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Reversible and Irreversible Emission of Methanethiol and Dimethyl Disulfide from Anaerobically Stored Broccoli

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The reversible and irreversible emission of methanethiol (MT) and dimethyl disulfide (DMDS) from broccoli florets was demonstrated during anaerobic storage at 20 °C for up to 24 h. Reversible emission of MT and DMDS was feasible only in broccoli stored for between 0 and 12 h under entirely anaerobic condition. Beyond that, the emission was completely irreversible. This irreversible process was demonstrated through significant reductions in the chlorophyll fluorescence values and rate of carbon dioxide production and significant increase in the membrane permeability of induced broccoli tissues after exposure to air and incubation. Irreversible emission was also demonstrated through significant change in color from the characteristic bright green to olive green as well as the conversion of chlorophyll *a* to pheophytin *a* and chlorophyll *a*′ contents of the induced florets after hot-water treatment. These findings suggest that the irreversible emission of MT and DMDS is a function of permanent membrane damage and loss of intracellular compartmentation in the broccoli tissues as a result of the anaerobic induction. The off-odor formation can still be reversed if the affected tissue is only temporarily impaired by anaerobic condition, thereby maintaining the quality of stored broccoli.

KEYWORDS: Broccoli; *Brassica oleracea* L. var. *italica* Plenck; methanethiol; dimethyl disulfide; anaerobic storage; reversible emission; irreversible emission; chlorophyll fluorescence; hot-water treatment

INTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica* Plenck) is a highly perishable fresh vegetable such that keeping the quality of this vegetable for an extended period of time often poses a serious problem. Yellowing of the florets and toughening of the stem are some of the most common storage disorders encountered.

Suggested methods for storage such as refrigeration and modified atmosphere packaging (MAP) have gained wide acceptance because they improve the quality and prolong the shelf life of broccoli. Off-odor and off-flavor developed in only 1 day of storage at 20 °C, but quality was maintained for up to 28 days of refrigerated storage at 0 °C (*1*). Packaging broccoli by means of polymeric films has also delayed the deterioration of quality and enhanced the retention of nutrients and color (*2*). Storage at temperatures of 5 or 7.5 °C and in atmospheres of 0.5−2 kPa of O_2 and/or 10 kPa of CO_2 reduced rates of respiration and inhibited yellowing of the vegetable (*3*, *4*).

However, if the permeability of the package to O_2 is insufficient, O_2 concentrations inside MAP may decrease to levels that result in the development of offensive odor during prolonged storage (*5*−*7*). An off-odor is usually emitted upon opening of the package prior to use or consumption. This can be removed by just exposing the stored sample to air or heat, but in other cases, the objectionable odor persists even after the broccoli has been subjected to aeration and hot-water dip treatment.

The primary chemical responsible for this offensive odor has been identified as methanethiol (MT) (*8*, *9*), described as an "intensely putrid, fecal-like aroma" (*10*) or that of rotten cabbage (*11*). In addition, dimethyl disulfide (DMDS) and other volatiles such as dimethyl sulfide, dimethyl trisulfide, ethanol, hydrogen sulfide, ethyl acetate, and acetaldehyde also contribute to this objectionable odor (*8*, *9*, *12*−*15*).

Volatile sulfur compounds are the critical factors limiting the market potential of broccoli in MAP. Although various research works have been conducted on the emission of these sulfur volatiles (*8*, *9*, *12*−*15*), no study has yet been conducted that could indicate whether the emission of the objectionable odors from anaerobically induced broccoli is still reversible or not.

Hence, the objective of this study was to demonstrate the reversible and irreversible emission of the two main volatile sulfur compounds, MT and DMDS, in broccoli during anaerobic storage by nondestructive measurement techniques and hot-water treatment. Furthermore, the extent to which the reversible and irreversible production of the unpleasant odors and off-flavors

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in broccoli can be attributed to temporary and permanent tissue breakdown or cellular membrane damage, respectively, during anaerobic induction was also evaluated.

MATERIALS AND METHODS

Plant Material. Fresh broccoli heads (*B. oleracea* L. var. *italica* Plenck) grown in the United States and weighing ~250−300 g were obtained from a local supermarket in Osaka, Japan. Broccoli buds and florets were used in the study. Broccoli florets measuring 2−3 cm in length were excised with a knife from the compact head. Broccoli buds (2−3 mm long) were cut further from the florets after they had been trimmed from the broccoli heads.

Chemicals. Authentic samples such as MT, DMDS, chlorophyll *a* (chl *a*), solvents including methanol, ethyl alcohol, and ethyl acetate, and all other chemicals used in the study were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Experimental Storage. Per storage period of 0, 6, 12, 18, and 24 h, six replicates each of 10 g of broccoli buds or florets in a 100-mL Erlenmeyer flask were prepared and placed inside a retortable pouch (20 × 30 cm) together with a silicon stopper. The pouches, made of nylon (15 μm) and polypropylene (70 μm) laminate (Toyo Canning Co., Tokyo, Japan), were alternately vacuumed and flushed with nitrogen gas before sealing with a Multivac A-300 vacuum sealer (Sepp Haggenmuller KG, Wolfertschwenden, Germany). The O_2 concentrations of the atmosphere inside the pouches were routinely checked after vacuum-sealing, and only pouches containing 0 kPa of O_2 were used. After sealing, the flasks inside the pouches were covered with silicon stoppers and stored at 20 °C in the dark.

After the indicated storage period, the flasks inside the first three replicate pouches were individually checked for sulfur-containing headspace volatiles and gas concentrations with gas chromatography. The remaining three replicate pouches were unsealed, and the stoppers were removed from the flasks. Each flask was flushed with air for 5 min at room temperature (~25 °C) to remove any off-odor accumulated during anaerobic storage. The same silicon stopper was used to reseal the flask prior to incubation for 2 h at 30 °C in a water bath. The headspace sulfur volatiles and the atmosphere inside the flasks were analyzed before and after incubation.

