Ethylene Biosynthesis and Polyamine Accumulation in Apples with Watercore

Shiow Y. Wang and Miklos Faust

Fruit Laboratory, Beltsville Agricultural Research Center Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705

Abstract. Ethylene biosynthesis and polyamine content were determined in normal and watercore-affected apple (Malus domestics Borkh. cv. Delicious). Fruit with watercore produced more ethylene and contained higher amounts of putrescine (PUT), spermidine (SPD), 1-aminoacyclopropane-1-carboxylic acid (ACC), and 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC). The activities of ACC synthase and ethylene-forming enzyme (EFE) in watercore-affected fruit were also higher than in normal fruit. The EFE activity in severely affected flesh was inhibited, resulting in ACC accumulation and low ethylene production. S-adenosylmethionine (AdoMet) was maintained at a steady-state level even when C2H and polyamides were actively synthesized in normal and affected fruit.

Watercore is a disorder that is usually associated with vascular bundles in apple fruit. Therefore, sorbitol, the major translocating carbohydrate in apple, cannot enter the cells; it remains in the intercellular spaces, where the higher osmoticum promotes water retention (Marlow and Loescher, 1984; Williams, 1966). As a consequence, the disorder appears as a translucent watersoaked area. In severe cases, the pitch adjacent to the core and all of the cortex may also be affected (Fishier, 1923; Marlow and Loescher, 1984; Olsen et al., 1962; Williams, 1966). The afflicted tissues have an elevated water content, decreased levels of reducing sugars and pectin, increased anaerobic products, and a higher sorbitol content (Atkinson, 1971; Faust et al., 1969; Marlow and Loescher, 1984; Williams, 1966) than normal tissue. Watercore may disappear after harvest if the symptoms are mild. However, a severe form of the disorder may cause internal breakdown during storage (Marlow and Loescher, 1984) and may result in severe economic losses.

Watercore usually occurs at an advanced stage of maturity. Kato and Sato (1978) found that affected apples had a higher concentration of internal C2H and higher rate of C2H evolution in whole fruit than unaffected fruit. The high C2H content in the watercore-affected fruit could be a result of stress caused by high sorbitol concentration. Osmotic stress caused by a high concentration of sorbitol has been found to enhance polyamine synthesis. Polyamines have multiple effects associated with senescence; it initiates fruit ripening, induces chlorophyll loss in leaves, and promotes senescence (Yang and Hoffman, 1984). On the contrary, polyamides are senescence inhibitors. They inhibit the rise in RNase (EC 3.1.27.5), protease (EC 3.4.24.4), and peroxidase (EC 1.11.1.7); reduce the rate of senescence of leaf protoplasts; induce DNA synthesis and mitotic activity; promote the synthesis of macromolecules; stabilize thylakoid membranes; maintain high protein content; and prevent the loss of chlorophyll in leaf disks (Galston, 1983). Since C2H and polyamides have opposing effects, the purpose of this study was to investigate the relation of these two types of compounds in watercored tissues to increase our understanding of this important disorder in apple fruit.

Materials and Methods

Plant material. ‘Delicious’ apples were harvested from ten 8-year-old apple trees in an orchard at Beltsville, Md., and stored in 0C air. The diameters of the fruit ranged from 6.5 to 7.5 cm. Nuclear Magnetic Imaging (NMI) was used to determine the severity of watercore and to group the apples into various classes (Wang et al., 1988). Whole apples and tissue

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slices with or without watercore were used in this study. Skin and flesh from normal fruit, and skin, nonaffected flesh, and flesh affected from fruit with watercore were used for chemical analyses. Samples were taken at harvest and at 3-week intervals throughout 15 weeks of storage.

**Ethylene determination.** Ethylene production of whole affected and normal fruit was determined by gas chromatography (GC) equipped with an alumina column and a flame ionization detector. Ten individual fruit were used for C\textsubscript{2}H\textsubscript{4} assay at each sampling. Before making the C\textsubscript{2}H\textsubscript{4} measurements, the fruit were warmed at 20°C for 1 day after removal from 0°C.

