Ascorbic Acid Oxidase Activity in Apple Buds: Relation to Thidiazuron-induced Lateral Budbreak

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Abstract. The activity of ascorbic acid oxidase (AAO) was studied in apple (*Malus domestica* Borkh.) buds during dormancy and thidiazuron-induced budbreak. In dormant buds, activity of AAO was low compared with buds that were treated with thidiazuron and had resumed growth. An increase in AAO activity began at the time of metabolic transition from dormancy to budbreak. The highest level of activity was reached 10 days after thidiazuron induction during the expansion growth phase. In vitro AAO activity of apple bud extract was increased by addition of Cu (CuSO_4) and inhibited by Cu-chelating agents, diethyldithiocarbamate (DDC), sodium azide (NaN_3), and 8-hydroxyquinoline (8-OH-Q). In vivo treatment of apple buds with Cu-chelating agents inhibited AAO activity and bud growth but not budbreak. Chemical name used: *N*-phenyl -*N'*-1,2,3-thiadiazol-5-ylurea (thidiazuron).

Many studies have demonstrated the marked physiological changes associated with differentiation and subsequent growth of plant cells. Ascorbic acid oxidase (AAO; EC 1.10.3.3.) is a Cu-containing enzyme that catalyzes the oxidation of ascorbate to 2-dehydroascorbate with the concomitant reduction of molecular oxygen to water (Ohkawa et al., 1989). Plants use ascorbate as an antioxidant in an ascorbate peroxidase (EC 1.11.1.11) reaction that produces dehydroascorbate. Ascorbate is then regenerated in a reduced glutathione (GSH)-dependent reaction catalyzed by dehydroascorbate reductase (EC 1.8.5.1). The oxidized disulfide

form of glutathione (GSSG) is then reduced back to GSH in a reaction involving glutathione reductase (EC 1.6.4.2) and NADPH (Dalton et al., 1986). Fuchigami and Nee (1987) speculated that glutathione is involved in overcoming rest in temperate woody perennials. We previously reported an increase in ascorbic acid, GSH, total glutathione, total nonprotein thiol, and nonglutathione thiol after thidiazuron-induced budbreak (Wang et al., 1991a). Thidiazuron-induced budbreak also increased the ratio of GSH : GSSG and activities of ascorbate free radical reductase (EC 1.6.5.4), ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase, and superoxide dismutase (EC 1.15.1.1) (Wang et al., 1991a, 1991b). These data suggest that the termination of dormancy and induction of budbreak may require the scavenging of superoxide anions and peroxide in bud tissue (Wang et al., 1991a, 1991b). AAO has been reported in

several higher plants (Newcomb, 1951), but its function in the metabolism of the plant cell is unclear. Newcomb (1951) and Morre et al. (1987) suggested that the AAO system might serve to generate energy-rich phosphates or alter properties of the plasma membrane. High activity of AAO is characteristic of diverse, actively growing tissue (Newcomb, 1951). High AAO activity apparently is associated with rapid growth of the primary wall (Honda, 1955; Newcomb, 1951; Waygood, 1950). We conducted our study to determine the changes in the activity of AAO in apple buds and to better understand the involvement of AAO in apple buds during budbreak and resumption of growth.

The growing conditions of 'York Imperial' apple seedlings and treatment methodology used in this study were described by Wang et al. (1986). The seedlings were planted in 3.5-liter pots and grown in a greenhouse with no chilling. Radiation sources

Fig. 1. Effect of pH on ascorbic acid oxidase activity (AAO) in apple buds; AAO was extracted from apple buds 10 days after treatment with 100 μM thidiazuron. Wall = cell wall fraction. Sup = supernatant fraction. Data are the means of three replicate samples. Vertical bars denote $\pm$ SE, bars smaller than the symbols are not shown (Expt. 1).

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Fig. 2. Changes of ascorbic acid oxidase activity and fresh bud weight associated with dormancy, thidiazuron-induced (T) budbreak, and growth resumption in apple buds compared with untreated buds (C). Stages of bud development were classified as follows: a, dormant bud; b, first swell; c, full swell; d, side green; e, green tip; f, bud expansion; g, bud open. Data are the means of three replicate samples. Vertical bars denote $\pm$ SE, bars smaller than the symbols are not shown (Expt. 2).

