Temperature-Dependent Autoxidation of Conjugated Trienols from Apple Peel Yields 6-Methyl-5-hepten-2-one, a Volatile Implicated in Induction of Scald

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Conjugated triene (CT) oxidation products of α-farnesene have long been thought to be involved in development of superficial scald in apple fruit. Early studies found that CT hydroperoxides and the volatile 6-methyl-5-hepten-2-one (MHO) are major in vitro autoxidation products of α-farnesene. However, it was recently shown that ≥99% of the oxidation products of α-farnesene that accumulate in apple peel are conjugated trienols (CTols), isomers of 2,6,10-trimethyldodeca-2,7,9,11-tetraene-6-ol. HPLC-purified CTols from fruit of two scald-susceptible cultivars, Granny Smith (GS) and Red Delicious (RD), were used to study autoxidation of these compounds in vitro. Incubation of CTols in sealed glass vials under air resulted in accumulation of MHO. Oxygen enrichment did not increase the amount of MHO produced. Regardless of which cultivar CTols were derived from, at 0 °C autoxidation yielding MHO was quite slow and linear, whereas at 20 °C MHO production was much more rapid, and after several hours the rate increased abruptly. However, CTols isolated from GS and RD fruit differed in the duration of the initial lag phase and the overall level of MHO generated at 20 °C. The sharp increase in MHO production occurred after 3 h with GS CTols and at about 12 h with RD CTols. Also, the yield of MHO from GS CTols after 6 h at 20 °C was nearly 6-fold greater than that from RD CTols after 20 h at 20 °C. The antioxidants butylated hydroxytoluene and diphenyamine reduced the yield of MHO by about 97%. Recent work has shown that MHO can induce scald-like symptoms in apple peel and that tissue sensitivity increases with time in storage. This may explain the correlation between high CTol levels and scald development, and why symptoms rapidly intensify when fruits are removed from cold storage.

Keywords: *α-Farnesene; conjugated triene; 6-methyl-5-hepten-2-one; autoxidation; apple fruit; superficial scald*

INTRODUCTION

Superficial scald is a storage disorder of apple and pear fruits that occurs after several months at low temperature (ca. 0−5 °C) and rapidly intensifies when the fruit are removed from storage. Susceptibility to scald is very much cultivar dependent, and several lines of evidence indicate that the death of hypodermal cells which precedes visual symptoms (Bain and Mercer, 1963) is caused by oxidative stress and loss of the ability to detoxify active oxygen species (Rao et al., 1998). A series of reports in the 1960s and 1970s established a connection between synthesis and oxidation of the sesquiterpene α-farnesene in apple peel during storage and subsequent development of superficial scald [reviewed in Ingle and D'Souza (1989)]. The primary oxidation products of α-farnesene were identified as "conjugated trienes" (CTs), which are readily detected in hexane-dip extracts of apple fruit due to their characteristic UV absorbance maxima at 259, 269, and 281 nm. Anet (1969) elucidated the structures of several hydroperoxide and epoxide CT products of in vitro autoxidation of α-farnesene, and proposed that free radicals generated during decomposition of these compounds are the toxic agents that induce scald. It was also determined in the course of these studies that a volatile ketone, 6-methyl-5-hepten-2-one (MHO), is a major breakdown product of α-farnesene (Anet, 1972a). Although it was well-known that accumulated apple volatiles exacerbate scald development, MHO was dismissed as a likely inducer of the disorder because a variety of volatile aldehydes and ketones failed to promote any symptoms resembling scald (Huelin and Kennett, 1958; Anet, 1972a,b).

There have been several important more recent findings regarding the role of α-farnesene oxidation in the mechanism of scald induction. Rowan et al. (1995) showed that over 99% of the CT species which accumulate in apple skin in vivo are major (7*E*,9*E*, ≥90%) and minor (7*E*,9*Z*, ≤10%) isomers of 2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol (conjugated trienols; CTols) rather than the CT hydroperoxides and epoxides identified by Anet (1969). In addition, Beaudry and co-workers found that MHO can induce scald-like symptoms in peel tissue of susceptible fruit, that sensitivity to the ketone increases dramatically with the duration of storage, and that a burst of MHO production ensues immediately after removal of scald-susceptible fruit from cold storage (Song and Beaudry, 1996; Mir and Beaudry, 1999; Mir et al., 1999). Here we demonstrate that autoxidation of HPLC-purified CTols in vitro yields MHO as a major product, and that this reaction is much more rapid at 20 than at 0 °C.

