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Effect of Nitric Oxide on Ethylene Synthesis and Softening of Banana Fruit Slice during Ripening

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The effects of nitric oxide (NO) on ethylene synthesis and softening of ripening-initiated banana slice were investigated. Fruit firmness, color, and contents of starch and acid-soluble pectin (ASP) were measured. In addition, ethylene production, 1-aminocyclopropane-1-carboxylic acid (ACC) content, expression and activities of ACC synthase (ACS) and ACC oxidase (ACO), and activities of cell-wall-modifying enzymes, polygalacturonase (PG), pectin methylesterase (PME), and endo-β-1, 4-glucanase, were analyzed. Application of NO reduced ethylene production, inhibited degreening of the peel and delayed softening of the pulp. The decrease of ethylene production was associated with the reduction in the activity of ACO and the expression of the *MA-ACO1* gene. Moreover, the NO-treated fruit showed a lower expression of the *MA-ACS1* gene but higher ACS activity and ACC content. In addition, NO treatment decreased the activities of PG, PME, and endo-β-1,4-glucanase and maintained higher contents of ASP and starch, which may account for the delay of softening. We proposed that the inhibition of ACO activity and transcription of gene *MA-ACO1* by NO resulted in decreased ethylene synthesis and the delay of ripening of banana slice.

KEYWORDS: Banana; nitric oxide; ethylene; ACC oxidase; cell-wall-modifying enzymes

INTRODUCTION

Fruits have classically been categorized as climacteric and nonclimateric on the basis of their patterns of respiration and ethylene production at the onset of ripening. Fruits that undergo a burst in ethylene production and an associated increase in respiration rate are referred to as climacteric (*1*). Banana is a typical climacteric fruit, where two systems of ethylene production operate. The low basal rate of ethylene production is due to system 1 ethylene synthesis. System 2 ethylene synthesis is responsible for the autocatalytic climacteric rise in ethylene production (*2*). Ethylene production is essential for ripening of banana fruits. An atmospheric ethylene concentration of 0.1 μL/L can trigger internal ethylene production and thus initiate ripening (*3*). Therefore, commercial strategies for banana fruit handling, transport, and storage are based on avoiding exposure to ethylene and/or minimizing ethylene production and action.

Nitric oxide (NO) is a relatively stable free-radical gas. Initially, it attracted attention as an environmental pollutant. However, recent research revealed that NO may be a multifunctional-signaling molecule in plants, mediating an array of pathphysiological and physiological processes, including responses to abiotic and biotic stresses, programmed cell death, stomatal closure, seed generation, and root development (*4*). Furthermore, evidence indicated that NO may have anti-ripening and senescence properties. NO treatment extends the postharvest life of some fresh horticultural produces (*5*−*7*). Possibly, the anti-ripening and senescence effects of NO are related to reduced ethylene biosynthesis and/or action. Leshem and Pinchasov (*8*) suggested that there may exist an antagonistic effect between NO and ethylene during fruit maturation and senescence. Zhu et al. (*9*) reported that exposure of peach fruit to NO partially inhibited ethylene biosynthesis during storage. However, the effect of NO on ethylene biosynthesis and ripening-associated processes is still not clear.

The objective of this study was to investigate the effects of exogenous sodium nitroprusside (SNP), a NO donor, on ethylene biosynthesis and fruit softening of ripening-initiated banana slice. The activities and expression of ethylene biosynthesis enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) and ACC content as well as fruit-softening-associated enzyme activities, including polygalacturonase (PG), pectin methylesterase (PME), and endo-β-1,4-glucanase, were evaluated.

