Ascorbic Acid Retention in Fresh-Cut Broccoli Florets during Hyperbaric Storage

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We investigated the efficacy of hyperbaric storing for preserving ascorbic acid (AsA) in fresh-cut broccoli florets. The samples were stored in a container pressurized at 0.3 and 2.1 MPa of air at 8 °C for 14 d. Florets stored under atmospheric pressure (0.1 MPa) were used as a control. We assayed AsA content, enzyme activities involved in AsA degradation and recycling, including ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), as well as antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Changes in partial pressure of O2 and CO2 in the storage container were also determined. AsA content was successfully maintained for 14 d under both of our hyperbaric treatments and was approximately twice as high as the AsA content in the control treatment. Activities of CAT, APX, GR and SOD increased at 0.3 MPa, except DHAR, whereas florets stored at 2.1 MPa showed almost no enzymatic activity. The respiration was slowed down in florets stored under hyperbaric conditions. Our results suggest that the physiological response of fresh-cut broccoli florets to the hyperbaric condition varied with the magnitude of pressure applied, especially the enhancement of CAT enzyme activity leads to the AsA retention at 0.3 MPa.

Keywords: ascorbic acid, antioxidant enzyme, fresh-cut broccoli, hyperbaric storage, respiration

INTRODUCTION

The market for fresh-cut produce has been growing continuously in response to an increase in consumer demand for convenience. At the same time, consumers have become aware of the importance of consuming a diet high in fresh fruits and vegetables. However, it is well known that fresh-cut produce has a short shelf life due to damage caused by minimally processing it (i.e., wounding during processing causes an increase in respiration rate and ethylene production); these changes lead to an acceleration of the senescence process and degradation of nutritional compounds (Abe and Chachin, 1995; Martiñon et al., 2014). Fresh-cut broccoli, an important minimally-processed vegetable, contains a high amount of bioactive compounds, especially ascorbic acid (AsA). However, a rapid decline in AsA content has been observed during storage (Rasheed et al., 2013). The decline in AsA content is the most important change associated with quality deterioration. Thus, proper postharvest treatments are essential for maintaining AsA content and thus extending the shelf life of fresh-cut broccoli.

Generally, low temperature can slow the reduction of AsA in intact and fresh-cut produce. The ability to preserve AsA content at low temperatures can be improved by combining with chemical treatment such as edible coating (Bal, 2013; Hassan et al., 2014; Sohail et al., 2015). However, the use of chemical treatments has been declining in response to consumer demand for safer foods. Rather than using chemical treatments, the physical postharvest techniques such as UV irradiation, hot water treatment, modified atmosphere packaging (MAP), and controlled atmosphere (CA) storage have been successfully applied to preserve AsA in fresh produce (Agar et al., 1997; Nunes et al., 1998; Barry-Ryan and O’Beirne, 1999; Moretti et al., 2003; Mirdehghan et al., 2006; Koukounaras et al., 2008; Zenoozian et al., 2011a; 2011b; Sucharitha et al., 2012).

Recently, storing fresh produces under high O2 conditions has been suggested as an alternative way to maintain AsA content, to reduce microbial growth and to inhibit enzymatic browning of intact and fresh-cut produce (Kader and Ben-Yehoshua, 2000; Jacxsens et al., 2001; Allende et al., 2002; Chunyang et al., 2010; Zhang et al., 2013; Bandia et al., 2015). Hyperbaric treatment is also involves the use of O2 at a partial pressure greater than atmospheric condition. This technique increases the partial pressure of O2 by injecting high-pressure air into a storage container to increase the total pressure of its gases. The use of hyperbaric treatment with compressed air has been reported to inhibit microbial growth, reduce weight loss of produce, reduce CO2 and ethylene production, and delay the ripening of fresh produce (Baba and Ikeda, 2003; Goyette et al., 2012; Liplap et al., 2013a; 2013b; 2014; Fernandes et al., 2015). However, only a few studies have been conducted on the effects of hyperbaric treatment on antioxidant compounds, such as AsA. Moreover, no studies have ever been conducted on fresh-cut produce.
In this study, fresh-cut broccoli florets were continuously stored under high-pressure condition at 8°C for 14 d. We investigated the usefulness of hyperbaric storage on AsA retention by measuring respiratory O₂ consumption and CO₂ production and the activities of enzymes involved in AsA degradation and recycling, such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), as well as antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT). Furthermore, we discuss the mechanism of AsA retention in light of these measured variables.

