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Inhibition of Apple Polyphenol Oxidase Activity by Sodium Chlorite

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Sodium chlorite (SC) was shown to have strong efficacy both as a sanitizer to reduce microbial growth on produce and as a browning inhibitor on fresh-cut apples in previous experiments. This study was undertaken to investigate the inhibitory effect of SC on polyphenol oxidase (PPO) and the associated mechanisms. The experiment showed that SC had a strong inhibition of apple PPO. The extent of inhibition was influenced by SC concentration and pH. Inhibition was most prominent at pH 4.5, at which ~30% of enzyme activity was lost in the presence of 10 mM SC, followed closely by that at pH 4.0 with a 26% reduction in PPO activity. The inhibition mode was determined using Dixon and Lineweaver−Burk plots, which established SC to be a mixed inhibitor of apple PPO for the oxidation of catechol. Preincubation of PPO with 8 mM SC for 8 min caused a maximum of 46% activity reduction compared to noninhibited control. However, preincubation of SC with catechol for 8 min resulted in no additional loss of PPO activity. These findings provide further evidence that the inhibition of PPO activity by SC is due to the inhibition of the enzyme itself rather than removal of the substrate.

KEYWORDS: Inhibition mode; sodium chlorite; polyphenol oxidase; apple; fresh-cut

INTRODUCTION

Fresh-cut apples have emerged as popular food snacks for consumers in both retail and food service sectors and are often included in school lunch programs (*1*). The production and consumption of fresh-cut apples are projected to continue growing as more consumers demand fresh, convenient, and nutritious foods. Browning is the main physiological disorder that impairs the sensory properties and discourages the consumer purchase of fresh-cut apples. Enzymatic browning reactions in fruits are catalyzed by polyphenol oxidases (PPO) followed by nonenzymatic formation of melanins (*2*). Extensive research has been focused on browning control of fresh-cut apples, and many approaches have been explored for this purpose (*3*). However, few browning inhibitors have shown potential for use in the food industry because of concerns over off-flavors and off-odors, food safety, economic feasibility, and effectiveness of inhibition (*4*, *5*).

The inhibitors of enzymatic browning most frequently used in industry include acid or brine dips, ascorbic acid (AA), and various forms of sulfite-containing compounds. The latter have applications for a broad range of produce and are strong antibrowning and antimicrobial agents. However, in addition to causing off-flavors, sulfites pose health risks to allergic individuals and, consequently, their application on fresh and fresh-cut produce was banned by the U.S. FDA (*6*). AA and its derivatives are frequently added to acidic dips used for the pretreatment of peeled or sliced fruit to prevent the oxidative browning of fruit juice prior to pasteurization (*7*). However, browning proceeds after the depletion of ascorbic acid (*4*). Furthermore, AA and its derivatives, unlike sulfiting agents, do not have antimicrobial activity, and thus a sanitizer should be used in conjunction with the antibrowning agent to reduce the potential pathogen contamination and spoilage microorganisms developed during the storage of produce. Unfortunately, most sanitizers used in the produce industry are incompatible with most antibrowning agents because they tend to be oxidizing agents, whereas most browning inhibitors tend to be reducing agents. Consequently, in combination they usually cancel out each other's desired effects, jeopardizing product safety. Recently, the largest fresh-cut apple processor experienced a costly recall of their products due to the detection of human pathogenic bacteria by the FDA (*8*). To maintain food safety and quality of fresh-cut apples, a sanitizer that is compatible with the currently widely used antibrowning solution or, better yet, a solution that can provide dual control of browning reaction and microbial growth is urgently needed.

Sodium chlorite (SC) is a strong oxidizing agent, which can generate chlorine dioxide under acidic conditions. In the food industry, SC is used for a variety of applications including disinfection of food products (*9*, *10*), purification of drinking water (*11*), and bleaching of brined cherries (*12*). Tests

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conducted in our laboratory demonstrated a strong efficacy of SC on the inactivation of the human pathogenic bacterium *Escherichia coli* O157:H7 on fresh-cut cilantro and apples. Follow-up studies also indicated that SC effectively inhibited the browning of fresh-cut apples. These findings suggest that SC may have the potential to become the much-needed dual control agent for both browning inhibition and pathogen inactivation. Because the browning of cut apple is caused primarily by PPO activity (*13*) and there is no published study regarding the effect of SC on PPO activity, this study was undertaken to investigate the inhibitory effect of SC on PPO and the associated mechanisms.

