Plant Physiol. (1991) 96, 159–165
0032-0889/91/96/0159/07/$01.00/0

Received for publication October 1, 1990
Accepted December 31, 1990

Expression of Ascorbic Acid Oxidase in Zucchini Squash (*Cucurbita pepo* L.)[1]

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ABSTRACT

The expression of ascorbic acid oxidase was studied in zucchini squash (*Cucurbita pepo* L.), one of the most abundant natural sources of the enzyme. In the developing fruit, specific activity of ascorbic acid oxidase was highest between 4 and 6 days after anthesis. Protein and mRNA levels followed the same trend as enzyme activity. Highest growth rate of the fruit occurred before 6 days after anthesis. Within a given fruit, ascorbic acid oxidase activity and mRNA level were highest in the epidermis, and lowest in the central placental region. In leaf tissue, ascorbic acid oxidase activity was higher in young leaves, and very low in old leaves. Within a given leaf, enzyme activity was highest in the fast-growing region (approximately the lower third of the blade), and lowest in the slow-growing region (near leaf apex). High expression of ascorbic acid oxidase at a stage when rapid growth is occurring (in both fruits and leaves), and localization of the enzyme in the fruit epidermis, where cells are under greatest tension during rapid growth in girth, suggest that ascorbic acid oxidase might be involved in reorganization of the cell wall to allow for expansion. Based on the known chemistry of dehydroascorbic acid, the end product of the ascorbic acid oxidase-catalyzed reaction, we have proposed several hypotheses to explain how dehydroascorbic acid might cause cell wall "loosening."

AAO[2] (EC 1.10.3.3) is a plant specific, copper-containing blue oxidase that catalyzes the aerobic oxidation of ascorbic acid to DHA via a free radical semidehydroascorbic acid (also known as monodehydroascorbic acid) intermediate. The enzyme has been known for several decades and its cell wall localization has been established for 40 years (19, 31, 44), but to date no definitive function has been assigned to it. This enzyme is of special interest because of the generally high concentrations of ascorbate in plant cells, the high reactivity of the ascorbate/DHA redox pair, and its cell wall localization. Being a complex and dynamic structure consisting of several classes of polysaccharides, proteins (enzymes as well as structural proteins), and small molecules, the cell wall offers ample opportunities for interaction with ascorbate/DHA. The more recent observations that AAO mRNA codes for a signal sequence (33) and that AAO is secreted into the culture medium in suspension-culture pumpkin cells (8, 9) further support the extracellular localization of AAO.

[1] This study was supported by U.S. Department of Energy grant DE-FG0284ER13255 to J.E.V.

[2] Abbreviations: AAO, ascorbic acid oxidase; DHA, dehydroascorbic acid.

Ascorbate is well known as a scavenger for active oxygen species, such as superoxide, hydroxyl radical, and hydrogen peroxide, especially in the chloroplast and in the nitrogen-fixing root nodules. That function, however, is mediated by a different enzyme, ascorbate peroxidase. Ascorbate peroxidase is the first enzyme in the peroxide-scavenging redox cycle that also involves glutathione and ultimately NADPH (6, 11, 16, 30). Ascorbate oxidase is unlikely to be involved in scavenging activity because the reaction consumes molecular oxygen, rather than hydrogen peroxide. It is of interest in this respect to note that ascorbate-dependent peroxidase activity has been reported in the extracellular fluid of *Phaseolus vulgaris* leaves (34).

The most abundant natural sources of AAO are probably the cucurbits such as cucumber and squash. To obtain a clue of the role of AAO in these plants, we have studied the expression of AAO in developing zucchini squash (*Cucurbita pepo* L.) fruits and in other organs and tissues of the zucchini plant. We report here the correlation of high growth rate and high AAO activity in both fruits and leaves.

MATERIALS AND METHODS

Plant Materials

Zucchini squash (*Cucurbita pepo* L. "Black Zucchini") plants were grown in the greenhouse from seeds. Female flowers were hand-pollinated on the day of anthesis. Fruit circumference (girth) at the thickest part and fruit length (from the pedicel attachment point to the corolla attachment point following the contour of the fruit) were measured with a string. Growth of leaf blades was monitored by marking dots (1 cm apart) on the three main veins with a marker pen and measuring the distances between the dots daily.