MATERIALS AND METHODS

Fruit Material and SNP Treatment. Hands of mature green banana (*Musa* spp., AAA group cultivar 'Brazil') were obtained from a local farm in Guangzhou, People's Republic of China. Fruits were cut into fingers, dipped for 3 min in 0.1% Sportak (prochloraz, Bayer) fungicide solution to control the postharvest diseases, and then allowed to air-dry. Banana fruits

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were selected for uniformity of shape, color, and size. Banana fruits were treated with 100 μL/L ethylene in sealed jars for 24 h at 24 °C to initiate ripening. Subsequently, the fruits were sliced with a sharp knife. From each banana, six slices (12 mm thick) were obtained. The slices were infiltrated with 5 mM SNP solution in a desiccator under an air pressure of 53 kPa for 3 min. Control slices were treated with distilled water. The slices were then placed in a plastic container (8 slices per container, 9 container per group) and maintained under controlled conditions [24 °C, 85% relative humidity (RH)] with ventilation of humidified air for 6 days. The samples were taken after ethylene treatment for 0, 2, 4, or 6 days and stored at −80 °C for analysis of expression and activities of enzymes and contents of ACC, starch, and pectin. Other measurements were conducted immediately after removing the samples from the plastic containers.

Ethylene Production. Nine fruit slices were sealed inside a 1.2 L glass jar for 2 h at 24 °C. Samples (1 mL) of headspace gas were withdrawn from the jar and injected into a gas chromatograph (GC-9A, Shimadzu, Kyoto, Japan) equipped with a 25 m HP-PLOT Q capillary column (Agilent Technologies, Santa Clara, CA) and a flame ionization detector (FID) to measure ethylene concentration. Rates of ethylene production were expressed as μL kg^{-1} h^{-1}.

Fruit Firmness. Firmness of each slice was determined with a penetrometer (model GY-1, Hangzhou Scientific Instruments, Hangzhou, China) by measuring force required for a 5 mm diameter flat probe to penetrate into the pulp to a depth of 5 mm.

Color and Chlorophyll Content. Peel color of each slice was measured with a Nippon Chromameter (model NR-300, Nippon Denshoku, Japan) in the CIE *L*a*b* mode. Peel chlorophyll content was measured according to the method of Blanke (*10*).

Contents of Starch and Acid-Soluble Pectin (ASP). Starch content in the pulp was measured according to the method described by Azelmat et al. (*11*). ASP was extracted according to the method by Cheng et al. (*12*). The uronic acid concentration of the ASP fraction was measured by the *m*-hydroxydiphenyl method (*13*) using galacturonic acid (GA) as a standard.

ACC Content. Frozen banana peel tissue (5 g) was extracted in 10 mL of 80% (v/v) ethanol at 4 °C, and the sample was centrifuged at 13000*g* for 25 min. The supernatant was evaporated under vacuum. Residues were dissolved in 5 mL of distilled water. ACC contents were measured by the method of Lizada and Yang (*14*).

Extraction and Activities of ACS and ACO. ACS activity in peel tissue was measured according to the method of Khan and Singh (*15*), with some modifications. Frozen peel tissue (5 g) was homogenized in 10 mL of 0.5 M K-phosphate buffer (pH 8.5) containing 5 μM pyridoxal phosphate, 5 mM dithiothreitol, and 2.5% polyvinylpyrrolidone. The homogenate was centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant was collected for the ACS activity assay. ACS activity was determined by incubating the mixture of 2 mL of enzyme extract and 1 mL of 500 μM *S*-adenosyl methionine in a sealed glass vial at 30 °C. After 1 h of incubation, 0.1 mL of $HgCl_2$ was added to terminate the reaction. The formation of ACC was measured by the method of Lizada and Yang (*14*). ACS activity was expressed as nmol of ACC (mg of protein)$^{-1}$ h^{-1}.

To determine the activity of ACO, 5 g of peel tissues was homogenized in 10 mL of 0.1 M Tris buffer (pH 7.5) containing 10% glycerol, 30 mM sodium ascorbate, and 2.5% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15000*g* for 20 min at 4 °C. The supernatant was desalted by passage through a Sephadex G-25 column. ACO activity was measured according to Moya-Leon and John (*16*), expressed as nmol of C_2H_4 (mg of protein)$^{-1}$ h^{-1}.