MATERIALS AND METHODS

Plant material and sample preparation

Fresh broccoli heads (Brassica oleracea L.) were obtained from a wholesale market. We selected broccoli heads that were of uniform color and had no visual defects. The heads were then cut into individual florets with a sharp knife.

Broccoli florets were subjected to three different, pressurized air storage treatments, (all at 8°C) 0.1, 0.3 and 2.1 MPa, in this study. Under the two hyperbaric storage treatments, approximately 21 g of fresh-cut broccoli florets were put into 1.5 × 10⁻⁴ m³ high-pressure container (TVS-1, Taiatsu Techno Co., Tokyo, Japan). The container’s lid was equipped with a pressure meter and needle valve. To enable high-pressure air injection, the intake port of the container was connected to a gas injection tube connected to a high-pressure gas cylinder. Then, air was forced into the container until the internal pressure reached the required magnitude (0.3 or 2.1 MPa, respectively). The needle valve was then closed and the gas injection tube was removed from the intake of the pressure container. For the control treatment, the fresh-cut broccoli florets were put in a beaker covered with plastic wrap punctured with small holes to prevent water loss under atmospheric condition (0.1 MPa). All samples were moved to an incubator (MIR-154-PJ, Panasonic Healthcare Holdings Co., Ltd., Tokyo, Japan), set at 8°C, for storage. After storage, the fresh-cut broccoli samples were immediately frozen in liquid nitrogen, and stored at −50°C for analysis of AsA content and the enzyme activity. This experiment was replicated three times.

Determination of the partial pressure of O₂ and CO₂ before and after hyperbaric treatment

Firstly, the total pressure in the high-pressure container was obtained from the reading of the pressure meter. Then, headspace gas in the container was collected in a glass bottle by using the water displacement method after 0 and 14 d of storage. A 0.2 mL sample of the gas was withdrawn from the bottle and injected into the GC analyzer (GC-14A, Shimadzu Co., Kyoto, Japan), set at 8°C. The heads were then cut into individual florets with a sharp knife.

Estimation of the total amount of respiratory O₂ consumption and CO₂ production during storage

For the fresh-cut broccoli florets stored at unpressurized condition (0.1 MPa), the rate of respiratory O₂ consumption and CO₂ production was measured by a closed system method after 14 d storage. Approximately 2.5 g (precisely weighted) of sample was put into a 5.5 × 10⁻³ m³ hermetic glass bottle and moved to an incubator at 8°C. A 0.2 mL of headspace gas was withdrawn by a gastight syringe at 30-min intervals for 2 h, and was injected to GC for the determination of O₂ and CO₂ concentrations. The free volume of the bottle was estimated by subtracting sample volume which was measured by water displacement. The rate of O₂ consumption and CO₂ production were calculated by using Eq. (1).

\[
R_{O_2,CO_2} = \frac{\Delta C_{O_2,CO_2}}{100} \times \frac{V_I}{W} \times \frac{P}{R T} 10^8 \tag{1}
\]

where \(R_{O_2,CO_2}\) is the rate of O₂ consumption and CO₂ production (mmol kg⁻¹ h⁻¹), \(\Delta C_{gas}\) is the change of the gas concentration in the bottle (O₂ and CO₂) (% h⁻¹), \(V_I\) is the free volume (m³), \(W\) is the weight of sample (kg), \(P\) is the atmospheric pressure (= 0.1 MPa), \(R\) is the universal gas constant (= 8.314 J K⁻¹ mol⁻¹), \(T\) is the absolute temperature (K).

