**Article**

**Induction and activity of terminal oxidases of electron transport chain in broccoli heads under controlled atmosphere storage**

Yoshio Makino 1,* Jun Inoue 1, Hsiao-Wen Wang 1, Masatoshi Yoshimura 1, Kensaku Maejima 1, Sachiko Funayama-Noguchi 2, Takeshi Yamada 3, and Ko Noguchi 4

1 Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657 Japan; jini19920701@gmail.com (J. I.; fyolxf@hotmail.com (H.-W. W.); ayoshimura@mail.ecc.u-tokyo.ac.jp (M. Y.); amaejima@mail.ecc.u-tokyo.ac.jp (K. M.)

2 Graduate School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; funayama@bs.s.u-tokyo.ac.jp (S. F.-N.)

3 P-Plus Project, Sumitomo Bakelite Co., Ltd., 5-8, 2-chome, Higashi-Shinagawa, Shinagawa-ku, Tokyo 140-0002, Japan; yamada@sumibe.co.jp (T. Y.)

4 School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan; knoguchi@toyaku.ac.jp (K. N.)

* Correspondence: amakino@mail.ecc.u-tokyo.ac.jp; Tel.: +81-(3)-5841-5361

**Abstract:** Controlled atmosphere (CA) storage, under atmospheres with low O₂ and high CO₂ concentrations, is effective for extending the shelflife of horticultural products. We investigated the influence of CA storage (O₂:CO₂: 2.5%/6.0% or 2.5%/0.0%) at 1°C for 21 d versus normoxia (normal air) on the physicochemical and biological properties of broccoli (Brassica oleracea var. italica, Plenck, 1794) via amounts and activities of terminal oxidases of electron transport chain. Mass loss, a sensitive index of freshness for broccoli heads under CA, was significantly lower under CA than under normoxia. The effect for depressing mass loss was observed 7 d earlier under CA including 6.0% CO₂ than under CA without CO₂. Environmental CO₂ was also effective for depressing loss of L-ascorbate. The alternative oxidase (AOX) level under CA was lower than under normoxia during storage, while the level of cytochrome c oxidase (COX), and the AOX/COX activity ratio (based on oxygen isotope discrimination), were stable during storage. Our results indicate that CA storage is effective for retaining freshness of broccoli heads during storage by depressing the induction of AOX. However, depression of AOX level was found to be independent of environmental CO₂.

**Keywords:** alternative oxidase; Brassica oleracea var. italica; cytochrome c oxidase; mass loss; oxygen isotope discrimination

1. Introduction

Controlled atmosphere (CA) storage is a useful method for prolonging the shelflife of many kinds of fruit and vegetables, involving storage under controlled atmospheres, including reduced O₂ and elevated CO₂ in a refrigerator [1].

Broccoli (Brassica oleracea var. italica, Plenck, 1794) is known to be rich in micronutrients, such as vitamins, minerals, flavonoids, etc. [2], and global production of this vegetable increases annually [3]. However, broccoli rapidly deteriorates after harvesting due to its high respiration rate [4]. CA storage at 0°C with 2%−5% O₂ and 10% CO₂ was reported to prolong the shelflife of broccoli heads 1.5-fold compared to those under normoxia [5]. Lipton and Harris [6] reported that shear resistance of broccoli heads was significantly retained by storing under 1% O₂ and 10% CO₂ at 5°C or 7.5°C for 3 d. Deschene et al. [7] reported that storage at 5°C or 10°C in a CA (O₂:CO₂: 3.0%/5.0%) strongly inhibited loss of chlorophyll of cut heads of broccoli. Makhlouf et al. [8], studying CO₂ production rate, color, chlorophyll concentration, soft rot and mold reported that an atmosphere including 2.5% O₂ and 6% CO₂ is suitable for retaining freshness in broccoli heads.
Shelflife is extended during storage by limiting metabolism, which is strongly affected by respiration [4]. During respiration, stored nutrients are transformed to substrates that drive electron flow through the electron transport chain (ETC). This suggests that the activities of terminal oxidases of ETC are associated with the shelflife of horticultural products.