Table 1. The effects of the Cu-chelating agents diethyldithiocarbamate (DDC), sodium azide (NaN_3), and 8-hydroxyquinoline (8-OH-Q), on bud growth and ascorbic acid oxidase (AAO) activity in apple buds (Expt. 2).^a

Treatment	Inhibitor concn (mM)	Fresh wt (mg/bud)	AAO activity (nmol O_2 /min per μg protein)
Control	0.0	13.2 $\pm$ 1.4	6.57 $\pm$ 0.21
DDC	0.1	9.2 $\pm$ 1.0	5.56 $\pm$ 0.18
	0.5	6.3 $\pm$ 0.7	4.33 $\pm$ 0.11
	1.0	4.2 $\pm$ 0.5	3.96 $\pm$ 0.07
	1.0	4.2 $\pm$ 0.5	3.96 $\pm$ 0.07
NaN_3	0.1	11.6 $\pm$ 1.1	6.09 $\pm$ 0.20
	0.5	7.9 $\pm$ 0.9	5.05 $\pm$ 0.10
	1.0	6.1 $\pm$ 0.8	4.64 $\pm$ 0.09
8-OH-Q	0.1	12.0 $\pm$ 1.2	6.31 $\pm$ 0.17
	0.5	10.6 $\pm$ 1.1	5.70 $\pm$ 0.16
	1.0	8.2 $\pm$ 0.8	5.19 $\pm$ 0.12

^aBuds were treated with indicated concentrations of the chelating agents followed by 100 μM of thidiazuron 4 h later. Samples were measured after 10 days. AAO activity was assayed with the cell wall fraction at pH 5.6.

^bMean of the three replicates $\pm$ SE.

in the greenhouse consisted of natural daylight and 400-W high-pressure sodium lamps (Energy Technics, York, Pa.); which provided a photosynthetically active radiation level of ≈ 400 to $500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 12 h-day⁻¹ (0700 to 1900 HR). Temperatures were $\approx 25/20^\circ\text{C}$ day/night, and relative humidity was 65%. Plants were ≈ 1 m high with a single stem. The buds were in paradormancy, and removal of apical dominance could have induced lateral budbreak. A completely randomized design was used for each of the four experiments. Thidiazuron (100 μM) and selected concentrations (0.1, 0.5, or 1.0 mM) of the Cu-chelating agents diethyldithiocarbamate (DDC), sodium azide (NaN_3), or 8-hydroxyquinoline (8-OH-Q) were prepared in 2.5% (v/v) dimethyl sulfoxide (DMSO) plus 0.5% (v/v) Tween 20 and applied to the buds with a brush until runoff. Control plants were treated with only DMSO and Tween 20. Only the upper seven buds were treated. Lateral buds were used,

and buds were analyzed as a whole for AAO activity and ascorbate content.

Ascorbic acid oxidase activity and pH (Expt. 1). To determine the effect of pH on AAO activity in apple buds, each of 75 apple seedlings was treated with 100 μM thidiazuron. AAO was extracted from apple buds 10 days after treatment. Triplicate bud samples of 0.2 g fresh weight were collected for each of eight pH values tested (range 5.0–6.4). Excised bud tissue (200 mg) was immediately ground in a cool mortar and pestle with 400 μl 100 μM leupeptin (Sigma, St. Louis). The homogenate was mixed with 1600 μl 0.1 M sodium citrate buffer, pH 5.6, and centrifuged at $800 \times g$ for 20 min at 0°C to obtain a clear supernatant. The ionic strength of the buffer was high enough to elute AAO activity from the wall residue (Lin and Varner, 1991). This supernatant fraction was designated as wall fraction and used for assaying AAO activity. The supernatant was further centrifuged for 30 min at $20,000 \times$

g. This supernatant fraction (SUP) was also used to assay AAO activity and to compare its activity with wall fraction. AAO was determined using an oxygen electrode (YSI model 53, YSI Inc., Yellow Springs, Ohio) by measuring O_2 consumption at 28°C . The reaction mixture contained 1.5 ml of 0.1 M sodium citrate buffer adjusted with 1 M HCl or NaOH to the desired pH value, 0.3 ml of 10 mM Na ascorbate, 1.15 ml ddH₂O, and 50 μl of crude enzyme extract. Enzyme activity was expressed as nanomoles of O_2 consumed per minute per microgram protein basis. Protein content was determined according to Bradford (1976), using bovine serum albumin as a standard.

Copper-chelating agents, bud growth, and AAO activity (Expt. 2). The effect of the Cu-chelating agents DDC, NaN_3 , and 8-OH-Q (0.1, 0.5, or 1.0 mM) on bud growth and AAO activity was studied in vivo. Thirty plants were used for each concentration of Cu-chelating agent. Their application was followed by 100 μM of thidiazuron 4 h later. Bud growth and AAO activity were measured after 10 days. Enzyme activity in the cell wall fraction was assayed at pH 5.6.