The total amount of respiratory O₂ consumption and CO₂ production under ambient pressure condition during storage was estimated by multiplying the obtained respiration rate at day 14 by total storage hours (= 336 h) assuming that the respiration rate is constant during 14 d of storage. For the hyperbaric condition, it was calculated using Eq. (2) assuming that the change of O₂ and CO₂ partial pressures in the high-pressure container came from the respiration of the sample.

\[
Q_{O_2,CO_2} = \left( \frac{P_{a,CO_2} - P_{a,CO_2}}{V_I} \right) \times \frac{1}{W} \times \frac{1}{R T} \times 10^9 \tag{2}
\]

where \(Q_{O_2,CO_2}\) is the total amount of O₂ consumption and CO₂ production by the produce during storage (mmol kg⁻¹), \(P_{a,CO_2}\) is the partial pressure of O₂ and CO₂ at storage period (\(i = 0 \) d, \(a = 14\) d) (MPa).

Determination of ascorbic acid content

AsA was extracted and analyzed, as described by Al-Ani et al. (2007) and Kapur et al. (2012), with some modifications. For the extraction of AsA, 2 g of frozen broccoli florets were placed into a 50 mL plastic centrifuge tube containing 8 mL of 5% (w/v) metaphosphoric acid, and then homogenized on ice using a physcotron homogenizer (NS-52K, Microtec, Chiba, Japan). The homogenate was then filtered through 5A quantitative filter paper.

To determine the total AsA in the extraction, 90 μL of 0.2% (w/v) 2,6-dichlorophenolindophenol was added into 1 mL of the extracted sample solution to transform L-ascorbic acid (L-AsA) to dehydroascorbic acid (DHAAs). Next, we added 1 mL of thiourea and 0.5 mL of 2% (w/v) 2,4-dinitrophenylhydrazine-sulfate solution. All samples and blank solutions were incubated in a water bath at 50°C for 30 min. After incubation, they were cooled on ice for 30 min. Then, 2.5 mL of cooled 85% (v/v) sulfuric acid was slowly added into all test tubes and mixed with a vor-
Dehydroascorbate reductase (DHAR) activity was determined according to Kato et al. (1997), with slight modifications. The reaction mixture contained 0.8 mL of potassium phosphate buffer (50 mM; pH 7), 0.1 mL of 0.1 mM EDTA disodium salt, 1 mL of 2.5 mM reduced glutathione, 1 mL of 0.2 mM DHAA, and 0.1 mL of crude enzyme extraction. The absorbance was monitored at 265 nm. The rate of non-enzymatic reduction of DHAA was determined as a blank. Specific activity was calculated using an extinction coefficient of 14 mM$^{-1}$ cm$^{-1}$.

Glutathione reductase (GR) activity was determined, as described by Hodges et al. (1997), with slight modifications. The reaction mixture contained 1.6 mL of 100 mM phosphate buffer (pH 7.8), 0.2 mL of 100 mM oxidized glutathione, 0.2 mL of 15 mM EDTA disodium salt, 0.04 mL of 10 mM NADPH in 1% (w/v) NaHCO$_3$, and 0.6 mL of crude enzyme extraction. Activity was determined by following the oxidation of NADPH at 340 nm. Specific activity was calculated using an extinction coefficient of 6.2 mM$^{-1}$ cm$^{-1}$.

Protein content was measured using a Pierce® BCA protein assay kit (Thermo Scientific, USA). Bovine serum albumin was used as the standard protein for calculating specific enzyme activity.

**Statistical analysis**

The AsA and enzyme assays were replicated three times per storage treatment. The effect of hyperbaric storage on each response variable was tested with one-way ANOVA (followed automatically by a Tukey’s post-hoc test at the 5% level of significance) in R software platform v.3.1.0 (R Foundation for Statistical Computing).