Wang et al. [9] reported that induction of the alternative oxidase (AOX) in cut broccoli florets was depressed under atmospheres with low O$_2$ and high CO$_2$. AOX, branching from the cytochrome c oxidase (COX) pathway, is a nuclear-encoded protein located in the inner mitochondrial membrane, forming the alternative pathway that consumes O$_2$ uncoupled from adenosine-5’-triphosphate (ATP) production [10].

The AOX induction phenomenon witnessed by Wang et al. [9] may have been occurring in the previous researches [5-8]. However, in the research by Wang et al. [9], the storage temperature was 25°C and they examined only the early stage of storage (within 50.5 h), conditions that differ from those used in practice for storage and transportation of broccoli heads. Furthermore, it has been unclear that the depression of AOX induction was caused by whether low O$_2$ or high CO$_2$ yet.

Our objective was to clarify the activities and amounts of the two terminal oxidases (COX and AOX) of ETC under the same CA conditions suggested by Makhlouf et al. [8] as suitable for storage of broccoli heads. Influence of low O$_2$ or CO$_2$ on freshness and terminal oxidase induction will also be separately investigated different from the research by Wang et al. [9]. Our results help clarify the reason why CA storage is effective for retaining freshness of horticultural products such as broccoli.

2. Materials and Methods

2.1. Samples

Fresh heads of broccoli (“Pixel” and “Ohayo” cultivars) were harvested at a farm in Hokkaido and Aichi prefectures, Japan, one day before use. Forty-two broccoli heads (twenty-one “Pixel” and twenty-one “Ohayo”) were prepared and each head was sealed in an oriented polypropylene-based, micro-perforated pouch (Sumitomo Bakelite Co., Ltd., Tokyo; O$_2$ transmission rate 7.75 × 10$^4$ ml m$^{-2}$ d$^{-1}$ atm$^{-1}$; surface area 0.175 m$^2$; thickness 25 μm). The micro-perforated pouch maintains gas composition inside the pouch the same as the ambient atmosphere while maintaining relative humidity (R.H.) at saturation point.

2.2. Controlled atmosphere storage methods

Storage experiments #1 (“Pixel” cultivar) and #2 (“Ohayo” cultivar) were conducted to investigate the effect of low O$_2$/high CO$_2$ or low O$_2$ on freshness or terminal oxidases, respectively. The storage apparatus is shown in Figure 1. In experiment #1, nine heads sealed in micro-perforated pouches were enclosed in a 7-L volume of acrylic chamber (V-7, Shin-ei Sangyou Co., Ltd, Daito, Japan), and gas mixture composed of O$_2$: 2.5% and CO$_2$: 6% (balanced to 100% using N$_2$) was passed through from the inlet to the outlet at a flow rate of 100 mL min$^{-1}$. Sealing in the pouch was effective for avoiding influence of air flow to the measured data as mass loss of samples. In experiment #2, the gas composition was changed to O$_2$: 2.5% and CO$_2$: 0% (balanced to 100% using N$_2$). In both experiments, another nine broccoli heads were sealed in micro-perforated pouches to retain R.H. at saturated point around the samples and stored under air (normoxia) without gas flow as a control. The atmosphere in experiment #1 was the same as reported by Makhlouf et al. [8]. Experiment #2 was conducted to investigate the influence of removal of CO$_2$ on the physicochemical and biological properties of the broccoli heads. These heads were stored at 1°C, and three heads were sampled from each acrylic chamber on days 7, 14 and 21 to measure physicochemical and biological properties.
2.3. \( \text{O}_2 \) uptake rate measurement