The protein content was determined according to the method of Bradford (*17*), with bovine serum albumin as the standard.

Extraction and Activities of PG, PME, and Endo-β-1,4-glucanase. A total of 5 g of pulp from banana fruit was homogenized with 20 mL of 50 mM sodium acetate buffer (pH 4.5) containing 7.5% NaCl (w/v) and 0.5 g of polyvinylpyrrolidone (insoluble) at 4 °C. The homogenate was centrifuged at 10000*g* for 20 min. The supernatant was used for assaying the activities of PG and endo-β-1,4-glucanase.

To measure PG activity, the supernatant was dialyzed overnight in 50 mM sodium acetate buffer (pH 4.5). The reaction mixture contained 0.4 mL of 200 mM sodium acetate (pH 4.5), 0.3 mL of polygalacturonic acid (PGA, 1% aqueous solution adjusted to pH 4.5), 0.2 mL of distilled water, and 0.1 mL of dialyzed enzyme extract. The reaction was initiated by the addition of the PGA substrate. The mixture was incubated at 37 °C for 1 h, followed by the addition of 3,5-dinitrosalicylate (DNS). The reaction was terminated by heating the reaction mix in a boiling water bath for 5 min. In control tubes, the substrate was added after the heat treatment. The formation of reducing groups was estimated using DNS reagent against galacturonic acid as the standard (*18*).

Endo-β-1,4-glucanase was assayed using CM-cellulose as the substrate, and the amount of reducing sugar released was determined using the DNS reagent (*18*).

PME activity was measured according to the method of Hangermann and Austin (*19*), with some modifications. A total of 5 g of pulp was ground with 20 mL of 8.8% NaCl (w/v) and 0.5 g of polyvinylpyrrolidone (insoluble) at 4 °C. The homogenate was centrifuged at 10000*g* for 30 min. The supernatant was collected, adjusted to pH 7.5, and assayed for PME activity. The activity was assayed in a mixture containing 2.0 mL of 0.5% (w/v) pectin, 0.15 mL of 0.01% bromothymol blue, 0.75 mL of water, and 0.1 mL of enzymatic extract. All solutions (pectin, indicator dye, and water) were adjusted to pH 7.5 with 2 M NaOH just before each trial was started. After the enzyme extract was added, the absorbance at 620 nm was monitored spectrophotometrically. The activity was calculated on the basis of the standard curve drawn as described by Hangermann and Austin (*19*).

Northern Blot Analysis. Total RNA from banana peel was extracted using the hot borate method (*20*). A total of 10 μg of total RNA was electrophoresed on a 1.2% agarose-formadehyde gel and capillary-blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blot-dried and cross-linked under UV at 280 nm. The primers used for Northern Blot analysis of *ACS* and *ACO* genes: MaACS-DIG-For, 5′-GCGAGTCGGGATTCATTGCT-3′; MaACS-DIG-Rev, 5′-GATGT-TTCAGGTGGCGGCTT-3′ and MaACO-DIG-For, 5′-GATGACCA-GAATGCGATGAA-3′; MaACO-DIG-Rev, 5′-CCACGCTCTTGTA-CTTGCCA-3′, were designed on the sequences registered in GenBank: *MaACS1* (accession number AF109927) and *MaACO1* (accession number AJ505611). The cDNAs of ACS and ACO were labeled with DIG-high prime DNA labeling and detection starter kit (Roche, Germany). The membrane was hybridized with the DIG-labeled probe for 16 h at 45 °C in high-sodium dodecyl sulfate (SDS) buffer [(7% SDS, 5× SSC, 50 mM sodium phosphate (pH 7.0), 2% blocking reagent, and 0.1% *N*-lauroyl-sarcosine) containing 50% deionized formamide (v/v)] (Roche, Germany). Blots were washed twice in 2× SSC and 0.1% SDS for 10 min at 37 °C, followed by washing twice in 0.1× SSC and 0.1% SDS for 30 min at 62 °C. All blots were exposed to X-rays for 30 min at 37 °C. The membrane was then subjected to immunological detection according to the instructions of the manufacturer (Roche Applied Science, Mannheim, Germany).

