Ethylene Binding Site Affinity in Ripening Apples

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Abstract. Scatchard plots for ethylene binding in apples (*Malus domestica* Borkh.), which were harvested weekly for 5 weeks to include the ethylene climacteric rise, showed C_{50} values (concentration of ethylene needed to occupy 50% of the ethylene binding sites) of 0.10, 0.11, 0.34, 0.40, and 0.57 μl ethylene/liter¹, respectively, for each of the 5 weeks. Higher ethylene concentrations were required to saturate the binding sites during the climacteric rise than at other times. Diffusion of ¹⁴C-ethylene from the binding sites was curvilinear and did not show any indication of multiple binding sites. Ethylene was not metabolized by apple tissue.

Ethylene binding sites have been studied in a variety of plants (Sisler, 1991), including beans (*Phaseolus vulgaris* L.) (Smith et al., 1987), tomatoes (*Lycopersicon esculentum* Mill.) (Sisler, 1982), tobacco (*Nicotiana tabacum* L.) (Sisler and Goren, 1984), citrus (*Citrus sinensis* L.) (Goren and Sisler, 1986), and *Arabidopsis thaliana* L. Heynh. (Bleecker et al., 1988). Whitehead and Bosse (1991) found that fatty acids affected ethylene binding in intact bananas (*Musa acuminata* Collar). Evidence has been presented that these binding sites meet criteria as physiological receptors (Sisler and Wood, 1987). Ethylene binding sites in the pulp of ripening apples and senescing morning glory [*Ipomea purpurea* (L.) Roth] flowers are present throughout these developmental changes (Blankenship and Sisler, 1989). In morning glory flowers, the total number of sites does not change, whereas in apple pulp, the total number decreased but not to a statistically significant degree (Blankenship and Sisler, 1989). Apples pass through a maturation phase when fruit sensitivity to ethylene increases and predisposes the fruit to ripening (Hartmann et al., 1987). What causes the change in ethylene sensitivity is unknown. Receptors could account for increased sensitivity through an increase in affinity for ethylene or increases in the concentration of receptors (Firn, 1986).

Little ethylene binding work on fruit has been done, because fruit is much more difficult to work with than other tissues. The high water content and low number of binding sites make it impossible to use current methodology on many kinds of intact fruit. Endogenous ethylene production also is a major problem in assaying intact tissues (Sanders et al., 1990a). Since fruit ripening is such an important ethylene effect, we wanted, within technical limits, to determine if there was any evidence that ethylene binding-site affinity might be changing with the initiation of fruit ripening. Dissociation curves have shown evidence of multiple binding sites in mung bean (*Vigna radiata* L.) sprouts (Sisler, 1990) and other tissues (Sanders et al., 1990a, 1990b). We were also interested in dissociation values in apples to see if there was any evidence of multiple sites, as this could also be a factor in increased ethylene sensitivity.

Materials and Methods

'Starkrimson' or 'Redchief Delicious' apples for the ripening displacement experiments were harvested from six lots of apples. Four lots came from commercial orchards in North Carolina, two locations in 1990 and two others in 1991. The remaining two lots came from dwarf trees in controlled environments at the Southeastern Plant Environment Laboratory at North Carolina State Univ., Raleigh. Each of these lots was sampled weekly for internal ethylene measurements, and tissue samples were taken for displacement plots. 'Golden Delicious' used for the dissociation experiments were harvested from a North Carolina experiment station. Immature apples (used either whole or ground in an experiment comparing the two) were picked 60 to 90 days after full bloom and produced no detectable ethylene.

Internal ethylene concentrations were measured by inserting a needle into the calyx end of the apple and withdrawing 1 ml of gas from the core. The gas was analyzed by gas chromatography on an alumina column. Means of 15 individual apples are presented (Fig. 1).

