Postharvest Biology and Technology 112 (2016) 105–113

Contents lists available at ScienceDirect

Postharvest Biology and Technology

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

Relationships between free and esterified fatty acids and LOX-derived volatiles during ripening in apple

Carolina Contreras[a], Henrik Tjellström[b], Randolph M. Beaudry[a,*]

[a] Department of Horticulture, Michigan State University, A22 Plant and Soil Sciences Building, Laboratory of Postharvest Physiology, East Lansing, MI 48824, USA

[b] Department of Plant Biology, Plant Lipid Metabolism Laboratory, Michigan State University, East Lansing, MI 48824, USA

ARTICLE INFO

Article history:
Received 20 April 2015
Received in revised form 22 September 2015
Accepted 16 October 2015
Available online 28 October 2015

Keywords:
Free fatty acid
Polar lipid
Aldehyde
LOX
Ester
Aroma
Flavor

ABSTRACT

Ripening in intact apple fruit is accompanied by an increase in the autonomous emissions of hexanol and esters derived from hexanol (hexyl esters). It is thought that these compounds are, to some extent, dependent upon hexanal synthesis by the action of lipoxygenase (LOX) on fatty lipids. To better understand the relationship between fatty acid substrates and their volatile products, we determined the content of oleic (18:1), linoleic (18:2), and linolenic (18:3) acids in free and polar (esterified) lipid fractions of the skin and subtending cortex of 'Jonagold' fruit throughout ripening. In the free fatty acid fraction, 18:1 and 18:2 content increased several-fold during ripening, but the 18:3 content remained low and unchanged. In the polar lipid fraction, ripening resulted in a modest increase in the 18:1 and 18:2 content and an extensive decline in the 18:3 content. The rise in 18:2 content of the free fatty acid fraction mirrored emissions of hexanol and hexyl esters in intact fruit and the decline in the 18:3 content of polar lipids reflected the drop in *cis*-3-hexenal emissions found for disrupted fruit tissue. For intact fruit, our results suggest that hexanal and its metabolites, hexanol and hexyl esters, may be derived from the action of LOX on 18:2 free fatty acid. For disrupted fruit tissue, the data suggest *cis*-3-hexenal and *trans*-2-hexenal are largely generated from the action of LOX on 18:3 in the polar lipid fraction. The mechanism whereby free 18:1 and 18:2 could accumulate without a concomitant increase in free 18:3 is not clear. To our knowledge, there is currently no synthetic or catabolic pathway characterized that might account for this finding.

1. Introduction

The lipoxygenase (LOX) pathway is one of several pathways known to contribute to the synthesis of aroma of fruits and vegetables (El Hadi et al., 2013; Muna et al., 2013; Sanz et al., 1997). In apple (*Malus* × *domestica* Borkh.), LOX pathway activity is responsible for many of the volatiles induced by cellular disruption, which are overwhelmingly represented by C-6 aldehydes (Dixon and Hewett, 2000; Fellman et al., 2000; Paillard, 1986; Paillard and Rouri, 1984). Disruption of tissues permits the comingling of lipases, lipids and the enzymes of the LOX pathway, leading to the formation of aldehydes from free fatty acids (Siedow, 1991). Oxidation of linolenic acid (18:3) by LOX enzymes that form hydroperoxides at the 13-position (13-LOX) yields *cis*-3-hexenal, which, in turn, can undergo isomerization to *trans*-2-hexenal (Contreras and Beaudry, 2013; Rowan et al., 1999). Oxidation of linoleic acid (18:2) by 13-LOXs yields hexanal. Oleic acid (18:1) lacks the 1- *cis*,4-*cis*-pentadiene structure of linoleic and linolenic acids and is not oxidized by LOX (Siedow, 1991), although a LOX-like enzyme has been found to produce hydroperoxides from oleic acid in *Pseudomonas* sp. (Guerrero et al., 1997). LOX enzymes that form hydroperoxides at the 9-position (9-LOX) yield C-9 aldehydes, which are important aroma compounds in cucumber (*Cucumis sativus*) (Takei and Ono, 1939).

