Effect of allyl isothiocyanate on antioxidants and fruit decay of blueberries

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The effect of allyl isothiocyanate (AITC) on radical scavenging capacity, and fruit decay of blueberries var. Duke (\textit{Vaccinium corymbosum} L.) was evaluated. Results from this study showed that AITC was effective in retarding blueberry decay during storage at 10 °C. However, AITC-treated fruit decreased the contents of total phenolics and anthocyanins. Compared to control, AITC-treated berries had lower scavenging capacities against radicals of oxygen radical absorbance capacity (pyroxyl radical; ORAC), hydroxyl radical scavenging capacity (‘OH) and 2,2-di(4-tert-octylphenyl)-1-picyrylhydrazyl (DPPH), but promoted the accumulation of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) radicals. The free radical scavenging properties of blueberry fruit with or without AITC treatment were also evaluated by electron spin resonance (ESR). Results of the ESR measurements confirmed that free radical scavenging capacities against ‘OH, DPPH and O\textsubscript{2} were lower in treated fruit than in control un-treated fruit. The results from this study indicate that AITC does not promote antioxidant property or scavenging of constitutive reactive oxygen species (ROS), but paradoxically generates additional amounts of ROS to inhibit the growth and proliferation of microbial cells, thereby reducing decay in fruit tissue.

1. Introduction

Blueberries are known to have a high content of antioxidants (Prior et al., 1998; Wang & Jiao, 2000), and thus they have the potential to inhibit oxidation of human low-density lipoproteins and prevent or alleviate various human diseases caused by oxidative stress (Ames, Shigena, & Hagen, 1993). However, blueberries are highly perishable, susceptible to rapid spoilage and have short market shelf-life (Hardenburg, Watada, & Wang, 1986). Therefore it is necessary to develop strategies to maintain their quality and increase their storage life. Proper postharvest handling of blueberries is critical to marketing success.

Fruit rots in blueberries are usually caused by fungi and have been considered to be a major problem in the blueberry industry. Anthracnose (\textit{Colletotrichum acutatum}) was the most common fruit rot in blueberries, followed by Alternaria (\textit{Alternaria} spp.) and Botrytis (\textit{Botrytis cinerea}). Captan is the most frequently used synthetic fungicide for controlling fruit rots (Hardenburg et al., 1986). However, there have been increasing concerns regarding the use of synthetic fungicides in agricultural products and their presence in the environment due to health risks. Therefore, the use of natural compounds with antimicrobial properties would be preferable.

Allyl isothiocyanate (AITC) is a natural compound which is present in all plants belonging to the \textit{Cruciferae} family such as \textit{Brassica nigra}, \textit{Lepidium sativum}, \textit{Wasabia japonica}, \textit{Raphanus sativus}, and \textit{Synapsis} spp. AITC is a hydrolysis product between the enzyme myrosinase and a glucosinolate known as sinigrin. It is responsible for the pungent taste of mustard, horseradish and wasabi. It is a colorless to pale yellow liquid that is slightly soluble in water, but well soluble in most organic solvents. AITC is a food additive present in many foods and is generally considered safe for human consumption and believed to be conducive to health (Kermanshai et al., 2001; Masuda, Harada, Inoue, Kishimoto, & Tano, 1999; Shin, Masuda, & Naohide, 2004; Troncoso, Espinoza, Sánchez-Estrada, Tiznado, & García, 2005). Although it is not considered to be a nutrient in the classical sense, AITC is bioactive and has been shown to have various biological effects including anti-oxidative, anti-bacterial, anti-fungal, anti-nematode and anti-insect activities (Kermanshai et al., 2001; Masuda et al., 1999; Shin et al., 2004). There is no known human health hazard associated with AITC consumption. AITC in wasabi has been shown to have a particularly strong antimicrobial effect against \textit{Escherichia coli}, \textit{Salmonella typhimurium}, \textit{Pseudomonas aeruginosa} and other pathogenic bacteria (Inoue et al., 1983; Nishida, 1958). Hasegawa, Matsumoto, Hoshino, and Iwashita (1999) and Shin et al. (2004) also reported that AITC inhibited the growth of \textit{Vibrio parahaemolyticus} and \textit{Helicobacter pylori}. It has been shown that blue mold (\textit{Penicillium expansum}) in pears was controlled by AITC vapour treatment (Mari, 2003).
Leoni, & Cembali, 2002). Furthermore, the antimicrobial activity of AITC against pathogens in iceberg lettuce, apples, and tomatoes has also been reported (Lin, Kim, Du, & Wei, 2000). Our previous studies (Chanjirakul, Wang, Wang, & Siriphanich, 2006, 2007) have also shown that AITC reduced decay of strawberries, blackberries, and raspberries. However, the relationship between AITC and antioxidant activity was unclear and has not been investigated.

