Biogenesis of Off-Odor in Broccoli Storage under Low-Oxygen Atmosphere

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Broccoli was stored for 10 days at 0 °C under modified atmosphere containing the following initial percentages of O_2/CO_2: (A) 1.1%/3%; (B) 18%/11%. Weight and color retention was better under modified atmosphere than in air, but under anaerobic condition broccoli produced severe off-flavor. In the headspace of low oxygen stored broccoli, methanethiol, dimethyl sulfide, and dimethyl disulfide were identified using GC/MS. A possible enzymatic pathway for the production of these compounds was investigated. In a cell-free system of fresh broccoli, methanethiol formation occurred in response to L-methionine and *S*-methylcysteine; dimethyl sulfide was formed from *S*-methylmethionine. An increase in free amino acids was observed in broccoli under anaerobic conditions and control; free sulfur-containing amino acids calculated were 10.4 and 2.2 μg/g, respectively. These findings suggest that broccoli has the enzymatic capacity to produce volatile sulfur compounds, which are contributors to the objectionable odor, but precursors, free sulfur-containing amino acids, seem to arise preferably in broccoli storage under low oxygen atmosphere.

Keywords: *Broccoli; storage; volatile compounds; off-odor biogenesis*

INTRODUCTION

Broccoli is a highly perishable product. To find favor with consumers, it must be green and have mild flavor and desirable texture. The more commonly encountered storage disorders are yellowing, toughening of stem, and developing undesirable odors. Refrigeration and modified atmospheres have been tested to improve broccoli storage.

Refrigeration at 0 °C prolonged the storage of broccoli up to 3 weeks, while it kept 1 day at ambient temperature (Wang and Hruschka, 1977). Oxygen concentrations under 1% are needed to retard completely the senescence of broccoli flowers head whereas a wide range of CO_2 concentrations was more effective than decreasing O_2 concentration (Leberman et al., 1968; Lipton and Harris, 1974; Wang, 1979). However, under certain modified atmospheres, at oxygen levels ca. <0.5%, offensive odor and off-flavors might occur (Kasmire et al., 1974).

The chemical nature of the odor produced by broccoli held under low oxygen atmosphere has been recently characterized. Forney et al. (1991) considered the methanethiol primarily responsible for the off-odor; Hansen et al. (1992), from the combination of threshold and concentration data, considered that the major contributors to the offensive off-odor included methanethiol, dimethyl trisulfide, and β-ionone. Because the potential production of this objectionable odor appears to be an important factor in marketing losses, considerable interest exists for approaches to minimize unpleasant flavors. An understanding of biogenesis of these volatile compounds could be appropriate to facilitate the development of strategies to prevent off-odor formation during broccoli storage.

Schmidt et al. (1985) have reported that in a cell-free system of pumpkin leaves methanethiol formation occurred in response to L-methionine. In bacteria and fungi, it is well-known that the formation of methanethiol from L-methionine is catalyzed by the enzyme methioninase (EC 4.41.11) by an α,γ-elimination reaction. (Lindsay and Rippe, 1986; Ruiz-Herrera and Starkey, 1969). In this study, we have focused our attention on enzymatic aspects and precursors that could be involved in the biogenesis of off-flavors in broccoli storage under modified atmosphere.

EXPERIMENTAL PROCEDURES

Materials. Broccoli florets were obtained from Las Lomas (Vejer de la Frontera, Cádiz, Spain) on the day of harvest, packed in ice, and transported to Seville. In the laboratory, ice was removed, and the florets were kept overnight at 0 °C with 90% relative humidity. After 24 h, the heads were selected, weighed, and evaluated for color using a Minolta CR-200 portable tristimulus colorimeter (Minolta, Ramsey, NY). The color was determined at the central part of the inflorescence, and the yellowing was expressed as the increase of the $b/L + |a|$ ratio (Olías et al., 1990).

