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Hydrogen Sulfide Prolongs Postharvest Shelf Life of Strawberry and Plays an Antioxidative Role in Fruits

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ABSTRACT: Accumulating evidence shows that hydrogen sulfide (H_2S) plays various physiological roles in plants, such as seed germination, root organogenesis, abiotic stress tolerance, and senescence of cut flowers. However, whether H_2S participates in the regulation of ripening and senescence in postharvest fruits remains unknown. In the present study, the effect of H_2S on postharvest shelf life and antioxidant metabolism in strawberry fruits was investigated. Fumigation with H_2S gas released from the H_2S donor NaHS prolonged postharvest shelf life of strawberry fruits in a dose-dependent manner. Strawberry fruits fumigated with various concentrations of H_2S sustained significantly lower rot index, higher fruit firmness, and kept lower respiration intensity and polygalacturonase activities than controls. Further investigation showed that H_2S treatment maintained higher activities of catalase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase and lower activities of lipoxygenase relative to untreated controls. H_2S also reduced malondialdehyde, hydrogen peroxide, and superoxide anion to levels below control fruits during storage. Moreover, H_2S treatment maintained higher contents of reducing sugars, soluble proteins, free amino acid, and endogenous H_2S in fruits. We interpret these data as indicating that H_2S plays an antioxidative role in prolonging postharvest shelf life of strawberry fruits.

KEYWORDS: *Hydrogen sulfide, strawberry fruits, postharvest shelf life, antioxidative enzymes, reactive oxygen species*

INTRODUCTION

Hydrogen sulfide (H_2S), strikingly similar to nitric oxide (NO) and carbon monoxide (CO), has recently been shown to be an endogenous gaseous signaling molecule in animal systems, where it is involved in varied physiological functions.[1,2] In the plant kingdom, NO and CO have already been identified as important signaling molecules regulating plant growth and development.[3,4] Since the 1970s, the phenomenon of H_2S emission from plants has been reported by many researchers.[5−7] More recently, accumulating evidence have shown that H_2S plays various physiological roles in plants, including seed germination, root organogenesis, abiotic stress tolerance, photosynthesis, guard cell movement, and senescence of cut flowers, implying that H_2S acts as an important gaseous regulator in plants, as do NO and CO.[8−15] NO, a well-known gaseous signal, has been shown to be an endogenous maturation and senescence regulating factor in postharvest fruits, vegetables, and horticultural plants.[16−18] In our previous work, we demonstrated a role of H_2S in delaying senescence of cut flowers and prolonging flower vase life in a wide spectrum of botanical species including herbaceous and woody plants, showing that this characteristic of H_2S might be a universal event in plant senescence.[11]

Strawberry (*Fragaria* × *ananassa* Duch.) is a nonclimacteric fruit with high edible and medicinal value and is highly appreciated by consumers for its excellent organoleptic properties. On the other hand, strawberry fruit is highly perishable during harvest and storage due to its soft texture, high softening rate, high sensitivity to fungal attack, and very short maturation period.[19] The postharvest shelf life of strawberry fruits is generally shorter than 1 week even under ideal conditions at 0 °C.[20] Prolonging postharvest shelf life of strawberry fruit has attracted researchers seeking to enhance shelf life. For instance, it has been shown that fumigation with NO significantly extends the postharvest life of strawberry.[18] Considering the similar functional characteristics of NO acting as an endogenous maturation and senescence regulating factor in plants,[16−18] we speculated that H_2S might also be involved in the regulation of postharvest shelf life in strawberry. In this work, we demonstrate that this is indeed the case.

MATERIALS AND METHODS

Plant Materials and Treatment. Strawberry (*Fragaria* × *ananassa* Duch., cv. Bao Jiao) used in this work was supplied by Strawberry Commercial Farm at Dawei, Anhui province, China. Fruits with similar size and color (diameter 3.0 ± 0.5 cm; about 70% superficial red color) were hand-harvested in the early morning and transported to the laboratory within 2 h. Fruits without physical damage and microbial and insect infection were selected for experiments. Sodium hydrosulfide (NaHS, Sigma) was used as a hydrogen sulfide (H_2S) donor. Aqueous NaHS solutions (200 mL) of 0.00, 0.20, 0.40, 0.60, 0.70, 0.80, 0.90, 1.00, or 1.25 mmol·L^{-1} were prepared in sealed containers (volume 3 L). These solutions could release H_2S gas rapidly, reach the highest levels within 30 min, and keep a constant concentration of 0.00, 0.05, 0.10, 0.15, 0.30, 0.60, 1.00, 1.50, or 2.00 × 10^{-10} mol·L^{-1} H_2S gas for 48 h, respectively. Strawberry fruits were exposed to treatment solutions in sealed containers containing the aqueous NaHS solutions. Each treatment

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unit comprised 30 strawberries and each experiment was repeated three times. The storage temperature was 20 ± 0.5 °C and the relative humidity was 85%~90%. Treatment solutions were renewed daily and the strawberry fruits were observed every 12 h.

Rot Index of Fruits. Rot index was measured by Zhu and Zhou[16] and Ayala-Zavala et al.[21] with modifications. Thirty fruits were used in each treatment for investigating rot index. Fruits were classified in five ranks according to the percentage of rotten surface area: 0, no rot; 1, rot surface less than 10%; 2, rot surface between 10% and 20%; 3, rot surface between 20% and 50%; 4, rot surface more than 50%. The rot index was expressed as the following equation: rot index = $[\sum 30(AB/4)] \times 100$, in which A represents the rot score of individual fruit, B is the number of rot fruit in each rank, 4 is the topmost rot rank (0−4), and 30 is the total number of fruit recorded. The experiment was repeated three times.

Determination of Fruit Firmness and External Color. Fruit firmness was measured at the equatorial part of each strawberry fruit by a 5 mm diameter flat probe with a texture analyzer (Model TA XT plus, SMS). The penetration depth was 5 mm and the cross-head speed was 5 mm·s^{-1}.[22] Fruit firmness values were an average of 10 strawberries.