The \( \text{O}_2 \) uptake rate of a broccoli head at \( 1^\circ\text{C} \) was measured according to the method of Makino et al. [11]. A head was sealed in a laminated high-barrier pouch (AS ONE Co. Ltd., Osaka), which provided a closed system (\( \text{O}_2 \) transmission rate, <1.0 ml m\(^{-2}\) d\(^{-1}\) atm\(^{-1}\); surface area, 0.086 m\(^2\); thickness, 150 \( \mu \text{m} \); polyethylene terephthalate/chlorinated polyethylene/aluminum/chlorinated polyethylene/polyethylene). \( \text{O}_2 \) concentration in the headspace of the pouch was measured using a gas analyzer (CheckMate 3, Dansensor A/S, Ringsted, Denmark). The headspace volume was measured by the water displacement method. Equation 1 was used to calculate the \( \text{O}_2 \) uptake rate, as follows:

\[
U = \frac{V(P_0 - P_t)}{100\rho T t M},
\]

where:
- \( M \) = mass of the broccoli head (kg)
- \( P \) = \( \text{O}_2 \) concentration in the pouch (%)
- \( t \) = incubation time
- \( T \) = incubator temperature (K)
- \( U \) = \( \text{O}_2 \) uptake rate (mmol kg\(^{-1}\) h\(^{-1}\))
- \( V \) = void volume in the pouch (mL)
- \( \rho \) = universal gas constant (L atm mol\(^{-1}\) K\(^{-1}\))
- Subscript 0 = initial (start) time, subscript \( t \) = incubation time

2.4. Mass loss measurement

Mass loss, \( M_L \) (%), was measured according to Equation 2 as:
\[ M_L = \frac{100(M_0 - M_t)}{M_0} \]  

2.5. L-ascorbate measurement

A 1-g sample of broccoli buds frozen in liquid N\textsubscript{2} was homogenized with 5 g of 3% metaphosphate for 1 min and then centrifuged at 3,000 \times g at 4°C for 20 min. The supernatant was used for measurement of L-ascorbate. Sensor area of Ascorbic Acid Test (Merck KGaA, Darmstadt, Germany) was immersed in the supernatant and the degree of blue color of the sensor area was measured using RQflex\textsuperscript{®} 10 Reflective brightness meter (Merck KGaA). Then the ascorbic acid concentration was observed in the display on the meter.

2.6. Determinations of AOX and COX protein amounts

The amounts of AOX and COX enzymes produced by the broccoli heads were measured according to the method of Wang et al. [9], to determine the relationship between O\textsubscript{2} consumption and storage atmosphere.

2.7. Determination of O\textsubscript{2} isotope discrimination

Guy et al. [12] found that the consumption of different O\textsubscript{2} isotopes differed between AOX and COX. Therefore, the ratio of AOX to COX activity can be expressed by O\textsubscript{2} isotope discrimination, hereafter abbreviated as “D” (Equation 3). The value of D for AOX is usually higher than for COX. Thus, to investigate the effect of various gas compositions on AOX and COX activities, the D value was measured by the method of Wang et al. [9].

\[ D = -\frac{\ln(R/R_0)}{\ln f} \]  

where:
- \( D \) = discrimination value (%)
- \( R = ^{18}\text{O}/^{16}\text{O} \) ratio of gas sample
- \( f \) = fraction of in-package O\textsubscript{2} concentration

2.8. Statistics

All results were analyzed with Tukey’s honest significant difference test using JMP\textsuperscript{®} Pro ver.13.2.0 (SAS Institute, Cary, NC).

