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Elucidation of the mechanism of enzymatic browning inhibition by sodium chlorite

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ABSTRACT

Sodium chlorite (SC) is a well known anti-microbial agent and its strong inhibitory effect on enzymatic browning of fresh-cut produce has recently been identified. We investigated the effect of SC on polyphenol oxidase (PPO) and its substrate, chlorogenic acid (CA), as it relates to the mechanisms of browning inhibition by SC. Results indicate that the browning reaction of CA (1.0 mM) catalyzed by PPO (33 U/mL) was significantly inhibited by 1.0 mM SC at pH 4.6. Two PPO isoforms were identified by native polyacrylamide gel electrophoresis, and both were inactivated by SC (3.0 mM). This suggests that SC serves as a PPO inhibitor to prevent enzymatic browning. Furthermore, the effect of SC on the stability of CA in both acidic (pH 4.5) and basic conditions (pH 8.3) was studied by UV–Vis scan and LC–MS analysis. The results showed that at the presence of SC (3.0 mM), CA (0.1 mM) degraded to quinic acid and caffeic acid as well as other intermediates. Hence, the anti-browning property of SC can be attributed to the two modes of action: the inactivation of polyphenol oxidase directly and the oxidative degradation of phenolic substrates.

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1. Introduction

Enzymatic browning is a widespread color reaction occurring in fruits and vegetables and tea leaves. The browning reaction requires the presence of oxygen, phenolic compounds and polyphenol oxidases (PPO) and is usually initiated by the enzymatic oxidation of monophenols into *o*-diphenols and *o*-diphenols into quinones, which undergo further non-enzymatic polymerization leading to the formation of pigments. Although enzymatic browning is beneficial to the color and flavor development of certain food items such as tea, coffee and cocoa, it impairs the quality and salability of fresh-cut produce (Hicks et al., 1996; Lopez-Nicolas, Perez-Lopez, Carbonell-Barrachina, & Garcia-Carmona, 2007; Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994). A variety of fruits and vegetables, such as lettuce, potato, apple, pear, banana and peach, are susceptible to enzymatic browning during processing and storage.

Extensive research (Chaisakdanugull, Theerakulkait, & Wrolstad, 2007; Komthong, Katoh, Igura, & Shimoda, 2006; Martinez & Whitaker, 1995) has focused on browning control by targeting PPO, substrates (oxygen and phenols) or the end products of browning reaction. Based on the working mechanisms, browning inhibitors can be categorized into six groups comprising reducing agents, acidulants, chelating agents, complexing agents, enzyme treatments and enzyme inhibitors (McEvily, Iyengar, & Otwell, 1992). Among all inhibitors tested, reducing agents, e.g., ascorbic acid and its derivatives, cysteine and glutathione, have been found to be effective in controlling browning, and a calcium ascorbate-based formula has been widely used by the fresh-cut apple industry. A major drawback associated with the use of reducing agents is that reducing agents are usually incompatible with sanitizers that are widely used in the fresh-cut industry to prevent pathogen contamination, because most sanitizers such as chlorine, ozone and chlorine dioxide are oxidative in nature. Since the browning-control solutions are often reused from batch to batch of apples, the lack of a proper sanitizing treatment may lead to potential contamination by food-borne human pathogens. In fact, one of the largest fresh-cut produce processors in the USA has recently experienced a costly recall of their produce due to the detection of *Listeria monocytogens* on their fresh-cut apple products. To maintain the safety and quality of fresh-cut apples, a browning inhibitor that is compatible with the currently widely used sanitizing treatment, or better yet, a solution that can provide dual control of both the browning reaction and microbial growth is urgently needed.

Acidified sodium chlorite (ASC) is a sanitizing agent recently approved by the FDA for dip or spray treatment of food items, including fresh and fresh-cut fruits and vegetables, and has shown a strong ability to control pathogens (*E. coli* O157:H7, *L. monocytogens*,