Data Handling. The experiments were arranged in a completely randomized design, and each was comprised of three replicates. Data were tested by analysis of variance using SPSS, version 7.5. Least significant differences (LSDs) were calculated to compare significant effects at the 5% level.

RESULTS

Firmness and Color. As was shown in **Figure 1A**, pulp firmness of banana slices decreased rapidly during ripening. However, NO treatment markedly delayed the decrease in pulp firmness. After 4 days of ripening, the firmness of control banana slices was less than 2 kg cm^{-2}, while it was 3.54 kg cm^{-2} in the NO-treated fruit slices. The firmness of NO-treated slices dropped to less than 2 kg cm^{-2} until after 7 days of ripening (data not shown).

A decrease in hue angle represents the change in peel color. The hue angle declined within 2 days of ripening in control fruit slice, reaching the value of 105.9 (yellow ripe) within 4 days (**Figure 1B**). The fruit slice treated with NO showed a much slower decline of the hue value. After 6 days of ripening, the hue value in NO-treated slices was 124.2 and the color still remained yellow−green. Until after 8 days of ripening, the hue value almost reached the consumption point (yellow ripe) (data not shown). The peel chlorophyll level generally mirrored the changes in hue vales. Chlorophyll degradation was significantly suppressed in the

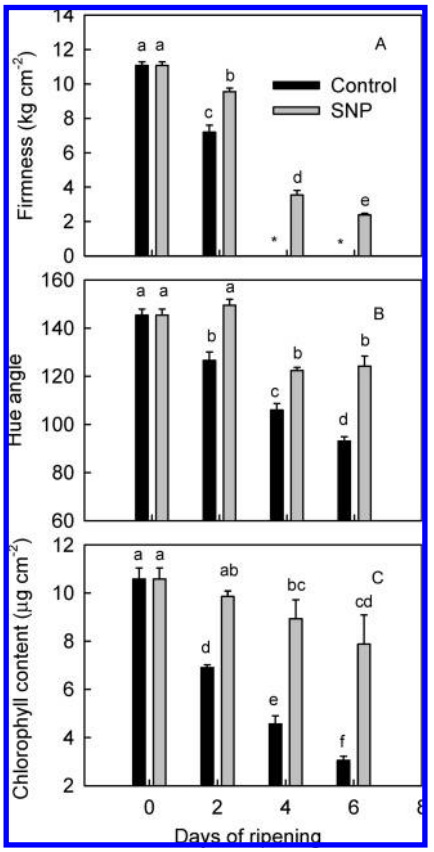

Figure 1. Effects of NO on (A) pulp firmness, (B) hue angle, and (C) chlorophyll content of peel of ripening-initiated banana slices at 24 °C. Fruits were treated with 100 μL/L ethylene for 24 h. After ethylene treatment, fruits were sliced and then treated with 5 mM SNP. Control fruit slices were treated with distilled water. Each data point represents a mean ± standard error (n = 3). The asterisk means that the firmness was less than 2 kg cm^{-2} in the control fruit slices. The values with different letters are significantly different (p < 0.05).

NO-treated fruit slices. After 6 days of ripening, the chlorophyll contents for control and NO-treated fruit slices were 3.05 and 7.88 μg cm^{-2}, respectively (**Figure 1C**).

Ethylene Production, ACC Content, and Activities of ACS and ACO. Ethylene production from ethylene-treated banana fruit slices increased rapidly until 4 days and then decreased. Application of NO, following ripening treatment with ethylene, markedly suppressed ethylene production. After 4 days of ripening, the ethylene production in fruit slices treated with NO was 58% of that in control fruit slices (**Figure 2A**).