We used only compact heads that had all buds closed and that weighed between 250 and 350 g. With these selected heads, three lots of 20 florets each were prepared for modified atmosphere treatment. Each lot (~6.5 kg) was placed in plastic containers of 60 × 40 × 40 cm; two of them were sealed with a methacrylate cover containing inlet and outlet 1/4-in. o.d. polyethylene tubes used for introducing the flow of modified atmospheres gases. The container used as a control was uncovered. The heads were stored for 10 days at 0 ± 0.1 °C under the following initial atmospheres: control, air; atmosphere A, O_2/CO_2: 1.1%/3%; atmosphere B, O_2/CO_2: 18%/11%.

The atmosphere of the container, once closed, was continuously moved by a small fan installed inside the container. The gas composition inside each chamber was regularly analyzed using an infrared gas analyzer for CO_2 and a paramagnetic gas analyzer for O_2 (Servomex 1400; S. Company, Norwood, MA). Samples of the gas were taken from coupling inlet and outlet polyethylene tubes in the cover of the container to the analyzer via a small pump.

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Headspace Analysis. To collect the volatile compounds that accumulated in the headspace of the closed container, a trap containing 150 mg of Tenax was attached to the outlet tube, and the inlet and outlet polyethylene tubes were connected to a pump to move the atmosphere inside the sealed container through the trap.

For sampling, a flow of 6 L/h passed through the Tenax trap for 1 h. To take the atmosphere of the control, the container was closed as stated above, and after 1 h the inlet and outlet tubes were connected as the other samples. The headspace was taken keeping the containers at 0 ± 0.1 °C.

Identification of compounds in the headspace was made by means of GC/MS. Trapped volatiles were introduced into the GC/MS system via thermal desorption using a 200 °C hot air gun and cryofocusing. The volatiles were separated using a GC equipped with a 30 m × 0.25 mm i.d. fused silica capillary column DB-5; carrier gas was He at 30 cm/s, the column was held for 15 min at 40 °C and then programmed at 2 °C/min to 160 °C. The mass spectrometer was a MS-30/70-VG directly coupled to the gas chromatograph described above. Identification was made by matching against the Wiley/NBS Library and by GC retention time against standards.

Extraction of Free Amino Acids. Three heads of each lot were coarsely chopped, and three samples of each were homogenized in a blender in batches of 10 ± 0.1 g with 20 mL of 95% ethanol. The residue was washed three times with 80% ethanol. Combined extracts were brought to a 50-mL final volume with 80% ethanol. A 10-mL sample of the extract was evaporated at 60 °C in an air-draft oven to remove ethanol. The aliquot was passed throughout a Dowex 50-X4 (H^+ form) to trap free amino acids. The column was eluted with 20 mL of 2 N NH_4OH, eluent was evaporated, and the residue was dissolved in 1 mL of 0.1 M sodium bicarbonate, pH 9. Aliquots of this extract were used for the amino acid analysis by HPLC and for colorimetric measurement of sulfur-containing amino acids.

Using D,L-aminobutyric acid as the internal standard 70% of free amino acid recovery was estimated.

Amino Acids Analysis. Amino acid derivatives were prepared according to the method described by Chang et al. (1981) with slight modifications. To 200 μL of amino acid solution was added 100 μL of 4-(dimethylamino)azobenzene-4′-sulfonyl chloride (DABS-Cl) solution. The mixture was heated at 70 °C for 12 min and dried, and the residue was dissolved in 1 mL of 70% ethanol. Amino acid derivatives were then chromatographed into a HPLC (HP 1090) equipped with an ODS 5 μm (2 mm × 250 mm) column and detection at 460 nm (Stocchi et al., 1985). The mobile phase utilized for the separation of DABS-containing amino acids consisted of two eluents: 25 mM potassium dihydrogen phosphate, pH 6.8 (solvent A), and acetonitrile:2-propanol (80:20) (solvent B). A 20-μL portion of the derivative was injected into the HPLC at constant flow of 0.5 mL/min. After 20 min of isocratic flow at 22% B, a linear gradient was begun that reached 34% B at 48 min, followed by a second gradient at 1% B/min to 84 min. Identification and quantification of each amino acid were carried out by preparing derivatives with authentic compounds.