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MATERIALS AND METHODS

Isolation of Conjugated Trienols. Peel tissue (including the epidermis and 2−3 mm of hypodermal cortex) from Granny Smith (GS) and Red Delicious (RD) fruit that were stored for 5−6 months in air at 0 °C was excised with a mechanical peeler, blotted, and immersed in liquid N_2. About 200 g of frozen tissue was then pulverized and extracted by stirring for 1 h in 500 mL of hexane at 5 °C under N_2 atmosphere. The extract was concentrated to 15 mL by rotary evaporation at 25 °C then held 2 h at −80 °C to precipitate most of the wax. After centrifugation, the supernatant was decanted and reduced to 5 mL under a gentle stream of N_2 without heating. The concentrate was then fractionated on a column containing 15 g of 60−100 mesh Florisil. The column was washed with 2,2,4-trimethylpentane, the hexane extract was applied, and the column was sequentially eluted with hexane (20 mL), hexane/diethyl ether, 29:1 (30 mL), hexane/ether, 9:1 (40 mL), and hexane/ether, 2:1 (30 mL). UV spectra of the column fractions showed that the bulk of the conjugated trienols (CTols) was in the hexane/ether 9:1 eluate (maxima at 259, 269, and 281 nm). Solvents were removed from the CTol-rich fraction with a stream of N_2 and the residue dissolved in 2 mL of cold methanol. This solution was passed through a 0.45-μm PTFE membrane filter prior to isolation of CTols by C_{18}-HPLC using methanol/acetonitrile/water, 90:5:5, as the mobile phase pumped at 0.8 mL min^{-1} (Whitaker et al., 1997). The pooled CTol HPLC fractions (from multiple 80-μL injections) were concentrated by N_2 evaporation, followed by addition of an equal volume of water and extraction of the purified CTols with hexane. The extract was concentrated under a gentle stream of N_2 to ca. 1 μg μL^{-1} (determined by absorbance at 269 nm; $\epsilon_{269nm} = 42\,500$). Purity and isomeric composition were checked by gas chromatography (Whitaker et al., 1997.)

Assay of CT Oxidation Products. Ten-microliter aliquots of the purified CTols in hexane (ca. 1 μg μL^{-1}) were dispensed in 4-mL screw-cap vials with Teflon-lined septa using a 10-μL Hamilton syringe. The vials were sealed for 1 min, opened, and flushed gently with N_2 for 10 s to remove most of the hexane, then left open on the bench for 2 min to equilibrate with air prior to resealing. In one experiment, two vials were flushed briefly with pure O_2 before resealing. In experiments where an antioxidant (BHT or DPA) or oxidant ($NaIO_4$) was used, these compounds were added to the vial in an appropriate solvent and taken to dryness under a flow of N_2 before adding the CTol aliquot. BHT (butylated hydroxytoluene) and $NaIO_4$ (sodium periodate) were obtained from Sigma, and DPA (diphenylamine) was purchased from Eastman Kodak. Zero time control vials were immediately placed in a −80 °C freezer. Otherwise, vials were incubated in the dark at either 0 or 20 °C for 1.5−20 h and then stored at −80 °C until analyzed. The 0−6 h time course of autoxidation of GS CTols at both incubation temperatures was repeated three times, and the 0−20 h time course of autoxidation of RD CTols was repeated twice, with replicate samples for each time point.