MATERIALS AND METHODS

Plant Materials. Red Delicious apples (*Malus domestica* Borkh.) from Washington state were obtained from a wholesale produce market in Jessup, MD. The fruits were stored at 0−1 °C and used within 4 months after harvesting.

Chemicals. SC, catechol, AA, sodium bisulfite (SBS), and other analytical grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). A series of SC solutions (5, 10, 15, 20, 40, 60, 80, and 100 mM) and catechol solutions (50, 100, 250, 500, and 750 mM) were prepared in 0.2 M acetate buffer (pH 4.0, 4.5, 5.0, or 5.5) before use and adjusted to the corresponding pH of the buffer using citric acid only if the difference among solutions and the buffer was >0.1.

PPO Extract Preparation and Assay. Apple PPO was extracted from an apple acetone powder following the method of Yoruk et al. (*14*). Enzyme activity was determined spectrophotometrically using catechol as the substrate. The 3 mL standard reaction mixture, prepared in a cuvette, contained 2.35 mL of acetate buffer, 0.3 mL of SC solution (or 0.3 mL acetate buffer for the control), 0.3 mL of catechol solution in acetate buffer, and 0.05 mL of PPO extract. The reference cuvette contained only the catechol solution and acetate buffer. The changes in absorbance at 420 nm and 25 °C for 3 min were measured using a Shimadzu PharmaSpec UV-1700 UV−vis spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). The enzyme activity was determined on the basis of the initial reaction rates.

Inhibition of PPO Activity by SC Concentration and pH. Inhibition of PPO activity was conducted in a disposable cuvette containing 3 mL of the standard reaction mixture. The final concentration of SC was 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, or 10.0 mM in an acetate-buffered reaction mixture with pH of 4.0, 4.5, 5.0, or 5.5, and PPO activity for the oxidation of catechol at a final concentration of 50 mM was determined. Activities were expressed as percent relative activity to that determined at pH 5.5 without SC.

Kinetic Study. Various concentrations of catechol (100, 250, and 500 mM) and SC (0, 5, 10, 20, 40, and 60 mM) were prepared in 0.2 M sodium acetate buffer (pH 5.0). The reaction mixture and PPO activity assay were the same as those for the standard reaction. The inhibition kinetics of SC on PPO activity were determined by Dixon and Lineweaver−Burk plots (*15*).

Preincubation of SC with PPO or Catechol. Preincubation of SC with PPO was performed by mixing a series of SC solutions (0, 10, 40, and 80 mM) prepared in 0.2 M sodium acetate buffer (pH 5.0) with PPO extract in a cuvette held at 25 °C for 0, 0.5, 1, 2, 4, and 8 min. The reaction was initiated by adding 0.5 M catechol to the SC and PPO mixture after the tested incubation time. For the preincubation study between SC and catechol, 80 mM SC and 50 mM catechol were mixed and held at 25 °C for 8 min. The reaction was initiated by adding PPO to the mixture, and the PPO activity was determined following the same procedure as described above.

Scanning Studies. To elucidate the effect of SC on the change in end product from the PPO and catechol reaction, a series of concentrations of SC (0, 10, 40, and 80 mM) prepared in 0.2 M sodium acetate buffer (pH 5.0) were added to the reaction mixture of PPO and 0.5 M catechol. Scanning of each reaction mixture was performed over a range of wavelengths from 700 to 220 nm at 0, 30, 60, and 120 min postaddition of SC. The reference cuvette contained only catechol. Well-characterized PPO inhibitors, that is, AA (56.8 mM) and SBS (9.6 mM), were also added to the reaction mixture replacing SC, followed by scanning at the wavelength range from 700 to 220 nm.

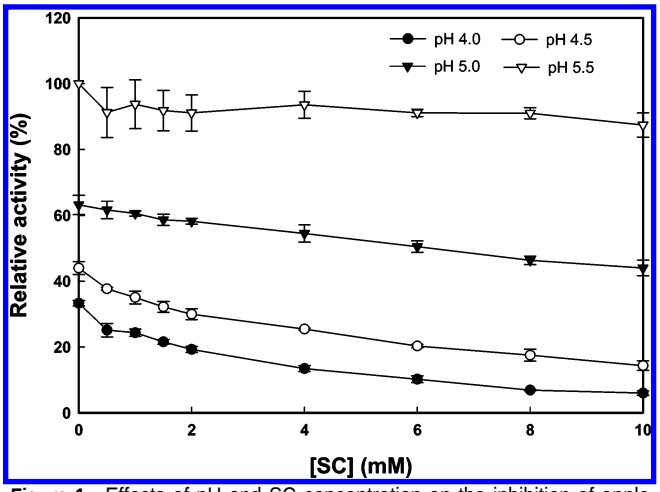

Figure 1. Effects of pH and SC concentration on the inhibition of apple PPO activity. PPO activity for the oxidation of catechol at a final concentration of 50 mM was determined in a standard reaction mixture buffered with 0.2 M sodium acetate with a targeted pH. Activities were expressed as percent relative activity to that determined at pH 5.5 without SC. The vertical bars represent the standard errors of three replicates.