Succeeding experiments were conducted following the same storage procedure described above. After each storage period, all six replicate pouches were unsealed and the stoppers covering the flasks in the first three replicate pouches were removed. Then, the anaerobically induced tissues inside the flasks were taken out for chlorophyll fluorescence and electrolyte leakage analyses. The flasks in the remaining three replicate pouches were flushed with air for 5 min. After flushing, the flasks were resealed with the same stopper and incubated for 2 h at 30 °C in a water bath. Chlorophyll fluorescence and electrolyte leakage analyses were then conducted after incubation.

For the third experimental run, the induced broccoli florets were all taken from the flasks after storage. Surface color and chlorophyll contents of the tissue samples from the first three replicate pouches were evaluated. The broccoli florets from the last three replicate pouches were subjected to hot-water test treatment. The tissues were immersed in boiling water for 1−2 min, and excess water was drained with a stainless strainer. Surface color and chlorophyll analyses of the tissues were subsequently conducted after the immersion process.

Headspace Sulfur Volatile Analysis. The headspace gas samples were analyzed on a Hitachi gas chromatograph model 163 (Hitachi Co. Ltd., Tokyo, Japan) equipped with a flame photometric detector (GC-FPD) and a Hitachi D-2500 chromato integrator. The volatile sulfur compounds were separated on two glass columns (GL Sciences, Inc., Tokyo, Japan) connected in series: (1) a 10 cm × 3 mm (i.d.) column packed with Porapak Q (Millipore Corp., Milford, MA); (2) a 3 m × 3 mm (i.d.) column packed with polyphenyl ether 5-rings [*m*-bis(*m*-phenoxyphenoxy)benzene], 5% coating on diasolid S (Nihon Kuromato Kogyo, Tokyo, Japan). The flow rate for nitrogen was 80 mL/min, that of oxygen, 25 mL/min, and that of hydrogen, 70 mL/min. The column temperature was maintained at 70 °C. Injector and detector temperatures were both held at 180 °C. MT and DMDS were identified and quantified by comparing their GC retention times with those of the authentic compounds.

Measurement of Gas Concentrations. One milliliter of gaseous sample was collected from the atmosphere inside the flask twice and analyzed for CO_2 and O_2 concentrations with a Yanaco gas chromatograph model G80 (Yanaco Co. Ltd., Kyoto, Japan) equipped with a thermal conductivity detector (GC-TCD) and two separate columns. Sample for CO_2 analysis was injected into a 2 m × 3 mm (i.d.) column packed with Porapak Q (Millipore Corp., Milford, MA), and for O_2 analysis, a gas sample was injected into an identical column containing Molecular Sieve 5A packing material (Nishio Industry Co. Ltd., Osaka, Japan). Argon was used as the carrier gas and pumped at a flow rate of 45 mL/min. The column temperature was maintained at 60 °C. The injector port was held at 140 °C, whereas the detector port was kept at 60 °C.

Measurement of Chlorophyll Fluorescence. Anaerobically induced broccoli florets were kept inside a box located in a dark room for 30 min. After holding, dark-adapted samples were quickly covered with a lightweight leafclip and immediately measured for chlorophyll fluorescence. The sample was covered with a leafclip, which contains a foam pad to allow the sample to rest while in the clip prior to measurement only to minimize damage to the structure of the floret tissues. A dim light was turned on to provide a low level of illumination during measurement. Chlorophyll fluorescence signals were measured using a Hansatech Plant Efficiency Analyser version 2.05 (Hansatech Instruments Ltd., Norfolk, U.K.). Light intensity was set to 100% and recording time was 5 s. The chlorophyll fluorescence signal received by the sensor unit during a recording and digitized in the control box was reported in terms of F_v/F_m, which is a ratio of the variable component of fluorescence, F_v, to the maximum fluorescence value, F_m, obtained from the same light intensity. Measurements of the chlorophyll fluorescence were conducted in triplicates with each measurement repeated at least three times.

Electrolyte Leakage Analysis. One gram of broccoli buds was immersed in a 100-mL Erlenmeyer flask containing 50 mL of deionized water for 1 h at 25 °C in a water bath. Electrical conductivity (EC) of the sample was periodically measured with an EC meter model CM-14P (TOA Electronics Ltd., Tokyo, Japan). Total electrolyte leakage was obtained by boiling fresh broccoli bud samples for 5 min. Results were expressed as percentage of the total electrolytes within a sample. Ion leakage was measured in triplicates with each measurement repeated at least three times.

Measurement of Surface Color. The color attributes, Hunter L^*, a^*, and b^* values, of the broccoli florets were measured with a Color Difference Meter model 1001DP (Nippon Denshoku Kogyo Co. Ltd., Tokyo, Japan) before and after hot-water treatment for 1−2 min following anaerobic storage. Color measurement was done in triplicates with each measurement repeated thrice. The measure of a surface color's lightness, Hunter L^*, was reported without further manipulation. Chroma (C^*) and hue angle ($h°$) values were calculated on the basis of the equation formulated by McGuire (*16*):

$$\text{chroma} = (a^{*2} + b^{*2})^{1/2}$$

$$\text{hue angle} = [(\tan^{-1} b^*/a^*)/6.2832] + 180$$

where L^* = lightness, which ranges from black = 0 to white = 100; a^* is a bluish green to red-purple hue on the horizontal axis; b^* is a yellow to blue hue on the vertical axis; C^* is chroma; and $h°$ is hue angle (0° = red-purple, 90° = yellow, 180° = bluish green, 270° = blue).