External color for individual fruit was measured with a color difference meter (model WSC-100, Konica Minolta), which provided CIE L^*, a^*, and b^* values, where L^* indicates lightness, a^* indicates chromaticity on a green (−) to red (+) axis, and b^* indicates chromaticity on a blue (−) to yellow (+) axis.[23]

Analysis of Respiratory Intensity and Polygalacturonase Activities. The respiration intensity was assayed according to the method of Wu et al.[24] In brief, about 1 kg of fruit was sealed in a 2.5 L glass container for 2 h, and 1 mL of gas sample was withdrawn from the headspace with a gastight hypodermic syringe and analyzed on a gas chromatograph (GC) equipped with a thermal conductivity detector. The respiration intensity was expressed as milligrams of CO_2 evolved per kilogram of fruit per hour. The gas sample withdrawn from the same volume of container without fruit was taken as the control. The data are presented as the mean ± SE of six replicates. Activities of polygalacturonase (PG) were determined by procedures described by Villarreal et al.[25] Frozen strawberries (10 g) were homogenized with 30 mL of sodium acetate/acetic acid buffer (pH 6.0, 50 mmol·L^{-1}, 1% PVPP). The mixture was centrifuged at 12000g for 30 min and the supernatant was discarded. The pellet was washed twice with 30 mL of sodium acetate/acetic acid buffer (pH 6.0, 50 mmol·L^{-1}). Then the sample was centrifuged at 12000g for 30 min, the supernatant was discarded, and the pellet was extracted with 30 mL of sodium acetate/acetic acid buffer (pH 6.0, 50 mmol·L^{-1}) containing 1 mol·L^{-1} NaCl. The mixture was stirred for 2 h and then centrifuged at 12000g for 30 min. The supernatant was dialyzed overnight with sodium acetate/acetic acid buffer (pH 6.0, 50 mmol·L^{-1}). All the steps were done at 4 °C. The dialyzed extract was used to determine PG activity, with polygalacturonic acid as substrate. A volume of 700 μL of enzymatic extract was incubated at 37 °C with 700 μL of 0.3% (w/v) polygalacturonic acid in sodium acetate/acetic acid buffer (pH 6.0, 50 mmol·L^{-1}). Aliquots (300 μL) were taken at 0, 6, 12, and 24 h from each reaction mixture. In the control reactions, 700 μL of sodium acetate/acetic acid buffer (pH 6.0, 50 mmol·L^{-1}) was added instead of enzymatic extract. The amount of galacturonic acid released was determined with 2-cyanoacetamide, and the PG activity was expressed as nanomoles of galacturonic acid released per second per kilogram of fruit.

Determination of Malondialdehyde, Hydrogen Peroxide, and Superoxide Anion. Contents of malondialdehyde (MDA), H_2O_2, and $^{\bullet}O_2^-$ were determined according to the methods described by Zhang et al.[11] Fruit samples (5.00 ± 0.05 g) were ground in 3 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10000g for 20 min, and 0.5 mL of the supernatant fraction was mixed with 2 mL of 20% TCA containing 0.5% thiobarbituric acid. The mixture was heated at 100 °C for 30 min, cooled, and centrifuged at 15000g for 10 min. Absorbance was recorded at 532 nm and the value for nonspecific absorption at 600 nm was subtracted. An extinction coefficient of 155 mM^{-1}·cm^{-1} was used to calculate MDA content.

For determination of H_2O_2, samples (5.00 ± 0.05 g) were ground and extracted in 2 mL of cold acetone. The homogenate was centrifuged at 10000g at 4 °C for 20 min, and then 0.5 mL of the supernatant was mixed with 1.5 mL of $CHCl_3$ and CCl_4 (1:3 v/v) mixture. Subsequently 2.5 mL of distilled water was added, the mixture was centrifuged at 1000g for 1 min, and the aqueous phase was collected for H_2O_2 determination. The reaction system included 0.5 mL of sample, 0.5 mL of 200 mmol·L^{-1} phosphate buffer (pH 7.8), and 20 μL (0.5 unit) of catalase (as controls) or inactive catalase protein (catalase was inactivated by incubating in boiling water for 5 min). After the mixture was incubated at 37 °C for 10 min, 0.5 mL of 200 mmol·L^{-1} titanium 4-(2-pyridylazo)resorcinol (Ti-PAR) was added. The reaction mixtures were incubated at 45 °C for another 20 min. Absorbance at 508 nm was measured, and the content of H_2O_2 in strawberry fruit was indicated as micromoles per gram.

Superoxide ($^{\bullet}O_2^-$) production was calculated with an extinction coefficient of 2.16 × 10^4 M^{-1}·cm^{-1}. Samples (5.00 ± 0.05 g) were ground with 1 mL of 200 mmol·L^{-1} phosphate buffer (pH 7.0), and the homogenate was centrifuged at 5000g at 4 °C for 10 min. The reaction mixture (1 mL) contained 50 mmol·L^{-1} Tris-HCl buffer (pH 7.5), 0.5 mmol·L^{-1} XTT [sodium 3′-(1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate], and 50 μL of sample extracts. Corrections were made for the background absorbance in the presence of 50 units of superoxide dismutase (SOD).

Assays of Catalase, Guaiacol Peroxidase, Ascorbate Peroxidase, Glutathione Reductase, and Lipoxygenase Activities. Activities of guaiacol peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), and lipoxidase (LOX, EC 1.13.11.12) were determined by procedures described by Zhang et al.[9,11] Frozen fruit samples (5.00 ± 0.05 g) were homogenized with 1 mL of 200 mmol·L^{-1} ice-cold phosphate buffer (pH 7.8) containing 1.0 mmol·L^{-1} ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 12000g at 4 °C for 20 min, and the supernatant was used for activity measurement. Analysis of guaiacol POD activity was based on the oxidation of guaiacol by hydrogen peroxide. The reaction mixture contained 2.6 mL of 50 mmol·L^{-1} phosphate buffer (pH 6.1), 1 mL of 3% H_2O_2, 1 mL of 1% guaiacol, and 100−200 μL of enzyme extract. The increase in absorbance at 420 nm was recorded. APX activity was determined in the presence of 0.5 mmol·L^{-1} ascorbic acid and 0.5 mmol·L^{-1} H_2O_2 by monitoring the decrease in absorbance at 290 nm. CAT activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm. The reaction mixture contained 2.8 mL of 50 mmol·L^{-1} phosphate buffer (pH 7.0), 100 μL of 3% H_2O_2, and 100 μL of enzyme extract. One unit of POD, APX, or CAT activity was defined as an increase or decrease of 0.01 in absorbance per minute under the assay conditions. The values of antioxidant enzyme activities are indicated as units per gram FW (fresh weight).

For LOX, samples (5.00 ± 0.05 g) were homogenized with 1 mL of 200 mmol·L^{-1} phosphate buffer (pH 6.0). The homogenate was centrifuged at 15000g at 4 °C for 10 min, and the supernatant was used for the enzyme assay. The assay mixture in a total volume of 3 mL contained 200 mmol·L^{-1} borate buffer (pH 6.0), 0.25% linoleic acid, 0.25% Tween-20, and 50 μL of enzyme extract. The reaction was carried out at 25 °C for 5 min, and the activity of LOX was determined in the presence of linoleic acid by monitoring the changes in absorbance at 234 nm.

