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Phenylalanine ammonia lyase inhibitors control browning of cut lettuce

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Abstract

Inhibitors of the first enzyme in the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL), were used to investigate the role of phenolic metabolism in browning of lettuce tissue. Excised 4×7-cm midrib segments were soaked for 1 h at 20°C in aqueous solutions of the PAL inhibitors, α-aminooxyacetic acid (AOA; 0.1–10 mM), 2-aminoindan-2-phosphonic acid (AIP; 50–100 μM), and α-aminooxi-β-phenylpropionic acid (AOPP; 200 μM). Browning of the cut ends and uncut surfaces was measured using a visual score, and CIE color values (L^*, a^*, b^*). Overall browning potential was measured as the absorbance at 340 nm of an aqueous extract of the tissue. The visual scores were more highly correlated with hue angle than with the a^* and b^* values; there was no correlation with the L^* values. Ethylene applied at 5 μl l^{-1} had no effect upon browning compared with the air treatments. AIP at 50 μM and AOPP at 200 μM effectively inhibited browning; AOA was less effective requiring 3–10 mM to reduce browning. These results confirm the view that for browning to occur in lettuce PAL activity is required to form phenolics that are subsequently oxidized and polymerized.

Keywords: 2-Aminoindan-2-phosphonic acid; α-Aminooxyacetic acid; α-Aminooxi-β-phenylpropionic acid; *Lactuca sativa*; PAL; Phenolic metabolism; Phenylpropanoid metabolism

1. Introduction

Lettuce quality and shelf-life are decreased by the development of tissue browning. Some of the most common postharvest browning disorders of whole head and cut iceberg lettuce tissue are russet spotting (RS), senescent browning (SB) and brown stain (BS) (Saltveit, 1997). Increased use of minimally processed lettuce, and increased restrictions on chemical treatments to prevent browning provide an impetus to better understand the causes of browning and how they can be controlled.

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Altered phenolic metabolism is thought to be involved in lettuce tissue browning. The first committed step in the phenylpropanoid pathway is the conversion of the amino acid L-phenylalanine to *trans*-cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL; EC 4.3.1.5) (for a review see Hahlbrock and Scheel, 1989). Subsequent reactions produce several new compounds, among which 5-caffeoylquinic acid (chlorogenic acid), 3,5-dicaffeoylquinic acid, caffeoyltartaric acid and dicaffeoyltartaric acid have been associated with increased browning in lettuce (Tomás-Barberán et al., 1997a,b). Both wounding (e.g. cutting) and exposure to the plant hormone ethylene (e.g. during storage and transport) stimulate the phenylpropanoid pathway and induce new enzymatic activity leading to increased production of the major phenolic compounds and the synthesis of new compounds (Ke and Saltveit, 1989a; Tomás-Barberán et al., 1997a).

Inhibition of PAL activity by compounds such as 2-aminoindan-2-phosphonic acid (AIP), α-aminooxyacetic acid (AOA), and α-aminooxi-β-phenylpropionic acid (AOPP) has been well characterized in several plant tissues (Amrhein and Gödeke, 1977; Amrhein, 1979; Hanson and Havir, 1981; Zón and Amrhein, 1992). The inhibitors are thought to directly affect PAL enzyme activity since AOPP and AIP did not affect the activity of phenylalanine–tRNA synthetase (Leubner-Metzger and Amrhein, 1994; Zón and Amrhein, 1992).

Several studies have described a relationship between PAL activity in lettuce leaf tissue and the development of RS and brown discoloration (i.e. SB and BS) (Hyodo et al., 1978; Ke and Saltveit, 1986, 1988, 1989a,b; Ritenour et al., 1996; Peiser et al., 1998). Increased PAL activity has also been correlated with a decrease in the subsequent shelf-life and overall visual quality of minimally processed lettuce (Couture et al., 1993; López-Gálvez et al., 1996). An increase in PAL activity was observed before the appearance of browning in minimally processed lettuce. It has been proposed that this increase in PAL activity could be used as a predictive index of shelf life (Couture et al., 1993; López-Gálvez et al., 1996). However, inhibitors of PAL activity have not been used to demonstrate a direct link between PAL activity and the development of any of these browning disorders.