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Salmonella poona etc.) on fresh-cut carrots and cilantro (Gonzalez, Luo, Ruiz-Cruz, & McEvoy, 2004; Inatsu, Bari, Kawasaki, Isshiki, & Kawamoto, 2005; Ruiz-Cruz, Luo, Gonzalez, Tao, & Gonzalez, 2006). Sodium chlorite (SC), the major component of ASC, also had strong anti-microbial ability against human pathogenic bacteria, *E. coli* O157:H7 on fresh-cut cilantro leaves. Lu, Luo, Feng, and Turner (2007) reported that SC strongly inhibited enzymatic browning on fresh-cut apples. Because of its dual role in browning inhibition and pathogen inactivation, SC may have the potential to become the dual control treatment that is needed by the fresh-cut industry to maintain food quality and safety. Interestingly, SC is an oxidizing agent and there is, to date, no report that enzymatic browning, an oxidizing process, can be inhibited by an oxidizing agent. Although Lu, Luo, and Feng (2006) suggested that SC has a mixed (competitive and non-competitive) type inhibition, the exact effects of SC on PPO and substrates were not fully studied. Herein, the working mechanism of SC to prevent enzymatic browning was investigated, where chlorogenic acid and tyrosinase were employed to emulate the browning reaction in fresh-cut apples.

2. Materials and methods

2.1. Reagents

Chlorogenic acid (CA), sodium chlorite (SC), catechol and mushroom tyrosinase (EC.1.14.18.1, 3320 U/mg) were obtained from Sigma Chemical Co (St. Louis, MO). A series of stock solutions, including CA (1.0, 10.0 mM), tyrosinase (3300 U/mL) and SC (3.0, 5.0, 10.0, 15.0, 20.0, 30.0, 50.0 mM), were prepared in acetate buffer (0.2 M, pH 4.6). The solutions were used for all experiments unless otherwise indicated. Precast polyacrylamide gels (10%), together with the running buffer (Tris–Glycine, pH 8.3) for electrophoresis, were purchased from Bio-Rad Laboratories (Hercules, CA). Formic acid (mass spectrometry grade), ammonia (HPLC grade) and acetonitrile (Optima® grade) were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were analytical grade. Deionized distilled water was used for making all solutions, and Optima® grade water (Fisher Scientific) was used for the mobile phase in LC/MS analysis.

2.2. Inhibition of enzymatic browning by sodium chlorite

Chlorogenic acid and tyrosinase, respectively, were employed as substrate and enzyme to emulate the enzymatic browning process. Experiments were carried out by continuously adding 1.0 mL CA (10.0 mM), 1.0 mL SC (5.0, 10.0 or 30.0 mM) and 0.1 mL tyrosinase (3300 U/mL) into a flask containing 7.9 mL acetate buffer (0.2 M, pH 4.6). The final concentrations in the reaction mixture were CA, 1.0 mM, tyrosinase, 33 U/mL, and SC, 0.5, 1.0 or 3.0 mM. The reaction solution was used immediately after mixing for UV analysis, and the absorbance evolution with time was recorded at 400 nm by using a Shimadzu PharmaSpec UV-1700 spectrophotometer (Shimadzu Scientific, Columbia, MD). Either SC or tyrosinase was replaced by a buffer for the control, and the CA solution (1.0 mM) was used for the reference measurement. All experiments were conducted at 25 °C.

2.3. Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was conducted to examine the effect of SC on tyrosinase activity. Tyrosinase, SC and acetate buffer (0.2 M, pH 4.6) were mixed in a tube to reach final concentrations of 33 U/mL tyrosinase and 0, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0 or 5.0 mM SC. The tube was vortexed and then incubated at 25 °C for 30 min. Next, bromophenol blue (0.001%, w/v) and glycerol (0.05%, v/v) were mixed in the tube, and 20 μL of the mixture was loaded onto the wells of a pre-cast gel. Electrophoresis was carried out at 4 °C using a Mini PROTEAN system (Bio-Rad, Hercules, CA), with Tris–Glycine (pH 8.3) as a running buffer. After running electrophoresis, the gel was dipped in a solution containing 0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.1 M citric acid and 20 mM catechol. The PPO activity was located in the gel, based on its reaction with catechol.

2.4. Oxidative degradation of chlorogenic acid by sodium chlorite

2.4.1. Scanning studies

Two modes of oxidation reactions between CA and SC were carried out under different pH conditions. One was conducted under acetate-buffered conditions (0.2 M, pH 4.6), and the other was carried out without a pH control, where CA and SC solutions were prepared and mixed in water. The oxidation reactions were performed at 25 °C by the addition of 1.0 mL CA (1.0 mM) and 1.0 mL SC (30 mM) in 8.0 mL buffer (or water). Subsequently, the reaction mixtures were analyzed for products at certain intervals using spectrophotometric scanning and LC–MS analysis. The scanning measurements were conducted over a range of wavelengths from 700 to 220 nm using water as the reference.