Protein Analyses

Developing fruits were harvested and peripheral tissue (including epidermis, peripheral vascular bundles, and outer mesocarp) near the distal end of the fruit (the corolla end) was removed with a razor blade and ground immediately in a chilled mortar in the presence of 100 μL 10 μM leupeptin. The homogenate was mixed with 200 μL 0.1 M NaOAc buffer, pH 5.6, and centrifuged to obtain a clear supernatant. The ionic strength of the buffer was high enough to elute AAO activity from the wall residue. Typically, 0.3 to 0.4 g fresh weight tissue was taken from each fruit. Protein in the supernatant was determined by the dye-binding method of Bradford (4) using BSA as the standard. SDS-PAGE was performed

as described by Laemmli (23), and nondenaturing (native) PAGE was as described by Sachs *et al.* (36). Unless otherwise noted, equal amounts of protein were loaded onto each lane of the gels. Proteins on the gel were visualized by staining with 0.1% Coomassie brilliant blue R-250 in 50% MeOH, 12% HOAc, and destaining in 7.5% HOAc with several changes.

For Western blot analyses, the gel was electroblotted in an Idea Scientific (Corvallis, OR) blot cell onto nitrocellulose membrane in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) containing 20% MeOH and 0.01% SDS. A 1 A battery charger operating at 6 V was used as the power supply, and blotting was performed in the cold room for approximately 17 h. Reaction with antibodies followed basically the procedure of Blake *et al.* (3) using alkaline phosphatase-conjugated sheep anti-rabbit immunoglobulin (IgG). Polyclonal antibodies against purified cucumber AAO as well as nonimmune serum from a different rabbit were kindly provided by Dr. A. Shinmyo, Osaka University, Japan. Dilution of sera was 1:1000.

AAO Assay

AAO was assayed using an oxygen electrode (YSI Inc., Yellow Springs, OH), measuring consumption of O_2. Reaction mixture (3 mL) consisted of 0.1 M NaOAc, pH 5.6, 10 mM Na ascorbate, and typically 2.5 μL of the supernatant of the tissue homogenate as described above. Reaction was started by adding either the tissue extract (with ascorbate already in reaction chamber) or ascorbate stock solution (with tissue extract already in the reaction chamber). The reaction chamber was kept at 25°C by a circulating water bath. Since zucchini extract was green, care was taken that photosynthetic O_2 evolution did not interfere with the O_2 consumption of the AAO reaction; this was done either by covering the reaction chamber with black cloth, or by allowing the reaction mixture to equilibrate in the absence of ascorbate until a stable, straight baseline was obtained. Enzyme activity is expressed as nmol O_2 consumed/min per μg protein or per mg tissue fresh weight.

Ascorbate Determination

Ascorbate was extracted as described by Foyer *et al.* (12) from the same region of the fruit where protein samples were taken. Ascorbate content was measured by the decrease in A_{265} (17) upon the addition of 1 unit ascorbate oxidase (Sigma) to an assay mixture containing 0.1 M NaOAc buffer, pH 5.6, and tissue extract in a final volume of 1 mL.

AAO Activity Staining

For activity staining, standard AAO (Sigma) and different amounts of zucchini fruit extract were electrophoresed in a native polyacrylamide gel (36). Following electrophoresis, the gel was equilibrated in 1 M NaOAc buffer, pH 5.6, for 10 min with one change of solution, and then incubated at room temperature in 6 mM Na ascorbate, pH 5.6, for 30 min with one change of solution. The gel was then rinsed in deionized water briefly, and nitroblue tetrazolium was added to a final concentration of 0.5 mg/mL. Nitroblue tetrazolium is reduced by ascorbate in the gel to a diformazan precipitate, and AAO activity shows as a clear band in a dark purple background.