Tissue disks at 10 mm diameter and 2-mm-thick flesh or 1-mm-thick skin were cut from each of 10 fruit for each treatment. Tissue disks were washed in 600 mM sorbitol and 10 mM Mes [2-(N-Morpholino) ethanesulfonic acid] buffer (pH 6.0). About 1 g of sample was placed in 25-ml flasks containing 3 ml 600 mM sorbitol and 10 mM Mes buffer (pH 6.0) solution. Flasks were incubated in a 30°C shaking water bath. After 3 to 4 h, C\textsubscript{2}H\textsubscript{4} in the headspace of the flask was determined by gas chromatography. Mature apple fruit already producing C\textsubscript{2}H\textsubscript{4} do not form wound C\textsubscript{2}H\textsubscript{4} upon slicing (Lieberman and Wang, 1982).

**ACC synthase extraction and assay.** ACC synthase was extracted as described by Bufler and Bangerth (1983). The extraction medium contained 100 mM Tricine-KOH (pH 8.5), 4 mM dithioerythritol (DTE), 5 mM pyridoxal phosphate, and 0.2% (v/v) Triton X-100. One gram of lyophilized tissue was extracted with 10 ml extraction medium using a cold mortar and pestle. The extract was first centrifuged for 15 min at 25,000 x g. The pellet was then extracted with 5 ml of extraction medium, centrifuged again as above, and the two supernatants combined. Two-milliliter samples of supernatant were purified on a Sephadex G-50 column (Pharmacia, Piscataway, N. J.) (14 x 1 cm), previously equilibrated with 5 mM Tricine-KOH (pH 3.0), containing 0.1 mM DTE and 1 mM pyridoxal phosphate. The protein fraction was collected and used in the enzyme assay. All steps were carried out at 4°C.

ACC synthase activity was determined according to Boiler et al. (1979). The reaction mixture contained 0.4 ml enzyme, 50 mM Tricine-KOH (pH 8.0), and 40 nmol AdoMet in a total volume of 0.6 ml. After the reaction mixture had incubated for 1 h at 30°C, the ACC formed was assayed by the method of Lizada and Yang (1979).

**Protein determination.** Protein concentration was determined by the method of Bradford (1976).

**Extraction of polyamines, AdoMet, ACC, and MACC.** Two grams of tissue (fresh weight) were extracted one at a time, with 15 ml of 5% ice-cold HClO\textsubscript{4} for 1 h. The extracts were centrifuged for 20 min at 5000 x g and the supernatants were analyzed for ACC, MACC, AdoMet, and free polyamines.

**Determination of polyamines.** The supernatants from the HClO\textsubscript{4} extracts were adjusted to pH 4.5 at 0°C with KHCO\textsubscript{3} and then centrifuged at 4°C, 6000 x g for 20 min. The supernatant (pH 4.5) was passed through a Bio-Rex 70 column (H’ form) that retained AdoMet and polyamides. AdoMet and polyamides were eluted from the Bio-Rex 70 column with 0.1 M HCl after neutralization, they were lyophilized and dissolved in 1.5 ml 5% HClO\textsubscript{4}, and used for free polyamine determination. Levels of polyamides were determined after dansylation (Wang and Steffens, 1985). The 10 µl of dansylated products was then analyzed using high-pressure liquid chromatography by the methods of Kramer and Wang (1989).

