LWT - Food Science and Technology 63 (2015) 383–390

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

The mechanism of ethanol treatment on inhibiting lettuce enzymatic browning and microbial growth

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ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form
22 February 2015
Accepted 1 March 2015
Available online 12 March 2015

Keywords:
Ethanol
Lettuce
Enzymatic browning
Phenylalanine ammonia lyase
Microbial growth

ABSTRACT

Tissue browning greatly affects the quality and consumer acceptance of fresh-cut lettuce. Unfortunately, effective antibrowning agents that either have antimicrobial activity or compatibility are hard to find. This study investigated the effects of ethanol treatment on enzymatic browning and microbial growth on lettuce stem discs. Lettuce stem discs were treated with ethanol (200 mL/L) for 2 min, drained by salad spinner, packaged in Ziploc® bags and stored at 5 °C. Stem discs treated with ethanol exhibited better appearance and lower microbial loads than untreated samples within 14 days of storage time. Phenylalanine ammonia lyase mRNA level and enzymatic activities in the ethanol treated group were significantly suppressed, and total phenols and quinones were also reduced. In addition, ethanol treatment increased CO_2 production and reduced O_2 level inside the package. In comparison to heat shock, trans-cinnamaldehyde and salicylic acid immersion, ethanol treated lettuce stem discs had the least browning and best quality attributes. These results indicated that 200 mL/L ethanol treatment was the most effective approach to inhibit enzymatic browning and control microbial growth on lettuce stem discs.

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1. Introduction

Enzymatic browning of fresh-cut fruits and vegetables is a costly problem for the fresh-cut produce industry because it has an adverse effect on appearance, aroma, flavor, and nutritional value (Toivonen & Brummell, 2008), detracts from customer acceptance, and reduces product shelf life. The main enzyme responsible for browning in fruits and vegetables is polyphenol oxidase (PPO), which catalyzes the oxidation of polyphenols to form colored quinones. In intact tissues, the enzyme is located in the cytoplasm while their substrates are located in the vacuoles (Chazarra, Garcia-Carmona, & Cabanes, 1999). Damaging lettuce tissue by cutting results in the mixing of the enzyme and substrates, and exposures to oxygen leading to the browning reaction (Degl'Innocenti, Guidi, Pardossi, & Tognoni, 2005). Saltveit (2000) suggested that altered phenol metabolism was involved in browning of lettuce leaf tissue. Polyphenols are produced by a complex set of interrelated metabolic reactions: 1) the shikimate pathway by which phenylalanine is produced from phosphoenolpyruvate and erythrose 4-phosphate; 2) the phenylpropanoid pathway by which cinnamic acid, a precursor for the biosynthesis of polyphenols are generated; and 3) browning reactions by which polyphenols are oxidized to form quinones. Phenylalanine ammonia-lyase (PAL), which catalyzes the conversion of L-phenylalanine to cinnamic acid, is the rate-limiting enzyme in this series of reactions. Cutting or wounding can induce the PAL expression and increase its activity which leads to the accelerated tissue browning (Hisaminato, Murata, & Homma, 2001; Llorach, Martínez-Sánchez, Tomasbarberan, Gil, & Ferrers, 2008).

Control of enzymatic browning has always been a challenge to the food industry (Altunkaya & Gökmen, 2009). Sulfites have long been used as both antimicrobial preservatives and antibrowning agents, because they alter products generated in enzymatic reactions by forming colorless adducts and inactivate PPO by binding to the enzyme irreversibly. However, sulfites alter flavor and aroma, destroy thiamine and possibly other dietary components, and are currently restricted due to the hazard of allergic-like reactions in sulfite-sensitive asthmatics (U.S. Food and Drug Administration, 1986, 1990). The reducing agents, ascorbic acid and L-cysteine were found to be competitive inhibitors of PPO in lettuce

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http://dx.doi.org/10.1016/j.lwt.2015.03.004
0023-6438/Published by Elsevier Ltd.

(Altunkaya & Gökmen, 2008). The inhibitory effect of ascorbic acid was temporary and declined as ascorbic acid was converted to dehydroascorbic acid (Altunkaya & Gökmen, 2008). Cysteine was found to be a more effective inhibitor of PPO (Altunkaya & Gökmen, 2008). Cysteine forms colorless cysteine-quinone adducts during enzymatic oxidation which are competitive inhibitors of PPO (Richard-Forget, Goupy, & Nicolas, 1992). Acidulants such as citric acid have also been used extensively as PPO inhibitors. Citric acid exerts a double inhibitory effect on PPO, by lowering the pH (below that necessary for optimal PPO activity) and by chelating copper (Ibrahim, Osman, Sasari, & Abdul Rahman, 2004). Sodium chlorite was demonstrated to inhibit PPO and inactivate *E. coli* O15:H7 on fresh-cut apples (Luo, Lu, Zhou, & Feng, 2011). However, it caused too extensive tissue damage in fresh-cut lettuce at concentrations that were effective to prevent browning (Luo, unpublished).