Statistical Analysis. All experiments were repeated three times with duplicate samples in each repetition. Data were analyzed for statistical significance using the Proc Mixed procedure of SAS (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Effect of SC Concentration and pH on PPO Inhibition. SC treatment displayed a significant ($P < 0.001$) inhibition on apple PPO activity (**Figure 1**). This inhibition was largely influenced by the SC concentration and the pH of the reaction medium. PPO activity was significantly ($P < 0.01$) reduced with decrease in pH and increase in SC concentration for pH values in the range of 4.0−5.0. Below pH 4.0, PPO activity was very low regardless of SC concentration (data not shown). Inhibition was most prominent at pH 4.5, at which ~30% of enzyme activity was lost, followed by that at pH 4.0 with a 26% reduction of activity in the presence of 10 mM SC.

Inhibition Kinetics. Inhibition of PPO by SC was determined in the presence of different concentrations of SC for three fixed concentrations of catechol at pH 5.0 (**Figure 2**). Dixon plots (**Figure 2a**) used to analyze inhibition kinetics show that the extrapolated lines for $1/V$ versus [SC] intersect one another near the x-axis, indicating that SC is a mixed (competitive and non-competitive mixed) type inhibitor (*15*). Mixed inhibitors can bind not only with free enzyme but also with the enzyme−substrate complex at a site other than the active site. The equilibrium constants of the two reactions occurring at the two separate sites are different. Catechol is able to bind PPO even in the presence of SC, but the affinity of substrate to enzyme and the velocity of reaction are reduced in the presence of the inhibitor. **Figure 2b** shows that as SC concentration increases, the slope of the $1/V$ versus 1/[catechol] curve increases, the value of K_m increases, and V_{max} decreases. This Lineweaver−Burk plot further supports the determination that SC is a mixed inhibitor.

To further investigate whether the inhibition of PPO activity by SC is attributable to the inhibitor's effect on PPO, the substrate, or both, preincubation of SC with PPO or catechol was carried out before the inhibition reaction started. **Figure 3** shows the effect of different durations of preincubation of the enzyme with SC at pH 5.0 and at three concentrations of SC. Preincubation time had a significant ($P < 0.01$) effect on PPO

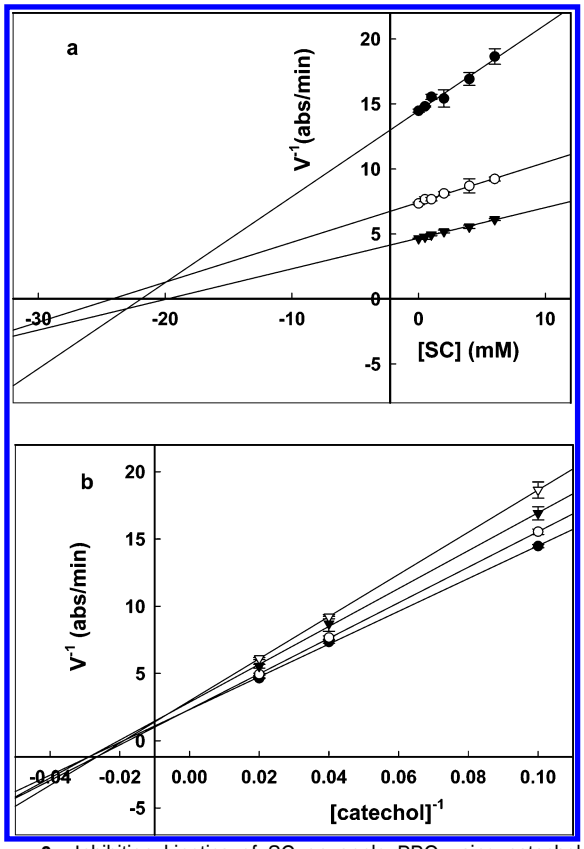

Figure 2. Inhibition kinetics of SC on apple PPO using catechol as substrate: (**a**) Dixon plots under three concentrations of catechol; (**b**) Lineweaver–Burk plots under four different concentrations of SC. PPO activity expressed as initial velocity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.2 M sodium acetate, pH 5.0.

