

Review

Enzymes of Ethylene Biosynthesis

Hans Kende

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

The properties of enzymes involved in ethylene biosynthesis are reviewed and progress toward the purification of these enzymes is described. The enzyme whose activity usually limits ethylene biosynthesis is 1-aminocyclopropane-1-carboxylate (ACC) synthase. Even though its level in plants is extremely low, it has now been purified from several sources. The enzyme that converts ACC to ethylene does not survive homogenization, apparently because it is membrane-bound and because its activity requires membrane integrity. Properties of this enzyme have been elucidated *in vivo* and in vacuolar preparations which possess the capacity to convert ACC to ethylene.

Our understanding of ethylene biosynthesis has grown substantially in recent years. This progress was based mainly on the finding that AdoMet¹ was a likely intermediate in ethylene formation (2) and on the discovery that ACC was the immediate precursor of ethylene (3, 13) (Fig. 1). Once the intermediates in ethylene biosynthesis had been described, interest turned to the purification and characterization of the enzymes that catalyze the individual reactions of the pathway. It became clear that such knowledge was required to elucidate the regulation of ethylene biosynthesis at the molecular level. This review aims at summarizing recent progress in the characterization and isolation of the ethylene biosynthetic enzymes.

AdoMet SYNTHETASE

Application of SeMet to plant tissues stimulates ethylene biosynthesis because SeMet is a better substrate for AdoMet synthetase than is methionine (12). Determining the kinetic parameters of the enzyme showed that the V_{max} with SeMet as substrate was twice as high as that with methionine. On the other hand, the affinity of the enzyme for methionine was higher (lower K_m) than for SeMet. As expected from these kinetic properties, the high rates of product formation with SeMet as substrate were competitively inhibited when methionine was added to the reaction mixture. Modulating *in vivo* the activity of AdoMet synthetase with SeMet and methionine was reflected in the rate of ethylene biosynthesis: stimulation

¹ Abbreviations: AdoMet, S-adenosyl-L-methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AEC, 1-amino-2-ethylcyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; EFE, ethylene-forming enzyme; SeMet, selenomethionine.

of ethylene formation by SeMet could be counteracted by the addition of methionine (12). These results indicated that AdoMet synthetase was an enzyme in the biosynthetic pathway of ethylene and that AdoMet was an intermediate, as proposed by Adams and Yang (2).

ACC SYNTHASE

Shortly after the discovery that ACC was the immediate precursor of ethylene (3, 13), Boller *et al.* (8) developed an assay for ACC, which was based on the *in vitro* conversion of ACC to ethylene, and identified ACC synthase in homogenates prepared from pericarp tissue of ripening tomato fruits. It was shown that AdoMet was indeed the substrate of ACC synthase (K_m 13 μM), that the enzyme activity was low in green tomatoes but increased with ripening, and that AVG inhibited its activity with a K_i of 0.2 μM (8). These findings were confirmed and extended by Yu *et al.* (26) who determined the pyridoxal phosphate requirement of ACC synthase and who showed that the enzymic reaction yielded, besides ACC, 5'-methylthioadenosine. ACC synthase was found to be induced by factors that promote ethylene formation, *e.g.* by IAA and by stress, such as wounding (24).

When it became clear that ACC synthase was involved in the regulation of developmental processes and stress responses in plants, several research groups began to purify this enzyme with the ultimate aim of elucidating the mode(s) of its control. The problem proved to be formidable because of the low abundance and lability of ACC synthase. Based on known kinetic parameters and molecular mass of the enzyme, Blecker *et al.* (6) estimated that the level of ACC synthase in ripening tomato pericarp tissue was <0.0001% of the total soluble protein. This low level can be boosted about 10-fold by wounding the tissue. Experience gained in the purification of an enzyme of such low abundance may be very useful for the purification of other enzymes involved in plant hormone biosynthesis whose levels are also expected to be extremely low.

ACC Synthase from Tomato Fruits

Most efforts have been concentrated on characterizing and purifying ACC synthase from tomato pericarp tissue. Conventional and HPLC gel filtration indicated that native ACC synthase had a molecular mass of 55 to 57 kD (1, 6, 23). ACC synthase was purified >6500-fold by conventional column chromatography and HPLC and identified as a protein of 50 kD by two-dimensional gel electrophoresis (6). The specific