Total RNA Isolation and Northern Blot Analyses

Total RNA was isolated from typically 10 to 15 g fresh weight of tissue according to a procedure described by Belanger *et al.* (2). The tissue was frozen immediately after harvesting in liquid nitrogen and kept at −80°C until RNA isolation. For Northern blot analyses, 5 μg of total RNA samples were separated by 1% formaldehyde-agarose gel electrophoresis as described by Sambrook *et al.* (37). After electrophoresis, the gel was soaked in 10× standard saline citrate (3 M NaCl, 0.3 M Na citrate) for 1 h and blotted onto "Gene Screen" membrane (New England Nuclear) using 10× standard saline citrate as the transfer medium. The filter was hybridized to a near full-length cDNA clone for cucumber ascorbate oxidase (pASO 11), a gift from Dr. A. Shinmyo, Osaka University, Japan (33). The cucumber AAO sequence shares extensive homology with the zucchini AAO sequence (28). Hybridization and washing were performed according to the method of Church and Gilbert (5). The probe was labeled by random hexamer priming using deoxycytidine-[^{32}P]triphosphate (New England Nuclear). After washing, the filter was blotted dry, wrapped in plastic wrap, and exposed to Kodak XAR-5 x-ray film with Dupont "Lightning Plus" intensifying screens at −80°C.

RESULTS

Growth of the Zucchini Fruit

The growth curve of a typical zucchini fruit from 0 to 20 d after anthesis is shown in Figure 1A. Both circumference and length of the fruit are plotted. Both dimensions showed a typical sigmoid growth pattern. A plot of the logarithms of circumference and length against time (Fig. 1B) shows a straight line up to 6 d after anthesis, indicating exponential growth during this period. Although not shown, estimated fruit volume (calculated from circumference and length) followed essentially the same growth pattern, which agrees well with the general growth pattern of cucurbit fruits analyzed in great detail by Sinnott (38–40). The fruit matured between 30 and 40 d after anthesis, as judged by the germinability of the seeds.

Time Course of AAO Activity in Developing Fruits

Figure 2 shows the specific activity of AAO (expressed as nmol O_2 consumed/min/μg protein) as a function of time. The highest specific activity was reached between 4 and 6 d after anthesis, which falls in the exponential growth phase (Fig. 1). Activity declined thereafter, and dropped to very low levels after 3 weeks. No clear relationship seems to exist between ascorbate content (about 1 nmol/mg fresh weight) and AAO activity over a wide range of AAO activity. However, the highest ascorbate contents were all associated with low AAO specific activity. The two organs with the highest ascorbate content (3–6 nmol/mg fresh weight) were leaves and petals.

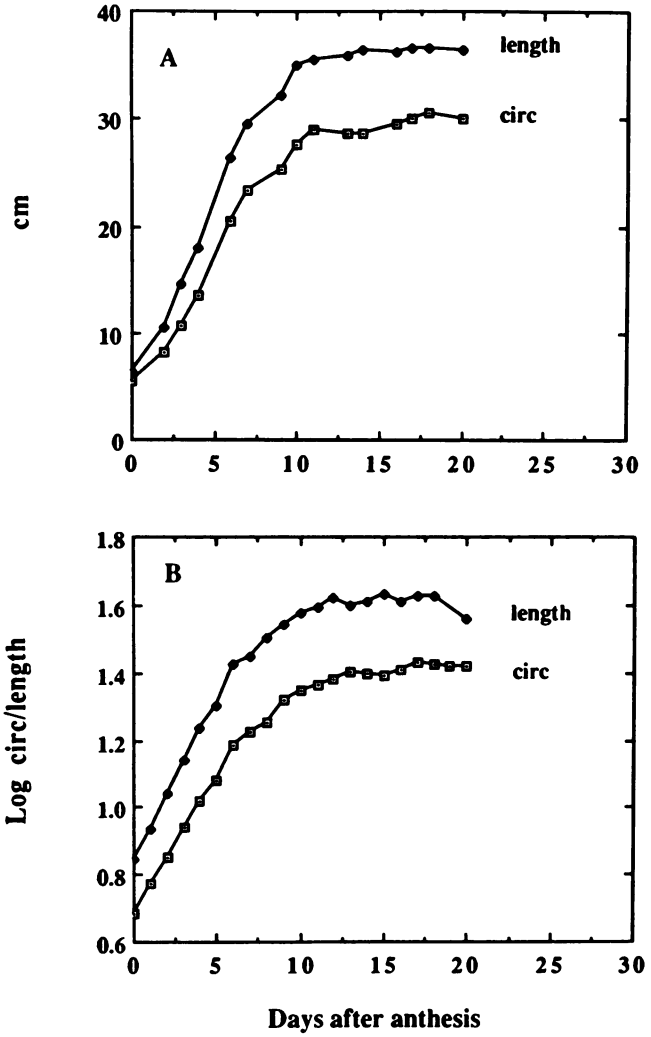

Figure 1. Growth of zucchini squash fruit. A, Growth curve of fruit circumference (circ) and length of a typical fruit. Circumference (at the thickest part of the fruit) and length were measured with a string. B, Plot of logarithms of circumference and length against time in days. In B, each time point represents average from a large number of measurements from different fruits.