In this study, we investigated the effect of AITC on controlling blueberry (Vaccinium corymbosum L., cv. Duke) fruit decay as well as its mechanism of the action and its effect on antioxidant property.

2. Materials and methods

2.1. Chemicals

Allyl isothiocyanate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, disodium fluorescein and trichloroacetic acid were purchased from Sigma-Aldrich (Milwaukee, WI). Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA). 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPPO) was purchased from Calbiochem, CA. Phenyl-tert-butylnitrone (PBN) was from Alexis Biochemicals, Lausen, Switzerland.

2.2. Fruit sample handling and treatments with natural volatile compound allyl isothiocyanate (AITC)

Blueberries var. Duke (V. corymbosum L.) used in this study were grown at a farm near Beltsville, Maryland, USA and were hand-harvested at a commercially mature stage, sorted to eliminate damaged, shriveled, and unripe fruit, and selected for uniform size and color. Selected berries were randomized and used for the experiments. Fifty fruit were placed into 1L polystyrene containers with snap-on lids. The volatile compound AITC (5 μL L−1) was spotted onto a piece of filter paper which was subsequently hung inside the plastic containers just before the lids were closed. The AITC was allowed to vaporize inside the containers spontaneously at 20 °C for 16 h. The containers were then stored at 10 °C. Each container contained 50 berries. Twenty-four containers each were used for control and AITC treatment. Control samples were handled similarly but omitting the volatile AITC treatment. Samples were taken initially and at 3, 7, 10, and 14 days during storage for chemical analysis. The samples were then frozen in liquid nitrogen and then stored at −80 °C until assayed for antioxidant capacity, anthocyanin and phenolic compounds. The development of decay was evaluated every three days for up to 21 days and the severity was expressed as percent of fruit showing fungal symptoms.

2.3. Total anthocyanin and total phenolic content

Triplicate samples of 5 g blueberries were extracted with 25 mL of 80% acetone containing 0.2% formic acid using a Polytron (Brinkmann Instruments, Inc., Westbury, NY). The homogenized samples from the acetone extracts were then centrifuged at 14,000 g for 20 min at 4 °C. The supernatants were transferred to vials, stored at −80 °C, and later used for determination of total anthocyanins, phenolics, and free radical scavenging capacity analysis.

Total soluble phenolics in the fruit extract were determined with Folin–Ciocalteu reagent by the method of Slinkard and Singleton (1977) using gallic acid as a standard. Results were expressed as mg gallic acid equivalents (GAE) per 100 g fresh weight.

Total anthocyanin contents in blueberry extract were determined by using the pH differential method (Cheng & Breen, 1991). Results were expressed as mg of cyanidin 3-glucoside equivalents per 100 g of fresh weight basis.

2.4. Free radical measurements

2.4.1. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). Final ORAC values were calculated using the regression equation between the Trolox concentration and the net area under the curve (AUC) and were expressed as μmol Trolox equivalents per g fresh weight.