For analysis of volatiles derived from the oxidative degradation of CTols, sample vials were first equilibrated 5 min at 20 °C when removed from the −80 °C freezer. A 1-cm-long solid-phase microextraction (SPME) fiber coated with a 100 μm thickness of poly(dimethylsiloxane) (Supelco) was inserted through the vial septum, and volatiles were sorbed to the fiber for 16 min. The volatiles were then desorbed 2 min at 250 °C in the splitless injection port of a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame-ionization detector and an 11 m × 0.1 mm i.d. HP-5 capillary column with 0.34-μm film thickness (Hewlett-Packard). Ultrapure H_2 was supplied as the carrier gas at a flow velocity of 52 cm s^{-1}, and the column oven was programmed to increase from 40 to 250 °C. The same procedures were used for identification of the volatiles by gas chromatography−mass spectrometry (GC−MS), except that ultrapure He was the carrier gas. Mass spectra were generated over a range of *m*/*z* 40−250 with a single quadrupole mass selective detector (Hewlett-Packard model 5971A) in the electron impact (EI) mode at 70 eV. A standard of 6-methyl-5-hepten-2-one purchased from Sigma was used to obtain a reference retention time and mass spectrum. Standard curves for MHO in the concentration range of 0−500 ng were generated both by SPME fiber adsorption/desorption and direct splitless injection. On the basis of these data, the adsorption efficiency of the SPME fibers was calculated to be 66%.

RESULTS

Identification and Purity of Conjugated Trienols. CTols isolated from peel tissue of air-stored GS and RD apples by column chromatography and C_{18}-HPLC were analyzed by GC-FID to assess purity and isomeric composition. The UV spectra and GC and HPLC retention times of CTols in the two preparations matched those of the 7(*E*),9(*E*) and 7(*E*),9(*Z*) isomers of 2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol previously identified by GC−MS and ^{1}H NMR (Rowan et al., 1995; Whitaker et al., 1997). The proportions of the two CTol isomers were nearly identical in the isolates from the two apple cultivars and were in agreement with previous determinations (Rowan et al., 1995; Whitaker et al., 1997); the 7(*E*),9(*E*) isomer was 92.1 and 92.2% and the 7(*E*),9(*Z*) isomer was 7.9 and 7.8%, in peel extracts from Granny Smith (GS) and Red Delicious (RD), respectively. In both CTol preparations, unidentified trace contaminants accounted for <2% of the total GC peak area.

Conjugated Trienol Autoxidation and Production of 6-Methyl-5-hepten-2-one. Incubation of purified CTols for several hours in a sealed vial in the presence of air resulted in production of MHO, which was identified by capillary GC and GC−MS. Over the range of *m*/*z* 40−250, the EI mass spectrum of MHO derived from CTol autoxidation was essentially identical to that of an authentic standard: *m*/*z* 126 (M^+, 8), 111 (18), 108 (44), 93 (13), 83 (11), 71 (18), 69 (46), 58 (24), 55 (44), 43 (100), and 41 (49). Enrichment of the oxygen concentration in the vial by flushing briefly with pure O_2 did not increase the amount of MHO produced with 10 μg of CTols from GS apple peel during 6 h at 20 °C (data not shown). Regardless of which cultivar the CTols were derived from, at 0 °C, autoxidation yielding MHO was quite slow and essentially linear, whereas at 20 °C, production of MHO was much more rapid, and after several hours, the rate increased abruptly (Figure 1). However, the CTol preparations from GS and RD fruit differed substantially with respect to the duration of the initial lag phase and the overall level of MHO generated during incubation at 20 °C. The sharp increase in the rate of MHO production occurred after 3 h with GS CTols (Figure 1a) and at about 12 h with RD CTols