**Determination of total AdoMet.** An aliquot of the Bio-Rex
which catalyses the conversion of AdoMet to ACC, the amount was determined via a liquid scintillation counter (Yu et al., 1980). Isotope dilution (Wang and Steffens, 1985) was also used to determine the specific radioactivity of ACC produced and the total radioactivity of nonradioactive AdoMet in the tissue could be calculated from the specific radioactivity of ACC produced and the total radioactivity of AdoMet added. ACC synthase was isolated from apples as described above and ACC was assayed according to the method of Lizada and Yang (1979), which is based on the chemical conversion of ACC to C\textsubscript{2}H\textsubscript{4} with NaOCl. The C\textsubscript{2}H\textsubscript{4} produced was then transferred to an evacuated 25-ml scintillation vial. A 2-ml gas sample was withdrawn from the vial for analysis by GC.

**Results and Discussion**

**Ethylene-forming enzyme activity.** One gram of flesh or skin tissues from watercore-affected or normal apples was incubated at 25\textdegree C in a 25-ml flask containing 3 ml of incubation medium (600 mm sorbitol, 10 mm Mes buffer, pH 6.0) in the presence (EFE activity) or absence (C\textsubscript{2}H\textsubscript{4} production) of 1 mm ACC. Incubations were carried out 1 h after the tissues were cut to allow for the escape of internal C\textsubscript{2}H\textsubscript{4}. The flasks were then sealed with serum caps, and gas samples were taken after 3 h for C\textsubscript{2}H\textsubscript{4} analysis by GC.

**Ethylene production, ACC and MACC content, ACC synthase, and EFE.** Ethylene production of intact apples from mid-October to February indicated that the apples were in the prec climacteric stage at the start of these experiments (Fig. 1). With increased time in 0\textdegree C storage, the apples showed the usual rise and fall of C\textsubscript{2}H\textsubscript{4} production associated with the climacteric. Ethylene in unaffected fruit reached its peak \(\approx 9\) weeks after harvest. In contrast, in apples with watercore C\textsubscript{2}H\textsubscript{4} production peaked 6 weeks after harvest. The total production of C\textsubscript{2}H\textsubscript{4} was slightly higher in affected than normal apples, but the rate of its increase was similar for the two groups of fruit (Fig. 1). Skin tissue produced more C\textsubscript{2}H\textsubscript{4} than either affected or nonaffected flesh of watercore-affected apples (Fig. 2). Similarly, skin tissue produced more C\textsubscript{2}H\textsubscript{4} than flesh tissue in normal apples. The peak of C\textsubscript{2}H\textsubscript{4} production and its peak time in each tissue was the same as in whole apples. The peak of C\textsubscript{2}H\textsubscript{4} production in every tissue of affected apples occurred 6 weeks after harvest, while in normal apples the peak was 9 weeks after harvest. Each tissue of apples with watercore produced more total C\textsubscript{2}H\textsubscript{4} than the corresponding tissue of normal fruit. After
9 weeks of storage, \( C_2H_4 \) production declined rapidly, especially in the affected flesh of affected fruit. The earlier peak of \( C_2H_4 \) indicates that apples with watercore are more advanced physiologically than normal apples (Brooks and Fisher, 1926).

The ACC content, ACC synthase, and EFE activities of all tissues were very low at harvest (Figs. 3–5), limiting \( C_2H_4 \) production. It has been reported that preclimacteric fruit tissue lacks the capability not only for ACC synthesis but also for the conversion of ACC to \( C_2H_4 \) (Hoffman and Yang, 1980).

The concentration of MACC increased in parallel with the high rate of ACC synthesis. The skin contained higher amounts of ACC and MACC than the flesh (Figs. 3 and 6). The content of MACC in skin or flesh was considerably higher than that of free ACC. The MACC level in all tissues increased during storage and did not show the pattern displayed by all other components examined. In general, EFE activity followed the same pattern as ACC synthase and ACC content (Figs. 3–5). The reduced ACC levels in normal tissue during later periods of storage (12 to 15 weeks) might be due to the rapid and steady conversion of ACC to \( C_2H_4 \), as reported by others (Lau et al., 1984; Lipton and Wang, 1987; Wang et al., 1985). Tissue with watercore contained more ACC and MACC than did normal tissue. The activities of ACC synthase and EFE were also higher in affected than in normal apples. After 12 weeks of storage, EFE activity in watercore-affected flesh was drastically inhibited (Fig. 5), resulting in ACC accumulation and low \( C_2H_4 \) production (Figs. 2 and 3). The system converting ACC to \( C_2H_4 \) may have been damaged in watercore-affected flesh at this stage. Also, internal \( O_2 \) concentration in watercore-affected tissue is low (Kato and Sate, 1978). Formation of \( C_2H_4 \) from ACC requires \( O_2 \) (Adams and Yang, 1979). If \( O_2 \) is limiting, conversion of ACC to \( C_2H_4 \) will be impaired and ACC accumulation and low \( C_2H_4 \) production are expected. EFE activity was not inhibited either in the skin or nonaffected flesh of fruit with watercore.