2.4.2. Liquid chromatography–mass spectrometry (LC–MS) study

Samples were analyzed using a LCQ Classic ion-trap mass spectrometer (Thermo/Finnigan, San Jose, CA) with an Agilent 1100 HPLC system consisting of a binary pump, a vacuum degasser, a thermostatted column compartment, an auto-sampler, and a diode array detector (DAD, Agilent Technologies, Palo Alto, CA). The injection volumes were 5.0 μL. The following MS conditions were used: sheath gas flow rate, 70 (arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); spray voltage, 4.50 kV; heated capillary temperature, 220 °C; capillary voltage, −4.0 V; tube lens offset, 25 V. The LC–UV/MS data was collected and processed by Xcalibur, which also served as the controlling software for the LC–UV/MS system.

A GL Wakosil (Ringwood, Australia) C18 reversed-phase column (150 × 2 mm, 5 μm) was used to separate samples obtained from the acetate-buffered (pH 4.6) reaction mixture. The mobile phases included 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The LC gradient used for elution was maintained at 5% B for 2 min, then increased linearly from 5% to 95% in 14 min, held for 2 min at 95% B before decreasing to 5% in 10 s, and then kept at 5% for 7 min. The flow rate was 0.25 mL/min. MS scans were performed in positive ion mode (ESI).

CA could be oxidized and converted into a quinone intermediate by SC, when the reaction was carried out without the pH control. In this case, a Supelco-RP C18 (250 × 3 mm, 5 μm) column (Supelco, Bellefonte, PA) was employed to detect the quinone intermediate. The mobile phases were 0.1% (v/v) ammonia in water (C) and 0.1% (v/v) ammonia in acetonitrile (D). The elution gradient was maintained at 0% D for 2 min, then increased to 95% D linearly in 14 min and returned to 0% D in 4 min. The flow rate was 0.50 mL/min. Ions were detected in negative ion mode (ESI^-).

3. Results and discussion

3.1. Inhibition of enzymatic browning by sodium chlorite

Chlorogenic acid (CA) is the major phenolic compound found in many fruits, including apples, pears, apricots and cherries. It is responsible for the enzymatic browning of these fruits during postharvest handling and processing. The browning process was

investigated using a model reaction where the oxidation of CA was catalyzed by tyrosinase (mushroom PPO) with or without the presence of sodium chlorite (SC). The effect of SC on CA browning was elucidated by analyzing the absorbance (400 nm) evolution of the reaction mixture with time.

As shown in Fig. 1A, the absorbance of the reaction mixture of CA (1.0 mM) and PPO (33 U/mL) at 400 nm increased rapidly, changing from 0.054 to 0.506 within 60 min, as a result of the enzymatic browning reactions. These changes in absorbance agreed with the visual observation of the color changes of the reaction mixture as it changed from colorless to light yellow and then darkened to brown. When 0.5 mM SC was added to the reaction mixture, the increase in absorbance was significantly reduced with the final absorbance only reaching 0.274 in 60 min (Fig. 1B). This indicates that the browning reaction of CA catalyzed by PPO was inhibited by the presence of SC, which agrees with our earlier findings that SC inhibited the browning reaction of fresh-cut cut apples (Lu, Luo, Feng, & Turner, 2007). Increasing the concentration of SC in the solution from 0.5 mM to 1.0 mM, further reduced the increase in absorbance over time, although no appreciable increase in the effect of SC was observed when the concentration of SC was increased from 1.0 mM to 3.0 mM (Fig. 1C and D). In the absence of PPO, there was no increase in the absorbance of the reaction mixture of CA and SC over time (Fig. 1E). This suggests that unlike PPO-catalyzed CA oxidation and subsequent polymerization, the reaction between SC and CA did not yield polymerized products with absorbance at 400 nm.