2.4.2. Hydroxyl radical scavenging capacity (OH; HOSC) assay

Five g of blueberries were extracted with 25 mL of 50% acetone. The OH in aqueous media is generated through the Fenton reaction. The HOSC assay was conducted with acetone solutions according to a previously published protocol (Moore, Yin, & Yu, 2006) with some modifications. The assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate fluorescence reader with a FL800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT). Fluorescence was measured every minute for 3 h with an excitation wavelength of 485 nm and emission wavelength of 535 nm. The plate reader was controlled by software KC4 3.0 (revision 29). Sample dilution was accomplished by a Precision 2000 automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments, Inc.). Reaction mixtures consisted of 170 μL of 9.28 × 10−6 M fluorescein prepared in 75 mM sodium phosphate buffer, 30 μL of standard or sample or blank, 40 μL of 0.1990 M H2O2, and 60 μL of 3.43 mM FeCl3. Trolox prepared in 50% acetone at concentrations of 20, 40, 60, 80, and 100 μM were used to prepare the standard curve for HOSC quantification. The HOSC values were determined by calculating the net area under the curve (AUC) of the standards and samples. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final HOSC values were calculated using the regression equation between Trolox concentration and the net AUC and were expressed as μmol Trolox equivalents (TE) per g of fresh weight.

2.4.3. Hydrogen peroxide (H2O2) assay

The assay for hydrogen peroxide in fruit extracts of blueberry was carried out following procedures previously described by Patterson, MacRae, and Ferguson (1981). The antioxidant capacity of fruit extract against H2O2 value was expressed as μmol of ascorbate equivalent per g fresh weight.

2.4.4. 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) assay

The method described by Hatano, Kagawa, Yasuhara, and Okuda (1988) was used for determining the antioxidant activity of blueberry fruit extracts on scavenging 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) radicals. The decrease in absorbance was measured at 515 nm against a blank without extract using a spectrophotometer (Shimadzu UV 1600U, Columbia, MD). The relative values of DPPH scavenging capacity of samples was expressed as μmol gallic acid (GA) per g of fresh weight.

2.5. Electron spin resonance (ESR) spectrometry assay of free radicals

ESR analysis was conducted using a Varian E-109X-Band ESR spectrometer (Varian, Inc., Palo Alto, CA) at ambient temperature in the Center for Food Safety and Applied Nutrition at the Food and Drug Administration (College Park, MD).
2.5.1. Hydroxyl radical (‘OH) scavenging activity

Hydroxyl radical (‘OH) scavenging capacities of the blueberry extracts were examined by the ESR method (Moore et al., 2006). The ESR assay is based on competition between the trapping agent and anti-oxidative activity in the extract. ‘OH was generated by the Fenton reaction, while 5,5-dimethyl N-oxide pyrroline (DMPO) was used as the trapping agent. The Fenton reaction (Fe^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^{-\cdot}), a well-known and defined generator of ‘OH, was used to examine whether AITC produced ‘OH radicals. The reaction mixture contained 25 \mu\text{L} of 1 mM freshly prepared FeSO_{4}, 25 \mu\text{L} of 1 mM EDTA, 25 \mu\text{L} of 250 mM DMPO, 25 \mu\text{L} of 1 mM \text{H}_2\text{O}_2, and 25 \mu\text{L} of diluted fruit extract or H_2O for the blank. The final amount of blueberry antioxidants used for measurement was 4.15 mg fresh weight equivalents per mL of reaction mixture. The ESR measurements were conducted at 4 min of each reaction at ambient temperature, using a Varian E-109X-Band ESR spectrometer (Varian, Inc., Palo Alto, CA), with the following spectrometer settings: microwave power of 10 mW, field modulation frequency of 100 kHz, and a modulation amplitude of 1 G.