ACC content was almost undetectable during the preclimacteric phase. A sharp increase in ACC content was observed after 2 days of ripening. There was no difference between control and NO-treated fruits. However, the NO-treated fruit slices exhibited much higher ACC content than control fruit slices after 4 and 6 days of ripening (**Figure 2B**).

The ACS activity from peel tissue (**Figure 2C**) increased in parallel with the increase in ACC content during ripening (**Figure 2B**). NO treatment resulted in a higher ACS activity compared to control fruit slices.

Except after day 2, activity of ACO in the peel tissue was much lower in fruit slices treated with NO than in control fruit slices, indicating that NO had an inhibitory effect on ACO activity of ripening-initiated fruit slices (**Figure 2D**).

Expression of *MA-ACS1* and *MA-ACO1* Genes. Figure 3 shows the Northern Blotting analysis for *MA-ACS1* and *MA-ACO1* genes related to ethylene biosynthesis in the peel of banana fruit slices. The amount of *MA-ACS1* mRNA was undetectable at the preclimacteric stage, was induced strongly by ethylene treatment, and reached the maximum at 4 days of ripening. Similar results were observed for the *MA-ACO1* mRNA. However, NO treatment significantly blocked the increases of *MA-ACS1* and *MA-ACO1* mRNA (**Figure 3**).

Contents of Starch and ASP. Figure 4 shows the effects of NO on the contents of starch and ASP from the pulp of banana slices during ripening. The contents of starch and ASP in the pulp of banana slices tended to decrease steadily with increasing ripening time. NO treatment decreased the degradation of starch and ASP compared to the control fruit slices. After 4 days of ripening, the

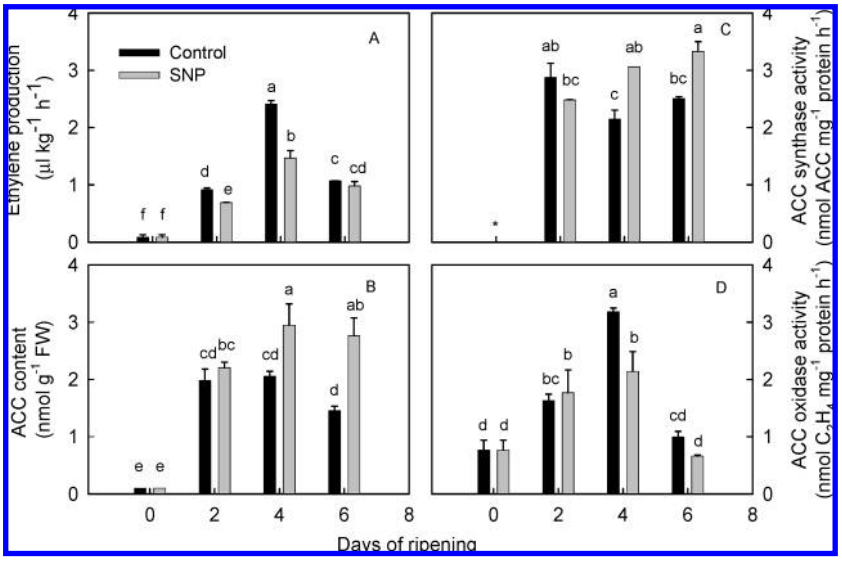

Figure 2. Effects of NO on (A) ethylene production, (B) ACC content, and activities of (C) ACS and (D) ACO in the peel of ripening-initiated banana slices at 24 °C. Fruits were treated with 100 μL/L ethylene for 24 h. After ethylene treatment, fruits were sliced and then treated with 5 mM SNP. Control fruit slices were treated with distilled water. Each data point represents a mean ± standard error (n = 3). The asterisk means that the ACS activity was undetectable. The values with different letters are significantly different (p < 0.05).