Chlorophyll Determination. *Extraction.* Chl pigments were extracted by grinding 2.5 g of hot-water-treated or untreated broccoli florets in 20 mL of chilled acetone and 2.5 mL of distilled water with a mortar and pestle. Homogenate was filtered out, and the residue was washed with 80% cold acetone until it became colorless. The filtrate was made up to 100 mL volume with 80% cold acetone. Aliquots of the combined extracts were passed through a 0.45-μm DISMIC filter unit, PTFE, (Advantec, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for HPLC analysis and spectrophotometric analysis.

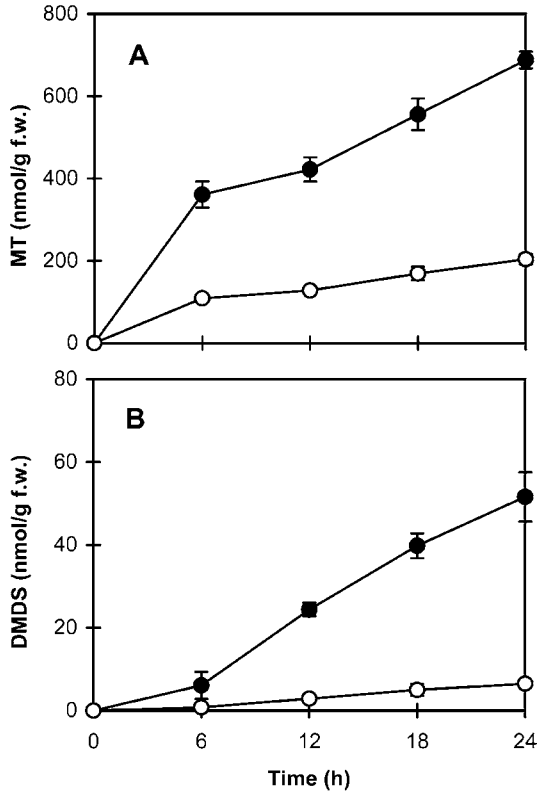

Figure 1. Emission of (A) MT and (B) DMDS from broccoli buds (●) and florets (○) in Erlenmeyer flasks after anaerobic storage at various holding times at 20 °C. Data shown are the mean values of three replicates ± SD.

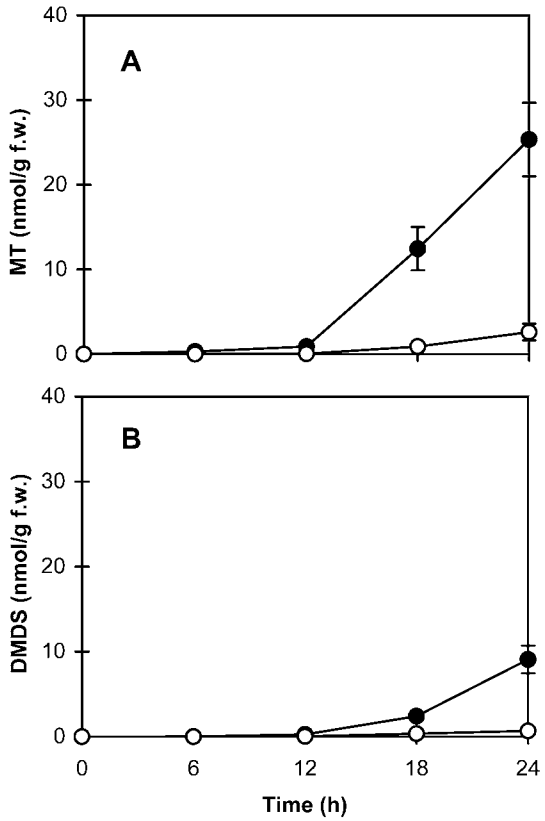

Figure 2. Irreversible emission of (A) MT and (B) DMDS from broccoli buds (●) and florets (○) in Erlenmeyer flasks after flushing with air and incubation for 2 h at 30 °C following anaerobic storage. Data shown are the mean values of three replicates ± SD.

Spectrophotometric Analysis. Total chl and chl *a* and *b* contents of broccoli florets before and after hot-water treatment were determined spectrophometrically according to the method of Arnon (*17*).

HPLC Analysis. Chl and its derivatives and isomers were determined according to a slightly modified procedure of Eskins and Harris (*18*). The HPLC system was equipped with a Hitachi model L-6200 pump with an automated gradient controller and a Hitachi UV−visible spectrophotometer model L-4200. Pigments were separated on a 4.0 mm × 250 mm (i.d.) Inertsil ODS-2 column (GL Sciences, Inc., Tokyo, Japan), 5 μm particle size, with solvent systems, A, 80% methanol, and B, ethyl acetate. A 25 μL sample was chromatographed at a flow rate of 1.0 mL/min under gradient conditions starting with 100% A and 0% B to reach 50% A and 50% B at 20 min and then kept isocratic for an additional 20 min. The absorption of the pigments was recorded at 665 nm with a Hitachi D-2000 chromato integrator. The spectra of the pigments were recorded using a Shimadzu photodiode array detector model SPD-M10AVP (Schimadzu Corp., Kyoto, Japan). Identification of chl and its derivatives was based on their retention times and visible absorption spectra. Initial verification was done according to the previous work of Yamauchi et al. (*19*). Retention times and visible absorption spectra were verified using commercially available and prepared standards.

Standards. Standards for chl derivative and isomer were prepared from authentic chl *a* sample. Pheophytin *a* (phy *a*) was prepared by adding one drop of 2 N HCl to the authentic sample. Preparation of chlorophyll *a*′ (chl *a*′), an isomer of chl *a*, was done by boiling the standard sample for 15 min.