Activity of glutathione reductase (GR, EC 1.6.4.2) was detected by procedures described by Knorzer et al.[26] Frozen fruit samples (5.00 ± 0.05 g) were homogenized with 1 mL of 200 mmol·L^{-1} ice-cold Tris-HCl buffer (pH 7.0) containing 1.0 mmol·L^{-1} ascorbic acid, 1.0 mmol·L^{-1} dithiothreitol, 1.0 mmol·L^{-1} glutathione (GSH), 5 mmol·L^{-1} $MgCl_2$, and 1.0 mmol·L^{-1} EDTA. The homogenate was centrifuged at 15000g at 4 °C for 20 min, and the supernatant was used for activity measurement. The reaction mixture contained 2.9 mL of 50 mmol·L^{-1} Tris-HCl buffer (pH 7.5) (including 5 mmol·L^{-1} $MgCl_2$, 0.5 mmol·L^{-1} GSSG, and 0.2 mmol·L^{-1} NADPH) and 100 μL of enzyme extract. The decrease in absorbance at 340 nm was recorded.

Detection of Reducing Sugar, Soluble Protein, and Free Amino Acid. Fruit samples (5.00 ± 0.05 g) were ground in 5 mL of phosphate buffer (pH 7.0, 200 mmol·L^{-1}), the homogenate was centrifuged at 10000*g* for 30 min, and the supernatant was used for detection of reducing sugar and soluble protein content. Reducing sugar was measured according to Miller via the dinitrosalicylic acid method.[27] The supernatant (0.2 mL) was mixed with 1.5 mL of 3,5-dinitrosalicylic acid and 1.8 mL of distilled water; the mixture was heated at 100 °C for 5 min, cooled, and brought to 25 mL with distilled water. Reducing sugars were determined spectrophotometrically at 540 nm and the results are expressed as milligrams per gram FW (fresh weight).

For soluble protein, 0.1 mL of supernatant was mixed with 0.9 mL of dH_2O and 5 mL of Coomassie brilliant blue. Absorbance was recorded at 595 nm after 5 min by the method described by Bradford.[28] The results are expressed as micrograms per gram FW (fresh weight).

For assay of free amino acid content, samples (5.00 ± 0.05 g) were ground in 10 mL of 80% ethanol, the homogenate was centrifuged at 10000*g* for 5 min, and the supernatant was heated at 80 °C and concentrated to 1 mL in order to remove the ethanol. Free amino acid content was measured according to Zhang et al.[9] on an automatic amino acid analyzer (model S433D, Syknm).

Measurement of Endogenous H_2S. H_2S was determined by formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4 according to the method described by Sekiya et al.[6] and Chen et al.[12] Fruit samples (5.00 ± 0.05 g) were ground and extracted in 5 mL of phosphate buffer solution (pH 6.8, 50 mmol·L^{-1}) containing 0.1 mol·L^{-1} EDTA and 0.2 mol·L^{-1} ascorbic acid. The homogenate was mixed in a test tube containing 100 mmol·L^{-1} phosphate buffer solution (pH 7.4), 10 mmol·L^{-1} L-cysteine, and 2 mmol·L^{-1} phosphopyridoxal at room temperature, and then H_2S was absorbed in a zinc acetate trap located in the bottom of the test tube. After 30 min of reaction, 0.3 mL of 5 mmol·L^{-1} dimethyl-*p*-phenylenediamine dissolved in 3.5 mmol·L^{-1} H_2SO_4 was added to the trap, and then 0.3 mL of 50 mmol·L^{-1} ferric ammonium sulfate in 100 mmol·L^{-1} H_2SO_4 was injected into the trap. The amount of H_2S in the zinc acetate trap was determined colorimetrically at 667 nm after the mixture was left for 15 min at room temperature. Blanks were prepared by the same procedures with unused zinc acetate solution, and known concentrations of Na_2S were used in a calibration curve.

Statistical Analysis. Statistical significance was tested by one-way or two-way analysis of variance (ANOVA), and the results are expressed as the mean values ± standard deviation (SD) of three independent experiments. Each experiment was repeated at least three times. Fisher's least significant differences (LSD) were calculated following a significant ($P < 0.01$ or $P < 0.05$) *t* test.

RESULTS

The H_2S Donor NaHS Prolongs Postharvest Shelf Life and Lowers Rot Index of Strawberry Fruits. In our experiments, H_2S gas released by the H_2S donor NaHS in aqueous solution was used to fumigate strawberry fruits. As shown in Figure 1A,C, H_2S fumigation treatment prolonged postharvest shelf life of strawberry fruits in a dose-dependent manner. Control strawberry fruits exposed to H_2O vapor matured quickly after 1 day of storage, while strawberries treated with a range of NaHS concentrations matured more than 2 days longer storage with the optimal concentration at 0.8 mmol·L^{-1} NaHS (Figure 1A). Fruits were classified in five ranks (0, 1, 2, 3 and 4, shown in the Rot Index of Fruits section under Materials and Methods) according to the percentage of rotten surface area, as shown in Figure 1B. On the basis of the method of Ayala-Zavala et al.,[21] we evaluated the postharvest shelf life according to rot index and the limit of shelf life at rot index equal to 40 (as shown in Figure 1C). From Figure 1C, it could be seen that the maximum postharvest shelf life was