We recently showed that PAL inhibitors affected phenylpropanoid metabolism and the color of RS lesions, but they did not prevent RS lesion development in lettuce midribs exposed to ethylene (Peiser et al., 1998). It appears that RS lesion development is independent of the increase in PAL activity and the accumulation of phenolic compounds that contribute to browning. In an analogous fashion, it is important to directly demonstrate a link between tissue browning and wound-induced stimulation of PAL activity since tissue browning may be relatively independent of a wound-induced stimulation of PAL activity. Therefore, the objective of this study was to further examine the role of PAL in the development of browning by using inhibitors of PAL activity. We present evidence indicating that a wound-induced increase in PAL activity is a prerequisite for the development of brown discoloration in cut lettuce tissue.

2. Materials and methods

2.1. Lettuce samples and tissue preparation

Iceberg lettuce (*Lactuca sativa* L.) was obtained from a local supermarket (unknown cultivar), a wholesale distributor ('Salinas', 'Honcho-2') or a shipper/processor in Arizona ('Desert Storm'). Whole lettuce heads were used immediately after arrival at the Mann Laboratory or stored at 2.5°C until used.

Lettuce wrapper and core leaves were discarded and the next five to eight leaves were gently removed from the core and the white midribs were excised from the green part of the leaf. Midrib segments were trimmed to produce 4×7-cm sections, and both ends of each section were cut with a razor blade at a 45° angle to provide a large wounded surface area for colorimeter readings. Sections were rinsed with chlorinated water (1:20 dilution of 5% commercial bleach) and centrifuged with a manual salad spinner to remove excess solution. Pieces were randomly mixed and

treated with the appropriate inhibitor solutions (see below). After treatment, ten midrib pieces were placed in 2-l plastic containers and connected to air flow-through systems to provide atmospheres of humidified air or air $+5\ \mu l\ l^{-1}$ ethylene at 5°C.

2.2. Treatments

Midrib sections were soaked for 1 h at 20°C in aqueous solutions of the PAL inhibitors α-aminooxyacetic acid (AOA) at 0, 0.1, 0.3, 1, 3 and 10 mM, 2-aminoindan-2-phosphonic acid (AIP) at 0, 50 and 100 μM, and α-aminooxi-β-phenylpropionic acid (AOPP) at 0 and 200 μM. AOA and AOPP were purchased from Sigma (St. Louis, MO) and Cambridge Research Biochemicals (Northwich, Cheshire, UK), respectively. AIP was kindly provided by Dr Nikolaus Amrhein. After treatments, pieces were centrifuged with a manual spinner to remove excess solution.

2.3. Respiration measurements

Carbon dioxide production from excised midrib lettuce sections was determined by taking 1-ml samples of the inlet and outlet air flow from the plastic containers and injecting them into an infrared gas analyzer as previously described (Saltveit and Strike, 1989). Production of CO_2 was determined from the difference in CO_2 concentration between the inlet and outlet flows, the rate of flow, and the fresh mass of the tissue.

2.4. Browning measurements

Midrib browning was measured by the following two methods. CIE color values (L^*, a^*, b^*) of the cut and uncut surfaces were measured using a Minolta model CR-2002 colorimeter (Minolta, Ramsey, NJ) with illuminant A and 10° viewing angle (McGuire, 1992). The colorimeter was calibrated using a white standard tile ($L^* = 97.63$, $a^* = -0.53$, $b^* = 2.38$). Subjective evaluation of the browning of the cut ends and uncut surfaces was determined visually using a 1–5 hedonic scale: 1, none; 3, moderate; and 5, severe browning. Overall browning potential was measured as the absorbance of an aqueous extract of the midrib tissue at 340 nm as described by Couture et al. (1993). This last method measured browning from the entire tissue and did not distinguish between the cut and uncut areas.

3. Results

We used three different methods to access the browning of the midribs: visual evaluation, measuring the CIE (L^*, a^*, b^*) color values, and measuring the change in absorbance at 340 nm of aqueous extracts of the midribs. Browning of the cut and uncut surfaces could be determined by visual evaluation and with the colorimeter, while the absorbance at 340 nm was essentially an average of the browning in all of the tissue.