AAO Protein and mRNA Levels during Fruit Development

Figure 3A shows a Coomassie brilliant blue-stained SDS gel of proteins extracted from developing zucchini fruits (4–32 d after anthesis). Many changes in protein profile occurred during this developmental period, with some polypeptides increasing, and others decreasing in intensity. The arrowhead points to a band that decreased with fruit age and that had the same molecular mass as AAO monomer (~68 kD). To see if this band was indeed AAO, a similar SDS gel was electroblotted to nitrocellulose membrane and probed with polyclonal antibodies against cucumber AAO. As preimmune serum from the same rabbit was not available to us, we used nonimmune serum from a rabbit different from the control. Figure 3B shows that a polypeptide with the same molecular mass (~68 kD) cross-reacted with the immune serum (arrowhead). This band was not recognized by the nonimmune serum, which instead cross-reacted with the most abundant polypeptide on the gel, probably the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. To further verify that the antibody cross-reacting band had AAO activity, native PAGE was performed. Half of the gel was electroblotted and probed with AAO antibodies, and the other half stained for AAO activity. An AAO standard from Sigma was included in this experiment. Figure 4A (Western blot) shows that the cross-reacting band in fruit extract co-migrated with the standard AAO. Figure 4B (activity staining) shows that the band co-migrating with AAO standard had AAO activity. Activity was detectable in as little as 0.5 μg of total protein.

AAO mRNA levels in the developing fruits, as detected by the cucumber AAO cDNA probe pASO 11, agreed well with the enzyme activity. Highest expression of the gene occurred around 6 d after anthesis. After 10 d, the message became almost undetectable. It is of interest to note that AAO protein was detectable for a much longer time than its mRNA (Fig. 3).

The distal end of the fruit (corolla end) had a slightly higher level of mRNA than the proximal end (pedicel end) (Fig. 5, lanes 2, 3). AAO activity was also higher in the distal end (data not shown). In this variety of zucchini squash, the distal end is thicker (with larger diameter) than the proximal part. Most of the mRNA was concentrated in the epidermal tissue (Fig. 5, lane 4), and nothing was detected in the central placental region where the seeds are borne (Fig. 5, lane 5). This localization of AAO mRNA correlated with AAO activity. Table I shows enzyme assay results from a 5 d old fruit. There was a clear gradient of AAO specific activity from outside in, with highest activity in the epidermis, and lowest activity in the seeds. Lanes 6 and 7 in Figure 5 compare two fruits of the same age (8 d) but different size. The size of a

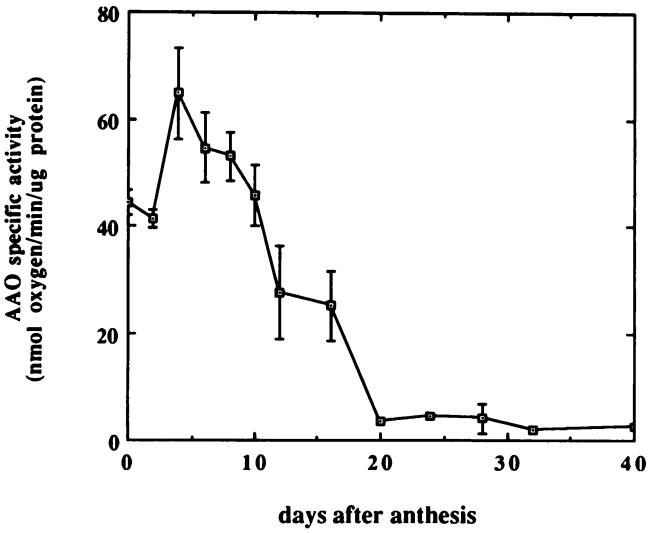

Figure 2. Ascorbic acid oxidase specific activity (nmol O_2 consumed/min/μg protein) as a function of fruit developmental stage. Each data point is the mean of at least three separate assays from different fruits. SE is shown for each timepoint. For d 20, 24, 32, and 40, the errors were too small to show on the graph.