**RESULTS AND DISCUSSION**

**Effect of hyperbaric storage on the ascorbic acid content of fresh-cut broccoli florets**

Figure 1 shows the change in AsA content of fresh-cut broccoli florets stored at the various pressure treatments at 8 °C for 14 d. After 14 d of storage, the broccoli florets stored under 0.1 MPa (unpressurized condition) showed a 60% reduction in AsA content, but the broccoli florets stored at 0.3 and 2.1 MPa conditions exhibited only 20% and 21% reduction in AsA, respectively. There was no significant difference in the AsA content between fresh-cut broccoli florets stored at 0.3 and 2.1 MPa. However, only DHAA content was observed in the broccoli florets stored at 2.1 MPa. In order to understand the mechanism of AsA retention underlying hyperbaric treatments, the activities of enzymes involved in AsA degradation and recycling system, and antioxidant enzymes were assayed.

**Effect of hyperbaric storage on activities of enzymes involved in AsA degradation and recycling system and antioxidant enzymes in fresh-cut broccoli florets**

The activities of enzymes involved in AsA degradation and recycling system and antioxidant enzymes in fresh-cut broccoli florets stored under three different hyperbaric pressures are shown in Fig. 2. The activities of APX, DHAR, GR, SOD and CAT enzymes in fresh-cut broccoli florets stored at 0.1 MPa (unpressurized condition) did not
In fact, the rate of O$_2$ species (ROS) in a plant tissue caused by high O$_2$ condition. The induction of CAT enzyme activity (approximately 4.5 times), that is more powerful to eliminate H$_2$O$_2$ than the activity of APX (2.1 times) and the action of L-AsA itself. Even if all enzymes activity had been suppressed at 2.1 MPa, we still observed the remaining DHAA content in broccoli florets (Fig. 1). It is caused by the difference between the L-AsA oxidation rate and the DHAA decomposition rate. Under the pressurized condition, H$_2$O$_2$ produced by high partial pressure of O$_2$ is non-enzymatically eliminated by L-AsA itself leading to the production of DHAA. Normally, DHAA is then hydrolyzed to 2,3 diketo-L-gulonate by L-dehydroascorbate lactonohydrolase. However, considering the suppression of all enzymes activities in broccoli florets stored at 2.1 MPa (Fig. 2), it can be assumed that the activity of L-dehydroascorbate lactonohydrolase is suppressed as well. In addition, DHAA cannot be converted back into L-AsA because of suppression of enzyme activity that involved in recycling system, resulting in the persistence of DHAA.

Based on our results, the response of enzymes to a given hyperbaric condition differs by the amount of pressure applied, which in turn leads to differences in retention mechanisms for AsA in fresh-cut broccoli florets.

**Effect of hyperbaric storage on respiration of fresh-cut broccoli florets**

Table 1 shows the partial pressures of O$_2$ and CO$_2$ in the high-pressure container containing fresh-cut broccoli florets stored at three partial pressures, before and after 14 d of storage. A decline in the partial pressure of O$_2$ and an increase in the partial pressure of CO$_2$ were observed in the pressure containers at the end of the storage period. These changes in partial pressures were caused by respiration in the fresh-cut broccoli florets. Normally, after harvesting, fruits and vegetables are still alive. The harvested fruits and vegetables respire to obtain the energy for continuing their metabolic processes. During the respiration process, glucose is oxidized to CO$_2$, while O$_2$, which serves as the electron acceptor, is then reduced to H$_2$O. Thus, CO$_2$ is released to the surrounding atmosphere, resulting in an accumulation of CO$_2$ in the container. In contrast, O$_2$ is reduced in the container.

The change in AsA content of broccoli after harvesting is related to the total amount of O$_2$ intake or CO$_2$ production by respiration (Techavuthiporn et al., 2008). Therefore, considering the relationship between respiration and AsA is also important for better understanding the mechanism for AsA retention under hyperbaric conditions. The total amount of O$_2$ consumption and CO$_2$ production of samples stored under the two tested hyperbaric conditions were significantly suppressed relative to samples stored at 0.1 MPa condition. Comparing between 0.3 and 2.1 MPa, there was no significant difference in the CO$_2$ production, whereas the O$_2$ consumption at 0.3 MPa was significantly change after 14 d of storage. Conversely, the activities of APX, GR, SOD and CAT enzymes in broccoli florets stored at 0.3 MPa were 2.1, 1.3, 2.3, and 4.5 times higher than those at 0.1 MPa, whereas DHAR activity decreased by half. When the broccoli florets were stored at 2.1 MPa, the activities of APX, DHAR, SOD and CAT were almost lost and the activity of GR also decreased.