Visual scores for browning on the cut ends and uncut surfaces were compared with the L^*, a^* and b^* values. There was a good correlation between the visual scores and hue angle ($\tan^{-1}(b^*/a^*)$) and the a^* values (Table 1). The correlation with the b^* values was lower and no significant correlation was found with the L^* values. Hue angle values decreased as browning occurred (Fig. 1). In the control samples, most of the browning on the cut and uncut surfaces occurred during the first 6 days. Both 50 and 100 μM AIP completely inhibited browning on the cut and uncut surfaces during the first 3 days. By days 6 and 9 there was a small amount of brown-

Table 1
Correlation coefficients between CIE color values a^*, b^* and hue angle ($\tan^{-1}(b^*/a^*)$) and visual quality scores of the cut and uncut surfaces of lettuce midrib tissue sections stored at 5°C

Tissue	Color values		
	Hue angle	a^*	b^*
Cut surfaces	−0.9237	0.7889	0.8132
Uncut surfaces	−0.9700	0.9212	0.3413

Coefficients were calculated from average values of five midrib sections measured at days 0, 3, 6 and 9 from the control, 50 and 100 μM AIP treatments held in air with and without 5 $\mu l\ l^{-1}$ ethylene.

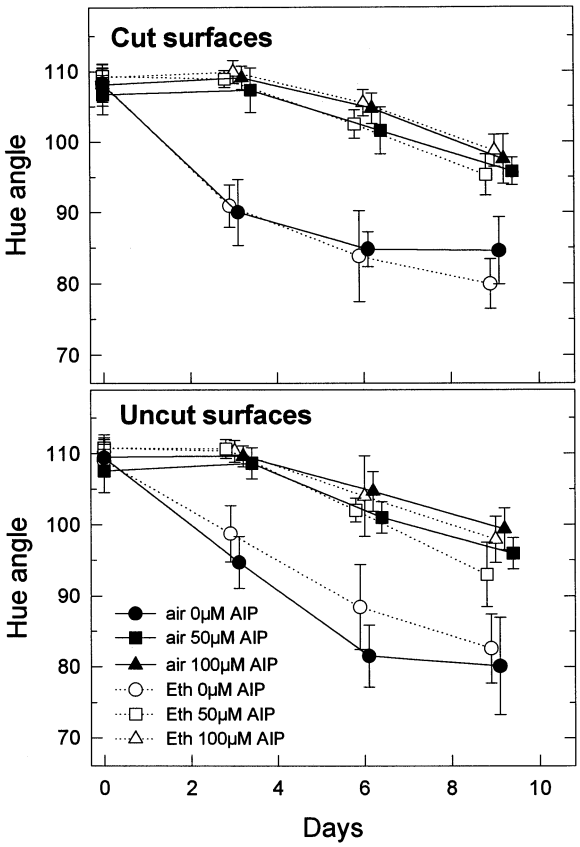

Fig. 1. Effect of 0, 50 and 100 μM 2-aminoindan-2-phosphonic acid (AIP) on hue angle ($\tan^{-1}(b^*/a^*)$) values measured on the cut ends and uncut surfaces of lettuce midrib sections held in air ± 5 μl l^{-1} ethylene at 5°C for 9 days. Each value is the average $\pm$ S.D. from five midrib sections.

ing in the inhibitor treatments, but this was still much less than in the control samples. In the experiment shown in Fig. 1, there were only slight differences between the development of browning in cut and uncut tissues for both control and treated tissue segments. In other experiments, however, the amount of browning of the uncut surfaces was much less than that of the cut surfaces even though the effect of the inhibitors on reducing browning was essentially the same as shown in Fig. 1 (data not shown).

Browning of the cut and uncut surfaces in the presence of ethylene was the same as that of air-stored pieces (Fig. 1). Browning was also similar for the air and ethylene controls in other experiments (data not shown).

AOPP at 200 μM and AOA at 3 mM also inhibited browning and resulted in a similar pattern for hue angle values as AIP (data not shown). Though AOA inhibited browning, a yellowish color developed on the cut and uncut surfaces of the midribs by day 6 and this was reflected as higher b^* values (i.e. more yellow) compared with the controls (data not shown). Because of this problem with AOA, it was only used in the preliminary experiments.

