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Effects of hydrogen sulfide on yellowing and energy metabolism in broccoli

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ABSTRACT

The effects of H_2S on yellowing and energy metabolism of broccoli florets treated with hydrogen sulfide (H_2S) or DL-propargylglycine (PAG) were investigated after four days of storage at 20 °C. Our study showed that H_2S treatment enhanced endogenous H_2S content by 28.19% and 49.78% in comparison to the control and PAG-treated group respectively. This result might be related to the increase of L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) activities. Meanwhile, H_2S treatment can maintain chlorophyll content at 0.329 g kg^{-1}, whereas the control at 0.298 g kg^{-1} and PAG-treated group at 0.275 g kg^{-1}. This led to an alleviation of the yellowing in broccoli florets. In addition, high endogenous H_2S content also activated the key enzymes, involved in energy metabolism, including ATPases, cytochrome C oxidase (CCO), succinate dehydrogenase (SDH), glucokinase, fructokinase, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). As a result, significantly higher energy charge was observed in H_2S-treated group ($p < 0.05$). These results suggest that H_2S can effectively inhibit the yellowing and maintain high energy charge, therefore prolong the shelf life of postharvest broccoli.

1. Introduction

Broccoli (*Brassica oleracea* var. *Italica*) is a high nutritive vegetable which contains significant content of vitamins, antioxidant substances, and anticarcinogenic compounds (Yuan et al., 2010). However, it turns to senescence quickly at ambient temperatures and thus reduces customer acceptance. Yellowing is a characteristic symptom of senescence in broccoli and occurs with chlorophyll breakdown (Fukasawa et al., 2010). Although several technologies such as 6-benzylaminopurine (Xu et al., 2012), sucrose treatment (Xu et al., 2016a) and 1-MCP (Xu et al., 2016b) have been reported to alleviate yellowing during storage, introduction and development of new methods to prolong the shelf life of broccoli are still in need.

H_2S is generally regarded as a toxic gas that smells like rotten eggs. However, accumulating evidences have indicated that only high sulfide concentrations cause toxic effects in cell, whereas low concentration of endogenous H_2S plays a variety of physiological functions in both plants and animals (Doeller et al., 2001; Hancock and Whiteman, 2014) Consequently, H_2S is qualified as the third gasotransmitter besides nitric oxide and carbon monoxide (Wang, 2003). Li et al. (2014) suggested that H_2S could modulate antioxidant defense by maintaining high levels of metabolites and low accumulation of reactive oxygen species and malondialdehyde in broccoli, which delayed the postharvest senescence as a result. In addition, the effect of H_2S on extending shelf life of grape (Ni et al., 2016), strawberry (Hu et al., 2012) and lotus root (Sun et al., 2015) has also been demonstrated.

Recently energy supply, as a vital factor in controlling ripening and senescence in postharvest, has been wildly noticed. It was suggested that inadequate supplies of energy might be associated with membrane damage, which caused physiological disorders of postharvest horticultural crops and thus led to senescence and deterioration (Chen and Yang, 2013; Wang et al., 2013). Furthermore, glycolysis, the pentose phosphate pathway (PPP), the mitochondrial tricarboxylic acid (TCA) cycle, as well as electron transport chain are the central respiratory pathways, related to the supply of energy in plants (Vanlerberghe, 2013). Our previous study showed that H_2S treatment enhanced the chilling tolerance of banana fruit by increasing the activities of enzymes, involved in energy metabolism in order to maintain energy charge during cold storage (Li et al., 2016a). However, to the best of our knowledge, H_2S function in the yellowing and energy metabolism in

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postharvest broccoli is still unclear. The aim of the present study was to investigate the possible roles of H_2S in yellowing of harvest broccoli as well as its regulation of energy status.

2. Material and methods

2.1. Plant material and treatment

Broccoli (*B. oleracea* L. var. *Italica*, cv. Lvyan) were harvested in Cixi District of Ningbo City and transported to the laboratory in Zhejiang University within two hours. After eliminating diseased and mechanical damaged ones, broccoli with uniform maturity and size were finally selected and randomly divided into three groups. In preliminary experiments, the concentration of H_2S were 0.2, 0.4, 0.6, 0.8 and 1.0 mM in which 0.8 mM H_2S was selected for this experiment according to the color evaluation. In this experiment, the first group was fumigated with 0.8 mM of H_2S (NaHS as a donor) for 30 min at 20 °C, while the second group was sprayed with 0.5 mM of DL-propargylglycine (PAG) under the same condition. Distilled water was used as control (the third group). Every three broccoli florets were put on one plastic tray and wrapped with polyethylene films. All samples were stored in incubator (SANYO MIR-254, Panasonic Co., Ltd., Japan) at 20 °C with 85–90% relative humidity for 4 days in dark. Physiological parameters were measured every day. There were fifteen trays for each treatment, and each treatment conducted independently for three biological replications.

2.2. Color evaluation

The value of L^*, a^* and b^* was measured by a colorimeter (KONICA MINOLTA, CR-400, Japan). Three broccoli florets from each replicate were randomly selected and each broccoli floret was measured three times on different areas. The value of H^* was calculated as $H^* = \arctan(b^*/a^*)$.