The induction of the enzyme activity in broccoli florets stored at 0.1 MPa could be resulted from storage at high partial pressure of O$_2$ condition. According to Duan et al. (2011), 0.1 MPa O$_2$ induces the activities of SOD, CAT, and APX in litchi fruit. Similarly, Liu and Wang (2012) found that a high activity of SOD and CAT occurred in mushrooms stored at 0.08 MPa O$_2$. This induction of antioxidant enzyme activity is a protective reaction corresponding to the accumulation of reactive oxygen species (ROS) in a plant tissue caused by high O$_2$ condition. In fact, the rate of O$_2^-$ and H$_2$O$_2$ production has been reported to increase when broccoli heads are stored at 0.1 MPa O$_2$ (Guo et al., 2013). On the other hand, the activities of all enzymes were suppressed when the broccoli florets were stored at 2.1 MPa, suggesting that there might have been an imbalance between ROS detoxification and ROS production, resulting in protein oxidation and enzyme inactivation.

In order to maintain substantial levels of L-AsA, both AsA degradation system including the antioxidant enzymes, and AsA recycling system including MDHAR, DHAR and GR enzymes play a crucial role as shown in Fig. 3. In our observation, the increased APX and the decreased DHAR activity were found in the broccoli florets stored at 0.3 MPa, however, these observations contradict the results of high L-AsA and low DHAA content shown in Fig. 1. These results lead to the hypothesis that MDHAR is also induced by the high partial pressure of O$_2$. The elevated activity of this enzyme may rapidly convert MDHA to L-AsA comparing to the disproportionation of MDHA into DHAA. Other possible reason for the preservation of high level of L-AsA in broccoli florets stored at 0.3 MPa is the inactivation of H$_2$O$_2$. Thus, CO$_2$ is released to the surrounding atmosphere, resulting in an accumulation of CO$_2$ in the container. In contrast, O$_2$ is reduced in the container.
significantly lower than that at 2.1 MPa (Table 1).

The decline in respiration of fresh-cut broccoli stored at 0.3 MPa is possibly due to an increase in the total pressure of the surrounding atmosphere. Inside the plant tissue, the intercellular spaces form a connected network and it is filled with the gas (Kuroki et al., 2004). These interconnected air spaces are necessary for the respiration of a plant tissue (Woolley, 1983). Under the hyperbaric conditions, the broccoli tissue is compressed by the pressure from all directions. It may cause a subdivision of gas-filled intercellular spaces in the broccoli tissue, causing limitation of diffusion of O₂ between the inside and outside cell and leading to lower rate of respiration. From this point of view, it can be imagined that the gas exchange rate between outside and inside is decreased with increase of pressure applied. But, in our observation, the total amount of O₂ consumption of broccoli florets stored at 2.1 MPa was higher than those at 0.3 MPa. This result cannot be explained by the subdivision of gas-filled intercellular space because of pressure. One possible reason might be an en-

Fig. 2 Changes in activities of ascorbate peroxidase (APX) (A), dehydroascorbate reductase (DHAR) (B), glutathione reductase (GR) (C), superoxide dismutase (SOD) (D), and catalase (CAT) (E) in fresh-cut broccoli florets stored at 8°C for 14 d under three different hyperbaric pressures (0.1, 0.3, and 2.1 MPa). (n = 3, vertical bars represent standard deviation. Symbols with the same letter are not significantly different between them, Tukey test 0.05). n.d. indicates not detected.
hancement of oxidative reactions such as auto-oxidation of lipid due to physical cell damage by excess pressure. However, overall it remains difficult to clearly explain the effect of hyperbaric pressure on the respiration rate.