Janovitz-Klapp, Richard, Goupy, and Nicolas (1990) reported that halide salts, such as NaF and NaCl, can inhibit PPO activity, although the exact mechanism is unknown. However, to our knowledge, no oxidant-based PPO inhibitor has ever been reported. Studies have shown that phenols are unstable and can be degraded in the presence of a strong oxidant (Antolovich et al., 2004; Fulcrand, Cheminat, Brouillard, & Cheynier, 1994; Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Given the fact that SC is a strong oxidant and the reaction of SC and CA did not produce polymerized products, it seems logical to hypothesize that the reaction between CA and SC may result in the oxidative degradation of CA and thus its removal from the reaction mix; and therefore the observed anti-browning property of SC might result either from direct enzyme inactivation, from the removal of substrate (or intermediates) due to oxidative degradation, or from both effects. To test this hypothesis, additional experiments were conducted to evaluate the effect of SC on PPO, as well as the effect of SC on CA at both acidic and basic conditions, and the results are presented in Sections 3.2–3.4, respectively.

3.2. Effect of sodium chlorite on PPO activity measured via native electrophoresis

Polyphenol oxidase activity is typically measured spectrophotometrically based on the changes in absorbance of enzyme–phenolic substrate mixture at 400 nm due to the formation of colored end products. When such enzyme assays were conducted in the presence and absence of an inhibitor, the effect of the inhibitor on PPO could be determined. However, a native polyacrylamide gel electrophoresis (PAGE) of PPO after incubation with SC, rather than a spectrophotometry analysis, was performed in this study in order to identify the exact effect of SC on PPO itself, considering the fact that SC could react with phenolic substrate. As shown in Fig. 2, two PPO isoforms (A, R_f 0.43; B, R_f 0.69) were identified and the patterns were similar to those reported by Zhang and Flurkey (1997). The staining of band A is more intense than band B, suggesting that isoform A might contribute more to the PPO-catalyzed browning process. Incubating PPO with SC prior to loading the samples on the electrophoresis had a considerable effect on the intensity of both bands A and B, indicating a significant inhibitory effect on PPO activity. Increasing the SC concentration from 0.3 to 5.0 mM considerably increased the inhibition of PPO activity, with complete inhibition of PPO activity of isoform A and B when the SC concentration reached to 1.5 mM and 3.0 mM, respectively. These observations proved that SC could serve as PPO inhibitor so as to prevent enzymatic browning.

Currently, the PPO inhibitory mechanisms of some proteins (peptides or amino acids) and compounds structurally similar to phenolic substrates were proposed. Proteins can exert an inhibitory effect on PPO activity by chelating the essential copper at the active site of PPO, and the substrate-similar compounds can work through competitive inhibition (McEvily et al., 1992). SC was expected to have a different mode of inhibition on PPO than either group mentioned above. PPO contains copper in its active site, which is essential for enzyme activity. The copper maintains an equilibrium between enzyme–Cu^{2+} and enzyme–Cu^{+} during enzymatic browning (McEvily et al., 1992; Whitaker, 1972). Therefore, SC might affect the oxidation level of copper so as to alter the catalyzing activity of PPO, in relation to the oxidizing property of SC.

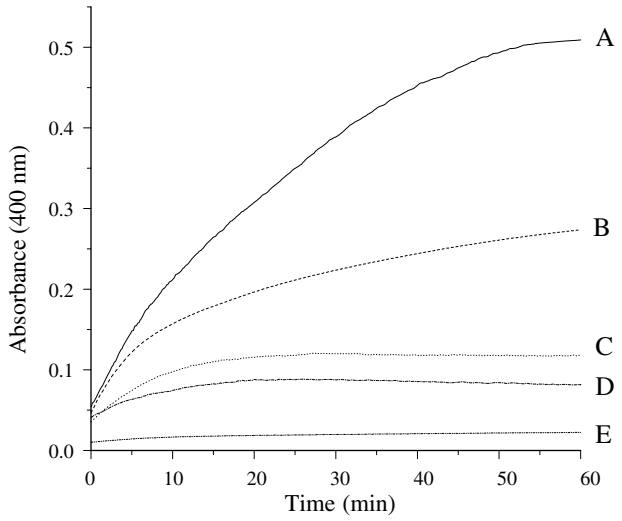

Fig. 1. Effect of varying concentration of sodium chlorite (SC) on the oxidative reaction of chlorogenic acid (CA) catalyzed by polyphenol oxidase (PPO) in a buffer (0.2 M acetate, pH 4.6) at 25 °C. The mixture of A–D contains 1.0 mM CA, 33 U/mL PPO and a varying amount of SC, where A = 0 mM SC; B = 0.5 mM SC; C = 1.0 mM SC; D = 3.0 mM SC. Reaction mixture E contains only 1.0 mM CA and 3.0 mM SC.