Procedures for preparing tissue and measuring binding sites have been described (Blankenship and Sisler, 1989; Sisler, 1979). Thirty apples were peeled and cored. About 100 g of flesh from each apple, excluding carpel tissue, were combined to make a 3-kg sample. Whole apples producing ethylene altered ethylene concentrations in the desiccators used for the assay; thus, ground apple pulp, which produced no ethylene, was used instead. During these experiments, the tissue was ground at 21C by three methods, which all gave similar results. The first method used grinding with an Oster blender (Oster Corp., Milwaukee). Tissue was put in the blender jar with ≈ 750 ml distilled water and ground for 3 min or until all large pieces of intact tissue were macerated. The second grinding method used an Oster juicer, which separated juice from pulp without the use of additional water. The third method used a food processor (Regal Co., Kewaskum, Wis.), which ground the tissue without the need for distilled water. The ground tissue was pressed within a nylon cloth to remove most of the liquid. We had previously assayed the liquid portion and found no measurable ethylene binding activity (Blankenship and Sisler, 1989). The remaining pulp was mixed with cellulose (2:1, v/v) as a spacer, divided into 24 samples (125 g fresh weight each), and frozen at -8C. Thawed, fluffy cellulose tissue mixtures were placed in cheesecloth bags for exposure to ethylene.

We began the general assay procedure by placing samples in each of two 2.5-liter desiccators containing 1.0 μCi [¹⁴C]ethylene-mercuric perchlorate complex (110 mCi/mmol) in a 25-ml Erlenmeyer flask. Filter paper containing 1 ml of 2 N NaOH was placed in the desiccator to absorb impurities that might be present in the

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released ethylene. After sealing the desiccator, 1 ml of saturated LiCl was injected through a silicone rubber stopper into the ethylene-mercuric perchlorate complex. A magnetic stir bar was used to stir the mixture for 10 min to ensure complete release of the ethylene. Unlabeled ethylene (1000 $\mu\text{l}\cdot\text{liter}^{-1}$) was included in one of the desiccators to determine nonspecific binding of ethylene, which was subtracted from all values. A sample of hydrated,

Fig. 1. Internal ethylene concentration of whole 'Red Delicious' apples used in the preparation of apple pulp/cellulose samples for the Scatchard plots during the 5 weeks surrounding the initiation of the ethylene climacteric. Mean $\pm$ SD.

Fig. 2. Scatchard plots of ethylene binding in 'Red Delicious' apple pulp over 5 weeks surrounding the initiating of the ethylene climacteric. Values have been corrected for nonspecific binding. Linear regression lines are: (A) week 1, $y = -17.7x + 1684$, $r^2 = -0.93$, $K_d = 0.06$; (B) week 2, $y = -14.0x + 1430$, $r^2 = -0.95$, $K_d = 0.07$; (C) week 3, $y = -3.3x + 365$, $r^2 = -0.96$, $K_d = 0.30$; (D) week 4, $y = -2.81x + 317.2$, $r^2 = -0.96$, $K_d = 0.36$; and (E) week 5, $y = -1.9x + 206.1$, $r^2 = -0.83$, $K_d = 0.53$.

ground mung bean seed (soaked overnight) mixed with cellulose was included in each desiccator as a standard. After 4 h, the desiccators were opened and a 2- to 4-min vacuum was applied to each to remove unbound ethylene from the samples. Preliminary experiments had shown that vacuum evacuation was necessary to reduce the background level quickly. Each sample (in the cheese-cloth bag) was placed in a 500-ml jar with 0.2 ml mercuric perchlorate on a piece of fiberglass filter in a liquid scintillation vial. After being sealed, jars were placed in a 60C oven for 6 h, and an additional 6 h were allowed for collecting the ethylene. Vials were then removed, scintillation fluid added, and the samples counted in a scintillation counter.

Scatchard plots (Segel, 1976; Venis, 1985) were obtained by exposing samples to various amounts of unlabeled ethylene in addition to labeled ethylene. The concentrations of unlabeled ethylene added were 0, 0.04, 0.1, 0.5, 1.0, or 1000 $\mu\text{l}\cdot\text{liter}^{-1}$. Both 1.0 and 1000 $\mu\text{l}\cdot\text{liter}^{-1}$ gave 100% saturation with unlabeled ethylene (or 100% ^{14}C -ethylene was displaced). From the disintegrations per minute (dpm) of each concentration, the percentage of displaced ^{14}C -ethylene was calculated (percent displaced is on the X axis, Fig. 2). The percentage displaced was divided by the concentration of ethylene (Y axis). The data was plotted and a line generated. K_d was equal to $1/\text{slope}$, and the relationship between K_d and C_{50} is expressed by $K_d = C_{50}/(1 + L^*/K_d^*)$, where L^* and K_d are the concentration and dissociation constants, respectively, of the labeled compound (Venis, 1985).