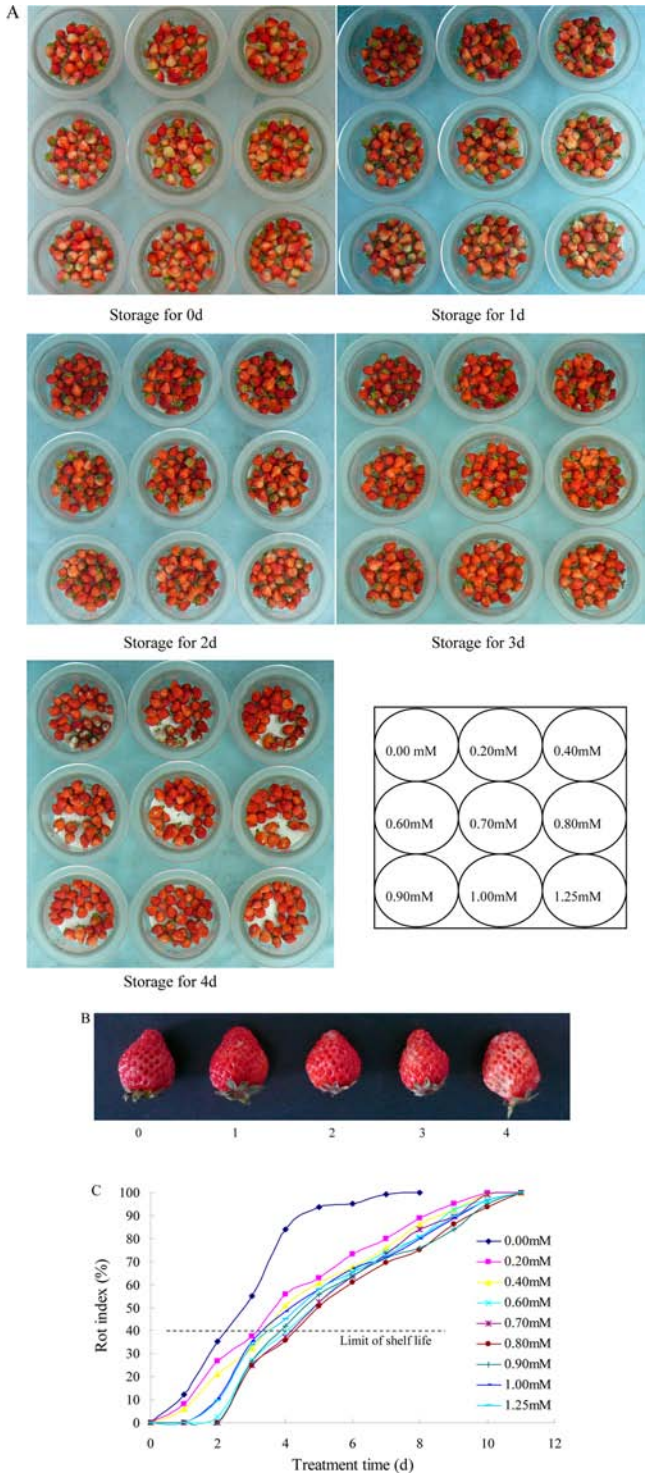

Figure 1. Effect of H_2S on postharvest shelf life and rot index in strawberry fruits. (A) Photographs of strawberries after exposure to 0, 0.2, 0.4, 0.6, 0.7, 0.8, 0.9, 1.0, or 1.25 mmol·L^{-1} H_2S donor NaHS for 0−4 days, respectively. Lower right rectangle indicates experimental treatments. (B) Photographs of classification standard for investigating rot index of strawberries. (C) Change in rot index of strawberries treated with H_2S donor NaHS at different concentrations (0, 0.2, 0.4, 0.6, 0.7, 0.8, 0.9, 1.0, and 1.25 mmol·L^{-1}).

obtained by exposure to a solution of 0.8 mmol·L^{-1} NaHS, prolonging postharvest life for 2 days in comparison with controls. Exposure to higher concentrations of NaHS (above 0.8 mmol·L^{-1}) did not significantly ($P > 0.05$) change the

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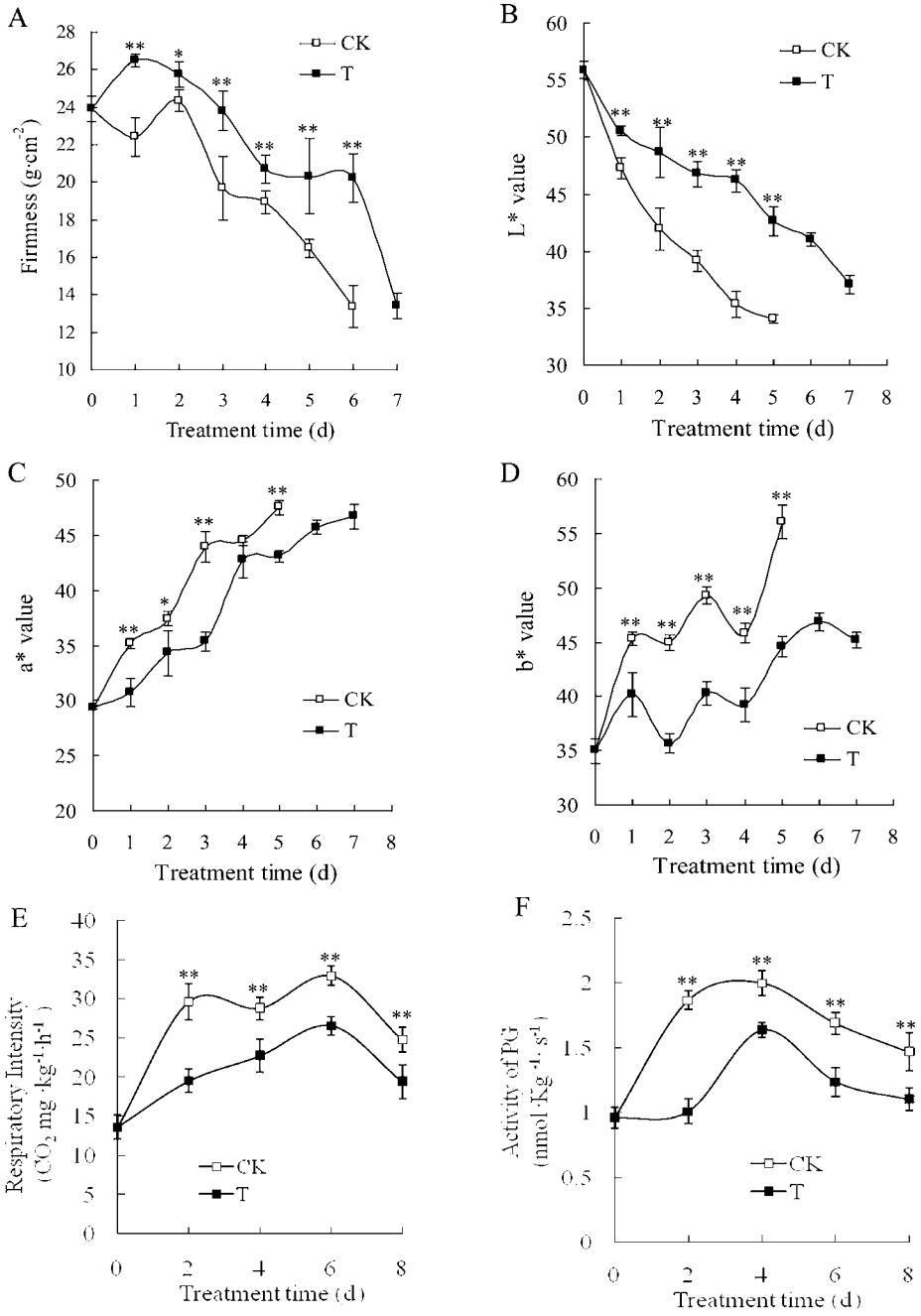