RESULTS AND DISCUSSION

Emission of the two main volatile sulfur compounds, MT and DMDS, from broccoli buds and florets was detected by GC-FPD in response to induction of anaerobic storage conditions (**Figure 1**). After just 6 h in storage, considerable amounts of MT compounds were identified in the headspace of the flasks containing broccoli buds and florets (**Figure 1A**). The accumulated MT in both samples increased linearly by 1.2-fold as storage time was increased. In contrast, the buildup of DMDS from both samples was observed in insignificant proportions in comparison with MT (**Figure 1B**). However, as the storage period was increased, the emitted DMDS from both samples also increased by an average of 2.3-fold, but these levels were still negligible when compared with MT.

The average emission of MT from broccoli buds was 3.3 times more than those from broccoli florets, whereas DMDS emission from the same buds was 8.1 times higher than those detected from the same florets (**Figure 1**). The results indicate that broccoli buds had the capacity to emit larger amounts of MT and DMDS than broccoli florets. Forney and Jordan (*15*, *20*) also reported that MT in broccoli was produced mainly in the buds, with lesser amounts of MT formed from stem and peduncle tissues. Peduncle tissues were found to produce only 1.5% as much MT and 0.3% as much DMDS on a fresh weight basis as the floret tissues (*13*).

MT and DMDS that accumulated in the headspace of the flasks after 6 and 12 h in storage dissipated completely upon exposure to air for 5 min at room temperature (~25 °C). Flushing the flask with air and incubation for 2 h at 30 °C in a water bath following storage at the same periods did not produce substantial amount of MT and DMDS (**Figure 2**). However, after 18 and 24 h in anaerobic condition, these two sulfurous compounds were measured in significant levels from broccoli buds and only in trace amounts from florets. In broccoli buds, the amount of MT measured after 2 h of incubation was 3.7% of that detected after 24 h (**Figure 2A**), whereas the measured DMDS from the same storage period was only 17.6% (**Figure 2B**).

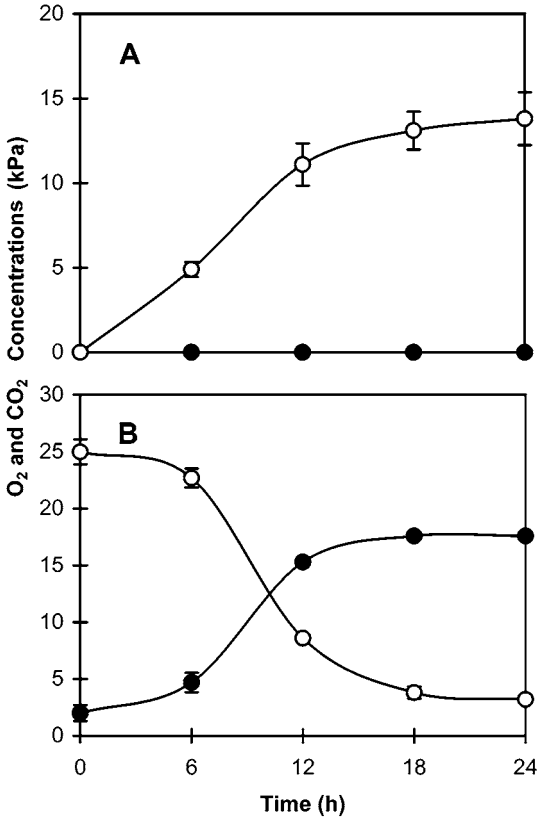

Figure 3. O_2 (●) and CO_2 (○) concentrations of the atmosphere inside the Erlenmeyer flask containing broccoli florets (A) after anaerobic storage at various holding times at 20 °C and (B) after flushing with air and incubation for 2 h at 30 °C following anaerobic storage. Data shown are the mean values of three replicates ± SD.

The results indicate that anaerobic induction of broccoli buds and florets led to two potential consequences in the emission of objectionable odors. The first was a reversible emission of MT and DMDS observed between 0 and 6 h of exposure to anaerobic conditions at 20 °C. The second consequence was an irreversible pattern that was observed between 18 and 24 h of exposure. The point at which the emission of this objectionable odor could still be reversed is assumed to be <12 h of anaerobic storage at 20 °C; however, that point was not determined in this work.

Reversible emission refers to the production of MT and DMDS during exposure to anaerobic conditions and the suppression of their formation during exposure to air and incubation, whereas irreversible emission means continuous production of MT and DMDS during anaerobic induction and even after exposure to air and incubation. These reversible and irreversible emissions of MT and DMDS from anaerobically induced broccoli florets were demonstrated through nondestructive (CO_2 production, chlorophyll fluorescence, and electrolyte leakage) and destructive (hot-water treatment) measurement techniques.

Nondestructive Measurements. *CO_2 Production*. Changes in the O_2 and CO_2 concentrations of the atmosphere inside the Erlenmeyer flasks containing fresh broccoli florets that were flushed with N_2 prior to storage at 20 °C at various holding times are presented in **Figure 3A**. A 0 kPa of O_2 concentration was maintained in all storage periods. In contrast, the rate of CO_2 production increased sharply to >10 kPa within the first 12 h of storage and then slightly as storage time was increased to 24 h.

The suppression of partial pressures of O_2 and enhanced partial pressures of CO_2 during storage had a dire consequence on the quality of stored broccoli. Anaerobic metabolism occurred, inducing the development of objectionable odors in stored broccoli buds and florets at 20 °C. In previous studies, it was established that the unpleasant odor usually occurred when broccoli tissues were held in atmospheres of >10 kPa of CO_2 and <0.5 kPa of O_2 (*5*−*7*).