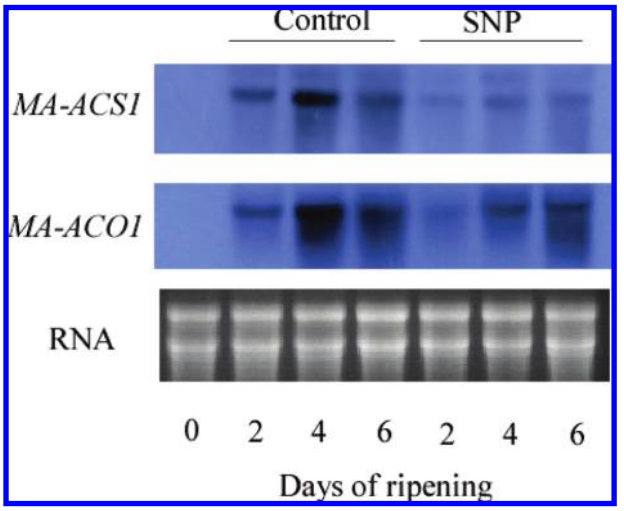

Figure 3. Effects of NO on the expression of *MA-ACS1* and *MA-ACO1* genes in the peel of ripening-initiated banana slices at 24 °C. Fruits were treated with 100 μL/L ethylene for 24 h. After ethylene treatment, fruits were sliced and then treated with 5 mM SNP. Control fruit slices were treated with distilled water.

contents of starch and ASP in NO-treated fruit slices were 1.78- and 1.45-fold those in control fruit slices, respectively.

Activities of PG, PME, and Endo-β-1,4-glucanase. PG, PME, and endo-β-1,4-glucanase are associated with cell-wall modification. PG activity in control fruit slices quickly increased, reached the peak value at day 2, and then deceased (**Figure 5A**). Treatment with NO partially inhibited PG activity of fruit slices, especially after 4 days of ripening.

Trends in the changes of PME and endo-β-1,4-glucanase activities were similar to that of PG. Fruit slices treated with NO exhibited lower activities of PME and endo-β-1,4-glucanase than the control fruit slices. For PME, the enzyme activities of SNP-treated fruit slices was 76 and 74% of the control fruit slices after 4 and 6 days of ripening, respectively (**Figure 5B**).

DISCUSSION

Banana ripening is accompanied by the changes in peel color from green to yellow, conversion of starch to sugar, flesh softening, and aroma development (*21*). In this study, NO treatment markedly inhibited the decrease of pulp firmness (**Figure 1A**), degreening of the peel (panels **B** and **C** of **Figure 1**), and starch degradation (**Figure 4A**) of ripening-initiated banana slice, indicating that NO delayed the ripening of banana fruit slices during storage. Previous studies have shown that fumigation with NO extended the postharvest life of strawberry (*5*), pear (*22*), and carnations (*6*). In addition, use of NO donor compounds was even more efficient in delaying the senescence of longan fruit (*7*).

Ethylene plays an important role in ripening of climacteric fruits, which signals an array of the ripening-associated process. Banana is a typical climacteric fruit. Ethylene production is essential for the ripening of banana fruit. In the present study, application of NO appears to significantly decrease the biosynthesis of autocatalytic ethylene (**Figure 2A**), which is responsible for delaying the ripening of banana fruit slices. Similar inhibitory effects of NO on ethylene biosynthesis were observed in pear (*22*), peach (*9*), and senescence-promoting pea leaves (*23*). Furthermore, evidence has shown that an antagonistic effect between NO and ethylene may exist in maturation and senescence of plant tissues, where endogenous NO and ethylene content maintain an inverse correlation during fruit ripening (*8*).