The volatile profile of intact apple tissues differs from that of disrupted tissue primarily by lacking unsaturated aldehyde products of the LOX reactions (Contreras and Beaudry 2013). In 'Jonagold' apple fruit, *cis*-3-hexenal production from disrupted tissue declines during ripening, even as the production of *trans*-2-hexenal and hexanal increases (Contreras and Beaudry, 2013; Paillard, 1986). In that *cis*-3-hexenal, *trans*-2-hexenal, and hexanal are all products of 13-LOX activity, their contrasting behavior is suggestive of changes in fatty acid (substrate) availability and/or enzymatic activity.

Abbreviations: LOX, lipoxygenase; TLC, thin layer chromatograph; GC-FID, gas chromatograph-flame ionization detector; FFA, free fatty acid; FAME, fatty acid methyl ester.

* Corresponding author. Fax: +1 517 353 0890.
E-mail address: beaudry@msu.edu (R.M. Beaudry).

http://dx.doi.org/10.1016/j.postharvbio.2015.10.009
0925-5214/

Previous analysis of fatty acids in 'Golden Delicious' apple indicated that, within neutral lipid and free fatty acid fractions, content of 18:1 and 18:2 increased as ripening progressed while 18:3 levels remained constant (Song and Bangerth, 2003). Song and Bangerth (2003) did not see trends in the various fatty acids of the polar lipid fraction. Earlier work by Galliard (1968), using an unknown cultivar, suggested that total 18:3 levels in total lipids declined by about 50% with ripening, primarily due to its loss in galactosyl diglycerides, but found little to no enhancement in the content of 18:1 and 18:2 in the various lipid fractions. Free fatty acids were not evaluated. Paillard (1986), using 'Golden Delicious', showed a marked decline in the content of 18:3 in the total lipids and a 3-fold increase in 18:2 content. Defilippi et al. (2005) also found an increase in 18:2 content in the total lipids in the peel of ripening 'Greensleeves' apple.

In apple, most fatty acids are found esterified to polar lipids, a modest portion in the neutral lipids, and a very small portion comprises the free fatty acids (Meigh and Hulme, 1965; Christie, 2003). In the polar lipid fraction, the most abundant saturated and unsaturated fatty acids are palmitic (16:0) and linoleic acid, respectively (Paillard, 1990; Wang and Faust, 1992). Linolenic acid is the predominant fatty acid in monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), while linoleic acid is a common constituent in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Phosphatidylglycerol (PG) contains relatively more palmitic acid (Wang and Faust, 1992). The content of phospholipids has been shown to increase slightly during ripening in apple; however, the rate of synthesis of phospholipids rises many-fold, suggesting synthesis is balanced to some degree by concomitant conversion of phospholipids to other cellular constituents such as waxes and oils (Bartley, 1985; Galliard, 1968; Kolattukudy, 1966; Samuels et al., 2008).

While LOX pathway activity has the potential to produce copious quantities of aldehydes in disrupted fruit tissues, the extent to which it contributes to autonomously-produced volatiles from intact fruit is not clear, although its contribution has been inferred from a number of enzymatic, volatile, genomic and biochemical studies (Altisent et al., 2009; Contreras and Beaudry, 2013; Defilippi et al., 2005; Park et al., 2006; Schiller et al., 2015; Sugimoto et al., 2008). It has been suggested that during ripening cell walls and membrane become more permeable, allowing the LOX pathway to activate without tissue disruption (Sanz et al., 1997). However, in intact fruit, the LOX product *cis*-3-hexenal and its ester products are not reported (Fellman et al., 2000; Contreras and Beaudry, 2013), which suggests that 18:3 fatty acids are not oxidized by LOX in vivo and argues against the permeable membrane hypothesis. On the other hand, the production of hexanal, also a LOX product, is present prior to and throughout ripening. Thus, a seeming paradox exists. If LOX activity is responsible for the synthesis of C-6 precursors for esters in intact fruit, then how can hexanal, an 18:2 product, be produced without concomitant production of *cis*-3-hexenal from the abundant 18:3 fatty acids?