3. Results and Discussion

3.1. Changes in O\textsubscript{2} uptake rate

The changes in O\textsubscript{2} uptake rate of broccoli heads under CA and normoxia are shown in Figure 2. In experiments #1 and #2, initial values were significantly higher than those during storage, possibly because the initial temperature of the samples was higher than during storage. The mean O\textsubscript{2} uptake rates in the low O\textsubscript{2} environment in both experiments were lower than those under normoxia except on day 21 in experiment #2. On individual sampling days, there were no significant O\textsubscript{2} uptake rate differences between low O\textsubscript{2} and normoxia. Values during storage at 1°C in the present study and those reported by Makhlouf et al. [8] were in the range 0.59–0.80 mmol O\textsubscript{2} kg\textsuperscript{-1} h\textsuperscript{-1} and 0.26–0.63 mmol CO\textsubscript{2} kg\textsuperscript{-1} h\textsuperscript{-1}, respectively. These are much lower than the values reported elsewhere: 2.5–11 mmol O\textsubscript{2} kg\textsuperscript{-1} h\textsuperscript{-1} at 25°C by Wang et al. [9]; 7.56 mmol O\textsubscript{2} kg\textsuperscript{-1} h\textsuperscript{-1} at 20°C by Makino et al. [13]; and 9.99 mmol CO\textsubscript{2} kg\textsuperscript{-1} h\textsuperscript{-1} at 20°C by Robinson et al. [4]. The low values exhibited in the present study were likely caused by the low storage temperature, which also made it difficult to detect significant influences arising from the different atmospheres.
Storage environments have been known to affect O\(_2\) uptake rate. It has been shown that O\(_2\) uptake rates of samples stored in CA environments become depressed during storage, due to the reduction in environmental O\(_2\) and the increase in CO\(_2\) level [14]. Reduced environmental O\(_2\) may have affected O\(_2\) consumption by AOX and COX even though a significant reduction was not observed under the CA (Figure 2).

Figure 2. O\(_2\) uptake rates of broccoli heads during storage at 1°C. (a) Experiment #1: O, normoxia; ●, O\(_2\)/CO\(_2\) = 2.5%/6.0% (+ N\(_2\) to 100%). (b) Experiment #2: O, normoxia; ●, O\(_2\)/CO\(_2\) = 2.5%/0.0% (+ N\(_2\) to 100%). Values are mean ± SE of three biological replicates. Significant differences (p < 0.05) are denoted by different letters.

3.2. Mass loss

Changes in mass loss of broccoli heads under CA and normoxia are shown in Figure 3. All the heads were sealed in micro-perforated pouches. Therefore, R.H. around the heads was maintained at saturated point. And also, gas flow in CA chamber did not affected to the data for mass loss because the pouch was effective for avoiding the influence by gas flow. According to the conditions, data for mass loss were affected by only the environmental atmospheres around the samples. Mass loss under the CA in experiment #1 was significantly lower than that under normoxia. In contrast, mass loss under the CA in experiment #2 was significantly lower than that under normoxia only on day 21. These results indicate that high CO\(_2\) combined with low O\(_2\) is effective for reducing mass loss in broccoli heads. Forney et al. [15] reported that mass loss in broccoli heads was reduced by 17% under CA (O\(_2\)/CO\(_2\): 14.0%/10.0%) at 5°C after 21 d storage. Makhlouf et al. [8] reported that mass loss in broccoli heads was reduced under CA (O\(_2\)/CO\(_2\): 2.5%/6.0%) at 1°C after 6 weeks storage. Wang et al. [9] reported that mass loss in broccoli florets under a modified atmosphere (O\(_2\)/CO\(_2\): 2.9%−6.1%/10.0%−11.0%) was reduced after storage at 25°C for 50.5 h. The decline in mass (as water) of broccoli heads leads to a reduction in nutritional quality, salability (due to wilting, shriveling, softening, increased flaccidity, limpness, loss of crispness, and juiciness), and economic loss (due to the loss of salable weight) [16]. Therefore, CA storage appears to be effective for keeping freshness of broccoli heads. In the present study, the decline of mass was significantly depressed on and after 14 d under CA including 6.0% CO\(_2\) though it was significantly depressed on 21 d under CA without CO\(_2\). This result indicated that environmental CO\(_2\) was effective for maintaining freshness of broccoli heads. Mass loss during storage has been shown to be strongly correlated with O\(_2\) uptake rate. A higher O\(_2\) uptake rate has been shown to enhance transpiration, which leads the higher mass loss [17-19].
Figure 3. Mass loss of broccoli heads during storage at 1°C. (a) Experiment #1: ○, normoxia; ●, O₂/CO₂ = 2.5%/6.0% (+ N₂ to 100%). (b) Experiment #2: ○, normoxia; ●, O₂/CO₂ = 2.5%/0.0% (+ N₂ to 100%). Values are mean ± SE of three biological replicates. Significant differences (p < 0.05) are denoted by different letters.