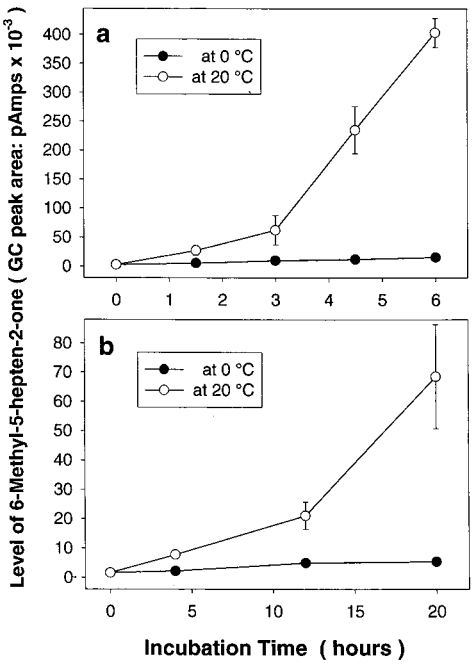

Figure 1. Production of the volatile ketone 6-methyl-5-hepten-2-one (MHO) during *in vitro* autoxidation of HPLC-purified conjugated trienols isolated from peel tissue of Granny Smith (a) and Red Delicious (b) apples. Ten-microgram samples were sealed in 4-mL glass vials under air and incubated at 0 or 20 °C. Values indicate the GC-FID response (peak area) in picoamps $\times$ 10^{-3} and represent the mean of replicate samples from two (RD) or three (GS) experiments. Vertical bars indicate $\pm$ SD (n = 4−6).

(Figure 1b). In addition, the yield of MHO from GS CTols after 6 h at 20 °C was about 6-fold greater than that from RD CTols after 20 h at 20 °C. Inclusion of 10 μg of the antioxidant BHT in the vial with 10 μg of GS CTols reduced the yield of MHO after 4.5 h at 20 °C by about 98%. Similarly, addition of 10 μg of either BHT or DPA with 10 μg of RD CTols inhibited production of MHO during 20 h at 20 °C by about 97% (data not shown). Thus, these antioxidants decreased the rate of autoxidation of CTols at 20 °C below that observed at 0 °C with no added antioxidant. Finally, when 10 μg of $NaIO_4$, a strong oxidizer, was included with 10 μg of RD CTols, the yield of MHO after 20 h at 20 °C was increased about 4-fold.

The data on the levels of MHO in Figure 1 are expressed in terms of GC peak area (picoamps $\times$ 10^{-3}). To relate these values to actual MHO concentration, standard curves were generated both by SPME adsorption/desorption and direct splitless injection (see Materials and Methods). Using the derived SPME fiber adsorption efficiency of 66% and the molecular mass ratio of MHO:CTols = 126/220 = 0.573, it was calculated that 11.7% of the GS CTols had oxidized to yield MHO after 6 h at 20 °C, while only 1.9% of the RD CTols had oxidized to yield MHO after 20 h at 20 °C. Recovery of intact CTols from the vials at the end of the experiments, based on UV absorbance spectra and A_{269nm} (ϵ_{269nm} = 42 500; Anet, 1969), was consistent with these levels of decomposition (86 $\pm$ 4 and 97 $\pm$ 3% recovery of GS and RD CTols, respectively.

DISCUSSION

A large body of correlative evidence links α-farnesene synthesis and oxidation with the occurrence of superficial scald, and in particular, accumulation of the CT oxidation products of α-farnesene is strongly correlated with subsequent scald development (Huelin and Coggiola, 1970a; Anet, 1972b; Anet and Coggiola, 1974; Meir and Bramlage, 1988; Whitaker et al., 1997). It has long been known that accumulation of apple volatiles during air storage exacerbates scald (Brooks et al., 1919; Meigh, 1970) and that MHO is a major product of α-farnesene oxidation (Filmer and Meigh, 1971; Anet, 1972a), but until recently MHO was dismissed as a possible causal agent (Anet, 1972a). However, two reports published last year showed that a burst of MHO production upon removal of Cortland apples from air storage had a close temporal correlation with the intensification of scald symptoms (Mir et al., 1999), and that a DPA treatment which prevented scald all but eliminated MHO production by the peel tissue (Mir and Beaudry, 1999). The findings in the present study, that autoxidation of the CTols which accumulate in apple peel during storage yields MHO and that the rate of this reaction is much greater at 20 than at 0 °C, provide a connection between the association of high CT concentrations with scald and the burst of poststorage MHO production coincident with worsening scald symptoms. It has been shown that the level of CTols in peel tissue declines sharply after rewarming of cold-stored fruit (Huelin and Coggiola, 1970b; Whitaker et al., 1997), and it is likely that this occurs due to much more rapid CTol autoxidation, which in turn produces MHO.