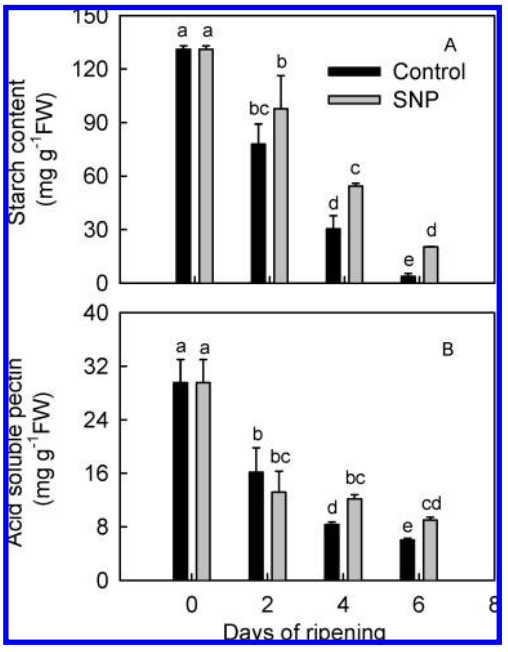

Figure 4. Effects of NO on the contents of (A) starch and (B) ASP in the pulp of ripening-initiated banana slices at 24 °C. Fruits were treated with 100 μL/L ethylene for 24 h. After ethylene treatment, fruits were sliced and then treated with 5 mM SNP. Control fruit slices were treated with distilled water. Each data point represents a mean ± standard error (n = 3). The values with different letters are significantly different ($p < 0.05$).

It is generally accepted that in higher plants ethylene is synthesized from methionine via a pathway in which ACS and ACO catalyze the conversion of *S*-adenosylmethionine to ACC and subsequent oxidation of ACC to ethylene, respectively (*24*). During ripening of climacteric fruits, both ACS and ACO are induced and contribute to the ethylene synthesis (*24*). In the present study, we found that, after ripening initiated by ethylene, activities of ACS and ACO were rapidly increased, concomitant with a sharp increase of ethylene production. NO treatment resulted in much lower ACO activity (**Figure 2D**) and ethylene production (**Figure 2A**) of banana fruit slices after 4 days of ripening, while higher ACC content (**Figure 2B**) and ACS activity (**Figure 2C**) were observed in NO-treated fruit slices than in control fruit slices, suggesting that the decrease of autocatalytic ethylene synthesis was mainly due to the inhibition of ACO activity. In biological systems, some transition metals are the targets of NO. NO can complex with iron in [Fe−S] cluster, haem, and other iron-containing proteins (*4*). ACO is a mononuclear nonheme ferrous enzyme that couples the oxidation of the co-substrate ascorbate to the oxidation of substrate ACC by dioxygen (*25*). It is possible that NO may mediate the modification of the active ferrous site in ACO, thus leading to the inhibition of the ACO activity.

We have also reported that NO treatment can block the expression of ACO genes in the peel of ripening-initiated banana slices. Basically, changes in the expression of the *MC-ACO1* gene reflect those in ACO activity. Therefore, we proposed that the inhibition of ethylene production by NO treatment could also be due to the suppression of *MA-ACO1* gene expression. Neil et al. (*4*) suggested that NO might interact directly with transcription factors or affect transcription by altering the activity of signaling pathways and ultimately affecting transcription factors. Similarly, the induction of the expression of *MA-ACS1* genes by ethylene was strongly inhibited by NO treatment. However, application of NO resulted in higher ACS activity in the peel of