It has been shown that there is a correlation between PAL activity and browning in lettuce leaves (Hisaminato et al., 2001; Murata, Tanaka, Minoura, & Homma, 2004; Tanaka et al., 2011). CO_2 can inhibit browning of physically damaged plant tissue by lowering the level of phenolic compounds (Siriphanich & Kader, 1985). Several other physical and chemical methods can control browning by affecting PAL. Heat shock (HS) or mild heat treatment at 50–60 °C inhibited enzymatic browning of cut lettuce by suppressing cutting-induced PAL activity (Murata et al., 2004). Salicylic acid (SA) (Peng & Jiang, 2006) and trans-cinnamaldehyde (Martiñon, Moreira, Castell-Perez, & Gomes, 2014) in appropriate concentration were also used to control browning in fruits and vegetables. However, there is no antibrowning agent with merit of effectively controlling microbial growth of spoilage and pathogen in fresh-cut produces. Ethanol is a generally recognized as a safe (GRAS) product and exits naturally in plants with low toxicity for fruits and vegetables. It has been used to retard tissue senescence (Perata & Alpi, 1991), maintain the quality of intact apples, grape, cherry, peaches, mango, fresh-cut eggplant (Chervin, Westercamp, & Monteils, 2005; Hu, Jiang, Tian, Liu, & Wang, 2010) and asparagus spears (Herppich, Huyskens-Keil, & Hassenberg, 2014). In this work we evaluated the effect of ethanol application to simultaneously control the browning and microbial growth of lettuce tissue after it was wounded by cutting. We also compared the antibrowning effects of ethanol treatment with other recently reported inhibitors.

2. Materials and methods

2.1. Lettuce stem discs and storage

Commercially grown and harvested crisp-head iceberg lettuce (*Lactuca sativa* L.) were purchased from a local retailer and stored at 1.5 °C. About 3000 stems were cored from the lettuce head. After removing the top and bottom portion of each stem, the middle portion was cut into discs with 2 mm thickness (about 2 g) each according to Tomás-Barberán, Gil, Castañer, Artés, and Saltveit (1997). About 5–7 discs were obtained from each stem.

2.2. Treatment of stem discs

Twenty five lettuce stem discs (about 50 g) for each treatment were immersed into 0, 100, 200 and 300 mL/L (v/v) ethanol solution (diluted with distilled water) at 15 °C for 2 min. Excess moisture was removed using a salad spinner. Untreated stem discs served as control. Treated and untreated discs were sealed in Ziploc® sealable polyethylene bags (18 cm × 16 cm) and stored at 5 °C for 21 days. Similarly, the stem discs were treated with 0.5 g/L *trans*-cinnamaldehyde solution (CD) at 15 °C for 30 min (Martiñon et al., 2014), heatshock (HS) by immersing in 50 °C water for 1.5 min (Murata et al., 2004), or 1 g/L salicylic acid solution (SA) at 15 °C for 1 min (Peng & Jiang, 2006).

2.3. Reagents

Ethanol was purchased from PHARMCO-AAPER (CT, USA), trans-cinnamic acid was purchased from Aldrich Chemical Company, Inc. (WI, USA), *trans*-cinnamaldehyde, phenylalanine and salicylic acid were purchased from Sigma–Aldrich, Inc. (MO, USA).

2.4. Evaluation of tissue browning

A Minolta Chroma Meter CR-300 (Minolta Corp., Osaka, Japan) was used to assess the color of lettuce stems discs. In order to account for within-stem and within-bag color variation among cut surfaces of lettuce stem discs, two measurements per lettuce stem disc and six measurements per bag were taken. The means of L^* a^*, and b^* from 12 readings were recorded for each replication on each sampling day. The Chroma meter was calibrated with a standard white plate ($Y = 94.00$, $x = 0.3158$, $y = 0.3322$). Color was measured on day 0, 4, 7, 14 and 20.

2.5. Enzyme extraction and assay

PAL activity was measured as described (Degl'Innocenti et al., 2005) with some modification. About two g frozen lettuce stem discs from the mixture of 25 discs were homogenized at 4 °C with 16 mL of 50 mmol L^{-1} borate buffer (pH 8.5) containing 5 mmol L^{-1} 2-mercaptoethanol and 0.2 g of polyvinylpyrrolidone. The homogenate was filtrated through 4 layers of cheesecloth and centrifuged at 20,000 × *g* at 4 °C for 10 min. The supernatant was assayed for PAL activity after the addition of 0.55 mL of 50 m mol L^{-1} L-phenylalanine and incubation at 40 °C for 1 h. The samples were measured at 290 nm using the Shimadzu PharmaSpec UV-160A UV–Vis spectrophotometer (Shimadzu Scientific Instrument, MD, USA) before and after incubation. One unit of PAL activity equals the amount of PAL that produced 10^{-3} mol of transcinnamic acid per kg fresh discs in 1 h. PAL activity was measured daily from day 0 to day 6 of storage.

2.6. Polyphenol compound determination

Phenolic compounds were extracted as previously described (Ke & Saltveit, 1988). Briefly, 2.5 g of tissue was homogenized with 5 mL of methanol using a VWR VDI 25 homogenizer (VWR International LLC., PA, USA) at high speed for 30 s. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 × *g* for 15 min. The supernatant was used directly to measure the browning potential and soluble o-quinones. The absorbance of an aliquot of the supernatant was measured at 320 nm to determine the browning potential, and at 437 nm to determine the relative concentration of soluble o-quinones using the Shimadzu UV-160A UV-VIS recording spectrophotometer. Measurements were taken daily from day 0 to day 6 of storage.

2.7. Analysis of microbiological profile

Lettuce stem discs (10 g) from each package were macerated in 90 mL sterile phosphate buffered saline (PBS) for 25 min and then homogenized for 1 min. The lettuce stem solution was then serially diluted with PBS, and plated on an appropriate medium using spiral plater (Microbiology International, MD, USA). The aerobic mesophilic bacterial count (AMB) and yeast and mold (YM) counts were determined by plating the samples on trypticase soy agar (BD, NJ, USA) incubating at 36 °C for 24 h, and on potato dextrose agar (BD,

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