g for 2 min to remove water and impurities in the sodium sulfate layer, and 100 µL of the supernatant was collected in a vial for HPLC analysis.

To determine DHA and DKG, the addition of the oxidant DCIP was omitted from the above procedure.

For determining DKG, DHA was reduced to ASC by adding 2 mM TCEP and incubating at room temperature for 15 min; DCIP treatment was omitted.[12]

Subtracting the value obtained for DKG from the DHA and DKG values gave the DHA content and subtracting the DHA and DKG values from the total ascorbic acid value gave the ASC content.

HPLC analysis

An HPLC system consisting of a Shimadzu LC10AD pump and an SL-6B autosampler (Shimadzu, Kyoto, Japan) was used. The DNPH derivatives were applied to a COSMOSIL 5SL-II column (4.6 mm i.d. x 150 mm, Nakarai tesque) and eluted isocratically with ethyl acetate/hexane/2-propanol/acetic acid (50:47:2:1) at 1.0 ml/min. The absorption at 495 nm was recorded using an SPD-10A UV-VIS (Shimadzu) spectrophotometer.

Data analysis

Experimental points represent the mean ± SD of at least three samples. For the statistical evaluation, student's t test was used to test the significance of the differences in the mean content of ascorbic acid and its derivatives between control and samples. Differences were considered significant at $p \leq 0.05$. All statistical analysis were undertaken using Microsoft Excel ver. 16.

3 Results

3-1 Changes in ascorbic acid content in broccoli florets during storage at low temperatures.

The total ascorbic acid content (ASA+DHA+DKG) in fresh broccoli was on average 95.7 mg/100 g fresh weight (FW) (Fig. 2). The total ascorbic acid level in broccoli generally ranged from 60.1 to 179.7 mg/100 g FW, with an average of 98.2 mg/100 g FW.[14-20] The current value obtained here was slightly lower; however, this variability can be related to cultivar, maturity at harvest, and preharvest conditions. The amount of total ascorbic acid in the broccoli did not change significantly over a period of 24 h when stored at 4°C and -20°C. Several studies have reported that the ascorbic levels in broccoli decreased when it was stored for more than 2 days.[18-21] However,

it has been reported that the amount of total ascorbic acid hardly decreased in a short period (approximately one day), which is consistent with our result.[18,21]

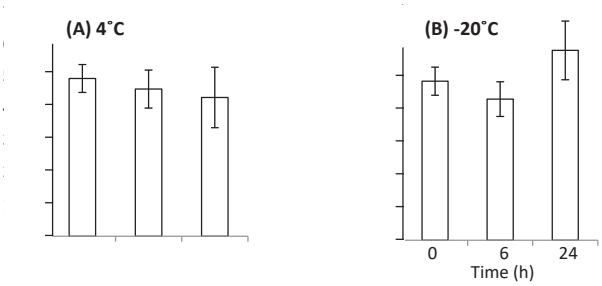

Fig. 2 Changes in ascorbic acid content in broccoli florets during storage at low temperatures.
A; stored at 4°C, B; stored at -20˚C for 0, 6, or 24 h. Data are the mean of three determinations.

3-2 Changes in DKG content in broccoli stored at 4°C and -20°C.

The proportions of ASC, DHA, and DKG in the total ascorbic acid in fresh broccoli were 75%, 7%, and 18%, respectively (Fig. 3). Furthermore, the sample stored at 4°C exhibited a 5% increase in DKG in 24 h, whereas DKG increased to approximately 60% of the total ascorbic acid, when stored at -20°C ($p = 0.03$). The ratio of DHA in the total ascorbic acid did not change owing to storage and was approximately 7%. These results suggested that the oxidation of ASC to DHA did not stop; however, DHA was rapidly broken down to DKG. As for the slight increase in the total ascorbic acid content after 24 h of storage at -20°C, it is possible that the DNPH derivatization method detected carbonyl compounds other than DHA, such as ketone bodies.[13] In addition, as there is almost no difference in the amount of the total ascorbic acid levels between 6 h and 24 h at -20°C, it is possible that some by-products were synthesized by the freezing process and detected as the total amount of ascorbic acid. The results showed that when fresh broccoli was frozen at -20°C, ASC was rapidly oxidized and degraded, and the ratio of active vitamin C (ASC+DHA) to the total amount of ascorbic acid decreased by approximately half.