Results from the 340-nm absorbance determinations of aqueous extracts of midrib pieces also showed that the overall browning potential was strongly inhibited by AIP over the 9-day storage

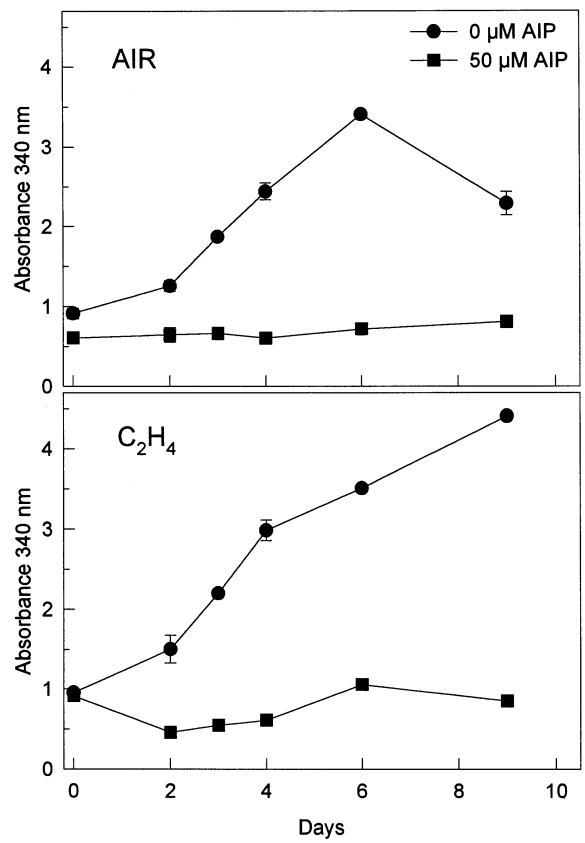

Fig. 2. Effect of 0 and 50 μM 2-aminoindan-2-phosphonic acid (AIP) on the absorbance at 340 nm of aqueous extracts of lettuce midrib sections held in air ± 5 μl l^{-1} ethylene at 5°C for 9 days. Each value is the average $\pm$ S.D. from three midrib sections.

period (Fig. 2). As demonstrated previously for hue angle, these measurements showed that the air and ethylene treatments were essentially the same. Although the 340-nm absorbance values in air control samples dropped at day 9 while they continued to increase in the ethylene treatment, this decreased in the air controls did not occur in all experiments (data not shown).

AIP had little to no effect upon the respiration rate of the lettuce midrib sections. Generally the rates of CO_2 production ranged from 10 to 15 ml kg^{-1} h^{-1}. Ethylene did not consistently affect respiration rates; only in one experiment were CO_2 production rates elevated to 25–30 ml kg^{-1} h^{-1} in the presence of ethylene.

4. Discussion

The inhibitors used in this study have been well characterized in terms of their affect on PAL activity in other plant tissues (Amrhein, 1979), and in lettuce (Peiser et al., 1998). An HPLC examination of the phenolic compounds produced by wounded and ethylene exposed lettuce revealed that the PAL inhibitors AIP and AOPP greatly reduced the accumulation of phenolic compounds (Peiser et al., 1998). At a concentration of 50 μM, AIP inhibited the formation of 5-caffeoylquinic and dicaffeoyltartaric acids in cut midribs of iceberg lettuce by 92 and 98%, respectively. These results indicate that the initial steps in phenylpropanoid metabolism in lettuce are inhibited by these compounds in vivo. AIP also greatly inhibited PAL activity in an in vitro assay of the supernatant fraction from lettuce midrib tissue homogenate with an apparent K_i of about 22 nM (Peiser et al., 1998).

The correlation between the visual browning scores and hue angle was slightly higher than that for the a^* values (Table 1). This is consistent with the results of Heimdal et al. (1995) using cut iceberg lettuce.

In the presence of AIP browning was strongly inhibited as determined by the visual scores, hue angle values (Fig. 1) and by the 340-nm absorbance of tissue extracts (Fig. 2). We have previously demonstrated that these concentrations of AIP inhibit PAL activity and the production of phenolics in lettuce midribs (Peiser et al., 1998). The results using the PAL inhibitors, particularly those with AIP, further support our view that browning of cut lettuce midrib tissue results from the increased production of phenolics via PAL and their subsequent oxidation and polymerization.