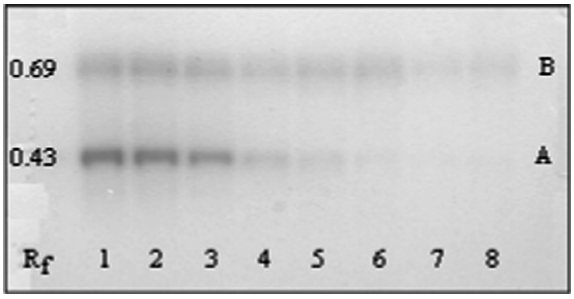

Fig. 2. Native PAGE of polypheol oxidase (PPO, 33 U/mL) after 30 min of incubation with sodium chlorite (SC). SC concentrations for (**1**), (**2**), (**3**), (**4**), (**5**), (**6**), (**7**) and (**8**) were 0, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0 and 5.0 mM (0.2 M acetate buffer, pH 4.6), respectively. The gel was stained for tyrosinase activity using catechol. See text for experimental details.

3.3. Oxidative degradation of chlorogenic acid by sodium chlorite

Chlorogenic acid contains a caffeic acid moiety esterified to a quinic acid in which the ester bond is unstable in acidic or basic environment, and the conjugation structure between the aromatic ring and the alkene double bond is sensitive to oxidants such as SC. The effect of SC (3.0 mM) on the stability of CA (0.1 mM) at pH 4.6, together with related reaction products, was studied herein. The reaction conditions used corresponded to an application study (Lu et al., 2007) in which SC achieved control of both enzymatic browning and microbial contamination in fresh-cut apples.

In the absence of SC, CA was stable and its scan spectrum remained almost unchanged at pH 4.6 for 12 h (data not shown). However, in the presence of SC, the typical peak absorbance of CA decreased gradually over time and nearly disappeared after 6.0 h (Fig. 3). This suggests that SC is capable of degrading CA under the test conditions and that the inhibitory effect of SC on the browning reaction of CA may also be attributed to the removal of CA, the substrate of the PPO-catalyzed browning reaction. In an acidic environment, SC is able to generate chlorine dioxide gas, which is also a strong oxidant (Ozawa & Kwan, 1987). Moreover, chlorine dioxide is already used as a decoloring treatment of phenol-containing wastewater (Xu, Xu, & Chen, 2003). So, the observed CA-removing effect may be attributed to both the oxidizing property of SC and the acidic condition used.

The HPLC chromatogram of CA displayed a single peak (Fig. 4A) with a retention time of 10.6 min (peak **1;** M^+ 355.3). After incubating CA with SC for 1.0 h, the HPLC chromatogram of the reaction solution displayed a total of six peaks (Fig. 4B), and peak **1** had a decreased concentration. Peaks **4** and **6**, respectively, were identified as quinic acid (M^+ 193.2) and caffeic acid (M^+ 181.1) based on their molecular weights and UV spectra, and they were most likely produced by the breakdown of the ester bond in CA. For peaks **2**, **3**, and **5**, although the exact chemical substances were not identified, it is expected that the compound responsible for peak **5** has an aromatic ring structure according to its UV spectra, and those responsible for peaks **2** and **3** might have structures similar to quinic acid, according to their retention times and UV spectra. After 12 h of reaction, the HPLC chromatogram of the reaction mixture of CA and SC displayed additional difference and that the peaks responsible for CA and caffeic acid decreased to a very low intensity and peak **5** disappeared completely (Fig. 4C). These findings suggest that CA, at pH 4.6, is unstable in the presence of SC and can be degraded to quinic acid and caffeic acid along with some phenolic intermediates, and the degradation products can be further degraded into other products. Therefore, it is indicated that, besides acting as a direct inhibitor for PPO, SC can also prevent enzymatic browning by removing phenolic substrates via oxidative degradation.