After each storage period, the accumulated off-odor from broccoli tissues was removed by flushing with air and subsequently subjected to incubation at 30 °C. The O_2 and CO_2 levels inside the flasks were 19−21 and 0.3−0.8 kPa, respectively, after flushing with air (data not shown). The partial pressures of O_2 and CO_2 of the atmosphere inside the Erlenmeyer flasks containing broccoli buds were analyzed after incubation for 2 h in a water bath, and the results are shown in **Figure 3B**. Initially, the detected O_2 concentration was only 2 kPa, but more O_2 remained between 6 and 12 h. At the end of the storage period, the O_2 level was approximately the same as in the air. On the other hand, the original CO_2 level detected was 25 kPa, but only 13% of this remained after 24 h.

Depleted O_2 levels after flushing with air and incubation for 2 h at 30 °C following storage for 0 and 6 h are presumed to be the results of the respiration process and metabolic activity of the broccoli buds. Therefore, the broccoli tissues are still in good condition because they consumed available O_2 and produced CO_2. Moreover, at these storage periods, another anaerobic condition did not occur because the remaining O_2 levels were still above the 0.5 kPa limit necessary to induce anaerobiosis (*5*−*7*). In contrast, high levels of O_2 and low levels of CO_2 after 18 and 24 h could be attributed to the permanent damage in the tissue during respiration process.

The increase in the emission of MT and DMDS in **Figure 1** seemed to be due to a parallel increase in the partial pressures of CO_2 during anaerobic storage in **Figure 3A**. However, these same sulfurous compounds were measured after incubation, as shown in **Figure 2**, even though the partial pressures of CO_2 were low (**Figure 3B**). We believe that O_2 deficiency condition, not the elevated CO_2 concentration, contributed to the cell poisoning or cell breakdown in broccoli, which was manifested during anaerobic metabolism in the form of emission of strong objectionable odors. Forney and Jordan (*15*) reported that the production of CO_2 under anaerobic condition was neither related to nor associated with the ability of the *Brassica* vegetables to produce MT.

Chlorophyll Fluorescence. The chlorophyll fluorescence signals of broccoli florets during storage at 20 °C were measured to demonstrate the reversal of MT and DMDS formation during anaerobic storage (**Figure 4**). The measured F_v/F_m values declined in storage at 20 °C over time. About 17% of the fluorescence signals were lost after 18 h and 27% after 24 h in storage. The F_v/F_m values of broccoli florets also decreased similarly after flushing with air and incubation for 2 h at 30 °C following storage in anaerobic conditions. However, after 18 and 24 h, a considerable reduction in the F_v/F_m ratio of the air-flushed and incubated broccoli florets was observed in which the amount of reduction reached 27 and 39%, respectively. The difference between the chlorophyll fluorescence signals emitted by broccoli florets during storage in nitrogen gas and after flushing with air was ~1.5-fold.

The results indicate that the considerable loss in the F_v/F_m values of stored broccoli florets is probably due to the anaerobic metabolism that causes membrane damage in the photosynthetic tissues during induced anaerobic storage. Hence, the irreversible

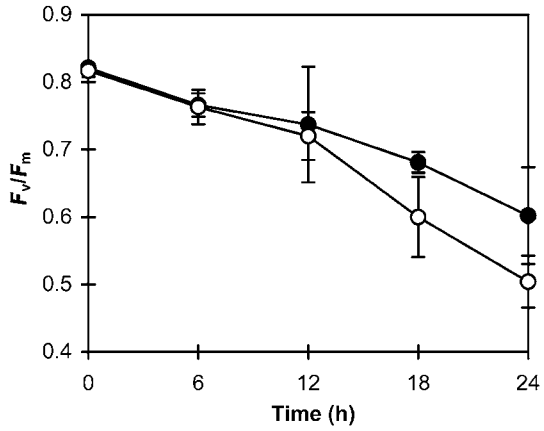

Figure 4. Chlorophyll fluorescence values in terms of F_v/F_m ratio of broccoli florets after anaerobic storage at various holding times at 20 °C (●) and after flushing with air and incubation for 2 h at 30 °C following anaerobic storage (○). Data shown are the mean values of three replicates with each measurement repeated at least three times ± SD.

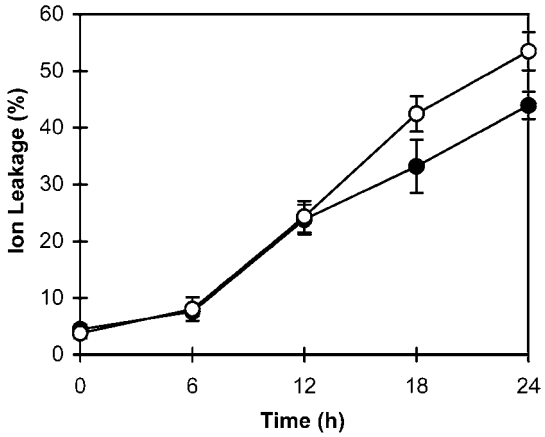

Figure 5. Electrolyte leakage in broccoli buds after anaerobic storage at various holding times at 20 °C (●) and after flushing with air and incubation for 2 h at 30 °C following anaerobic storage (○). Data shown are the mean values of three replicates with each measurement repeated at least three times ± SD.

emission of sulfurous odor from broccoli florets occurred as a result of this damage in the tissue. DeEll and Toivonen (*21*) reported that the chlorophyll fluorescence technique, which is a good indicator of anaerobic metabolite levels in broccoli during MAP storage, showed that broccoli held in bags had developed physiological problems even though the appearance was not affected. Values for chlorophyll fluorescence measurement were known to be modified by changes in cellular function as indicated by changes in respiration rates for broccoli (*22*, *23*).

Furthermore, Tian et al. (*24*) showed that F_v/F_m values could be a sensitive indicator of responses of broccoli to hot-water treatment before visual changes were noted. A similar result was obtained in this study in which the F_v/F_m values of anaerobically induced broccoli florets decreased remarkably after exposure to air following anaerobic storage. The severe damage in anaerobically induced tissues that manifested after aeration was also investigated elsewhere (*25*, *26*).