The answer may lie in the concentration and/or distribution of the fatty acids and 13-LOXs. In that LOXs primarily act on free fatty acids rather than esterified fatty acids (i.e., mono- and diacyl polar lipids) (Siedow, 1991), we hypothesize the selective production of hexanal can be explained by the de novo synthesis of 18:2 and its accumulation in the free fatty acid pool. Similarly, the aforementioned decline in *cis*-3-hexenal production by disrupted apple fruit tissue suggests a decline in the pool of lipids containing18:3 fatty acids. The aim of this work is to further clarify the relationship between developmental changes in LOX substrates and their products and the subsequent incorporation of these products into esters in both intact and disrupted tissues. Our objective is to document changes in the pool of fatty acids that exist as free, unesterified, fatty acids and fatty acids that are constituents of esterified membrane lipids, particularly polar lipids, and relate these changes to the production of aldehydes, alcohols and esters derived from these fatty acids in ripening apple fruit.

2. Materials and methods

2.1. Plant material and developmental study

'Jonagold' apples (*Malus* × *domestica* Borkh.) from a commercial orchard in Sparta, MI (43°06′13.9″N 85°41′56.0″W) were used to investigate the relationship between stage of development, volatile production, and the lipid profile and content of apple peels for intact fruit and for disrupted fruit tissue. The study was repeated in 2009, 2010, and 2012. The first harvest took place on 04 Sept. 2009, 08 Sept. in 2010 and 06 Sept. 2012. After the initial harvest, in 2009 and 2010, ripening fruit were harvested every three to four days, and in 2012 fruit were harvested on a weekly basis. Harvest continued until ripening was imminent as judged by two criteria: the average internal ethylene concentration (IEC) being greater than 0.1 $\mu L L^{-1}$ and 3–4 fruit of 20-fruit sample having an IEC greater than 10 $\mu L L^{-1}$. At that time, (09 Oct. 2009, 01 Oct. 2010, and 17 Sept. 2012), approximately 400 additional fruit were harvested and thereafter allowed to ripen in a controlled environment chamber at 15 °C. This was done to avoid damage in the field due to freezing and fruit drop. These fruit were covered with plastic bags to minimize moisture loss and subsequently examined every three to four days in 2009 and 2010, and once per week in 2012 until the conclusion of the study on 27 Oct. (day 53) in 2009, 20 Oct. (day 42) in 2010 and 29 Oct. 2012 (day 53). One day prior to each assay date, fruit were moved to the laboratory to equilibrate to room temperature (22 ± 1 °C). During this adjustment period, fruit were covered with black plastic bags to exclude light and minimize moisture loss as described previously.

On each evaluation date, 40 apples were used for analysis. Maturity was assessed on 20 randomly selected fruit by measuring internal ethylene ($\mu L L^{-1}$) as described in Contreras and Beaudry (2013), and fruit weight (g), red skin coloration (%), background color (1–5 scale), firmness (N), soluble solids content (%), and starch index (1–8) according to Mir et al. (2001). These data were used to assess the readiness of the fruit to ripen and accordingly schedule the final harvests as noted previously.

2.2. Ethylene, respiration rate and volatile analyses of whole and crushed apple tissue

For each assay date, the internal ethylene content of each of the remaining twenty fruit was measured and, of these, the five fruit having internal ethylene content nearest the median were selected for analysis of CO_2 production as described by Contreras and Beaudry (2013). After respiratory analysis, the inlet and outlet ports of the respirometer were sealed for 20–30 min and the accumulated volatiles were analyzed and quantified using the methodology of Contreras and Beaudry (2013). Identification of all quantified compounds was by comparison of the mass spectrum with authenticated reference standards where possible and with spectra in the national institute for standard and technology (NIST) mass spectral library (Search Version 1.5) (Anon, 2011). Volatile compounds were quantified as previously described by calibrating with a standard mixture of 28 aroma compounds prepared with authenticated compounds (Sigma–Aldrich Co., and Fluka Chemika) of known purity. The standard mixture contained equal volumes of each compound (ethanol, butanol, 1-hexanol, cis-3-hexenol, 2-methyl butanol, 3-methyl butanol, 2-methyl propanol, propanol, *trans*-2-hexen-1-ol, butyric acid, 2-methyl butyric, hexyl acetate, butyl acetate, butyl butanoate, butyl hexanoate, ethyl acetate, ethyl