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Pinking of lettuce

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ABSTRACT

The mid-ribs of whole and processed (i.e., fresh-cut) lettuce can develop a pink discoloration that reduces consumer acceptance. Both biotic and abiotic stresses stimulate the phenylpropanoid pathway in lettuce to produce a number of *o*-diphenol compounds. Caffeic acid, an intermediate in this pathway, usually reacts with quinic or tartaric acid to form the *o*-diphenols chlorogenic and isochlorogenic acids, or caffeoltartaric and dicaffeoltartaric acids, respectively. The concentration of these higher molecular weight *o*-diphenols is usually low in unstressed lettuce, but they can accumulates to levels that can produce tissue discoloration after the tissue has been stressed (e.g., wounded). The constitutive enzyme polyphenol oxidase (PPO) converts *o*-diphenols into reactive *o*-quinones. Diversion of caffeic acid from the phenylpropanoid pathway by reaction with PPO produces caffeic acid *o*-quinone that is pinkish in color, while PPO activity on the accumulated higher molecular weight *o*-diphenols (e.g., chlorogenic acid) produces *o*-quinones that are brownish in color. Some of these higher molecular weight *o*-diphenols (e.g., chlorogenic acid) can act as antioxidants and reduce the formation of pink *o*-quinone from the action of PPO on caffeic acid. The interplay among the phenylpropanoid pathway, the accumulation and sequestering of the phenolic compounds produced, the activity of PPO, the mixing and reaction of the *o*-diphenols with PPO, and the reaction of the *o*-quinones produced can account for the variability in pinking in lettuce.

1. Introduction

Tissue discoloration (e.g., brown stain, edge browning, pinking) reduces the quality and consumer acceptance of whole and processed lettuce. Biotic and abiotic stresses encourage the synthesis and accumulation of phenolic compounds, and some of these compounds can participate in reactions leading to tissue discoloration. Unlike produce that quickly discolor when cut because of high endogenous levels of phenolic compounds, lettuce is usually low in such compounds and only acquires the ability to quickly discolor upon wounding when some stress had previously induced the synthesis and accumulation of specific phenolic compounds (Garcia et al., 2017; Saltveit et al., 2016). Browning of the cut edges is the usual response of lettuce to wounding. However, in some incidences, lettuce tissue will develop a pink discoloration in the field, or over time in storage after processing (Monaghan et al., 2017). This pink discoloration may progress to a darker color, or it may disappear during addition time in storage.

The first enzyme in the pathway for the synthesis of many phenolic compounds in lettuce is phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) (Tomás-Barberán et al., 1997; Saltveit, 2017). In wounded or stressed lettuce tissue, increased activity of PAL and the subsequent phenylpropanoid pathway produces a number of hydroxycinnamic acids (i.e., phenylpropanoid compounds having a C6–C3 skeleton) (Fig. 1). This pathway proceeds from phenylalanine to *trans*-cinnamic acid to *p*-coumaric acid to caffeic acid (CA, 3,4-dihydroxy cinnamic acid). CA is one of the pivotal intermediates in the phenylpropanoid pathway. The enzyme involved in the conversion of *p*-coumaric acid into CA is a plant-specific cytochrome P450 dependent monooxygenases; *p*-coumarate 3-hydroxylase (C3H) (Lin and Yan, 2012). Due to its instability and membrane-bound property, the purification and characterization of C3H is difficult (Kim et al., 2011).

An ester bond formed between CA and quinic or tartaric acid produces chlorogenic acid (CHL; 5-caffeoylquinic acid), or caffeoyltartaric acid (5-caffeoyltartaric acid), respectively. CHL is formed when the caffeoyl moiety of caffeoyl-CoA is transferred to quinic acid (Stockigt and Zenk, 1974). Further condensation of these compounds with an additional CA yields isochlorogenic acid (3,5-dicaffeoylquinic acid), and 3,5-dicaffeoyltartaric acid. The predominant phenolic compound accumulated in wounded Romaine lettuce is CHL (Tomás-Barberán et al., 1997).

The most common oxidation products of CA and related *o*-diphenols (i.e., caffeic acid derived phenylpropanoid molecules containing two adjacent (*ortho*) hydroxyl groups) by polyphenol oxidase (PPO, EC 1.14.18.1, also called tyrosinase and laccase) are *o*-quinones (e.g., CAQ,

Abbreviations: CA, caffeic acid; CAQ, caffeic acid *o*-quinone; CHL, chlorogenic acid; CHLQ, chlorogenic acid *o*-quinone

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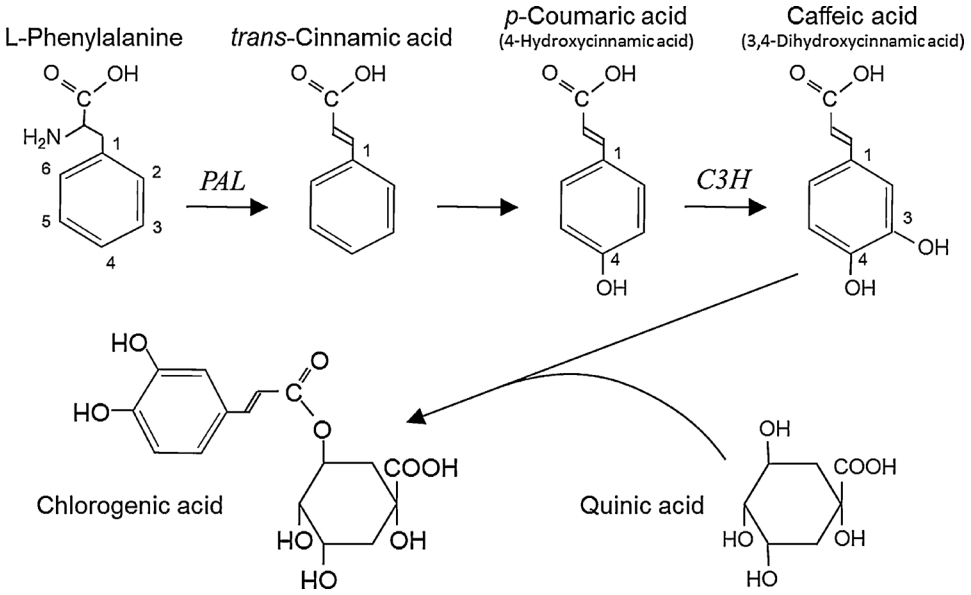