Figure 2. Effect of H_2S on changes in firmness, external color, respiratory intensity, and PG activity in strawberry fruits treated with H_2O (shown as CK) and 0.8 mmol·L^{-1} H_2S donor NaHS (shown as T). (A) Change of firmness in strawberries during storage at 20 °C. Data are presented as means ± SD (n = 5). (B) Change of L^* value in strawberries during storage at 20 °C. L^* indicates lightness. Data are presented as means ± SD (n = 10). (C) Change of a^* value in strawberries. a^* indicates chromaticity on a green (−) to red (+) axis. Data are presented as means ± SD (n = 10). (D) Change of b^* value in strawberries during storage at 20 °C. b^* indicates chromaticity on a blue (−) to yellow (+) axis. Data are presented as means ± SD (n = 10). (E) Changes of respiratory intensity in strawberries during storage. (F) Changes of PG activities in strawberries during storage. Data are presented as means ± SD (n = 3). * and ** mean significance of difference between CK and T at $P < 0.01$ and $P < 0.05$, respectively.

effectiveness of the treatment in increasing fruit shelf life. Shelf life of fruits exposed to 0.2−0.6 mmol·L^{-1} NaHS solutions was significantly greater than that of untreated fruits, but it was much lower than treatment with 0.8 mmol·L^{-1} NaHS (Figure 1A, C). Since 0.8 mmol·L^{-1} NaHS was shown to be optimal, it was used in all subsequent experiments.

Changes in Fruit Firmness, External Color, Respiratory Intensity, and Polygalacturonase Activity of Strawberry Fruits. As shown in Figure 2A, strawberry fruits showed a decreased in firmness over time during storage in both controls and NaHS treatment. However, fruits treated with 0.8 mmol·L^{-1} NaHS were significantly firmer than the control group during the storage period (Figure 2A). Changes in skin color of strawberries during storage were evaluated via L^*, a^*, and b^* values. Figure 2B shows a significant loss of lightness in both untreated and treated fruits, while the external L^* value of treated fruits decreased more slowly and was maintained higher than that of controls (Figure 2B).

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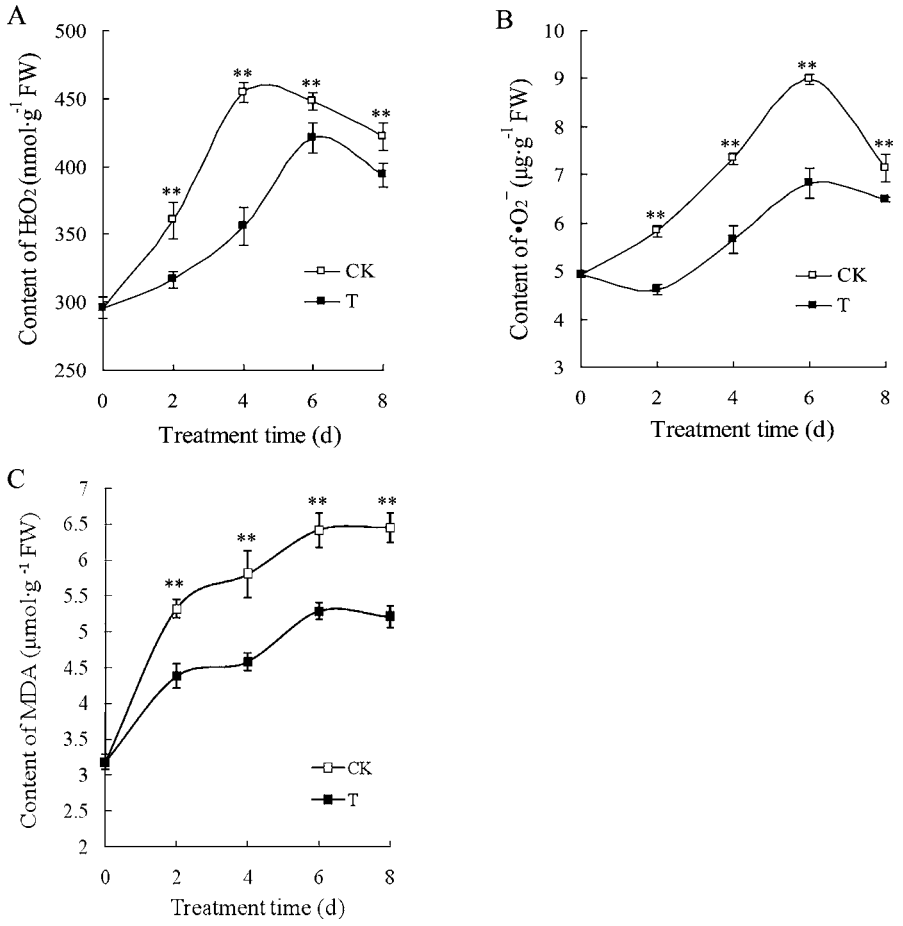

Figure 3. Effect of H_2S on the contents of (A) hydrogen peroxide, (B) superoxide anion, and (C) malondialdehyde in strawberries during storage at 20 °C. Data are presented as means ± SD (n = 3). * and ** mean significance of difference between CK and T at $P < 0.01$ and $P < 0.05$, respectively.

Furthermore, both a^* and b^* values of treated fruits increased more slowly and were lower than that of untreated controls during the storage period (Figure 2C,D).

In order to understand the elongated shelf life and increased firmness of NaHS-treated fruits, we determined the respiratory intensity in strawberry fruits exposed to 0.8 mmol·L^{-1} NaHS and untreated controls (Figure 2E). The CO_2 production of NaHS-treated fruits was lower than that of controls. As Figure 2E shows, NaHS exposure maintained lower respiratory intensity compared with controls during the entire storage period. An increase of CO_2 production in both treatments was observed at 2 days and a maximum of respiratory intensity appeared at 6 days, followed by a decline at 8 days, while the respiratory intensity in NaHS-exposed fruits was always significantly lower than that of controls during the whole storage time. To further understand the increased shelf life and firmness of NaHS-treated fruits, we measured the activity of polygalacturonase (PG) in strawberry fruits exposed to 0.8 mmol·L^{-1} NaHS and untreated controls. As shown in Figure 2F, similar change patterns of PG activity in NaHS-treated and untreated fruits were observed; PG activity increased with storage period and peaked at 6 days, and then a decline of PG activity was observed at 8 days in both treatments. However, PG activity in NaHS-treated fruits was always significantly lower than that of controls during the storage period (Figure 2F), suggesting the role of H_2S in downregulation of PG activity and the delay of the softening process.