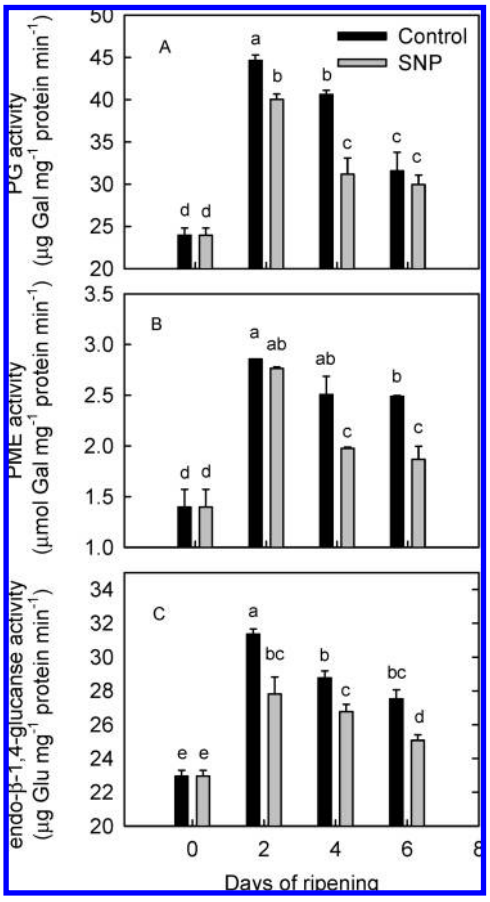

Figure 5. Effects of NO on activities of (A) PG, (B) PME, and (C) endo-β-1,4-glucanse in the pulp of ripening-initiated banana slices at 24 °C. Fruits were treated with 100 μL/L ethylene for 24 h. After ethylene treatment, fruits were sliced and then treated with 5 mM SNP. Control fruit slices were treated with distilled water. Each data point represents a mean $\pm$ standard error (n = 3). The values with different letters are significantly different ($p < 0.05$).

ripening-initiated banana slices. The inconsistency between gene expression and activity of ACS may be associated with post-transcriptional modification of the enzyme.

Banana fruit softens rapidly once ripening is initiated (*26*). In this study, the NO-treated fruit slice maintained a higher firmness compared to the control fruit slice (**Figure 1A**). Fruit softening is generally attributed to cell-wall disassembly, particularly because of pectin solubilization (*27*). Application of NO resulted in higher ASP content (**Figure 4B**) in the pulp of banana slices after 4 and 6 days of ripening, which may account for the inhibition of banana slice softening. In our previous work, the inhibition of pectin solubilization and softening by NO was also observed in plum fruit stored at 2 °C (Duan and Jiang, unpublished data). Moreover, starch is the bulk polysaccharide present in banana, and its enzyme hydrolysis results in pronounced loosening of the cell structure (*28*). The inhibition of starch degradation by NO treatment is beneficial in maintaining the firmness of banana fruit slices (**Figure 4A**).

Involvement of PG and/or PME in enzymatic disassembly of the cellular wall has been widely reported (*28*, *29*). PG catalyzes the cleavage of pectin backbones, while PME de-esterifies methoxylated pectin present in the cellular wall, which results in released sites accessible to further degradation by PG (*28*). In the present study, PG and PME activities increased first and subsequently decreased during ripening. Lower PG activity was found in the fruit slices treated with SNP after 2 and 4 days of ripening (**Figure 5A**), while SNP treatment suppressed PME activity after 4 and 6 days of ripening (**Figure 5B**). It seems that the suppression of PME activity by NO contribute more to delaying the decrease of pectin degradation in the treated fruit slices at late stage of ripening. In addition, endo-β-1,4-glucanase may also play a role in cell-wall disassembly during fruit softening. The endo-β-1,4-glucanases or cellulases, which are proposed to degrade hemicellulose, are associated with fruit softening (*30*). In this study, we found that endo-β-1,4-glucanase activity in NO-treated banana slices was lower than that in control banana slices throughout ripening. Possibly, the decreases in activities of PG, PME, and endo-β-1,4-glucanases in the NO-treated fruit slices were responsible for the inhibition of pectin and hemicellulose degradation, consequently, leading to delayed softening of banana fruit slices.

In conclusion, treatment with NO effectively decreased ethylene biosynthesis and pulp softening of ripening-initiated banana slice. The inhibition of the ethylene production by NO was related to the reduced activity and expression of ACO, while lower activities of PG, PME, and endo-β-1,4-glucanases resulted in the delay of softening.

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