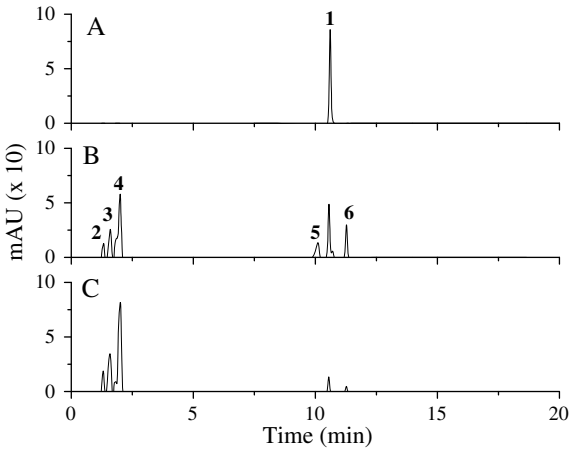

Fig. 4. HPLC chromatograms of a chlorogenic acid solution (0.1 mM) when reacted with sodium chlorite (3.0 mM) for 0 h (A), 1.0 h (B) and 12.0 h (C). The reaction occurred in a buffer (0.2 M acetate, pH 4.6) at 25 °C. Peaks: (**1**), chlorogenic acid, retention time 10.6 min, *m/z* 355.3 (M^+), absorption maxima 216, 248, 302 and 326 nm; (**2**), unknown, retention time 1.3 min, absorption maximum 262 nm; (**3**), unknown, retention time 1.6 min, absorption maximum 260 nm; (**4**), quinic acid, retention time 2.0 min, *m/z* 193.2 (M^+), absorption maxima 208, 220 nm; (**5**), unknown, retention time 10.1 min, absorption maxima 208, 250 and 304 nm; (**6**), caffeic acid, *m/z* 181.1 (M^+), retention time 11.3 min, absorption maxima 240, 302 and 323 nm. See text for LC/ESI–MS details.

3.4. Formation of quinone of chlorogenic acid

It is well documented that CA can be easily oxidized to its quinone form in a basic environment, and that subsequent polymerization of the quinones will result in the browning process (Antolovich et al., 2004). However, in our previous study (Lu, Luo, Feng, & Turner, 2007), an aqueous SC solution at pH 8.5 also exhibited certain anti-browning effects on fresh-cut apples, although the inhibitory effect was weaker than that of SC in acidic condition (pH 4.6). Thus, the reaction of CA (0.1 mM) with SC (3.0 mM) was further investigated without adjusting pH. The initial

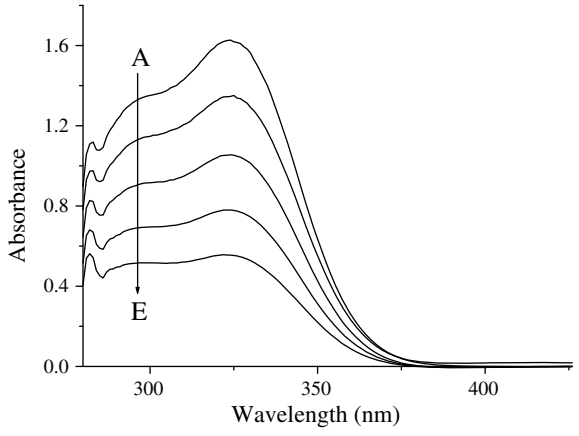

Fig. 3. Spectrophotometric scan of chlorogenic acid solution (0.1 mM) in the presence of sodium chlorite (3.0 mM) for 0 h (A), 0.5 h (B), 1.0 h (C), 3.0 h (D) and 6.0 h (E). The reaction was carried out in a buffer (0.2 M acetate, pH 4.6) at 25 °C.

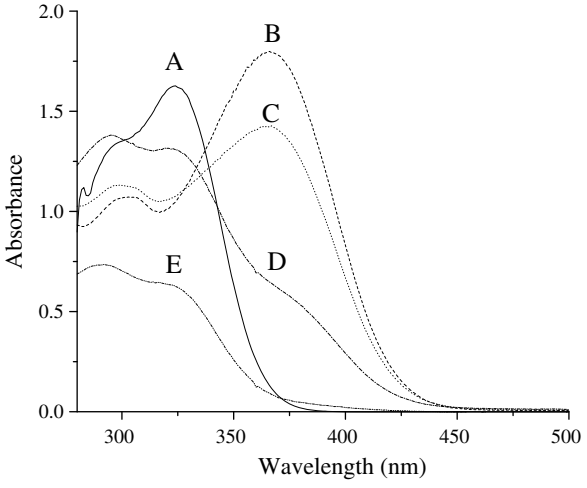

Fig. 5. Spectrophotometric scan of chlorogenic acid solution (0.1 mM) when reacted with sodium chlorite (3.0 mM) for 0 min (A), 10 min (B), 1.0 h (C), 3.0 h (D) and 10.0 h (E). The reaction occurred in water without pH control at 25 °C.

