# Mode of Oxygen and Carbon Dioxide Action on Strawberry Ester Biosynthesis

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Abstract. 'Chandler' strawberries (*Fragaria ananassa* Duck.) were kept in air,  $0.25\% O_2$ ,  $21\% O_2 + 50\% CO_2$ , or  $0.25 O_2 + 50\% CO_2$  (balance N<sub>2</sub>) at 5C for 1 to 7 days to study the effects of controlled atmospheres (CAs) on volatiles and fermentation enzymes. Concentrations of acetaldehyde, ethanol, ethyl acetate, and ethyl butyrate were greatly increased, while concentrations of isopropyl acetate, propyl acetate, and butyl acetate were reduced by the three CA treatments compared to those of air-control fruit. The CA treatments enhanced activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) but slightly decreased activity of alcohol acetyltransferase (AAT). The results indicate that the enhanced PDC and ADH activities by CA treatments cause ethanol accumulation, which in turn drives the biosynthesis of ethyl esters. The increased ethanol concentration also competes with other alcohols for carboxyl groups for esterification reactions. The reduced AAT activity and limited availability of carboxyl groups due to ethanol competition decrease production of other acetate esters.

Controlled atmospheres (CAs) can be used to delay senescence and control decay and insects of strawberries (Aharoni et al., 1979; Couey and Wells, 1970; Couey et al., 1966; El-Kazzaz et al., 1983; Harris and Harvey, 1973; Ke et al., 1991; Li and Kader, 1989; Prasad and Stadelbacher, 1974; Smith, 1957; Woodward and Topping, 1972). Exposure to CAs reduced respiration and ethylene production rates and retarded softening of strawberries (El-Kazzaz et al., 1983; Ke et al., 1991; Li and Kader, 1989; Woodward and Topping, 1972). Keeping strawberries in <1% O<sub>2</sub> and/or >20% CO<sub>2</sub> caused accumulations of certain volatile compounds (Ke et al., 1991; Li and Kader, 1989; Prasad and Stadelbacher, 1974; Smith, 1957; Ueda and Bai, 1993; Woodward and Topping, 1972).

Volatiles are important contributors to flavor and odor of fruit and vegetables. Pyruvate decarboxylase (PDC) and alcohol acetyltransferase (ADH) are two important enzymes responsible for acetaldehyde and ethanol production, and their activities were increased by some CA treatments (Ke et al., 1993). Esters are the volatiles formed by esterification of alcohols and carboxyl groups. Ethyl acetate is produced through an esterification reaction catalyzed by the enzyme alcohol acetyltransferase (AAT), using ethanol and acetyl CoA as substrates (Harada et al., 1985). Fellman et al. (1993a, 1993b) reported that AAT activity increased with advanced maturity of apples but was suppressed by exposure to 0.5% or 1% O<sub>2</sub>. Mattheis et al. (1991) found that storing apples in 0.05% O<sub>2</sub> caused accumulations of several ethyl esters, a condition that was associated with a decrease in concentrations of other esters requiring the same carboxylic acid group for synthesis. Hansen et al. (1992) showed that keeping apples in 1% to 4% O<sub>2</sub> decreased propyl acetate and butyl acetate. Treating oranges with <1% O<sub>2</sub> for 24 h increased concentrations of ethyl acetate, ethyl butyrate, methanol, and ethanol (Shaw et al., 1990). Ke et al. (1991) found that strawberries kept in < 0.25% O<sub>2</sub> or > 50% CO<sub>2</sub> for >6 days developed off-flavor, which was correlated with the accumulation of acetaldehyde, ethanol, and ethyl acetate. Ueda and Bai (1993) found that evolution of ethyl acetate and ethyl

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butyrate was increased by exposing strawberries to 20%  $CO_2$  for 2 days at 1C.

Applying ethanol postharvest caused accumulation of ethyl esters and decreased concentrations of butyl esters and hexyl esters in apples (Berger and Drawert, 1984). Exposing feijoa fruit to acetaldehyde vapor enhanced taste and volatile concentrations; however, the same treatment caused off-flavor and too-high concentrations of ethanol, ethyl acetate, and ethyl butyrate in strawberries (Pesis et al., 1989).

In this research, we studied the regulation of ester biosynthesis in strawberries kept in 0.25%  $O_2$  and/or 50%  $CO_2$ , with emphasis on the mode of CA action on acetate ester production.

## **Materials and Methods**

*Materials and treatments.* 'Chandler' strawberries were obtained on the day of harvest from a commercial shipper in Oxnard, Calif. Damaged and nonuniform fruit were eliminated, and good fruit were matched by color to remove unripe and overripe fruit. Twenty selected fruit were placed in a 4-liter glass jar at 5C and ventilated with humidified air or a specified gas mixture at a continuous 50-ml·min<sup>-1</sup> flow rate for up to 7 days. The gas mixtures included 0.25%  $O_2$  + 99.75%  $N_2$ , 21%  $O_2$  + 50%  $CO_2$  + 29%  $N_2$ , and 0.25%  $O_2$  + 50%  $CO_2$  + 49.75%  $N_2$ . Daily, half of the fruit was used to extract juice to measure volatiles and the other half was used for each treatment.