Electrolyte Leakage. Irreversibility of off-odor production in anaerobically induced broccoli buds was further demonstrated through electrolyte leakage analysis. The ion leakage in broccoli buds stored in nitrogen gas and after flushing with air and incubation increased sharply following the accumulation of off-odor (**Figure 5**). No marked change was noticed in the electrolyte leakage between the two treatments after the first 12 h of storage. However, after 18 and 24 h, the ion leakage of broccoli buds reached 42.4 and 53.5%, respectively, after flushing with air and incubation for 2 h at 30 °C. At the same storage periods, the rates of ion leakage measured in anaerobically induced broccoli buds were only 33.2 and 43.9%, respectively.

The increased leakage from the tissues was highly likely due to increased permeability of the membrane as a result of anaerobic induction. The pattern of changes in the electrolyte leakage was similar to that found earlier for broccoli held in polyethylene bags at 20 °C (*14*). Salveit and Morris (*27*) reported that increased ion leakage, which is one of the many symptoms of chilling injury, is due to a failure of the cellular membranes to maintain compartmentalization of the cellular contents. In addition, a slight increase in membrane permeability of chilling sensitive crops is due to phase separation (transition) of polar lipids in the membranes and/or lowering of active transport of solutes into the cells. A sudden increase in membrane permeability at the onset of chilling injury may be the result of denaturation of membranes such as tonoplast and plasmalemma (*28*). Hence, the increased permeability of membranes may cause the promotion of enzyme−substrate interaction in the anaerobically induced broccoli bud tissues, resulting in the irreversible emission of MT and DMDS.

Hot-Water Treatment. *Color*. Reversible and irreversible trends in the production of MT and DMDS were demonstrated by immersing the induced broccoli florets in boiling water for 1−2 min after storage at 20 °C. Initially, broccoli florets had a pale yellowish green color after the tissues were induced by anaerobic conditions. However, upon hot-water treatment, the color of the broccoli florets turned bright green. In samples stored for 6 h, a bright green color was still observed in the florets after hot-water treatment (**Figure 6A**). Then, a slight blackening of the buds became visible in the florets stored for 12 h (**Figure 6B**). However, when MT and DMDS emission became irreversible between 18 and 24 h, treated samples turned olive green, with most individual buds within the florets severely blackened, but the color of the stems remained intensely green (**Figure 6C**,**D**). In contrast, the color of the untreated samples remained pale or dull green color even though the odor was already offensive and the GC-detected volatile emission was already irreversible.

The surface color of anaerobically induced broccoli florets was measured using a Color Difference Meter, and the results are shown in **Table 1**. Hot-water-treated and untreated tissues had considerable differences in L^* and C^* values, but their $h°$ values were almost in the same range. In addition, treated samples were darker and more chromatic than untreated florets in terms of L^* and C^* values. In treated samples, L^*, C^*, and $h°$ values decreased moderately after storing for 24 h in an anaerobic condition. Considerable reduction of these values, however, was not gauged until after 18 and 24 h in storage.

The sharp changes in the surface color of the treated samples were considered to be reliable indicators of the reversal process of MT and DMDS emission in anaerobically stored broccoli. As the formation of objectionable odor was reversed, the surface color of broccoli florets remained intensely green (**Figure 6A**,**B**), whereas irreversibility was demonstrated by the blackening of the florets' surface color, particularly within the individual buds (**Figure 6C**,**D**). The severe color breakdown of these broccoli buds was easily visible right after hot-water treatment.

Chlorophyll. The reversibility and irreversibility of the objectionable odor formation were further demonstrated by

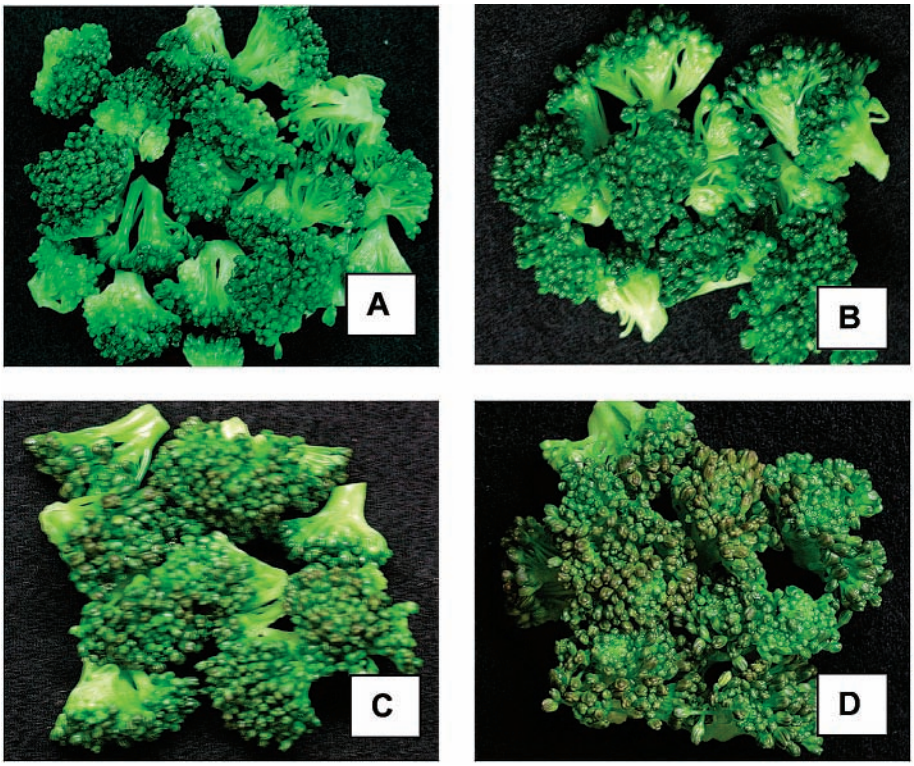

Figure 6. Broccoli florets stored under anaerobic conditions for (A) 6, (B) 12, (C) 18, and (D) 24 h at 20 °C after hot-water treatment.