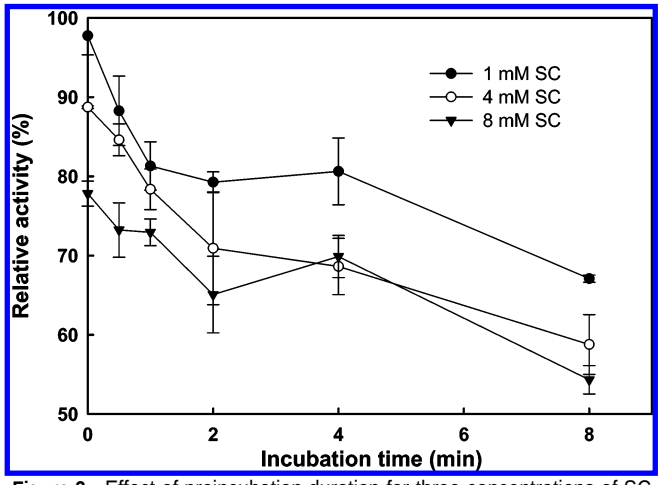

Figure 3. Effect of preincubation duration for three concentrations of SC with PPO on the inhibition of apple PPO activity. PPO activity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.2 M sodium acetate, pH 5.0, after different incubation times at a final concentration of 50 mM catechol. Activities were expressed as percent relative activity to that determined without SC and incubation. The vertical bars represent the standard errors of three replicates.

activity at each SC concentration. An 8-min preincubation with8 mM SC resulted in a 46% loss in PPO activity compared to that without the incubation. Interestingly, preincubation of SC with catechol for 8 min resulted in no additional loss of PPO activity compared to that without incubation (**Figure 4**). This finding suggests that SC inhibits PPO activity by acting directly on the enzyme rather than on the substrate.

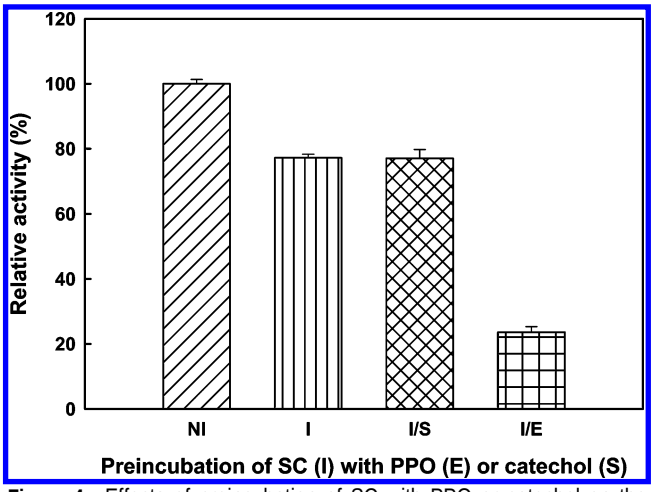

Figure 4. Effects of preincubation of SC with PPO or catechol on the inhibition of apple PPO activity. PPO activity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.2 M sodium acetate, pH 5.0, after preincubation for 8 min by mixing either PPO or catechol (50 mM final concentration) with 8 mM SC. Activities were expressed as percent relative activity to that determined without SC or preincubation: no SC or preincubation (NI); 8 mM SC, no preincubation (I); preincubation with catechol (I/S); preincubation with PPO (I/E). The vertical bars represent the standard errors of three replicates. Bars are not shown when masked by the symbols.