*Measuring volatiles*. Frozen fruit juice was thawed and a 5-ml sample was put in a 15-ml screw-cap test tube. The tube was closed with an elastic cap and incubated at 60C. After 60 min, a 1-ml headspace gas sample was taken with a 1-ml glass syringe. Concentrations of acetaldehyde, ethanol, ethyl acetate, ethyl butyrate, and other esters were measured by injecting the sample into a gas chromatograph (HP5890A; Hewlett Packard, Palo Alto, Calif.) with a flame ionization detector (at 250C) and a glass column (2 mm  $\times$  1.0 m) containing 5% Carbowax on a 60/80 Carbopack as the stationary phase (Supelco, Bellafonte, Pa.) at 120C.

Determining fermentation enzymes. To extract PDC and ADH, 3 g of tissue was sliced from three strawberries and homogenized in 10 ml of 100 mm 2-(*N*-morpholino)ethane-sulfonic acid (MES)

The AAT extraction and assay method was modified from that of Fellman et al. (1993b). To extract AAT, 6 g of tissue was sliced from three fruit and frozen in liquid N<sub>2</sub>. The sample was ground in 12 ml of 100 mM potassium phosphate buffer (pH 7.5) and 2 g polyvinylpolypyrolidone using a precooled mortar and pestle. After thawing, the sample was filtered through four layers of cheesecloth and centrifuged at  $27,000 \times g$  for 15 min at 4C. The supernatant was decanted and set on ice as enzyme extract. AAT activity was assayed by mixing 0.75 ml of 100 mM potassium phosphate buffer (pH 7.5), 0.1 ml of 10 mM 5,5' dithiobisnitrobenzoic acid (DTNB), 0.01 ml of  $1 \text{ M} \text{MgCl}_2$ , 0.1 ml of enzyme extract, 0.02 ml of 20 mM isoamyl alcohol, and 0.02 ml of 50 mM acetyl CoA. The increase in absorbance at 412 nm over time was measured by a spectrophotometer to follow the production of a yellow thiophenol product (2-nitro-5-thiobenzoic acid) formed by the reaction of DTNB with free CoA released from the AAT esterification reaction.

Enzyme activities were expressed as millimoles of substrate used or product formed per minute per gram fresh weight. For in vitro studies of enzyme kinetics, several assay pH values were used to study the changes in PDC, ADH, and AAT activities. For PDC and ADH, 100 mM MES buffer was used for pH 4.5 to 7.5; 100 mM tris buffer was used for pH 7.5 to 8.0. For AAT, 100 mM potassium phosphate buffer was used for pH 6.0 to 7.5; 100 mM tris buffer was used for pH 7.5 to 8.5. The activities at pH 7.5 were the averages of the two buffers used.

#### **Results and Discussion**

Exposing 'Chandler' strawberries to 0.25%  $O_2$ , 21%  $O_2$  + 50%  $CO_2$ , or 0.25%  $O_2$  + 50%  $CO_2$  at 5C for 1 to 7 days greatly increased concentrations of acetaldehyde, ethanol, ethyl acetate, and ethyl



Fig. 1. Changes in concentrations of acetaldehyde, ethanol, ethyl acetate, and ethyl butyrate of 'Chandler' strawberries kept in air, 0.25%  $O_2$ , 21%  $O_2$  + 50%  $CO_2$ , or 0.25%  $O_2$  + 50%  $CO_2$  at 5C for 1, 3, 5, or 7 days. The vertical bars represent LSD at P = 0.05.

butyrate over those of air-control fruit (Fig. 1). While 21%  $O_2$  + 50%  $CO_2$  caused the highest acetaldehyde concentration (Fig. 1A), 0.25%  $O_2$  caused the greatest accumulations of ethyl acetate (Fig. 1C) and ethyl butyrate (Fig. 1D). The three CA treatments generally resulted in lower concentrations of other acetate esters (isopro-

pyl acetate, propyl acetate, and butyl acetate) compared to those of air-control fruit (Fig. 2).

Changes in concentrations of volatiles may influence flavor. At relatively low concentrations, acetaldehyde, ethanol, ethyl acetate, and ethyl butyrate may enhance fruit flavor (Pesis et al., 1989;





Fig. 2. Changes in concentrations of isopropyl acetate, propyl acetate, and butyl acetate of 'Chandler' strawberries kept in air, 0.25%  $O_2$ , 21%  $O_2$  + 50%  $CO_2$ , or 0.25%  $O_2$  + 50%  $CO_2$  at 5C for 1, 3, 5, or 7 days. The vertical bars represent LSD at P = 0.05.