Polyamine content. In the skin of apples with watercore, PUT and SPD increased while SPM decreased with time in storage, but all remained higher than in normal apples (Fig. 7). Similar results were observed in the flesh (Fig. 8). Polyamine levels were generally lower in the flesh than in skin tissue. Affected flesh contained higher levels of polyamides than nonaffected flesh of affected apples, but both were higher than in the flesh of normal fruit. The greater accumulation of polyamides in watercore tissue is unexplained, but may be due to a higher amount of sorbitol in watercored apples (Williams, 1966). Flores and Galston (1984) have shown that a higher than normal concentration of sorbitol causes osmotic stress, which, in turn, increases PUT and SPD levels. Increases in polyamine levels in rice seedlings have also been shown under \( O_2 \)-deficit stress (Reggiani and Bertani, 1989; Reggiani et al., 1989). Since tissue of apples with watercore has a low \( O_2 \) concentration (Kato and Sate, 1978), this may have also contributed to the higher content of polyamides in watercore-affected tissue.

AdoMet content. Fruit with watercore have higher ACC and polyamine contents than normal fruit. However, AdoMet levels in skin or flesh were the same for both types of fruit (data not shown). These data indicate that the step between methionine and AdoMet is not rate-limiting. No change in the level of
AdoMet was observed even when C\textsubscript{2}H\textsubscript{4} and the polyamides were actively synthesized. AdoMet is the substrate for both ACC and polyamine synthesis, but its consumption apparently did not alter the AdoMet steady-state level. Similar results were reported in water-stressed apple leaves (Wang and Steffens, 1985). Kushad et al. (1988) also demonstrated that the polyamine and C\textsubscript{2}H\textsubscript{4} biosynthetic pathways are not actively competing for AdoMet at any given stage of avocado fruit development and ripening.

Collectively, the data presented here indicate that watercore-affected fruit contains higher concentrations of PUT, SPD, ACC, and the polyamides and C\textsubscript{2}H\textsubscript{4} than normal fruit. The ACC synthase activity in affected fruit was also higher than in normal fruit, probably due to osmotic stress induced by the high sorbitol content in fruit with watercore. The EFE in severely watercore-affected flesh was inhibited, possibly due to low internal O\textsubscript{2} levels that may result in ACC accumulation and low C\textsubscript{2}H\textsubscript{4} production. No change in the level of AdoMet was observed between normal and watercore-affected fruits, although the polyamides and C\textsubscript{2}H\textsubscript{4} were actively synthesized. Our results detail several metabolic aspects of watercore in apples—a complex disorder. Further research is needed to elucidate the interaction between polyamides, C\textsubscript{2}H\textsubscript{4}, sorbitol, and general metabolic state of the overmature apple fruit, as exemplified by watercore-affected tissue.

**Fig. 8.** Polyamine content of flesh tissue from normal and watercore-affected ‘Delicious’ apples at harvest and periodically after removal from 0C storage. NF = normal flesh; WNF = apples with watercore, nonaffected flesh; WAF = apples with watercore, affected flesh. Vertical bars denote ±se, bars smaller than the symbols are not shown.

### Literature Cited


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