Contents of Hydrogen Peroxide, Superoxide Anion, and Malondialdehyde in Strawberry Fruits. Determination of H_2O_2 and $^{\bullet}O_2^-$ content of strawberry fruits showed that NaHS treatment maintained lower levels of H_2O_2 and $^{\bullet}O_2^-$ during postharvest storage (Figure 3A,B). Significantly lower levels of H_2O_2 were observed in fruits treated with 0.8 mmol·L^{-1} NaHS compared with controls during the entire storage period (Figure 3A). Control fruits had as much as a 2-fold increase in H_2O_2 compared with NaHS-treated fruits at 4 days, and thereafter H_2O_2 content in control fruits decreased gradually due to senescence and rot with prolonged storage time. The production of H_2O_2 in NaHS-treated fruits was delayed for the first 2 days of storage, with the level of H_2O_2 always being significantly lower than controls during the entire storage period (Figure 3A).

Figure 3B shows that $^{\bullet}O_2^-$ production in postharvest fruits was rapidly enhanced during the first 6 days of storage irrespective of treatment. During the 6 day storage period, significantly lower $^{\bullet}O_2^-$ production was observed in fruits exposed to NaHS compared to controls (Figure 3B).

As shown in Figure 3C, MDA showed rapid accumulation during the first 6 days of storage in the presence or absence of NaHS, but the MDA level of strawberry fruits exposed to NaHS was significantly lower than that of controls (Figure 3C). Thereafter, the contents of MDA in fruits sustained at the constant levels, while the lower levels of MDA in NaHS-treated fruits were sustained during the entire storage period in comparison with the water controls (Figure 3C).

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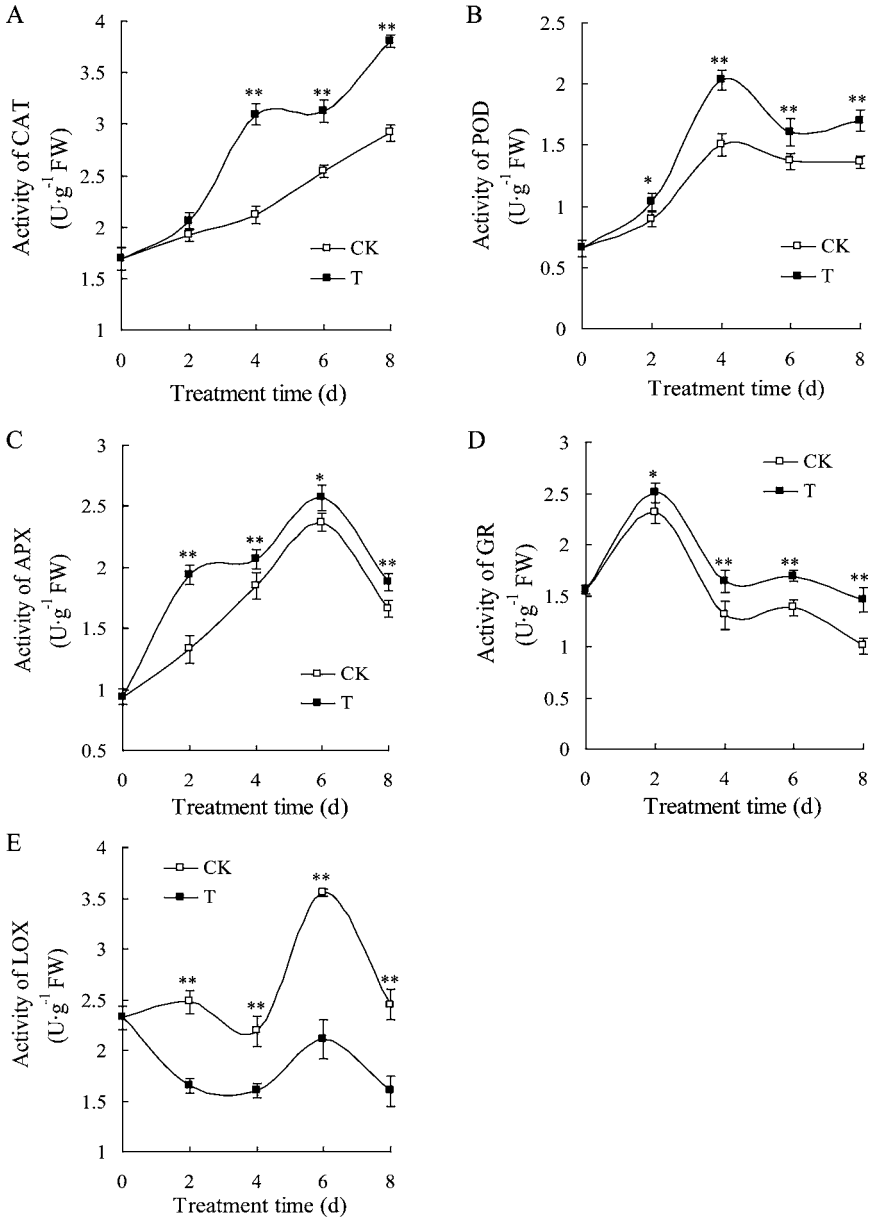

Figure 4. Effect of H_2S on the activities of (A) catalase, (B) guaiacol peroxidase, (C) ascorbate peroxidase, (D) glutathione reductase, and (E) lipoxygenase in strawberries during storage at 20 °C. Data are presented as means ± SD (n = 3). * and ** mean significance of difference between CK and T at $P < 0.01$ and $P < 0.05$, respectively.

Changes in the Activities of Catalase, Guaiacol Peroxidase, Ascorbate Peroxidase, Glutathione Reductase, and Lipoxygenase. The activities of enzymes involving in oxidative metabolism in plants, such as catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), and lipoxygenase (LOX), were measured in strawberry fruits exposed to 0.8 mmol·L^{-1} NaHS and untreated controls (Figure 4). Figure 4A shows changes in CAT activities after the two different treatments. During the first 2 days of storage, CAT activities in fruits increased slightly regardless of treatment. With prolonged storage, CAT activities of fruits exposed to NaHS increased rapidly, while those of controls increased slowly. After 4 days of storage, CAT activities in fruits exposed to NaHS were about 2-fold higher than controls. Though the CAT activities in both treatments increased gradually, significantly higher activities were maintained in fruits exposed to NaHS compared with controls (Figure 4A). Changes in the activities of POD are illustrated in Figure 4B and show that NaHS exposure maintained higher POD activities compared with controls during the entire storage period. During the first 4 days of fruit storage, a rapid enhancement of POD activity occurred in both treatments. At later storage time (4−8 days), POD activities in fruits exposed to NaHS decreased slightly but were maintained at a high level until the end of the storage period. POD in control fruits did not increase after 4 days of storage and was always lower than POD in fruit exposed to NaHS (Figure 4B).