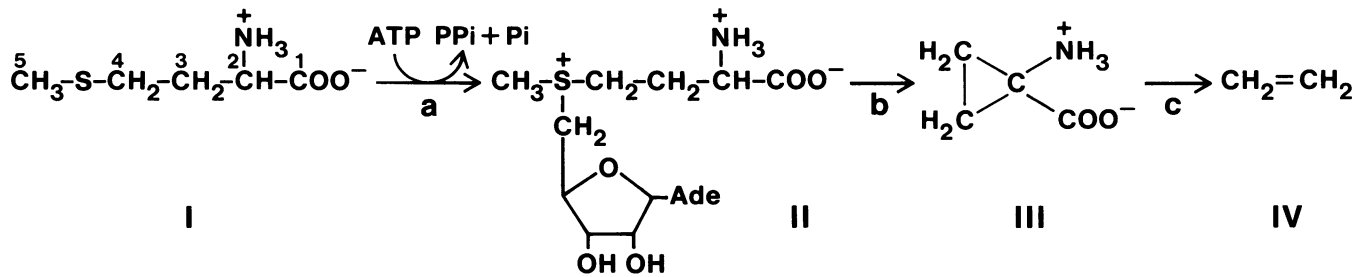


Figure 1. The pathway of ethylene biosynthesis. The intermediates are (I) methionine, (II) S-adenosyl-L-methionine (AdoMet), and (III) 1-aminocyclopropane-1-carboxylic acid (ACC). The enzymes mediating individual steps of this pathway are (a) AdoMet synthetase (ATP:methionine S-adenosyltransferase, EC 2.5.1.6), (b) ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), and (c) the ethylene-forming enzyme (EFE) that has not yet been isolated. Reaction (b) yields, in addition to ACC, methylthioadenosine; reaction (c) requires molecular O_2 and yields, in addition to ethylene (IV), CO_2 and HCN. Ethylene is derived from carbon atoms 3 and 4 of methionine.

activity of purified ACC synthase was estimated to be 2 to 4 $\times 10^5$ units per mg protein (1 unit = 1 nmol ACC produced per h at 30°C). A partially purified ACC-synthase preparation was used to induce antibody production in mice. Following fusion of spleen cells from an immunized mouse and of myeloma cells, 26 hybridoma lines producing antibodies against ACC synthase were obtained. These lines were cloned out twice by limiting dilution. For five of the cell lines, all single colonies tested positively for antibody formation after the second cloning (4, 6). Monoclonal antibodies thus obtained immunoprecipitated native ACC synthase but did not recognize denatured enzyme. Immunoaffinity gels prepared by coupling monoclonal antibody to Sepharose or Affigel were able to remove 90 to 98% of the ACC-synthase activity from crude or partially purified enzyme preparations. The immunopurified protein was shown to have a molecular mass of 50 kD by SDS-PAGE (5, 6). The same molecular mass was observed when the protein preparation was obtained in the presence of proteinase inhibitors (5). Two monoclonal antibodies recognizing different epitopes on the ACC-synthase protein were used to develop a sensitive ELISA for ACC synthase (5). Immunoassays and radioactive labeling showed that ACC synthase was *de novo* synthesized in wound-induced tissue (5, 6), confirming earlier results of density labeling experiments (1).

Privalle and Graham (19) labeled ACC synthase from tomato pericarp tissue by reducing the double bond between pyridoxal phosphate and ACC synthase with sodium borotriide. Analysis by SDS-PAGE showed that the radioactivity was associated with a protein of 50 kD. Satoh and Yang (20) discovered that substrate inactivation of ACC synthase was accompanied by covalent attachment of at least a fragment of AdoMet to the enzyme. When a partially purified enzyme preparation was incubated with [^{14}C]AdoMet and subsequently analyzed by SDS-PAGE, only one radioactive protein of 50 kD was observed. Satoh and Yang (20) also isolated the substrate-labeled protein from the reaction mixture with the immunoaffinity gel of Bleeker *et al.* (6). Taken together, these results constitute strong evidence that ACC synthase in homogenates of tomato fruit pericarp is a 50 kD protein.

Mehta *et al.* (16) also prepared monoclonal antibodies against ACC synthase from wound-induced tomato pericarp tissue. The antibodies from two cloned hybridoma cell lines

recognized the native enzyme and precipitated the enzyme activity. On immunoblots, three polypeptides of 73, 82, and 90 kD molecular mass were identified as ACC synthase. Subsequently, Mehta *et al.* (15) reported that a monoclonal antibody against ACC synthase from wounded pericarp tissue of tomato fruits reacted on immunoblots with a 67 kD protein which was termed a new isoform of the enzyme. It is not clear what the relationship between the 67, 73, 82, and 90 kD polypeptides is and how these relate to ACC synthase which was identified by others (5, 6, 19, 20) as a protein of 50 kD molecular mass.