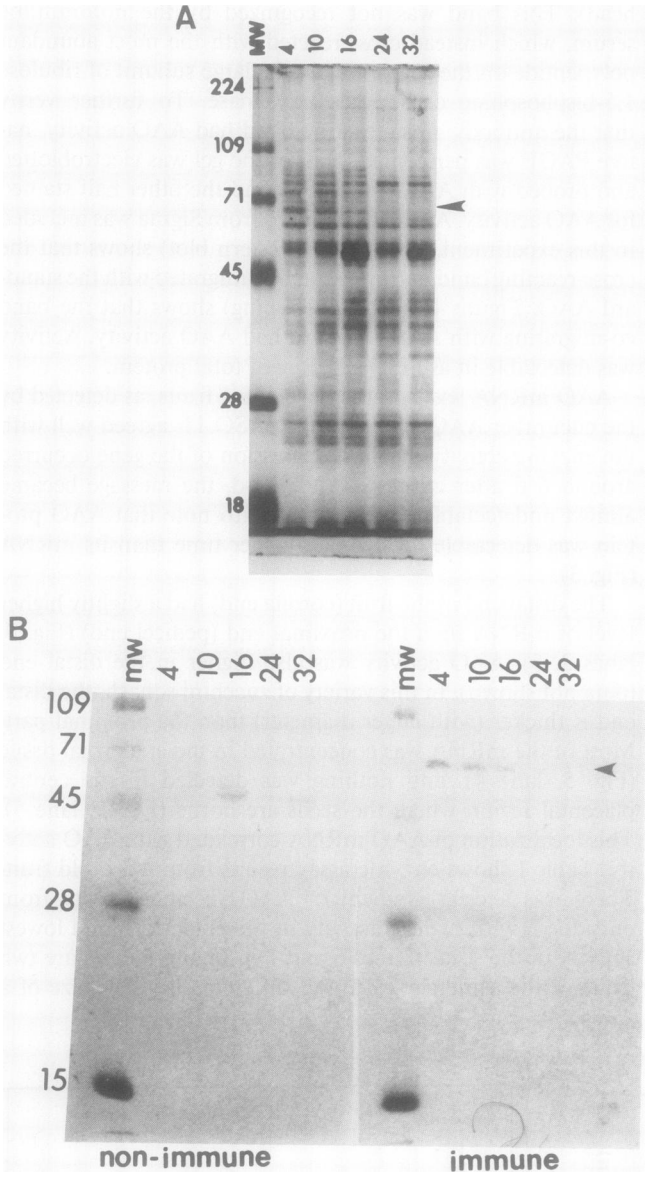

Figure 3. Coomassie brilliant blue-stained SDS gel of proteins extracted from developing zucchini fruits (A), and Western blot of a similar gel probed with antibodies against purified cucumber AAO (B). Numbers on top indicate days after anthesis. Arrows point to a polypeptide that decreased with developmental stage and that cross-reacted with the antibodies. mw = mol wt markers.

given zucchini fruit at a given time depends greatly on the sink distribution as well as the general health of the plant; for example, a fruit growing on a plant where two or more other fruits are developing at the same time will be smaller than a fruit of the same age but developing on a plant without competition. The 8 d small fruit (Fig. 5, lane 6) had a much higher mRNA level than the 8 d large fruit (Fig. 5, lane 7). The same trend was observed in enzyme activity in most cases (data not shown). Thus, AAO expression seems to be a function of "growth potential," or a combination of age and size, rather than of either factor alone.

AAO Activity in the Leaf

As AAO activity was correlated with high growth rate in the fruit, we decided to assay for AAO in the leaf to see if the same correlation existed. Table II shows the AAO activity in three leaves of different developmental stages on the same plant. The specific activities were low in the leaves because of the high protein content. When expressed as activity per mg tissue fresh weight (Table II, last column), the activities were comparable to those found in developing fruits. AAO activity, expressed in either way, decreased with leaf age and size.