Copper-chelating agents and AAO activity in vitro (Expt. 3). The effect of Cu, DDC, NaN_3 , and 8-OH-Q on the activity of AAO oxidase was studied in vitro. Seventy-five plants were treated with 100 μM thidiazuron. AAO was extracted from apple buds 10 days after they had been treated with thidiazuron. The test compounds, at various concentrations, were preincubated with crude cell wall AAO extract at pH 5.6 for 3 min before the Na ascorbate substrate was added. The measurement of enzyme activity was conducted as described in Expt. 1.

AAO activity, ascorbate, and bud characteristics (Expt. 4). To study the changes of AAO activity, ascorbate content and fresh bud weight associated with dormancy, thidiazuron-induced budbreak, and growth resumption in apple buds, 1050 apple seedlings were used. One-third of the plants were used as control and two-thirds were treated with thidiazuron. Buds from control and thidiazuron-treated plants were sampled at day 0 and then at 2-day intervals over 12 days. Triplicate bud samples of 0.2 g fresh weight were collected at each sampling time. Depending on their size, the number of buds required ranged from ≈ 10 to 125. AAO activity was assayed as described in Expt. 1.

Ascorbate was determined using the method of Arakawa et al. (1981) and Nakagawara and Sagisaka (1984). This assay is based on the reduction of ferric ion to ferrous ion by the ascorbic acid solution followed by formation of a red chelate between ferrous ion and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) that absorbs at 534 nm. Bud tissue (200 mg) was homogenized in 2 ml of cold 5% (w/v) trichloroacetic acid containing 80 mg polyclar AT (polyvinylpyrrolidone; Sigma) and 400 mg sea sand. The homogenate was filtered through four layers of Miracloth and centrifuged at $16,000 \times g$ for 10 min at 4°C . The supernatant was used for the ascorbate assay. The ascorbate assay

Fig. 3. Relation of ascorbate content (micro-moles ascorbate per gram dry weight) to ascorbic acid oxidase activity (nanomoles O_2 per minute per microgram protein) in apple buds (Expt. 4).

Table 2. The effects of Cu ($CuSO_4$) and the Cu-chelating agents diethyldithiocarbamate (DDC), sodium azide (NaN_3), and 8-hydroxyquinoline (8-OH-Q), on relative ascorbic acid oxidase (AAO) activity in apple bud extract (Expt. 3).^{a,b}

Treatment	Inhibitor concn (μM)	AAO activity (% of control)
$CuSO_4$	0.05	513 $\pm$ 9.8
	0.1	795 $\pm$ 11.9
	1	994 $\pm$ 14.3
DDC	10	84.9 $\pm$ 7.3
	25	58.4 $\pm$ 4.7
	50	16.0 $\pm$ 2.4
NaN_3	50	57.8 $\pm$ 3.9
	100	19.4 $\pm$ 2.5
8-OH-Q	50	78.8 $\pm$ 6.1
	100	62.6 $\pm$ 4.7

^aAscorbic acid oxidase was extracted from apple buds 10 days after treatment with 100 μM thidiazuron. The experiments were conducted with the cell wall fraction at pH 5.6. Absolute value for AAO activity in the control was 6.50 nmol O_2 /min per μg protein.

^bMean of three replicates $\pm$ SE.

mixture contained 0.1 ml of the sample extract, 0.5 ml of absolute ethanol, 0.6 M trichloroacetic acid, 3 mM bathophenanthroline, 8 mM H_3PO_4 , and 0.17 mM $FeCl_3$. The final total volume was 1.5 ml. The solution was kept at 30°C for 90 min to allow the Fe^{2+} -bathophenanthroline complex to develop. The absorbance of the colored solution was measured at 534 nm, using a spectrophotometer (Shimadzu UV-160A, Columbia, Md.). A standard curve in the range 0–10 μmol ascorbate was used to calculate ascorbate content in apple buds.

Thidiazuron stimulated budbreak; 2 days after its application, the treated buds began to swell. As previously reported, untreated buds from the same plant and buds from control plants did not swell (Wang et al., 1991a), indicating that thidiazuron was not translocated in apple stems.