APX activities increased gradually in stored fruits regardless of treatment up to day 6 of storage and then decreased at the end of storage time, but APX levels in fruit exposed to NaHS were always higher than in controls (Figure 4C). As shown in Figure 4D, the activity of GR exhibited a rapid increase in strawberry fruit during 0−2 days of storage followed by a gradual decrease during 2−8 days regardless of treatment. As

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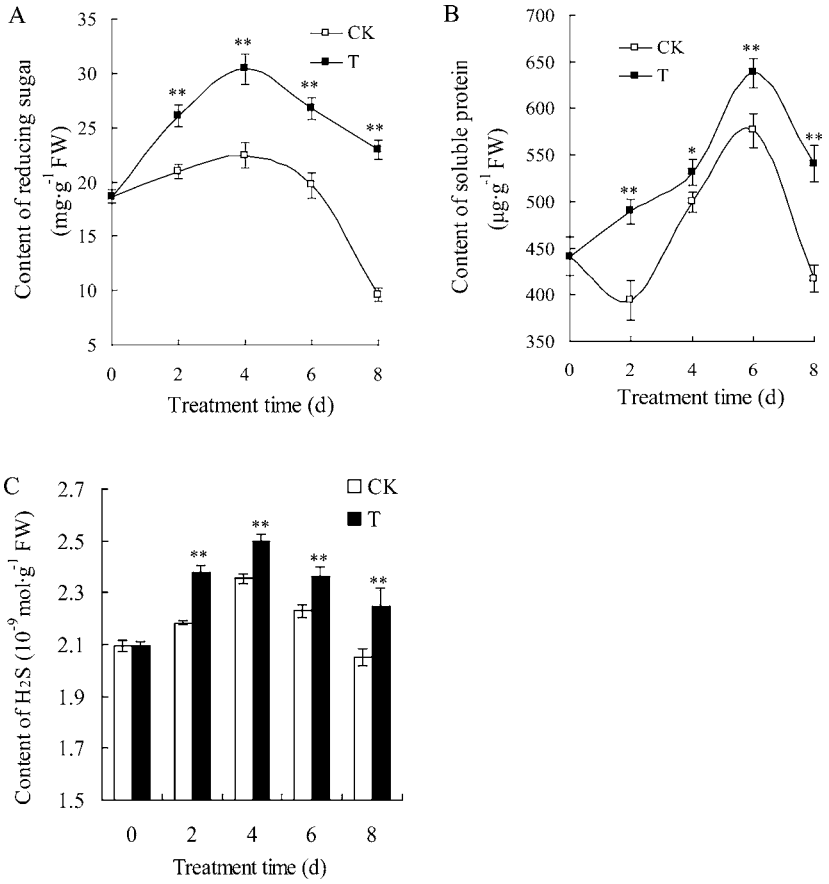

Figure 5. Effect of H_2S on the contents of (A) reducing sugars, (B) soluble proteins, and (C) endogenous H_2S in strawberries during storage at 20 °C. Data are presented as means ± SD (n = 3). * and ** mean significance of difference between CK and T at $P < 0.01$ and $P < 0.05$, respectively.

was the case for APX, the levels of GR in fruit exposed to NaHS were always higher than controls (Figure 4D).

Lipoxygenase (LOX) is an index of lipid peroxidation. Results in Figure 4E show that the activities of LOX in strawberry fruits exposed to NaHS were maintained at a lower and stable level during the entire experimental period, whereas those in control fruits showed wider fluctuation and a high peak around 6 days of storage (Figure 4E).

Reducing Sugar, Soluble Protein, Free Amino Acid, and Endogenous H_2S Contents in Strawberry Fruit. Figure 5 shows that exposure of fruit to NaHS elevated the content of reducing sugars (Figure 5A) and soluble proteins (Figure 5B). The content of reducing sugars in NaHS-exposed fruit increased dramatically during the first 4 days of storage and then declined but was still higher than that on the first day of storage. The content of reducing sugars in controls increased slightly during the first 4 days of storage and thereafter declined steeply to significantly lower levels (Figure 5A). The content of soluble proteins in control fruits fell significantly during the first 2 days of storage and thereafter increased to a maximum at 6 days of storage. With prolonged postharvest time, the content of soluble proteins declined dramatically to lower levels due to senescence and rot. By contrast, NaHS exposure inhibited the degradation of soluble proteins in fruits at the early stage of storage and maintained higher content of soluble proteins during the entire storage period compared with the controls (Figure 5B).

Free amino acid levels in freshly harvested and treated and untreated stored strawberry fruit are shown in Table 1. Softening and senescence induce a rapid decrease of the total amino acid contents regardless of treatment, although exposure to NaHS alleviated the decrease. With the exception of Gly and Phe, the content of all free amino acids in NaHS-treated and untreated fruit was lower than in freshly harvested fruit. In addition, Leu was detected only in freshly harvested and NaHS-treated fruit, and Lys was detected only in NaHS-treated fruits (Table 1). Many free amino acids including Thr, Cys, Met, Ile, Tyr, and Arg were not detected in stawberry fruits.

As shown in Figure 5C, the rapid accumulation of endogenous H_2S in strawberry fruits was observed during the first 4 days of storage in the presence or absence of NaHS, but endogenous H_2S was slightly elevated in the fruit exposed to NaHS treatment compared to the control groups. After 4 days of storage, the concentrations of H_2S in both treatments gradually decreased, but endogenous H_2S concentrations in NaHS-exposed fruits was sustained at higher levels than those in control fruits (Figure 5C).