In another experiment, growth rate was determined on a

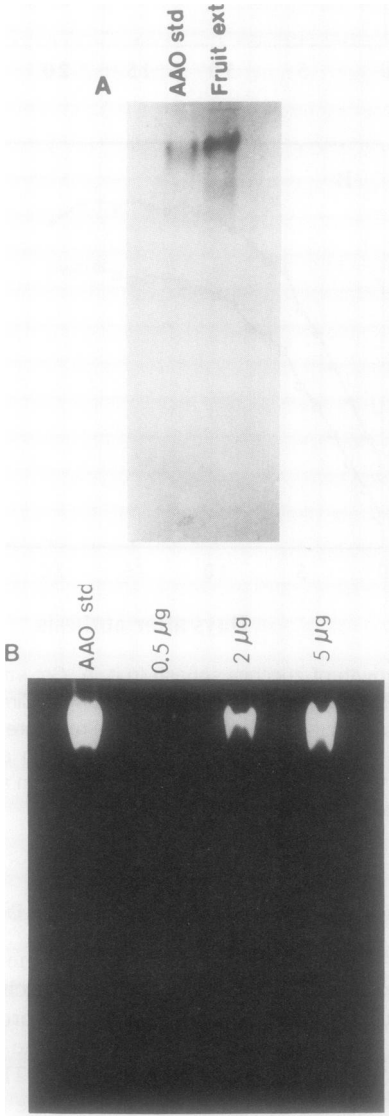

Figure 4. Western blot of a native polyacrylamide gel probed with cucumber AAO antibodies (A), and AAO activity staining of zucchini fruit extract (B). Standard AAO from Sigma (isolated from *Cucurbita* sp.) was included in both panels. Two units of Sigma AAO were used in A, and one unit was used in B. One unit of the Sigma enzyme was approximately 0.5 μg protein. Three different amounts of buffer-extracted zucchini fruit protein (0.2, 0.5, and 5 μg) were used in the activity gel, and 20 μg was used in the Western blot.

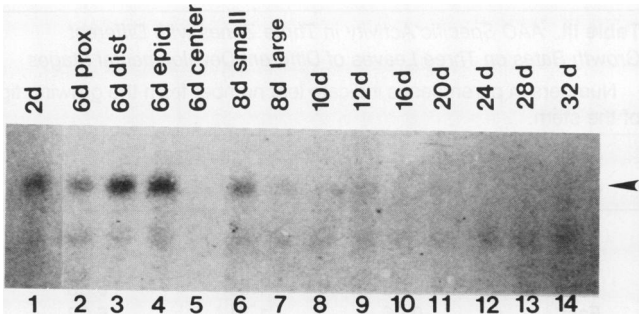

Figure 5. Northern blot of total RNA from developing zucchini fruits probed with cDNA clone (pASO 11) for cucumber AAO. Prox = proximal end of fruit (pedicel end); dist = distal end (corolla end) of fruit; epid = fruit epidermal tissue. Two different sizes of 8 d fruits were included (8 d small and 8 d large). Arrow at right points to the AAO transcript. This band migrates between the two ribosomal RNA bands and is the same size as the cucumber AAO transcript (33).

given leaf, as described in "Materials and Methods." It was found that most elongation of the leaf occurred at the basal third of the blade, and that the least was near the tips of the lobes. In Table III, three leaves of different developmental stages (old, intermediate, young) were assayed for AAO activity in the three growth zones (slow, intermediate, and fast, with intermediate being the region between lower third and leaf apex). As in Table II, the young leaf had highest activity in all three regions. Within a given leaf, regardless of age, activity was highest in the fast-growing region, and lowest in the slow-growing region. Thus, similar correlation of high AAO activity and high growth rate was observed in the leaf.

Since AAO activity was found in green tissues (fruit epidermis and leaves; also in other green organs including petioles and pedicels of male and female flowers) and was very low in nongreen tissues (petals and roots; data not shown), a question was raised as to whether AAO was associated with Chl content of the tissue. This was found not to be the case because in another squash variety ("Gold Bar"), in which the fruit has no trace of green color, AAO activity in the fruit epidermis was comparable to that found in the green zucchini.

DISCUSSION

The association of ascorbic acid and its oxidase with the cell walls of cells undergoing rapid growth has been reported in early literature (21, 27, 35). In cultured cells, AAO activity also rapidly increases after transferring the callus to fresh culture media, *i.e.* when the callus begins to grow (9). Newcomb (31) reported stimulation of AAO activity by auxin in cultured tobacco pith cells and suggested that the AAO system might serve to generate energy-rich phosphate or to alter the properties of the plasma membrane, with the oxidized form of ascorbate at the cell surface and the reduced form in the cell interior. A more recent view links the auxin-induced cell wall acidification to the activity of the plasma membrane-associated NADH-ascorbate free radical oxidoreductase, or monodehydroascorbate reductase (29). The ascorbate free radical (monodehydroascorbate) in this model is generated by the wall-bound ascorbate oxidase.