The Scatchard plots represent combined means generated from six separate 5-week experiments. Results from the two 'Red Delicious' strains were similar and thus combined. Within each of these experiments there were three replicate samples in each desiccator each week. In two of the six experiments, there were three replicate runs of the displacement curve for each week. Linear regressions of the combined means are presented (Fig. 2).

In the dissociation experiments, immature whole or pulped apple flesh ground and processed as described above was exposed to labeled ethylene in the presence or absence of 1000 μl unlabeled ethylene/liter. After exposure, desiccators containing samples were exposed to a 2-min vacuum to remove unbound ethylene. Whole apples were cut in half before being subjected to the vacuum to facilitate gas exchange. Samples were then aired up to 90 min (minus the vacuuming time), after which they were sealed in jars as described above. Dissociation experiments from whole or ground immature apples were repeated four or three times, respectively. Means and pooled SD of experiments are presented (Fig. 3).

^{14}C -ethylene metabolism and binding were measured simultaneously using procedure 2 of Sanders et al. (1989a). Vials of 1.0 M H_2SO_4 or 3 M NaOH were used during the assay to trap ethylene

Table 1. Dpm bound in mercuric perchlorate, sulfuric acid, or sodium hydroxide traps for ^{14}C -ethylene metabolism and binding assay in immature 'Delicious' apples (whole or pulp). Values have been corrected for nonspecific binding.

Trap vial	Whole apple ^z (dpm/g FW) ^x	Apple pulp ^y (dpm/100 g FW)
HgClO ₄ ^w	258 $\pm$ 27	281 $\pm$ 45
H ₂ SO ₄	1	1
NaOH	0	13

^zMean of three experiments.

^yMean of two experiments.

^xdpm, disintegration per minute; FW, fruit weight.

^wMean $\pm$ SD.

Fig. 3. ^{14}C -ethylene diffusion from 'Golden Delicious' whole immature apples (A) or pulp of immature apples. Dpm values have been corrected for nonspecific binding. Means of four or three experiments. Pooled SD = 374 (A) or 59 (B).

oxide and carbon dioxide, respectively. Carbon disulfide was not used as it appeared to interfere with the assay procedure. Experiments were done in duplicate or triplicate, and means are presented (Table 1).

Results

'Red Delicious' apples used in the Scatchard plots showed a rise in internal ethylene at week 4 of the experiment (Fig. 1). From the displacement data a K_d value was calculated and Scatchard plots constructed (Fig. 2). The K_d value was then used to calculate a C_{50} value, which gives the concentration of ethylene required to saturate half of the binding sites (Venis, 1985). In preclimacteric apples (weeks 1 and 2), the K_d values were the lowest (0.06 and 0.07 $\mu\text{l}\cdot\text{liter}^{-1}$, respectively). In the week before the climacteric rise and as the climacteric rise began (weeks 3, 4, and 5), the K_d values rose from 0.30 to 0.36 to 0.53 $\mu\text{l}\cdot\text{liter}^{-1}$. The rise in the K_d values during the study period indicated a higher concentration of ethylene was required to saturate 50% of the binding sites as the climacteric started. C_{50} values were calculated to be 0.10, 0.11, 0.34, 0.40, and 0.57 μl ethylene/liter, respectively, for each of the 5 weeks. The r^2 values of the displacement data (Fig. 2) range from -0.83 to -0.96, indicating good linear correlations. Nonlinear Scatchard plots often are indicative of multiple binding sites. We attribute the minor fluctuations from a straight line to the difficulty in working with fruit tissue and the low number of binding sites present. Fruit from week 5, which produced considerable ethylene, yielded pulp that was difficult to assay due to high backgrounds; therefore, there is a missing data point in that graph (Fig. 2E). The lower r^2 value also is indicative of the difficulty in assaying climacteric apple tissue.