With our current limited knowledge of the mechanism of scald induction, the correlative evidence from which it is inferred that oxidation of α-farnesene and production of MHO are involved in scald development is subject to at least the following three interpretations: (1) susceptibility to scald is strictly a function of weak antioxidative defenses in the peel tissue (Rao et al., 1998), and α-farnesene oxidation yielding MHO is merely a secondary consequence of runaway oxidative reactions; (2) free-radical species generated during α-farnesene oxidation contribute substantially to the oxidative cascade leading to tissue damage and scald symptoms, but MHO is not involved (Anet, 1972a); and (3) oxidative stress, including free radicals derived from CT autoxidation, in some way sensitize the peel tissue to MHO, and MHO toxicity plays a role in cell death (Mir et al., 1999). Recent results with the White Angel $\times$ Rome Beauty apple hybrids described by Rao et al. (1998) indicated that fruit of at least one line accumulate relatively little α-farnesene and CTols yet consistently develop mild scald symptoms (Whitaker and Watkins, unpublished). Although this suggests that α-farnesene oxidation is not important in scald induction, one cannot rule out the possibility that α-farnesene and CTols are rapidly oxidized and degraded in these fruits and/or they are hypersensitive to MHO. On the other hand, largely unpublished work by Song and Beaudry showed that MHO can induce scald-like symptoms in peel tissue disks of scald-susceptible apples and that the tissue sensitivity to MHO increased dramatically after several months of storage in air at 0 °C (see Song and Beaudry, 1996). Clearly, further innovative studies will be required to either prove or disprove the role of α-farnesene oxidation products in the induction of scald.

While the results of the present study show unequivocally that CTols derived from α-farnesene can autoxidize in air at 20 °C and release MHO as a degradation

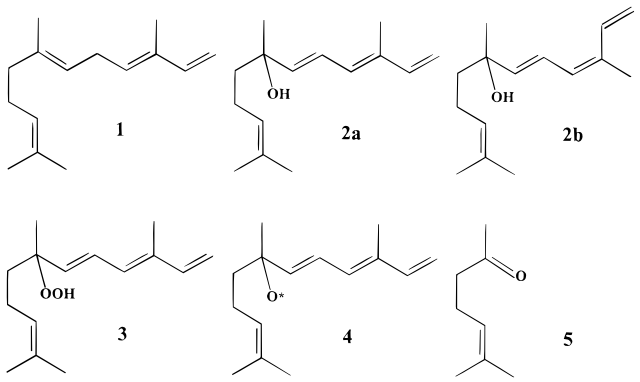

Figure 2. Structures of identified and hypothesized oxidation products of the sesquiterpene α-farnesene (**1**). Major (≥90%) 7*E*,9*E* isomer (**2a**) and minor (≤10%) 7*E*,9*Z* isomer (**2b**) of conjugated trienol (2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol), in vivo oxidation product of α-farnesene that accumulates in peel tissue of apple fruit after 1−2 months of storage at 0 °C in air (Rowan et al., 1995). Conjugated triene 6-hydroperoxide (**3**) identified by Anet (1969) as a major in vitro autoxidation product of α-farnesene, and the conjugated triene 6-oxyradical (**4**) hypothesized by Anet (1972a) to be an intermediate in decomposition of **3** that yields the volatile 6-methyl-5-hepten-2-one (**5**).

product, it is unclear why the rate of autoxidation differed so markedly in the two, ostensibly pure, CTol preparations from peel tissue of Granny Smith and Red Delicious apples. It is interesting to note that the more reactive CTols were isolated from GS fruit which subsequently scalded much more severly than the RD fruit, but this may have been purely coincidental. One explanation for the observed difference in oxidation rate is that during HPLC purification, small amounts of a potent prooxidant or antioxidant coeluted with the GS or RD CTols, respectively (Anet, 1974). There are many alternative explanations, such as use of different brands of solvents, HPLC columns, or freezer storage under somewhat different levels of O_2 (after N_2 flushing). Future use of synthetic CTol (Brimble et al., 1994) would eliminate most of these variables. In any case, further experimentation will be necessary to elucidate the apparent free radical-mediated reaction mechanism involved in CTol autoxidation. Of particular interest are whether conjugated trienol (2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol) is converted to the corresponding 6-hydroperoxide described by Anet (1969), and whether the 6-oxyradical hypothesized by Anet (1972a) is an intermediate in the decomposition that yields MHO (Figure 2).

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