2.3. Total chlorophyll content assay

Total chlorophyll content was measured using the method described by Zhang et al. (2015) with some modifications. Five grams of frozen samples from each tray were ground in liquid nitrogen and extracted by adding 10 mL of 80% chilled acetone for 30 min in darkness. After centrifugation at 12,000 × *g* for 10 min, the supernatant was collected. This procedure was repeated until the residue turned white. Finally, the volume was made up to 25 mL using 80% acetone and the absorbance at 663 nm and 645 nm was measured by ultraviolet and visible spectrophotometer (UV-1750, Shimadzu Co., Ltd., Japan). The total chlorophyll content was presented as $20.29A_{663} + 8.05A_{645}$.

2.4. Endogenous H_2S content assay

The endogenous H_2S was determined according to the method of Sekiya et al. (1982). Five grams of frozen samples from each tray were ground in liquid nitrogen, followed by an addition of 20 mL of 50 mM phosphate buffered saline (PBS, pH 6.8), containing 0.2 M ascorbic acid and 0.1 M EDTA. After centrifugation at 10,000 × *g* for 20 min, the supernatants were mixed in a test cube containing 0.1 M PBS (pH 7.4), 2 mM phosphopyridoxal and 10 mM L-cysteine. The released H_2S was absorbed in a zinc acetate trap which located at the bottom of the test tube. After 30 min of reaction, 0.3 mL of 5 mM dimethyl-*p*-phenylenediamine in 3.5 mM H_2SO_4 was injected into the trap. Afterwards, 0.3 mL of 50 mM $NH_4Fe(SO_4)_2$ in 0.1 M H_2SO_4 was injected to form methylene blue. The absorbance at 667 nm was measured after an incubation of 15 min at ambient temperature and Na_2S solution was used to prepare a calibration curve. The endogenous H_2S content was presented as μmol kg^{-1} fresh weight.

2.5. L-Cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) activities assay

The LCD and DCD activities were measured using the method described by Riemenschneider et al. (2005) with some modifications. Two grams of frozen samples from each tray were ground in liquid nitrogen, followed by an addition of 8 mL of 20 mM Tris-HCl (pH 8.0). After centrifugation at 12,000 × *g* for 20 min, the soluble protein content of supernatant was collected and used for the following assay.

For LCD activity measurement, 1 mL of supernatant was mixed with 1 mL of mixture solution containing 0.1 M Tris-HCl (pH 9.0), 2.5 mM dithiothreitol (DTT) and 0.8 mM L-cysteine and incubated at 37 °C for 15 min The reaction was terminated by adding 100 μL of 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride, dissolved in 7.2 M HCl and 100 μL of 30 mM $FeCl_3$, dissolved in 1.2 M HCl. The absorbance of methylene blue at 667 nm was measured and Na_2S solution was used to prepare a calibration curve. Similarly, the DCD activity was analyzed following the same methods with slight modifications: L-cysteine was replaced by D-cysteine in mixture solution, and the pH of the Tris-HCl was adjusted to 8.0.

2.6. Assay of enzymes involved in energy metabolism

The procedures of enzyme extract were conducted at 4 °C. For ATPase, cytochrome C oxidase (CCO) and succinate dehydrogenase (SDH), 20 g of frozen samples from each tray were ground in ice-bath with 30 mL of 50 mM Tris-HCl containing 0.25 M sucrose, 0.3 M mannitol, 1 M EDTA, 0.1% bovine serum albumin, 0.1% cysteine and 5 g L^{-1} polyvinyl pyrrolidone. After centrifugation at 4000 × *g* for 10 min, the supernatant was collected and centrifuged again at 12,000 × *g* for 10 min to precipitate the mitochondria. The sediment was washed twice with 5 mL of 10 mM Tris-HCl containing 1 mM EDTA, 0.3 M mannitol and 0.25 M sucrose, and centrifuged at 12,000 × *g* for 10 min Finally, the sediment was collected and dissolved with 4 mL of washing buffer. For glucokinase and fructokinase, 3.0 g of frozen samples from each tray were ground in ice-bath with 6 mL of 0.1 M Tris-HCl (pH 8.0) containing 10 mM KCl, 5 mM EDTA, 2 mM DTT, 1 mM $MgCl_2$ and 10% glycerine, and centrifuged at 12,000 × *g* for 20 min The supernatant was collected. For glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), 2.0 g of frozen samples from each tray were ground in ice-bath with 6 mL of 0.1 M Tris-HCl (pH 8.0) containing 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 1% PVP, and centrifuged at 12,000 × *g* for 20 min The supernatant was collected.

H^+-ATPase and Ca^{2+}-ATPase activities were assayed according to the method of Jin et al. (2013). One unit of H^+-ATPase or Ca^{2+}-ATPase was defined as production of 1 μmol phosphorus/min.

CCO activity was determined using the method of Jin et al. (2013). One unit of CCO was defined as an increase of 0.01 in absorption at 510 mm/min.

SDH activity was assayed by the method of Acevedo et al. (2013). One unit of SDH was defined as an increase of 0.01 in absorption at 600 mm/min.

Glucokinase and fructokinase activities were estimated according to the method of Schaffer and Petreikov (1997). One unit of glucokinase and fructokinase was defined as the amount of enzyme that causes a change of 0.01 in absorption at 340 nm/min when fructose and glucose were used as the substrate respectively.

G6PDH and 6PGDH activities were carried out using the Sgherri et al. (2002) method. One unit of G6PDH and 6PGDH was calculated from the change of 0.01 in absorption at 340 mm/min when glucose-6-phosphate and 6-phosphogluconate were used as the substrate respectively.

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