3.3. L-ascorbate

Changes in L-ascorbate levels in broccoli heads under CA and normoxia are shown in Figure 4. This micronutrient is required to prevent the disease “scurvy” in humans and is well known as an indicator of freshness because its content decreases during storage. Barth et al. [20] reported that L-ascorbate concentration in broccoli spears decreased over time at 10°C, and modified atmosphere (O₂/CO₂: 10.0/8.0%) packaging was effective for retaining the concentration. In the present study, L-ascorbate concentration on day 21 under normoxia in experiment #1, and on and after day 14 both under CA and normoxia in experiment #2, were significantly lower than that in fresh heads. Only in heads stored under CA in experiment #1, the L-ascorbate level did not become significantly reduced during storage (Figure 4A), indicating that a high CO₂/low O₂ CA may be effective for retention of L-ascorbate level in broccoli heads. This result indicated that environmental CO₂ was effective for maintaining nutritional value of broccoli heads.

Figure 4. L-ascorbate concentration in broccoli heads during storage at 1°C. (a) Experiment #1: ○, normoxia; ●, O₂/CO₂ = 2.5%/6.0% (+ N₂ to 100%). (b) Experiment #2: ○, normoxia; ●, O₂/CO₂ = 2.5%/0.0% (+ N₂ to 100%). Values are mean ± SE of three biological replicates. Significant differences (p < 0.05) are denoted by different letters.

3.4. Changes in amounts of AOX and COX enzymes during storage
Changes in amounts of AOX and COX enzymes in broccoli heads under CA and normoxia are shown in Figures 5 and 6, respectively. In experiment #1, AOX amounts did not significantly change during the storage period (Figure 5A), although the amount of AOX under CA on day 21 was significantly smaller than that under normoxia. Also, the amount under CA on day 14 was not significantly different from that under normoxia on day 7, though that under normoxia on day 14 was significantly larger than that under CA on day 7. In experiment #2, AOX amounts under CA were consistently smaller than those under normoxia. These results indicate that a CA can have the effect of depressing the induction AOX. AOX is an enzyme that consumes O₂ molecules taken into plant cells, and this small amount of AOX induction may be sufficient for the broccoli head under the low O₂ environment. Wang et al. [9] reported that amounts of AOX in broccoli florets under modified atmosphere (O₂/CO₂: 2.9%–6.1%/10.0%–11.0%) increased 2.83-fold while those under normoxia increased 6.18-fold during 50.5 h storage at 25°C, a tendency reflected in the present research. In contrast, Wang et al. [9] reported that AOX levels under CA (O₂/CO₂: 6.0%/10.0%) at 32.5 h were almost the same as in fresh florets. This result from an early storage stage using cut samples is also reflected in the present research on long and cold storage using intact samples. Influence of environmental CO₂ on induction of AOX was not clear in the present study. Therefore, induction level of AOX was found to be affected by only environmental O₂ in the present study.

Figure 5. Relative amount of alternative oxidase (AOX) in broccoli heads during storage at 1°C. (a) Experiment #1: ○, normoxia; ●, O₂/CO₂ = 2.5%/6.0% (+ N₂ to 100%); (b) Experiment #2: ○, normoxia; ●, O₂/CO₂ = 2.5%/0.0% (+ N₂ to 100%). Values are mean ± SE of three biological replicates. Significant differences (p < 0.05) are denoted by different letters.

The levels of COX were relatively stable during the storage period (Figure 6).

During respiration, stored nutrients such as carbohydrates, lipids, organic acids, etc. are transformed to substrates that drive H⁺ and e⁻ flows through the ETC. O₂ molecules taken into a plant cell are consumed for the oxidation of H⁺ and e⁻ to H₂O, catalyzed by AOX and COX. Therefore, consumption of O₂ by these enzymes promotes a reduction in stored nutrient levels, an effect that is one of the main causes of deterioration in horticultural products such as broccoli. In the present study under CA, mass loss and induction of AOX were indices of deterioration in broccoli heads.