Table 1. Surface Color Values of Anaerobically Induced Broccoli Florets before and after Hot-Water Treatment

	color characteristics[a]				
time (h)	L^{*b}	a^{*c}	b^{*d}	C^{*e}	$h^{\circ\,f}$
	Before Hot-Water Treatment				
0	36.8 ± 1.2	–8.4 ± 1.5	10.0 ± 0.8	12.7 ± 1.3	130.0 ± 5.4
6	36.4 ± 2.1	–8.8 ± 0.9	11.5 ± 1.4	14.6 ± 1.1	127.4 ± 5.0
12	36.3 ± 1.4	–8.0 ± 1.8	11.7 ± 2.3	14.3 ± 2.7	124.2 ± 4.7
18	36.0 ± 2.0	–8.2 ± 0.9	12.4 ± 2.1	14.9 ± 2.0	123.9 ± 4.2
24	36.6 ± 2.1	–8.1 ± 2.1	13.6 ± 1.9	15.3 ± 1.8	120.5 ± 3.5
	After Hot-Water Treatment				
0	28.6 ± 1.9	–17.2 ± 2.3	22.8 ± 2.2	28.6 ± 2.6	127.0 ± 3.6
6	27.0 ± 2.0	–15.9 ± 1.8	21.7 ± 2.3	26.9 ± 2.5	126.2 ± 3.3
12	26.1 ± 1.8	–14.3 ± 2.1	20.4 ± 3.3	24.9 ± 3.5	125.1 ± 4.1
18	24.0 ± 3.1	–11.7 ± 1.4	19.2 ± 2.3	22.5 ± 2.6	121.3 ± 1.9
24	22.9 ± 1.5	–9.6 ± 1.3	18.4 ± 1.2	20.8 ± 1.3	118.4 ± 3.9

[a] Data shown are the mean values of three replicates with each measurement repeated at least three times ± SD. [b] L^* = lightness, which ranges from black = 0 to white = 100. [c] a^* = bluish green to red-purple hue on the horizontal axis. [d] b^* = yellow to blue hue on the vertical axis. [e] $C^* = (a^{*2} + b^{*2})^{1/2}$ = chroma. [f] h° (from arctangent b^*/a^*) = hue angle (0° = red-purple, 90° = yellow, 180° = bluish green, 270° = blue).

analyzing the chlorophyll loss in hot-water-treated and untreated broccoli florets by using spectrophotometer and HPLC. **Figure 7** shows the quantitated total chl together with the chl *a* and *b* contents of blanched broccoli florets, which were not very much different from those of untreated tissues. The chl contents of both samples decreased linearly after storage for 24 h in anaerobic condition. The total chl losses in treated florets alone were 51% after 18 h and 60% after 24 h, about 74 and 78%, respectively, of which were due to chl *a* loss and the remaining due to chl *b* loss. The ratio of chl *a* and *b* in treated florets was 3.1:1, whereas untreated tissues had a ratio of 2.9:1.

On the basis of the quantitative estimation with a photodiode array detector, the chl *a* content of stored broccoli florets, whether hot-water-treated or not, decreased in linear fashion as

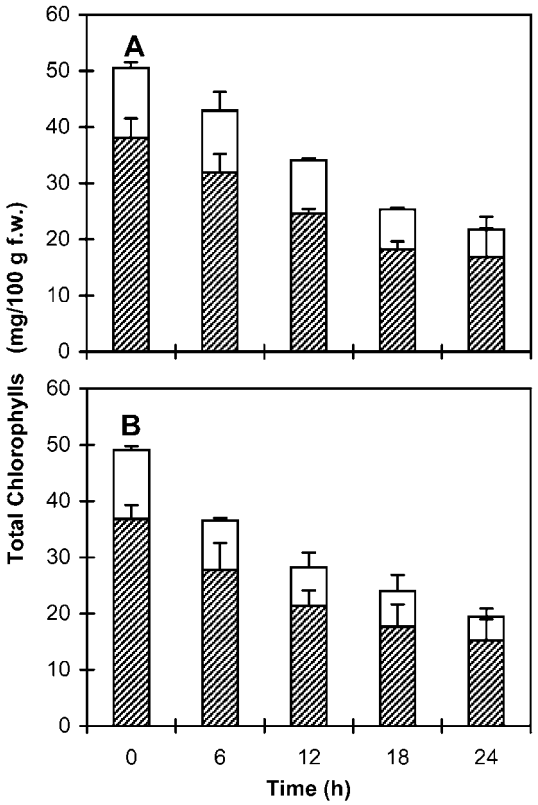

Figure 7. Total chlorophyll contents of broccoli florets (A) before and (B) after hot-water treatment following anaerobic storage at 20 °C: chl *a* (slashed bars); chl *b* (white bars). Data shown are the mean values of three replicates ± SD.

storage time was increased (**Figure 8A**). The pattern of chl reduction here is basically similar to the results obtained in the spectrophotometric analysis described above. In addition, a slower rate of chl *a* loss was accompanied by the formation of