The enzyme extract of apple bud tissue from the cell wall fraction contained higher

levels of enzymatic activity than the supernatant fraction (Fig. 1), which supports reports that AAO is structurally associated with cell walls (Hansl, 1955; Honda, 1955; Newcomb, 1951; Waygood, 1950). The optimal pH for the enzymatic oxidation of ascorbic acid by the cell wall or supernatant fraction of apple bud tissue was within the pH range of 5.5 and 6.0 (Fig. 1), with a rather sharp decline in activity above pH 6.0. This pattern agrees with the pH optimum for AAO reported by others (Dawson and Magee, 1955; Mertz, 1961). All subsequent experiments were conducted with the cell wall fraction at pH 5.6.

Copper-chelating agents DDC, NaN_3 , and 8-OH-Q did not affect thidiazuron-induced budbreak (data not shown) but evidently inhibited bud growth and AAO activity (Table 1). The degree of inhibition increased with increased inhibitor concentration. The decrease of AAO activity was probably due to the inhibition of bud growth by DDC, NaN_3 , and 8-OH-Q. It seems unlikely that AAO was involved in scavenging activity during budbreak, because the oxidation of ascorbate to 2-dehydroascorbate by AAO consumes molecular oxygen rather than hydrogen peroxide (Ohkawa et al., 1989). Very little is known about the specific functions of Cu in fruit trees (Faust, 1989). However, fruit trees respond to Cu deficiency with severe dieback, which is a more severe reaction than their response to other nutrient deficiencies.

AAO activity increased during budbreak, and growth resumed following thidiazuron application (Fig. 2). The control buds remained dormant and had low enzyme activity throughout the experiment. An increase in AAO activity began at the time of metabolic transition, when buds emerged from paradormancy induced by thidiazuron, and resumed growth. AAO activity increased rapidly after induction, and the highest level of activity was reached 10 days later, during the expansion growth phase (Fig. 2). AAO activity was correlated with the growth rate in bud tissue ($r = 0.902$); its activity declined by day 12, probably due to a general increase in protein content. The association of AAO with the walls of cells undergoing rapid growth has been reported in root tips of various plants (Honda, 1955; Jensen and Kavaljian, 1956; Mertz, 1961; Reid, 1941) and in fruits and leaves of zucchini squash (*Cucurbita pepo* L.) (Lin and Vamer, 1991). AAO activity also increased rapidly during the growth of cultured pumpkin (*Cucurbita* sp.) and tobacco (*Nicotiana tabacum* L.) pith cells (Esaka et al., 1988, 1989; Newcomb, 1951).

The ascorbate content was positively related to AAO activity (Fig. 3). Since intracellular ascorbate localization was not determined in this study, we do not know the distribution of ascorbate within the cell. Ascorbic acid acts as an antioxidant and also functions as a co-substrate of plant peroxidases, such as the ascorbate peroxidase or oxidase system that produces dehydroascorbate (Halliwell, 1982). Increased ascorbic acid content in apple buds was associated with

thidiazuron-induced budbreak (Wang et al., 1991a).

AAO activity in apple bud extract was markedly increased by Cu ($CuSO_4$) and was inhibited by the Cu-chelating agents in vitro (Table 2). The degree of increase or inhibition was related to the concentration of Cu or Cu-chelating agents. Copper sulfate at 1, 0.1, and 0.05 μM increased enzyme activity by 10, 8, and 5 times over the control, respectively. At 50 μM , DDC, NaN_3 , and 8-OH-Q inhibited AAO activity in apple bud extract by 84%, 42%, and 21%, respectively. At 100 μM , NaN_3 , and 8-OH-Q inhibited enzyme activity 80% and 37%, respectively. These data suggest the participation of a Cu enzyme in the oxidation of ascorbic acid in apple buds. Esaka et al. (1989) showed a marked increase in AAO activity in pumpkin callus when Cu was added to the culture medium, and the amount of AAO protein in pumpkin callus was controlled by Cu, a prosthetic metal of the enzyme (Esaka et al., 1988).

These studies indicate that AAO is largely localized in the cell walls of apple buds; it is unlikely to be involved in scavenging activity during budbreak because in vivo Cu-chelating agents showed no inhibiting effect on thidiazuron-induced budbreak. The changes in AAO activity that occur in thidiazuron-treated apple buds appear to be associated with the resumption of growth. The rapid increase in enzyme activity parallels rapid cell growth, suggesting that AAO might be involved in the reorganization of the cell wall in apple buds to allow for the buds' expansion. Whether the changes in enzyme activity in the thidiazuron-treated buds are similar to those during dormancy and bud growth under natural conditions warrants further study.

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