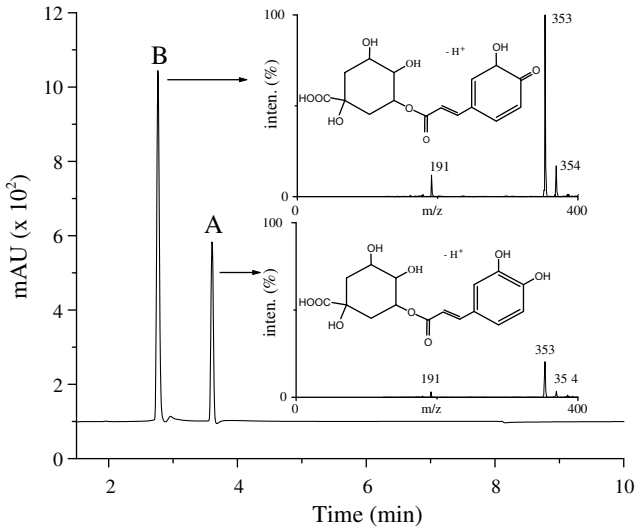

Fig. 6. LC/ESI–MS chromatogram (ESI⁻ mode) of a chlorogenic acid solution (2.0 mM) after 2 min of reaction with sodium chlorite (0.3 mM). The reaction occurred in water without pH control at 25 °C. Peaks: (A), chlorogenic acid, retention time 3.6 min, *m/z* 353.0 (M^-), absorption maxima 216, 248, 302 and 326 nm; (B), mono-quinone of chlorogenic acid, retention time 2.8 min, *m/z* 353.0 (M^-), absorption maxima 210, 230, 300 and 368 nm. See text for LC/ESI–MS details.

pH value of the reaction mixture was 8.3 and it subsequently decreased slightly. As shown in Fig. 5A and B, under the basic conditions, the characteristic CA peak (326 nm) disappeared and a new peak formed at 368 nm after 10 min of reaction of CA with SC. This increase of maximum absorption wavelength suggests that CA may be oxidized by SC to its corresponding quinone under the basic reaction conditions. As shown in Fig. 5C–E, the formed quinone of CA did not undergo further polymerization leading to the formation of pigments. Contrarily, it disappeared gradually with the prolonged reaction time due to oxidative degradation, as shown in Fig. 3. Thus, even in a basic environment (pH 8.3), SC leads to the degradation of CA and is consequently able to inhibit browning.

In order to confirm the quinone formation, LC–MS analysis was further conducted and the results are shown in Fig. 6. The LC–MS spectra of a mixture, obtained 2 min after the reaction of SC (0.3 mM) with an excessive amount of CA (2.0 mM), displayed two peaks. Peak A was identified as unreacted CA based on its molecular weight (M^- 353.0) and UV spectra (absorption maxima 216, 248, 302 and 326 nm). The UV absorption maxima (210, 230, 300 and 368 nm) of peak B corresponds to the new peak formed after the reaction of SC with CA as shown in Fig. 5 and which was thought to be a quinone of CA. The identical molecular weights of the quinone and CA (M^-, 353.0) as shown in Fig. 6, indicate that this oxidation product might be the mono-quinone of CA. No observation showed that this mono-quinone of CA could be further oxidized by SC to its di-quinone form which is reported to have a maximum absorption at 400 nm (Antolovich et al., 2004).

4. Conclusions

This work characterized the inhibitory effects of sodium chlorite on enzymatic browning and revealed the associated inhibitory mechanisms. The browning reaction of chlorogenic acid catalyzed by PPO was inhibited by sodium chlorite. It was further found that the incubation of SC and PPO resulted in enzyme inactivation, and that the reaction occurred between SC and CA led to the degradation of CA. Therefore, the anti-browning property of sodium chlorite could be attributed to two modes of action, i.e., direct inactivation of PPO and oxidative degradation of the phenolic substrates. We expect that by providing an understanding of the mechanism by which SC inhibits enzymatic browning, this research will help to accelerate the adoption of SC as a dual control by the fresh-cut produce industry, thus promoting improved quality and safety of fresh-cut produce.

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