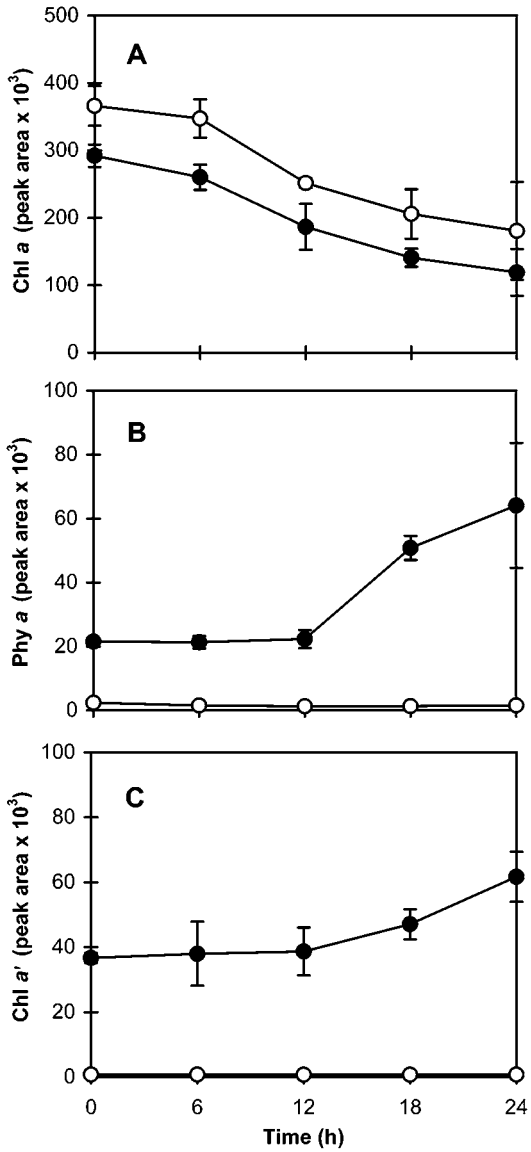

Figure 8. (A) Chl *a*, (B) pheo *a*, and (C) chl *a*′ of broccoli florets before (●) and after (○) hot-water treatment following anaerobic storage at 20 °C. Data shown are the mean values of three replicates ± SD.

its derivative and isomer in treated florets but not in untreated samples. The production of phy *a* and chl *a*′ did not change between 0 and 12 h but suddenly increased between 12 and 24 h as the MT and DMDS emission reached its irreversible point. Phy *a* increased by 3-fold, whereas chl *a*′ increased by 1.7-fold after 24 h of storage (**Figure 8B**,**C**).

Anaerobic condition affects metabolic processes associated with the quality of the treated commodity, among which are pigment metabolism and volatile compound metabolism. Among the pigments affected by atmospheric modification, chl is most broadly associated with fruit and vegetable quality (*29*). The result of the chl analysis here is in agreement with this observation. Color degradation in broccoli during anaerobiosis was due to the conversion of chl *a* to phy *a* and chl *a*′. Yamauchi et al. (*19*) noted the presence of pheophytin at the outset of an in vitro study of chlorophyll degradation in broccoli. The color change of broccoli florets from blue-green to olive green alongside blackening of the buds is produced primarily by the conversion of chls *a* and *b* to their respective pheophytins. This is the most widespread alteration that takes place during cooking, thermal processing, freezing preservation, or storage of vegetables (*30*).

It is postulated that the conversion of chlorophyll to pheophytin in hot-water-treated broccoli florets is highly likely due to the breakdown in the integrity of the cellular membranes during anaerobic storage, resulting in the lowering of the tissue pH. Once the chlorophyll in the chloroplasts is exposed to the acidic medium, the broccoli turns olive green and blackish green. Gunawan and Barringer (*31*) reported that the production of acidic metabolic byproducts lowers the pH and causes pheophytinization. They also asserted that the color change is due to the acid converting chlorophyll to pheophytin, by replacing the magnesium with hydrogen, and this conversion under acidic conditions is the first step in the degradation of chlorophyll to pheophytin and pheophorbide.

Mechanism of MT and DMDS Emission. The results of this paper strengthen the general view that the development of MT and DMDS in broccoli held under anaerobic conditions is enhanced by cellular decompartmentation and membrane damage. Dan et al. (*14*) also reported that MT and DMDS were formed as a result of the deterioration of cellular membrane lipids and loss of intracellular compartmentation, allowing the enzyme−substrate reaction to proceed during anaerobic induction.

The mechanism for the formation of MT and DMDS under anaerobic condition is likely similar to the proposed mechanism of formation of these two volatile sulfur compounds in gradually disrupted broccoli tissues through manual crushing (*32*). Loss of intracellular compartmentation and breakdown of cellular membrane during anaerobic induction allow the cysteine sulfoxide lyase to act on *S*-methyl-L-cysteine sulfoxide, a nonprotein, nonvolatile sulfur-containing amino acid. Methanesulfenic acid (MSI), pyruvate, and ammonia were formed as a result of this enzymatic reaction. This highly reactive and unstable primary intermediate, MSI, undergoes chemical disproportionation to form MT and methanesulfonic acid. Then, the formation of DMDS is rationalized by the subsequent oxidation of MT (*33*, *34*).

The emission process of MT and DMDS is still reversible if the tissue damage is only temporary. However, emission is considered to be irreversible if the tissue impairment is already permanent. The irreversibility trend was demonstrated by nondestructive measurement techniques through significant reductions of chl fluorescence signals as well as CO_2 production and increase in the membrane permeability. Furthermore, a destructive hot-water treatment also indicated the same trend through significant change in the characteristic bright green to olive green or blackish green color as well as the loss of chl *a* concomitant to formed phy *a* and chl *a*′. The critical control point between the reversible and irreversible odor production under anaerobic condition may vary among different storage conditions and temperatures including different broccoli cultivars.

ABBREVIATIONS USED

Chl, chlorophyll; CO_2, carbon dioxide; GC-FPD, gas chromatography−flame photometric detector; GC-TCD, gas chromatography−thermal conductivity detector; HPLC, high-performance liquid chromatography; DMDS, dimethyl disulfide; MSI, methanesulfenic acid; MT, methanethiol; O_2, oxygen; phy, pheophytin.

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