2.5.2. Radical DPPH* scavenging activity

Radical DPPH* activity of blueberry extracts (un-treated vs AITC-treated for 7 days) were determined by an electron spin resonance (ESR) spectrometer method, using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH*) (Yu et al., 2002). Aqueous blueberry extract was mixed with DPPH* stock solution to initiate the fruit extract–radical reaction. The final concentration was 250 \mu\text{M} for DPPH* in all reaction mixtures. The final amount of blueberry fruit used for measurement was 0.25 mg fresh weight. The control reaction contained no fruit extract. DPPH* was prepared with 50% ethanol in water (v/v). ESR signals were recorded at 1, 5, 10, 20, and 30 min following the start of the reaction, with 20 mW incident microwave power, 100 kHz field modulation of 2 G (Zhou, Yin, & Yu, 2006). The ESR spectra recorded at 30 min of reaction were presented.

2.5.3. Superoxide anion radical (O_2^{-}) scavenging activity

O_2^{-} scavenging activity was determined by the ESR method and the xanthine/xanthine oxidase system was used to generate the O_2^{-} (Noda, Kohno, Mori, & Packer, 1999). The antioxidant-radical reaction was initiated by addition of xanthine oxidase solution (XOD), whereas 5-tert-butoxy carbonyl 5-methyl-1-pyrroline N-oxide (BMPO) was used as the trapping agent (Zhou et al., 2006). The total volume of the reaction mixture was 125 \mu\text{L} and the concentrations were 5 mM for xanthine, 125 mM for BMPO, 0.5 mM for diethylenetriaminepentaacetic acid (DTPA), and 0.25 units/mL for XOD. The final amount of blueberry fruit used for measurement was 12.5 \mu\text{g} fresh weight. The ESR spectra were recorded at 5 min of reaction at ambient temperature with 10 mW incident microwave power and 100 kHz field modulation of 1 G.

2.6. Statistical analysis

Data presented were the means ± SD values. All statistical analyses were performed with the NCSS Statistical Analysis System (Statistical Analysis and Graphics, Kaysville, UT, USA) (NCSS, 2007).

3. Results

AITC was effective in retarding blueberry decay during storage at 10 ºC (Fig. 1). In addition to reducing decay, fruit treated with AITC had decreased amounts of total phenolics, and anthocyanins, and lowered antioxidant activities against radicals of DPPH*, ROO*, and ‘OH (Figs. 2 and 3A, B, and D). However, the level of H_2O_2 in blueberry fruit increased after treatment with AITC (Fig. 3C). As shown in Fig. 3C, AITC treatment significantly (p < 0.05) promoted the accumulation of H_2O_2 over the 14 days of treatment, and the content of H_2O_2 in AITC-treated fruit was around 20% higher than that in control fruit.

The scavenging activities of blueberry fruit against ‘OH, DPPH* and O_2^{-} from ESR measurements were shown in Fig. 4. Fruit treated with AITC had markedly higher radical signals for ‘OH, DPPH*, and O_2^{-} than un-treated fruits, indicating that the treated fruit had lower scavenging activities for these radicals compared to un-treated fruits. ESR measurements showed that AITC generated the ‘OH radical by the Fenton reaction when DMPO was used as the trapping agent (Fig. 5). AITC with spin trap α-(4-pyridyl-1-oxide)-N-tert-butyl nitronitrone (4-POBN) in water or with α-phenyl-tert-butyl nitronitrone (PBN) in chloroform generated no radicals.
Fig. 3. Effect of allyl isothiocyanate (AITC) treatment on (A) oxygen radical absorbance capacity (ORAC), (B) 2,2-di[(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH*)], (C) hydrogen peroxide (H₂O₂) and (D) hydroxyl radical scavenging capacity (·OH) in ‘Duke’ blueberries after 3, 7, 10 and 14 days of storage at 10°C. Data were expressed as mean ± SD, n = 3.