Apart from the pressure effect, the decreased respiration may have also resulted from the elevated partial pressure of CO₂ at both 0.3 MPa and 2.1 MPa. A reduction in respiration rate was observed in broccoli stored at 0.06 MPa CO₂+0.02 MPa O₂+0.02 MPa N₂ compared to broccoli stored under an ordinary air condition (Kubo et al., 1989). Gun et al. (2001) also found that an increase in partial pressure of CO₂ from 0 to 0.03 MPa resulted in a suppression of respiration rate and ethylene production of fresh-cut apple slices. During the respiration process, it is well known that ROS is produced (Tripathy and Oelmüller, 2012) and it is subsequently scavenged by L-AsA. In our study, fresh-cut broccoli florets stored under hyperbaric conditions showed the decrease in amount of O₂ consumption and CO₂ production, suggesting that the total pressure and the elevated high partial pressure of CO₂ under pressurized air storage treatments slow down the respiratory activity of fresh-cut broccoli florets which is one of the major causes of AsA degradation in fresh produce.

CONCLUSION

In this study, we investigated the effect of hyperbaric storage on AsA retention in fresh-cut broccoli florets. AsA was successfully maintained by storing broccoli under pressurized air storage conditions, which also suppressed the respiration of the broccoli florets during storage. In contrast, AsA content was 60% less when the broccoli florets were stored under ambient pressure (0.1 MPa). The response of enzymes involved in AsA degradation and recycling, and antioxidant enzyme differed, depending on the magnitude of pressure applied. A hyperbaric pressure of 0.3 MPa has the potential to maintain L-AsA content by promoting the activity of CAT enzyme and reducing the respiratory activity. Furthermore, the advantage of hyperbaric storage may provide a means for the fresh-cut produce industry to improve the nutritional value of their produce.

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Table 1 Changes in partial pressure of O₂ and CO₂ in a storage container and the total amount of O₂ consumption and CO₂ production by fresh-cut broccoli florets stored at 8°C for 14 d under three different hyperbaric pressures (0.1, 0.3, and 2.1 MPa).

| Gas   | Treatments | Partial pressure (MPa)          | Total amount of O₂ consumption and CO₂ production during 14 d of storage (mmol g⁻¹) |
|-------|------------|--------------------------------|-----------------------------------------------------------------------------------|
|       |            | Before storage                  | After 14 d storage                                                                 |
| O₂    | 0.1 MPa    | 2.1±0.0×10⁻²                   | 2.1±0.0×10⁻²                                                                       | 8.2±0.5×10⁻¹² |
|       | 0.3 MPa    | 6.3±0.0×10⁻²                   | 2.8±0.2×10⁻²                                                                      | 9.3±0.6×10⁻¹² |
|       | 2.1 MPa    | 43.9±0.0×10⁻²                  | 34.6±2.0×10⁻²                                                                      | 2.4±0.5×10⁻¹³ |
| CO₂   | 0.1 MPa    | 3.8±0.0×10⁻⁷                   | 3.8±0.0×10⁻⁷                                                                      | 9.7±1.8×10⁻¹⁵ |
|       | 0.3 MPa    | 1.2±0.0×10⁻⁴                   | 3.5±1.2×10⁻²                                                                      | 9.3±3.2×10⁻²¹ |
|       | 2.1 MPa    | 8.1±0.0×10⁻⁴                   | 3.9±0.3×10⁻²                                                                      | 1.0±1.0×10⁻¹⁴ |

*The 0.1 MPa treatment was the unpressurized condition. Total amount of O₂ consumption and CO₂ production during 14 d of storage were estimated by multiplying obtained respiration rate by the storage hours (see the “material and method” for the explanation).

Values represent the mean of three replicates with standard errors (SD). Values within columns followed by the same letter are not significantly different at P = 0.05 (ANOVA with a Turkey’s post-hoc test).
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