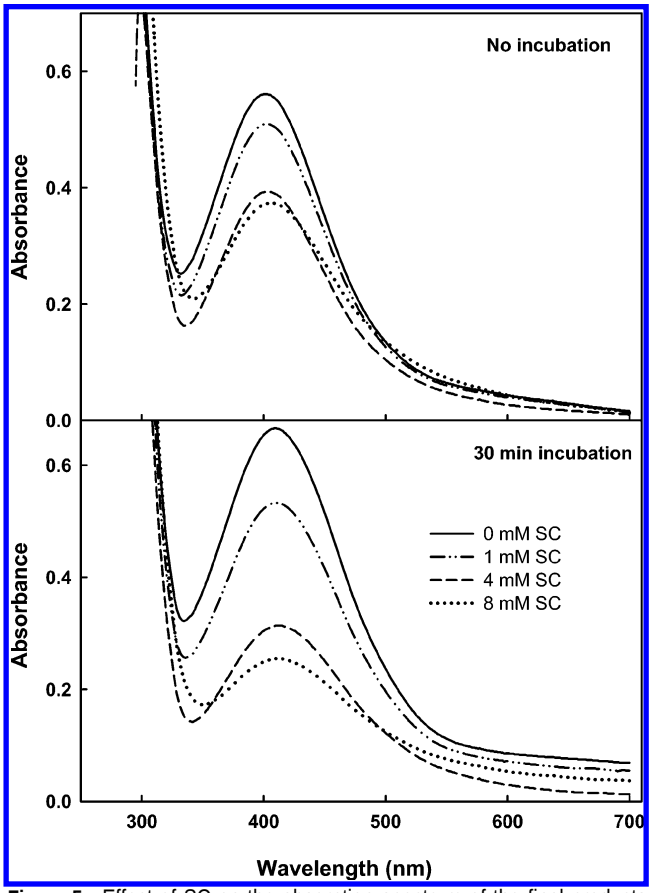

Figure 5. Effect of SC on the absorption spectrum of the final products of PPO–catechol reaction at 0 and 30 min. Scans conducted at 0 min (**a**) and 30 min (**b**) after the reaction were initiated by mixing PPO, 0.5 M catechol and 0, 10, 40, or 80 mM SC in a 3 mL standard reaction mixture buffered with 0.2 M sodium acetate, pH 5.0, at 25 °C.

Effect of SC on the End Product of PPO and Catechol Reaction. In the absence of inhibitor (SC), the increase in absorbance over time indicates the continuation of the PPO and catechol reaction at 25 °C (compare panels **a** and **b** of **Figure 5**). However, the addition of 4 or 8 mM SC to the reaction

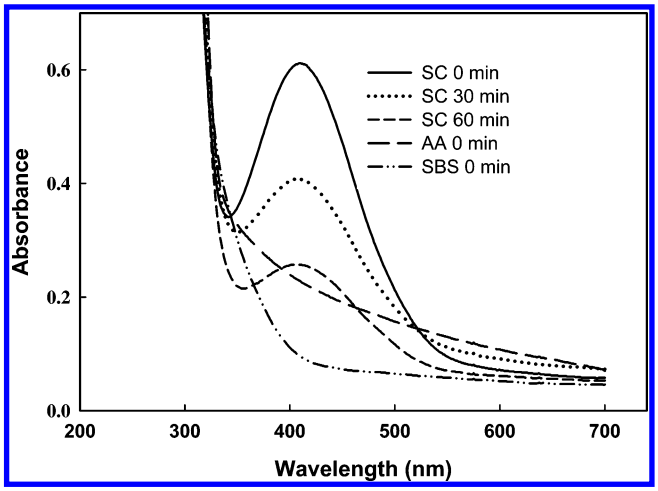

Figure 6. Effect of SC, AA, and SBS on the final products of PPO–catechol reaction. The scanning mixture in a cuvette containing PPO and 50 mM catechol in a standard reaction was incubated for 30 min at 25 °C before addition of SC (1, 4, 8 mM), AA (56.8 mM), or SBS (9.6 mM).

mixture reversed this trend as indicated by the decrease in absorbance over time, with no change in the absorption spectrum, suggesting that SC can also decrease the amount of end products formed during PPO reaction. To further investigate the role of SC in PPO inhibition, well-established PPO inhibitors, AA and SBS, were added to the reaction mixture followed by scanning at 700−200 nm (**Figure 6**). The addition of 56.8 mM AA or 9.6 mM SBS to the reaction mixture immediately rendered the solution colorless and caused a significant reduction in the absorbance, whereas nearly 2 h of reaction was needed for 8 mM SC to achieve a completely colorless solution (data not shown). AA is known for its ability to change the absorption spectra by reducing quinones back to the original colorless diphenol (*4*) and also to partially irreversibly inactivate PPO (*16*). SBS, on the other hand, acts on the quinones formed by PPO-catalyzed oxidation of *o*-dihydroxyphenols and on the enzyme itself by irreversibly binding to the "met" and "oxy" forms of binuclear copper at its active site (*17*). The exact manner in which SC affects the end product of the PPO catechol reaction is unknown. However, the slow changes in the absorbance after the addition of SC to the PPO reaction mixture may suggest that SC can also alter the quinones formed in the reaction.

Conclusion. This study demonstrates that SC exhibits a strong inhibition of apple PPO activity. The inhibition of PPO activity is pH and SC concentration dependent. Kinetic studies via Dixon and Lineweaver−Burk plots indicate that SC is a mixed inhibitor of PPO activity.

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