Presumably browning of the uncut surfaces resulted from the stress of wounding during preparation that induced PAL activity and compromised membrane integrity which disrupted cellular compartmentalization and allowed poly-phenol oxidase to come into contact with the phenolics produced by PAL. In healthy cells, polyphenol oxidase is in the cytoplasm and the phenolic compounds are in the vacuole (Mayer, 1987). We have shown that wounding can cause an increase in PAL activity up to 2.5 cm from the wound site in lettuce tissue (Ke and Saltveit, 1989a). Since a chain of physiological events is known to occur in the uncut surfaces before browning appears, variables such as conditions in the field, handling during transport, and variety of the lettuce could have influenced these events and caused a variability in browning of the uncut surfaces in the different experiments. For example, we observed that the amount of browning on the uncut compared to the cut surfaces was similar (Fig. 1) to much less (data not shown). Since browning of the injured tissue at the cut surfaces is more strongly induced by the adjacent wound than is browning of cells on the surface that are distant from the wound, the pre-treatment variables should have much less of an effect upon cut surface browning. Indeed, we observed that the extent of edge browning, as indicated by the hue angle values, was quite consistent for the same treatment in all three experiments.

The inhibitors strongly inhibited browning in both cut and uncut tissue. As indicated by the lack of a change in the hue angle values, no browning occurred by day 3 in either the cut or uncut surfaces in the presence of both 50 and 100 μM AIP (Fig. 1). However, by days 6 and 9 there was a small amount of browning (lower hue angle). Similar results were also obtained with 200 μM AOPP. The small amount of browning on

days 6 and 9 may have resulted from the dilution or metabolism of the inhibitors to such an extent that they were unable to completely inhibit PAL which was continually being synthesized by the tissue. Levels of PAL enzyme (Amrhein and Gerhardt, 1979; Noé and Seitz, 1982) and PAL mRNA (Bolwell et al., 1988) are known to increase when PAL activity is inhibited. This increase is due to the lack of feedback regulation on PAL synthesis resulting from the very low cinnamic acid levels in the presence of inhibitors (e.g. in the presence of AOPP) (Bolwell et al., 1988; Mavandad et al., 1990). Presumably AIP would have a similar effect on PAL enzyme and mRNA levels.

Couture et al. (1993), using 340-nm absorbance measurements, found that minimally processed lettuce exposed to ethylene had a somewhat higher browning potential than the air controls. We did not observe an effect of ethylene upon browning as measured by either the hue angle or absorbance at 340 nm. The ethylene effect on browning is probably related to tissue sensitivity to ethylene and the degree of wounding; both of which varied from the preceding study.

Although we measured no effect of ethylene upon browning per se, ethylene does play a role in wound responses and leaf senescence (Abeles et al., 1992). Ethylene is produced by wounded tissue (Yang and Pratt, 1978; Ke and Saltveit, 1989a), and we have measured ethylene in commercial bags of Garden Salad at concentrations ranging from 0.2 to 0.9 μl l^{-1} (López-Gálvez et al., 1997). These concentrations are high enough to be physiologically active and potentially could cause increased senescence. However, in these particular bags, there may have been little or no effect of ethylene upon senescence since O_2 concentrations were low (0.15–0.8%) and CO_2 concentrations were high (6–20%). Ethylene action is inhibited at these concentrations of O_2 and CO_2 (Abeles et al., 1992).

Browning of cut lettuce greatly reduces its visual quality, and therefore it is very important to prevent these reactions. Although the PAL inhibitors used in this study were very effective at inhibiting browning, these chemicals are not approved for food use. Other compounds permitted as treatments on food, such as calcium chloride and acetic acid, have been found to reduce browning reactions in cut lettuce, but they are presently not being used commercially (Ke and Saltveit, 1986; Tomás-Barberán et al., 1997b). Commercially, browning in cut lettuce is inhibited principally by using modified atmosphere packaging that develops low O_2 and moderate CO_2 concentrations. Yet when O_2 concentrations become too low off-odor volatiles can be produced (Bolin and Huxsoll, 1991; Heimdal et al., 1995; López-Gálvez et al., 1997). Therefore, safe and effective chemical means to augment the benefits of less extreme modified atmospheres could be useful to prevent browning. Elucidation of the pathways and control points regulating browning in plant tissue could direct more fruitful research into their beneficial modification through genetic engineering.

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