Quantitative Analysis of Sulfur-Containing Amino Acids. The measurement of the sulfur-containing amino acids, in the free amino acids extract, was carried out according to the method described by Awwad and Adelstein (1966), using methionine as standard.

Preparation of Cell-Free Extract. Broccoli buds (25 g) were homogenized in a chilled Waring Blendor in three volumes of grinding buffer, 50 mM sodium phosphate, pH 6.8. Grinding was done in three 30-s periods. The homogenate was passed through four layers of cheesecloth, and the filtrate was centrifuged at 15000*g* for 30 min at 4 °C. To remove inactive material, the supernatant solution was then placed on Sephadex G-25 disposable column (PD-10 column, Pharmacia) and eluted with grinding buffer. The active fraction was passed through a 0.2-μm nylon filter before assaying enzyme activities. Protein was measured according to the method described by Bradford (1976), using the Pierce Coomassie protein assay reagent with crystalline BSA as the standard protein.

Table 1. Volatile Compounds Identified in the Headspace of Broccoli Stored under Low-Oxygen Atmosphere[a]

compound[b]	RRt[c] (min)	major MS ions (*m*/*z*)
methanethiol	1, 38	47, 48, 45, 46
acetaldehyde	1, 40	44, 29, 43, 42, 48
dimethyl sulfide[d]	1, 42	62, 47, 45, 46, 61
acetone	1, 50	43, 58, 42, 44
methyl acetate	1, 52	43, 74, 42, 59
ethyl acetate	1, 62	43, 61, 45, 70, 29
ethanol	1, 81	31, 45, 29, 27, 43
ethyl propionate	1, 88	29, 57, 45, 75, 74, 102
ethyl thioacetate	2, 12	43, 90, 45, 47, 75
dimethyl disulfide[d]	2, 28	94, 79, 45, 46, 47

[a] Broccoli heads were stored at 0 °C for 10 days; O_2/CO_2: 1.1%/3% was the initial gas composition. [b] Mass spectrum and RRt were consistent with that of the authentic compound. [c] Retention time relative to CO_2. [d] Traces of these compounds were detected in the headspace of broccoli control.

Enzyme Assay. The standard assay mixture was prepared according to a method described by Lindsay and Rippe (1986) with slight modifications. The reaction mixture consisted of 50 mM sodium phosphate buffer, pH 7.6, 20 μM pyridoxal 5′-phosphate, 4 mM L-methionine, and 50 μL of enzyme solution (1.1 mg of protein/mL) in a total volume of 3 mL. The mixture was incubated at 35 °C for the desired time in an 11-mL vial. Sulfur volatile compounds accumulated in the headspace of the sealed vial were analyzed by gas chromatography. Headspace samples of 0.25 mL were injected into a HP 5890 gas chromatograph equipped with a flame photometric detector and a 2 m × 2 mm glass column packed with 5% SE-30; N_2 20 mL/min was used as the carrier gas. An isothermal oven temperature of 35 °C was used, and both injector and detector were maintained at 200 °C.

RESULTS

The atmosphere inside each container was regularly sampled. Respiration of the broccoli heads held in atmosphere A (O_2/CO_2: 1.1%/3%) during the 10 days of storage obviously was anaerobic, oxygen concentration at 5 and 10 days was 0.7% and 0.4%, respectively, at the same time dioxide concentration increased up 9% and 15%, respectively. Acetaldehyde, acetone, methyl acetate, ethyl acetate, and ethanol are fairly common products of anaerobic respiration (Karaoulanis, 1983). In the headspace of broccoli held in atmosphere A (Table 1) these compounds in addition to the volatile sulfur compounds were identified, which clearly indicate the anaerobiosis in these broccoli heads.

In atmosphere B (O_2/CO_2: 18%/11%) oxygen concentration at 10 days dropped to 7.5%, and dioxide concentration increased up to 14%. During storage, aerobic respiration seemed to predominate, and no volatile compounds associated with anaerobiosis were identified in the headspace of these broccoli.