Our present study shows correlation of high AAO activity with high growth rate in zucchini squash, one of the most abundant natural sources of the enzyme. The correlation existed in both fruits and leaves. Within a given fruit, AAO activity was highest in the epidermal tissue, and lowest in the placental region. The distal end of the fruit, with a larger diameter than the proximal end, also had higher AAO activity. It appears that cells that are under greatest tension during rapid increase in fruit diameter have the highest AAO activity. According to Sinnott (41), in cucurbit fruits, at about the time of flowering, cell division in most of the young fruit ceases, and nearly all later fruit growth is by cell expansion, although cell division persists for a longer time in the epidermis (38). In the leaf, growth is presumably diffuse in nature, involving both cell division and cell elongation (41). Thus, it is not entirely clear whether high AAO activity in zucchini is associated with cell division or cell expansion, or both. Early literature (21, 27, 31) suggests association of AAO activity with cell enlargement, but not cell division.

It is somewhat surprising to note that in an old leaf (25th leaf from the apical meristem), AAO was still expressed differentially in the three different regions corresponding to the fast-, intermediate-, and slow-growing zones in a younger leaf (Table III). Perhaps some degree of cell expansion was still occurring at this late developmental stage. Another possibility is that some senescence-related process involving AAO takes place at different rates in the three regions.

Arrigoni *et al.* (1) reported that many plant tissues lacked AAO activity, even though they had DHA reductase and ascorbate free radical reductase activities. It is not clear whether AAO is universally associated with cell growth in plants, or is confined to certain plant species. A survey of AAO activity in the growing regions of several plant species not closely related should yield answers to this question.

The overall ascorbate content did not seem to have a relationship with AAO specific activity. The fruit appeared to maintain a more or less constant level of ascorbate regardless of AAO activity. Since intracellular ascorbate localization was not determined in this study, we do not know the distribution of ascorbate within the cell; perhaps high ascorbate levels in the chloroplasts masked the differences of ascorbate content in or near the cell wall (green tissue close to the epidermis was used for ascorbate determination). Another remote possibility is that ascorbate is not the *in vivo* substrate for AAO although the enzyme is most active towards L-ascorbate. It has been reported that higher plant AAO oxidizes leuco-2,6-dichloroindophenol to the blue quinoid dye (7) as well as compounds containing functional *o*-diphenolic group like the naturally occurring (+)-catechin (26). The optimal pH for

Table I. *AAO Specific Activity in Different Tissues of a 5 d Fruit*

Tissue Type	AAO Activity
	nmol O_2/min/μg protein
Epidermis	53.8
Peripheral vascular tissue	20.9
Mesocarp	4.9
Placental region	1.0
Seeds	0.3

catecholoxidase activity, however, is 6.7 (26). We found no activity of the zucchini extract towards catechol at pH 5.6, the optimal pH towards ascorbate. Mertz (27) and Dayan and Dawson (7) also found no activity of maize root tip gross homogenates and squash AAO preparation, respectively, towards hydroquinone and catechol at pH 5.7 to 5.8. The physiological pH of the cell wall is probably closer to 6 than 7. Furthermore, *o*-diphenolic compounds normally do not come in contact with AAO *in vivo,* unless destruction of normal cellular structure occurs, such as through wounding or during senescence. It is not known to what extent AAO interacts with the small amounts of phenolics present in the growing cell wall.