Dissociation of ^{14}C -ethylene from the binding sites in whole

immature apples showed a gradual loss of bound ethylene over time ($t_{1/2} = 34$ min) (Fig. 3A). In immature apple pulp there was a more abrupt loss of bound ethylene in the first 10 min, giving a $t_{1/2}$ of ≈ 21 min (Fig. 3B). There was a 10-fold difference in the amount of binding between whole apples (Fig. 3A) and pulp (Fig. 3B), although the kinetics of diffusion were similar. We were unable to assay whole mature apples due to interference in the assay by natural ethylene production, and, therefore, had to use immature apples for a comparison of whole and pulped apples. Attempts to reduce ethylene production, using ethylene production inhibitors in intact apples, were unsuccessful (data not shown). Inhibitors did reduce ethylene production, but it was not enough to allow for an undisturbed assay.

The possibility exists that ethylene metabolism influenced our results, even though Sanders et al. (1989b) concluded that ethylene metabolism is not linked to ethylene action. There was no evidence of the ethylene metabolites ^{14}C -ethylene oxide (H_2SO_4 trap) or ^{14}C -carbon dioxide (NaOH trap), in assays involving whole immature apples or apple pulp from mature, ethylene-producing apples (Table 1). The HgClO_4 trap, which contained the majority of the radioactivity, is where ^{14}C -ethylene would be trapped.

Discussion

The K_d value of 0.06 $\mu\text{l}\cdot\text{liter}^{-1}$ for preclimacteric apples in this study is very close to the K_d of 0.09 $\mu\text{l}\cdot\text{liter}^{-1}$ previously reported (Blankenship and Sisler, 1989). As preclimacteric apples start into the climacteric rise in ethylene production, the K_d and C_{50} of the ethylene binding sites present in the pulp increase, requiring a higher concentration of ethylene to saturate 50% of the binding sites. However, the total number of binding sites in apple pulp did not change significantly during this time (Blankenship and Sisler, 1989), and it may be that affinity of the binding sites is changing. Possibly, the binding sites are losing their affinity for ethylene as they age, or a compound is competing with ethylene for the sites.

There possibly are several types of sites, and the composition of the total binding site population is changing, thereby accounting for a change in K_d . We know virtually nothing about multiple sites in fruit or the capacity of the tissue to generate sites. There is evidence in other tissues of multiple binding-site types with different association and dissociation properties (Sanders et al., 1990a, 1990b; Sisler, 1990). We found no evidence of a very fast dissociating site in the dissociation studies in apples. However, this possibility should not be dismissed since dissociation from multiple sites could overlap and mask each other in the curve. Also, apples may not show the magnitude of dissociation that mung beans do (Sisler, 1990) making multiple sites more difficult to detect. Further, apples may contain a very slow dissociating site as noted in mung beans (Sisler, 1990). In mung beans, there is an obvious loss of short-time binding sites with grinding (Sisler, 1990). In the absence of discernible multiple sites in apples, it appears that the total number of sites simply decreases with grinding. Further, the sites lost in apples and mung beans may be of major physiological importance, as their properties may be different from the remaining sites.

Some changes in the ethylene binding sites are taking place with the onset of ripening. The concentration of ethylene necessary to saturate the sites increases. If residual ethylene from endogenous ethylene production were present on the sites, it should take less ethylene to saturate the sites. The increased sensitivity of apples to ethylene with maturation would argue for an increased affinity of the sites for ethylene. This appears not to be the case, at least in the sites present in ground apple flesh, as they show a lower affinity for

ethylene.

Other changes are occurring that make the fruit more difficult to assay with the onset of ethylene production. Immature apples, ground or whole, assay more consistently than more mature apples. In intact apples, even small amounts of endogenous ethylene production during the assay can influence the results. Sites that require a long time to associate and sites on which ethylene remains for extended periods may not be apparent. If ethylene is on such sites, ¹⁴C-ethylene cannot bind to them. The current assay procedure may only identify sites with a relatively short exchange time. Very rapidly exchanging sites would also be missed. The excessive water content in intact fruit can give a high background masking results, whereas tissue maceration causes loss of binding sites. Before the issue of binding site changes in ripening fruit can be resolved, techniques to assay intact fruit, or to isolate the sites in an active form, need to be developed. A complete understanding of ethylene action in fruit will require information about the ethylene receptor and the subsequent steps necessary to initiate an ethylene response. Possibly, ethylene action can be manipulated through ethylene binding to the receptor.

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