DISCUSSION

Decay, softening, and discoloration are common changes that accompany ripening and senescence of strawberry fruit. By analyzing parameters such as rotting index, firmness, and external color, it was found that when strawberry fruit was exposed to the H_2S donor NaHS, senescence was delayed and postharvest shelf life was prolonged (Figure 1). The high softening rate of strawberry contributes to its fast postharvest decay. Respiration is an important factor in determining the postharvest deterioration of fruits. Increased respiratory

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Table 1. Effect of H_2S on Free Amino Acid Content of Strawberries during Storage at 20 °C[a]

	free amino acid content (nmol·g^{-1} FW)										
	Asp	Ser	Glu	Gly	Ala	Val	Phe	His	Leu	Lys	total AA
0	549.6 ± 6.9a	9660.5 ± 37.2a	559.2 ± 7.8a	57.3 ± 0.8a	2162.3 ± 12.9a	176.0 ± 2.1a	37.9 ± 0.7a	120.5 ± 2.0a	78.6 ± 1.4a	ND	13401.8 ± 46.3a
CK	310.9 ± 4.4b	5223.5 ± 24.5b	94.3 ± 1.3b	81.2 ± 1.2b	462.9 ± 2.6b	32.7 ± 0.6b	77.3 ± 0.9b	28.6 ± 0.5b	ND	ND	6311.4 ± 31.4b
T	460.9 ± 8.2c	7954.5 ± 36.8c	112.2 ± 1.7c	183.3 ± 3.5c	1375.6 ± 7.6c	64.3 ± 1.1c	94.9 ± 1.5c	43.9 ± 0.9c	31.6 ± 0.7b	3.3 ± 0.1	10324.6 ± 41.5c

[a]Strawberry fruits were treated with H_2O (shown as CK) or 0.8 mmol·L^{-1} NaHS solution (shown as T) for 4 days and prepared for amino acid determination. 0 represents freshly harvested fruits; CK represents H_2O control, and T represents treatment with NaHS. ND, not detected. Thr, Cys, Met, Ile, Tyr, and Arg cannot be detected in fruits. Different letters mean significance of difference between the treatments ($P < 0.01$, ANOVA, P-test LSD).

intensity is frequently associated with decreased quality and shortened shelf life. Furthermore, polygalacturonases (PG) catalyze the hydrolytic cleavage of galacturonide linkages of homogalacturonans, and can be of the exo- (EC 3.2.1.67) or endo- (EC 3.2.1.15) acting types. PG is the primary enzyme playing an important role in pectin dissolution and fruit maturation and softening. The increased firmness (Figure 2A) and postharvest shelf life of strawberry (Figure 1) probably resulted from the decreased respiratory intensity and PG activities in NaHS-treated fruits (Figure 2E,F). Recently, hydrogen sulfide (H_2S) has been proved as the third endogenous gaseous signaling molecule in animal and plant systems involving in varied physiological functions after nitric oxide (NO) and carbon monoxide (CO).[1,2,9−15] While approval of the use of H_2S gas on foods has not yet been granted, this work provided data of interest to research about the basic biology of H_2S in plants and provided valuable insights into the endogenous regulation of postharvest physiology of horticultural produce during storage. At present, application of exogenous regulators or reagents is frequently used to extend the postharvest life of fruits, while manipulating endogenous regulators has not yet found wide application. Ethylene and nitric oxide (NO) are two gases that are exceptions. NO, which was considered to be a toxic gas, has been found to act as a signal regulatory substance in plants and is widely used in storage and preservation of fruits and vegetables.[16−18] Considering the successful cases where NO has been used, H_2S, the well-known toxic small gas, also could be act as a regulatory substance that can be used to enhance postharvest fruit storage. In this paper, the concentration of the applied signal donor and the level of endogenous H_2S (Figure 5C) are quite low, thus the fumigation of fruits with H_2S gas released from NaHS solution could be safe.

An inevitable result of mitochondrial, chloroplast and plasma membrane-linked electron transport is production of reactive, toxic oxygen species or ROS.[29] The reactive nature of ROS makes them potentially harmful to all cellular components. Reactive oxygen species overproduction and oxidative damage is a universal event in postharvest strawberry fruits during storage. Fortunately, plants have evolved the capacity to eliminate these ROS with an efficient ROS-scavenging system,[30,31] and antioxidant enzymes such as SOD, POD, APX, GR, and CAT are among the most effective ROS scavengers. Furthermore, ascorbate−glutathione cycle is the recycling pathway of ascorbate and glutathione. In the cycle, APX and GR are the key enzymes and play vital roles in maintaining the homeostasis of ascorbate and glutathione in plants.[32] Recently, evidence has shown that H_2S could increase the activities of SOD, POD, CAT, APX, or GR; inhibit lipoxygenase (LOX) activities; decrease the overproduction of ROS; and maintain higher levels of ascorbate and glutathione, counteracting abiotic stresses in many plant species.[9−15,33,34] In this paper, lower H_2O_2 and $^{\bullet}O_2^-$ content and elevated activities of CAT, POD, APX, and GR in strawberry fruit exposed to the H_2S donor NaHS was observed (Figures 3A,B and 4A−D), suggesting that H_2S might protect fruit tissue against damaging effects of ROS via upregulation of antioxidant enzymes during fruit ripening and senescence. The ripening and senescence of postharvest fruits is a complex and highly regulated process that involves lipid peroxidation, resulting in the loss of integrity of the plasma membrane. LOXs are a family of enzymes that catalyze oxygenation of polyunsaturated fatty acids and formation of lipid hydroperoxides during responses to various

environmental stresses.[35,36] It showed that the activity of LOX and production MDA were downregulated in H_2S-treated strawberry fruits compared with controls, indicating that H_2S could provoke a reduction in lipid peroxidation. In summary, the data support the hypothesis that the H_2S donor delays senescence in strawberries by increasing the activities of ROS scavenging enzymes that bring about a suppression of the production of ROS such as H_2O_2 and $^{\bullet}O_2^-$, leading to a reduction in lipid peroxidation and membrane damage.

The ripening and senescence of postharvest fruits are regulated by many internal and external factors in which endogenous signaling molecules and hormones are involved, including ethylene, ABA, auxin, IP_3, Ca^{2+}, H_2O_2, and NO.[16−18,37,38] H_2S could be produced endogenously in plants and higher levels of endogenous H_2S were observed under the exposure to abiotic stresses, suggesting that H_2S is an important molecule in plant growth and development.[5−7,9−15] In our recent work, we showed that H_2S is involved in preventing senescence of cut flowers and prolonging flower vase life in a wide spectrum of botanical species, including herbaceous and woody plants.[11] In this work, we demonstrate the role of H_2S in delaying senescence of strawberry fruits and prolonging their postharvest shelf life, implying that H_2S might be an indispensable endogenous maturation and senescence regulating factor in plants. However, whether and how H_2S is involved in cross-talk with other hormones and signaling molecules, such as ethylene, ABA, and NO, requires further elucidation.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

H_2S, hydrogen sulfide; NaHS, sodium hydrosulfide; CO, carbon monoxide; NO, nitric oxide; CAT, catalase; POD, guaiacol peroxidase; APX, ascorbate peroxidase; GR, glutathione reductase; LOX, lipoxygenase; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; $^{\bullet}O_2^-$, superoxide anion; ROS, reactive oxygen species; ABA, abscisic acid

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