If AAO indeed is actively involved in cell growth, it might in some way cause cell wall "loosening." Although there are theories that link the activity of AAO to cell growth (29, 31), we propose that DHA, the end product of the AAO reaction, probably plays an important role in this respect. DHA possesses a number of interesting chemical properties. First, it is structurally analogous to ninhydrin and undergoes the same Strecker reaction with amino acids: two molecules of DHA react with one molecule of amino acid to form CO_2, an aldehyde, and a chromophore (43). In a polypeptide, DHA can presumably react with the amino terminus and lysine sidechains. In fact, we have evidence that DHA changes the behavior of poly-L-lysine in an SDS gel (L.-S. Lin and J. E. Varner, unpublished results). Second, DHA is also an analog of a group of dicarbonyl compounds (2,3-butanedione, 1,2-cyclohexanedione, and phenylglyoxal) that are commonly used to react with arginine sidechains in a protein (for example, Refs. 15, 22, 32). These reagents form a complex with the guanidinium group on arginine residues at slightly alkaline pHs (42). These complexes are often stabilized by borate. The complex formed between DHA and arginine has in fact been studied using x-ray diffraction (24). Finally, DHA is a precursor for oxalic acid. Although there are a number of pathways for oxalate biosynthesis (from ascorbate, glyoxalate, or oxaloacetate; Ref. 14), the ascorbate pathway appears to be preferred in many plants including some oxalate accumulators (25). Ascorbate is converted to DHA before the two-carbon unit cleavage occurs that leads to oxalate formation. Oxalate is best known for its role in calcium oxalate crystal formation in the vacuoles. The older view that these crystals are either metabolic waste products or for protection against predators is probably an underestimation of their importance. The more recent view is that these crystals regulate calcium traffic between the vacuole and the wall, the two largest calcium reservoirs of the cell. Vesicles containing wall-like materials have been observed to move from plasmalemma to vacuole, possibly carrying calcium (associated with wall pectin) to the crystal forming sites in the vacuole in stomium cells of *Capsicum* anthers (20). Calcium oxalate formation has also been reported to be reversible in *Lemna* (13), where crystals form rapidly in the vacuole in response to high apoplastic calcium. In this respect, it is of interest to note that calcium stimulates secretion of AAO in cultured pumpkin cells (10).

Based on the known chemistry of DHA described above, it is conceivable that DHA could cause cell wall loosening by one or more of the following mechanisms: (a) prevention of wall protein covalent cross-linking by modifying lysine sidechains, which are likely to be involved in Schiff base formation with the reducing ends of polysaccharides; (b) prevention of electrovalent interactions between wall proteins and pectin by modifying the positively charged lysine and arginine residues, which could otherwise interact with the negatively charged pectin (polygalacturonate); and (c) active removal of calcium from the calcium-pectin complex in the wall by enhancing oxalate formation. Removal of calcium from the wall should result in a less rigid wall that accomodates growth. A similar mechanism is used by anther stomium cells to weaken the wall and facilitate dehiscence (20). Cell growth can also result from increased turgor caused by accumulation of solutes in the vacuoles, including calcium and oxalate (13). A related discovery is that ascorbate free radical, an intermediate of the AAO reaction, enhances vacuolization, and hence cell elongation in onion root tip cells (18). Calcium oxalate formation is not implicated in the latter report. For the calcium oxalate mechanism to operate, either oxalate needs to compete with pectin for calcium, or some changes (*e.g.* pH, methylesterification) occur in pectin to release calcium, which then combines with oxalate. It is not known whether calcium and oxalate travel to the vacuole independently, or calcium oxa-

Table III. *AAO Specific Activity in Three Zones with Different Growth Rates on Three Leaves of Different Developmental Stages*

Numbers in parentheses indicate leaf number from the growing tip of the stem.

Growth Rate	Developmental Stage		
	Old (25th)	Intermediate (9th)	Young (4th)
	nmol O_2/min/μg protein		
Slow	0.43	0.78	5.16
Intermediate	0.66	1.05	5.53
Fast	0.96	1.71	6.28

Table II. *AAO Activity in Three Leaves of Different Developmental Stages*

Leaf number refers to fully expanded leaf number from the shoot apical meristem.

Developmental Stage	Leaf No.	Size (width × length)	AAO Specific Activity	AAO Activity
		cm × cm	*nmol O_2/min/μg protein*	*nmol O_2/min/mg tissue fresh weight*
Young	3	12 × 9.5	7.3	87.0
Intermediate	6	18 × 13.5	3.8	43.3
Old	17	38.5 × 31.5	0.3	2.0

late is formed in the wall first and then transported to the vacuole.

ACKNOWLEDGMENT

We thank Dr. A. Shinmyo, Department of Fermentation Technology, Osaka University, Japan, for his generous gift of cucumber ascorbate oxidase antibodies and cDNA clone that we used in this study.

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