Fig. 4. Effect of allyl isothiocyanate (AITC) treated blueberry fruit on ·OH, DPPH* and O₂· radicals measured by ESR. Blueberry fruit with and without AITC treatment were stored at 10°C for 7 days. Fruit extract (2.5 mg mL⁻¹) was used for the assay. For ·OH radicals, the reaction of ESR contained 0.5 mM DEPMO, 0.1 mM FeSO₄, 1.0 mM H₂O₂ (3455G/200G/1G/2 min) and the following reactants: (A) without berry extract (B) blueberry extract without AITC treatment (C) blueberry extract with AITC treatment, for DPPH* radicals, the reaction contained 0.125 mM DPPH* in 50% ethanol and the following reactants: (A) without berry extract (B) blueberry extract without AITC treatment (C) blueberry extract with AITC treatment. For O₂· radicals, the reaction contained 1.0 mM xanthine and 0.05 U/mL xanthine oxidase, 10 mM DEPMP (3455G/200G/1G/ 10 min/room temperature) and the following reactants: (A) without berry extract; (B) blueberry extract without AITC treatment and (C) blueberry extract with AITC treatment.
ties, AITC or related compounds have been reported to exhibit antibacterial, antiviral, antiinflammatory, antitoxigenic, antiparasitic, and insecticidal properties (Shin et al., 2004; Suh, Moon, & Kim, 2006). AITC enhanced \( \text{H}_2\text{O}_2 \) production in blueberries as shown in Fig. 3C. In our study, ESR data showed that AITC can induce the formation of \( \cdot\text{OH} \) and blueberry fruit treated with AITC produced higher amounts of DPPH, \( \cdot\text{OH} \) and \( \cdot\text{O}_2^- \) radicals than un-treated fruit (Fig. 4). Hydroxyl radical \( \cdot\text{OH} \) is a highly reactive molecule that will react at diffusion-limited rates with various biomolecules, including lipids, proteins, and DNA (Miller & Britigan, 1997). It is possible that blueberry fruit treated with AITC produced high amounts of ROS that resulted in an intolerable level of high oxidative stress in fungi cells. When the critical threshold for fungi cells to cope with the induced oxidative stress has been reached, key cellular components such as DNA are damaged irreparably. Therefore, the possible mechanism for the antimicrobial activity of AITC might be via an indirect effect of its pro-oxidant action. Recently, increasing evidence has shown a close relationship between ROS accumulation and decreased fruit susceptibility to decay after harvest. Liu, Jiang, Bi, and Luo (2005) found higher levels of \( \text{H}_2\text{O}_2 \) content were correlated with lower decay incidence in peach fruit treated with benzo-(1,2,3)-thiadiazole-7-carboxothioic acid 5-methyl ester (BTH). Similarly, lower susceptibility of early harvested apple fruit to \( P. \text{expansum} \) infection was closely related to increased \( \text{H}_2\text{O}_2 \) content (Torres, Valentines, Usall, Vinas, & Larrigaudiere, 2003). Salicylic acid treatment increased \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) contents and enhanced resistance to anthracnose rot of mango fruit (Zeng, Cao, & Jiang, 2006). In the present study, AITC-treated fruit had a significantly higher level of \( \text{H}_2\text{O}_2 \) compared to controls. These results suggest that enhanced \( \text{H}_2\text{O}_2 \) generation may be one of the major factors that trigger the disease resistance in AITC-treated blueberry fruit.

In conclusion, our results indicate that AITC-treated blueberries reduced decay, but did not increase the amounts of phenolic compounds, anthocyanins, antioxidant capacity, or the capacity of the tissue to scavenge excess ROS in fruit tissue. The reduction of decay in fruit tissue by AITC may be due to its pro-oxidant action by paradoxically generating additional amounts of ROS to inhibit the growth and proliferation of microbial cells. This treatment may be a potential substitute for commercial fungicides to control pathological rots on blueberries. Further studies are necessary to determine the effect of AITC on the sensory quality of fruit.

References