Five days after the broccoli heads were sealed, in container A an objectionable odor could be detected through olfactory evaluation of the headspace. In sample B, no off-odor was detected. Ten days following sealing, the atmosphere of sample B was unobjectionable, while in sample A a strong off-odor was developed. At this time the headspace of the container was taken, and once opened, broccoli bunches were individually rated for weight and color.

The heads were weighed for each sample, and loss of weight was expressed as a percentage of the initial fresh weight. Weight loss from broccoli heads during storage was less under modified atmosphere (7.1% and 7.2% atmospheres A and B, respectively) than in air (9.4%). Makhlouf et al. (1989) explained this difference by the

lower rate of respiration under modified atmosphere, since relative humidity was high in all treatments.

Retardation of yellowing, expressed as a percentage of the initial chromaticity value, was more noticeable in broccoli stored under modified atmosphere (3.2 and 3.0% in atmospheres A and B, respectively) than in air (7.7%), which agrees with previous reports (Leberman et al., 1968; Kasmire et al., 1974; Lipton and Harris, 1974). In conclusion, the visual quality of broccoli was maintained inalterable in both modified atmospheres and was better than the one stored in air (control); however, broccoli held at low oxygen level, as has been shown previously (Kasmire et al., 1974; Lipton and Harris, 1974; Makhlouf et al., 1989), developed strong off-odors.

In two recent studies by Forney et al. (1991) and Hansen et al. (1992), it has been indicated that the major contributors to the offensive odor of broccoli storage at low oxygen concentration included methanethiol, dimethyl trisulfide, and β-ionone. In this work, the major sulfur components identified using the Tenax trap procedure included methanethiol, dimethyl sulfide, methyl thioacetate, and dimethyl disulfide.

The simplest explanation for methanethiol formation in broccoli would be an inducible methioninase similar to the systems found in pumpkin leaves (Schmidt et al., 1985) and in microorganisms (Lindsay and Rippe, 1986; Ruiz-Herrera and Starky, 1969).

In order to check this possibility, a cell-free extract of fresh broccoli was incubated with L-methionine in sealed vials for 3 h; at various times after this interval, 0.25 mL headspace gas was withdrawn from the vial with a gas-tight syringe, and the sulfur volatiles were analyzed by GC. At early stages of incubation, methanethiol was only detected in the headspace; 30 min later dimethyl disulfide, a product of methanothiol oxidation, and methanethiol were detected; and after 2 h, only dimethyl disulfide was observed. Using *S*-methylcysteine as substrate, a similar pattern of formation was obtained.

Lindsay and Rippe (1986) working with methioninase obtained from *Pseudomonas putida* showed that under anaerobic condition only methanethiol was yielded while aerobic conditions favored conversion of substantial amounts of methanethiol to dimethyl disulfide. To study this possibility in a cell-free extract of broccoli, vials with standard reaction mixtures were purged with purified oxygen or nitrogen to produce aerobic and anaerobic conditions before adding enzyme solution. Reactions carried out in both atmospheres yielded methanethiol and dimethyl disulfide. In a recent study, it has been shown that ascorbate and transition-metal catalyze methanethiol oxidation to dimethyl disulfide, under aerobic and anaerobic conditions (Chin and Lindsay, 1994). This reaction could probably account for the formation of dimethyl disulfide in broccoli storage under low oxygen; however, we cannot exclude in broccoli the presence of the enzyme cysteine sulfoxide lyase, which in cruciferous vegetables (Mazelis, 1963) has been generally recognized as responsible for the formation of dimethyl disulfide.

Thermal decomposition of *S*-methylmethionine sulfonium salt has been shown to yield dimethyl sulfide (Sumitani et al., 1991); similar cleavage has been shown with an enzyme fraction from leaves of cabbage (Lewis et al., 1971). When fresh broccoli cell-free extract was incubated with this amino acid, dimethyl sulfide was detected in the headspace of the vial.