Fig. 1. A simplified phenylpropanoid pathway showing the synthesis of caffeic and chlorogenic acid from L-phenylalanine. Enzymes involve are phenylalanine ammonia-lyase (PAL) and *p*-coumarate 3-hydroxylase (C3H). Intervening steps are not shown to improve clarity.

caffeic acid quinone; CHLQ, chlorogenic acid quinone) (Rawel and Rohn, 2010; Yoruk and Marshall, 2003). PPO can catalyze the hydroxylation of monophenols to *o*-diphenols, and the oxidation of *o*-diphenols to *o*-quinones (Toivonen and Brummell, 2008). These oxidative reactions requite molecular O_2, and the rate of these reactions is reduced as O_2 availability becomes limiting. The *o*-quinones produced by PPO have the two hydroxyl groups on the benzene ring of the *o*-diphenol converted to the double bonded oxygen characteristic of quinones; producing a much more reactive molecule (Oszmianski and Lee, 1990).

Lettuce contains several isozymes of PPO in both active and latent forms (Chazarra et al., 1996), and PPO's greatest substrate specificity is for CA and CHL (Altunkaya and Gokmen, 2008). PPO is localized in plastids (e.g., chloroplast), and is released into the cytosol upon wounding or senescence. They both found that a pH of 7.0 was optimal for lettuce PPO.

The oxidation of *o*-diphenols to *o*-quinones can be reversed by antioxidants (e.g., ascorbic acid, L-cysteine) that reduce the *o*-quinones to their colorless *o*-diphenol precursors. A number of intermediates and end products of the phenylpropanoid pathway exhibit antioxidant properties (Shahidi and Chandrasekara, 2010) with CHL being one of the most potent antioxidative phenolic acids (Chen and Ho, 1997; Curveier et al., 1992). CA is also reported to have antioxidant capacity (Niggeweg et al., 2004). The antioxidant capacity of lettuce increases after wounding (Kang et al., 2002) in concert with an increase in the concentration of phenolic compounds (Tomás-Barberán et al., 1997).

The polymerization of *o*-quinones produces complex brown products of high molecular weight (Pierpoint, 1969; Hunter et al., 2017). The *o*-quinone of CA is pink and the products of its reactions with some amino acids can produce additional pink colored compounds (Bittner, 2006; Pierpoint, 1966; Nicolas et al., 1994). Many natural and artificial coloring substances (e.g., dyes and pigments) are quinone derivatives (e.g., Alizarin or 1,2-dihydroxyanthraquinone has been isolated from the Madder root and used as a red dye since antiquity). Pierpoint (1969) observed that the colors produced during the oxidations of CA and CHL were more characteristic of the phenol being oxidized than of the amino acid present. In the presence of amino acids, CHLQ radicals reacts to form green pigments (Pierpoint, 1969; Namiki et al., 2001), while in the presence of PPO, CHL solutions first turned brown and later darken during the next few hours.

The extent of tissue discoloration is not directly related to the concentration of phenolic compounds in the tissue or the activity of their oxidizing enzymes (Cantos et al., 2001; Mai and Glomb, 2013). Interaction among various metabolic pathways, and the compartmentation of products and reactants could account for the lack of a direct relationship between phenolic content, enzyme activity and tissue discoloration.

An assay (wherein excised lettuce leaf disks are laid on wet filter paper and subsequently produces a pink border around the disks on the wet filter paper) has been used to select cultivars of lettuce with reduced browning potential (Van Dun, 2014). After 4 days at 5 °C the formation of a pink border at the edges of the excised leaf disks became apparent, reaching a maximum intensity after one week. Individuals from a mutated population of lettuce plants were selected that exhibited reduced discoloration about the disk's border. Mature lettuce plants selected from these populations exhibited reduced edge browning upon processing.

If the subsequent reactions leading from CA to CHL formation and possible subsequent tissue browning were curtailed, the accumulation of CA and its conversion to CAQ in lettuce could produce pinking. Research reported in this paper was undertaken to examine the participation of phenolic metabolism in the development of pink discoloration in lettuce tissue. Spectra of solutions containing CA and/or CHL with lettuce tissue extracts having PPO activity showed several characteristic changes in absorbance at specific wavelengths. These wavelengths were used to follow the production of *o*-quinones from their respective *o*-diphenols and the corresponding changes in color of the solutions.

All experiments were performed with both a PPO active lettuce tissue extract and with purchased purified mushroom PPO (also called tyrosinase or laccase). The lettuce tissue extract probably contained many active enzymes. Experiments that used the purchased purified mushroom PPO were used to show that the observed spectral changes caused by the lettuce tissue extract were consistent with PPO activity. While similar changes were observed with both lettuce tissue extracts and purified mushroom PPO, results from the PPO active lettuce tissue extracts are presented to maintain the focus of this paper on the cause of pinking in lettuce.