Fig. 3. Changes in activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and alcohol acetyltransferase (AAT) of 'Chandler' strawberries kept in air, 0.25%  $O_2$ , 21%  $O_2$  + 50%  $CO_2$ , or 0.25%  $O_2$  + 50%  $CO_2$  at 5C for up to 7 days. The vertical bars represent LSD at P = 0.05.

Shaw et al., 1990). However, too-high concentrations of these fermentation volatiles may cause off-flavor (Ke et al., 1991; Pesis et al., 1989). Decreased concentrations of other acetate esters may also result in lack of characteristic flavor.

Storing strawberries in 0.25%  $O_2$ , 21%  $O_2$  + 50%  $CO_2$ , or 0.25%  $O_2$  + 50%  $CO_2$  increased PDC and ADH activities but slightly decreased AAT activity compared to those of air-control fruit (Fig. 3). The 0.25%  $O_2$  treatment enhanced ADH activity the most (Fig. 3B) and reduced AAT activity the least (Fig. 3C). The in vitro studies



Fig. 4. Effects of pH on relative activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and alcohol acetyltransferase (AAT) extracted from fresh 'Chandler' strawberries.

indicated that PDC and ADH had an optimal pH of 6.0, while AAT had an optimal pH of 8.0 (Fig. 4). It has been found that exposing fruit and vegetables to low  $O_2$  and/or high  $CO_2$  reduced cytoplasmic pH (Chavez, 1991; Nanos and Kader, 1993; Siriphanich and Kader, 1986); high  $CO_2$  affected pH more than low  $O_2$  (Hess et al., 1993). In most plant tissues, cytoplasmic pH is  $\approx$ 7 and CA treatments reduce the pH by 0.2 to 0.8 units. If strawberries are affected similarly, then a reduced pH by CA treatments may have activated PDC and ADH activities but inhibited AAT activity (Fig. 4).

The induction and/or activation of PDC and ADH (Fig. 3) by CA treatments was consistent with the increased acetaldehyde and ethanol concentrations (Fig. 1). Ethanol and acetyl CoA are the substrates for ethyl acetate biosynthesis. Since AAT activity was slightly reduced by CA treatments (Fig. 3C), it seemed that the dramatic increase in ethanol concentration (Fig. 1B) was the major driving force for ethyl acetate accumulation (Fig. 1C). The slight reduction in AAT activity may have partly counteracted the effect of ethanol on ethyl acetate accumulation. It was the combination of AAT activity and ethanol concentration that determined ethyl acetate concentration.

AAT activity and ethanol concentration play a major role in determining the concentrations of acetate esters. AAT catalyzes the esterification reactions to produce acetate esters. The reduced AAT activity by CA treatments was a major reason for the decreased concentrations of isopropyl acetate, propyl acetate, and butyl acetate.

Ethyl acetate and the other three acetate esters share acetyl CoA as one of their substrates. The dramatic increase in ethanol used a lot of acetyl CoA to produce ethyl acetate, which may have competed with the other alcohols for acetyl CoA and limited the availability of this substrate for the biosynthesis of other acetate esters. The increased ethanol concentration also caused the accumulation of ethyl butyrate (Fig. 1D), since ethanol is one of its



Fig. 5. Proposed mode of  $O_2$  and  $CO_2$  action on ester biosynthesis of strawberries. AAT = alcohol acetyltransferase; ADH = alcohol dehydrogenase; PDC = pyruvate decarboxylase; PDH = pyruvate dehydrogenase; + is induction and/or activation; - is reduction and/or inhibition.

substrates. It has been reported that CA treatments in apples and oranges also caused accumulations of ethyl esters but decreased concentrations of acetate esters (such as propyl acetate and butyl acetate) and other esters requiring the same carboxylic acid group for synthesis (Hansen et al., 1992; Mattheis et al., 1991; Shaw et al., 1990). This result indicates that ethanol competes with other alcohols for carboxyl groups in esterification reactions. This idea was further supported by the observation that applying ethanol or acetaldehyde vapor postharvest to apples and strawberries caused accumulations of ethyl esters but reduced concentrations of other esters (Berger and Drawert, 1984; Pesis et al., 1989).

### Conclusions

Using the results from this study and the cited references, a proposed mode of CA action on ester biosynthesis in strawberries is presented in Fig. 5. In this model, low  $O_2$  and/or high  $CO_2$  concentrations decreased cytoplasmic pH. The CA treatment (directly) and the decrease in pH (indirectly) enhance PDC and ADH activities but reduce AAT activity. As a result, acetaldehyde and ethanol accumulate. The great increase in ethanol concentration causes the accumulation of ethyl acetate and ethyl butyrate. The reduced AAT activity and limited availability of acetyl CoA due to competition by ethanol decrease concentrations of other acetate esters (such as isopropyl acetate, propyl acetate, and butyl acetate). Also, CA treatments induce ethanol accumulation, which causes the production of ethyl esters and the reduction of other esters. These changes in volatile profiles may influence fruit flavor.

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