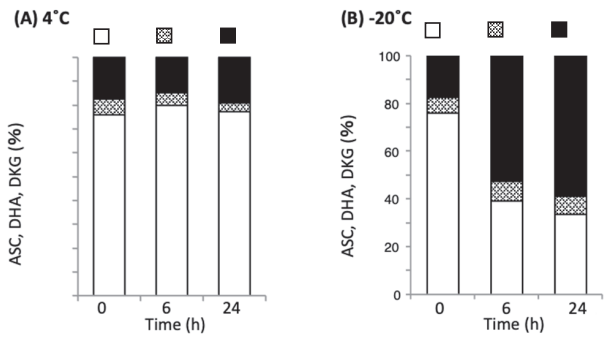

Fig. 3 Changes in DKG content at different storage temperatures.
A; stored at 4°C, B; stored at -20˚C for 0, 6, or 24 h.

3-3 Changes in DKG content in broccoli due to cooking

The change in total ascorbic acid levels following different cooking treatments of the broccoli florets are shown in Fig. 4. Boiling resulted in a significant loss of total ascorbic acid, whereas steaming and microwaving resulted in less than a 50% loss. In addition, boiling and steaming yielded more than 50% DKG, and microwaving resulted in the highest amount of vitamin C remaining. The reason for this may be that the rapid rise in the internal temperature of the food quickly deactivated the ascorbate oxidase, which in turn suppressed the oxidation and degradation of vitamin C. Furthermore, Vallejo et al.,[19] reported that the total amount of vitamin C decreased by 40% when broccoli was heated in a microwave oven, which is consistent with this result. Vallejo et al.,[19] revealed that boiling for 5 min or less suppressed the decrease in total vitamin C, whereas Lisiewska et al.,[15] concluded that the total vitamin C decreased to 0% after 15 min of boiling.

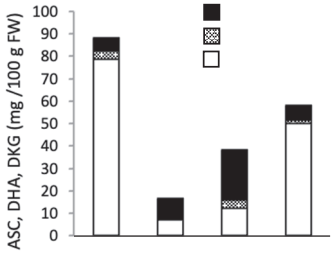

Fig. 4 Effect of different cooking methods on the DKG contents of broccoli.
The meanings of the abbreviations are as follows: fresh broccoli without cooking; boil, boiling; steam, steaming; microwave, microwave oven.

4 Discussion

Owing to the diversity and importance of its physiological effects, it is crucial to obtain the required amount of vitamin C from the diet. It is known that ASC, together with its oxide DHA, is always maintained at a constant ratio by the glutathione cycle, and ASC continues to be supplied even under transient stress conditions.[6] However, such homeostasis is not maintained in foods, and the fluctuation of the intracellular level of ASC has not been comprehensively investigated to date. In the standard tables of food composition compiled in Japan, the amount of vitamin C is calculated using the DNPH method, implying that the measured values include DKG, which does not have vitamin C activity.[26] It has recently been reported that DHA has a poor absorption rate in the intestinal tract.[13] In addition, as DKG has a dicarbonyl structure, it is a substrate for the Strecker reaction and is involved in the production of melanoidins, implying that it may be involved in the production of carcinogens.[22] In this context, it is important to elucidate the detailed profile of ascorbic acid in foods and the metabolic pathways involved. In this study, we analyzed the changes in the

accumulation of ASC, DHA, and DKG resulting from various storage and processing treatments. The results showed that DKG accumulated in broccoli up to approximately 50% after storage at -20°C. Although the reason for this is not clear at this time, it may be that the degradation of DHA is accelerated at -20°C compared to 4°C,[27] and that the accumulation of by-products detected as DNPH derivatives is increased at -20°C. The latter is currently being analyzed using gas chromatography or liquid chromatography-mass spectrometry. It was also suggested that this phenomenon differs depending on the plant species (data not shown). We believe that these findings provide lucid information on the storage of food materials and highlight the importance of comprehensive food material analysis by metabolome analysis.

Acknowledgment

The authors thank Prof. Kazutaka Miyatake for his valuable discussions and interest.

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