COX is one of the crucial terminal oxidases in oxidative phosphorylation (OXPHOS), which consumes O₂, and is coupled with ATP synthesis [21,22]. ATP generated via OXPHOS is required to maintain the biological activity of plant cells [23]. These findings suggest that COX is important for maintaining metabolic activities in horticultural products even after harvest. Therefore, the amount of COX may be maintained at a level suitable for maintaining metabolic activities.

In contrast, induction of AOX may be adjusted in relation to the environmental O₂ level. There have been no reports of the measurement of terminal oxidases of ETC in horticultural products under long term CA storage, despite the effectiveness of CA storage being recognized more than a century ago by Kidd [24-26]. The reason why horticultural products need a lower level of O₂ than
mammals is suggested from the results of the present study. Since plants do not locomote, like mammals, lower ATP concentrations and COX induction levels are needed. Furthermore, when horticultural products are stored under normoxia, excessive O$_2$ is taken into cells promoting induction of AOX, and hence causing deterioration. Therefore, the effectiveness of CA for the retention of freshness may arise from the depression of excessive induction of AOX. This hypothesis is suggested from the data for AOX and COX induction observed in the present study. However, whether this hypothesis could be applied universally needs further investigation using other kinds of horticultural product.

**Figure 6.** Relative amount of cytochrome c oxidase (COX) in broccoli heads during storage at 1°C. (a) Experiment #1: ☐, normoxia; ●, O$_2$/CO$_2$ = 2.5%/6.0% (+ N$_2$ to 100%). (b) Experiment #2: ☐, normoxia; ●, O$_2$/CO$_2$ = 2.5%/0.0% (+ N$_2$ to 100%). Values are mean ± SE of three biological replicates. Significant differences (p < 0.05) are denoted by different letters.

3.5. *Changes in O$_2$ isotope discrimination during storage*

Changes in the $D$ values of broccoli heads under CA and normoxia are shown in Figure 7. In experiment #1, $D$ values were stable during storage independent of atmosphere (Figure 7A). In experiment #2, the mean $D$ value on day 21 under CA was significantly higher than that of fresh heads (day 0), and higher than that under normoxia on the same day (but not significantly). These results suggest that the influence of atmosphere on $D$ values is minor, which reflects the results of Wang et al. [9]. According to Guy et al. [12], the $D$ value is equivalent to the activity ratio of AOX to COX and is higher with increasing AOX activity.

The influence of atmosphere on AOX amount was clearly observed in this study (Figure 5B), in contrast to the results for $D$ (Figure 7). This suggests that the $D$ value is less sensitive than AOX levels for evaluating the effects of CAs on the biological properties in stored horticultural products.
4. Conclusions

AOX levels seemed to be dependent on the storage atmosphere, but COX levels did not. The amount of AOX produced under hypoxia was significantly lower than that produced under normoxia, while the amount of COX produced was stable and independent of the storage atmosphere. Meanwhile, induction of terminal oxidases was found to be independent of environmental CO₂ in the present study. Product degradation, such as mass loss, which occurs during the storage of broccoli, was accompanied by an increase in the amount of AOX. These results suggest that broccoli heads adapt to atmospheric changes, not by changing induction of COX, but by induction of AOX. Knowledge of the AOX-to-COX ratio under different storage environments may provide the basis for improving postharvest storage strategies. The use of storage conditions that depress AOX induction may be useful for reduction of postharvest losses of horticultural products. In addition, results of this study may contribute to developing improved strategies for selecting cultivars that produce a minimal amount of AOX postharvest.


Funding: The research foundation was partly supported by Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research (A) 25225045 and (B) 15KT0026.

Acknowledgments: The authors thank Dr. Katsuhiro Sakano and Professor Ichiro Terashima for the use of the vacuum line and mass spectrometry, respectively.

Conflicts of Interest: We have the following interests: Takeshi Yamada is employed by Sumitomo Bakelite Co., Ltd. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the Foods policies on sharing data and materials, as detailed online in the guide for authors.

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