Table 2. Free Amino Acids in Broccoli Heads after Holding for 10 Days at 0 °C under Modified Atmosphere

	control	oxygen 1.1% + carbon dioxide 3%	oxygen 18% + carbon dioxide 11%
Gly	–[c]	17.6[a]	–
Arg	30.6	18.9	–
Ile	7.9	12.2	–
Leu	–	3.8	1.4
Phe	14.9	7.9	0.5
Pro	55.2	122.5	4.4
S-AA[b]	2.2	10.4	–
total	110.8	193.3	6.3

[a] μg per 1 g fresh weight; average of three determinations. [b] All sulfur amino acids are included in the determination. [c] Not detected.

Table 3. Volatile Sulfur Compounds Found in the Headspace of a Cell-Free Extract of Broccoli in Response to Sulfur Amino Acids

substrate[a]	sulfur-containing compounds found
L-methionine	CH_3SH and CH_3SSCH_3
S-methylcysteine	CH_3SH and CH_3SSCH_3
S-methylmethionine	CH_3SCH_3

[a] The reaction mixture, as stated in the experimental section, with appropriate substrate was incubated at 35 °C in an 11 mL vial. After 4 h, volatile sulfur compounds in the headspace were analyzed by CG using FPD.

With the experiments described above, we checked the capacity of the enzyme present in fresh broccoli cell-free extract to metabolize, in vitro, *S*-methylcysteine, L-methionine, and *S*-methylmethionine sulfonium salt, but as we stated before, the objectionable odor was only developed in broccoli stored under low oxygen atmosphere. The explanation for this difference could be in the availability of the precursors.

The free amino acids profile of broccoli after holding for 10 days at 0 °C under modified atmospheres is shown in Table 2. In the broccoli heads stored under atmosphere B (O_2/CO_2: 18%/11%) only 6.3 μg/g free amino acids was detected, and free sulfur compounds were not observed. Free amino acid content in control and broccoli held under low oxygen atmosphere was elevated, suggesting an increase in proteinase activity during storage, being more important in broccoli under anaerobic conditions. Free sulfur-containing amino acids were detected in both samples (Table 2), which suggest that potential precursors are available to produce volatile sulfur compounds.

DISCUSSION

Three main factors could be involved in the formation of volatile sulfur compounds, which are contributors to the objectionable odor in broccoli: (1) the availability of substrates, free sulfur amino acids; (2) the present of enzyme with specificity to cleave these amino acids; and (3) the accessibility between substrates and enzymes. As has been proved in this work, fresh broccoli has enzyme or enzymes with the capacity to metabolize free sulfur-containing amino acids (Table 3); the absence of sulfur volatiles in broccoli storage in atmosphere B (O_2/CO_2/18%/11%) could be explained for the lack of precursors, free sulfur-containing amino acids. The increase in free sulfur-containing amino acids in broccoli produced by low oxygen atmosphere could be the cause of the development of the off-flavor in broccoli storage under anaerobic conditions, but we cannot exclude induced enzyme activity (i.e., C-S lyases) and physiological disorders produced by this atmosphere.

In broccoli control stored under air at 1 °C, both free amino acids and enzyme were present, but only traces of sulfur volatiles were analyzed in the headspace. Kasmire et al. (1974) studying the effects of aeration rate on the production of off-odors by broccoli heads during an simulated transit period concluded that adequate ventilation is essential to prevent accumulation of offensive odors. As stated in the experimental section, plastic containers of 60 × 40 × 40 cm were used to prepare modified atmospheres, and broccoli controls were stored in a cool room of 2 × 2 × 3 m. Analyzing the relative weight of broccoli/volume of chamber, we could conclude that the absence of an objectionable odor in the sample control could be a consequence of the adequate ventilation in the cool room, which could avoid accumulation of volatiles produced by broccoli stored under air control; although the absence of physiological disorders preventing substrate–enzyme interaction could be another possibility.

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