ACC Synthase from Other Sources

The pitfalls of isolating an enzyme of extremely low abundance are evident from reports on the purification of ACC synthase from mesocarp tissue of winter squash (11, 17, 18). It has to be recognized that ACC synthase may copurify with a relatively abundant protein which is then mistaken for ACC synthase on SDS-PAGE. Antibodies against such contaminated protein preparations are, in all likelihood, not monospecific. They may immunoprecipitate the enzyme in question but may also bind to the major protein on immunoblots. Even with monoclonal antibodies, one has to verify carefully that they are indeed produced by hybridoma cell lines derived from single cells. Nakajima and Imaseki (17) reported to have purified to homogeneity ACC synthase from winter squash. The molecular mass of the enzyme was shown to be 160 ± 10 kD by gel filtration; based on SDS-PAGE, it was thought to be composed of two subunits of 84 kD molecular mass. In a subsequent publication Imaseki *et al.* (11) reported that an antibody prepared against the 84 kD protein did not bind ACC synthase. However, another antibody raised against ACC synthase did immunoprecipitate the enzyme and bound to a 60 kD protein on immunoblots. Hence, the molecular mass of the ACC synthase subunit was revised from 84 to 60 kD. *In vitro* translation of poly(A) $^+$ RNA from winter squash mesocarp followed by immunopurification showed that translatable mRNA encoding the 60 kD protein was not present in freshly cut tissue but increased in level with time after wound induction. More recently, Nakajima *et al.* (18) found that the polyclonal antibodies used to identify the 60 kD polypeptide as ACC synthase were probably not monospecific. A new antibody preparation was used, therefore, to immu-

nopurify ACC synthase from winter squash. The molecular mass of the purified enzyme estimated by SDS-PAGE was now reported to be 50 kD, that of the *in vitro* translation product recognized by the new antibody 58 kD.

Yu and Yang (25) were the first to show the presence of ACC synthase, albeit at very low specific activity, in homogenates of IAA-treated mung bean hypocotyls. Tsai *et al.* (21) reported that a 1050-fold purification of ACC synthase from mungbean hypocotyls yielded a homogeneously pure enzyme of 65 kD molecular mass on SDS-PAGE. The molecular mass of the native enzyme was found to be 125 kD by gel filtration.

In summary, based on gel filtration and determinations of enzyme activity, there appear to be significant differences in the molecular mass of ACC synthase from various sources. Great caution has to be exercised in the identification of ACC synthase by SDS-PAGE. Because the enzyme is present at such low levels, there is a real danger that an abundant contaminant is mistaken for ACC synthase. Antibodies against such impure preparations will immunoprecipitate ACC synthase but will also recognize the contaminant on immunoblots.

ETHYLENE-FORMING ENZYME

Identifying EFE proved to have its pitfalls as well because any system (enzymic or nonenzymic) that contains or produces oxidants also converts ACC to ethylene (24). This is probably the basis for ACC-dependent ethylene production in a number of cell-free systems (22, 24). However, Hoffman *et al.* (10) described a test that permits one to distinguish between artifactual and natural ACC-dependent ethylene-forming activities. The ring structure of ACC contains four hydrogen atoms which can be replaced, one at a time, with an ethyl group to yield four stereoisomers of the ACC analog AEC. One of these, (1R,2S)-AEC, is converted preferentially to 1-butene by the same enzyme that oxidizes ACC to ethylene. Artifactual ethylene-forming systems do not show this stereospecificity (22).

In efforts to identify subcellular compartments that contain components of the ethylene biosynthetic pathway, Guy and Kende (9) found that vacuoles isolated from pea leaves produced 80% of the ethylene evolved by protoplasts. The EFE activity of isolated vacuoles exhibited the same stereospecificity as did the *in vivo* enzyme. Just as EFE activity of intact tissues was destroyed by homogenization so was the activity of the vacuolar enzyme by lysis of the vacuole (9, 14). Further work using isolated vacuoles of *Vicia faba* provided evidence that EFE was associated with the inside face of the tonoplast and that the activity of this enzyme depended on membrane integrity, probably because of the requirement for a transmembrane ion gradient (14). While it seems very likely that the vacuole constitutes one compartment where ethylene is formed in the cell, it is not known whether it is the only one. Conceivably, other membranous structures may also convert ACC to ethylene, but their integrity is destroyed when the tissue is homogenized or when protoplasts are lysed.

Serious technical problems have prevented isolation and *in vitro* characterization of EFE. Reconstitution of this activity, e.g. from lysed vacuolar preparations, and determination as to why its functioning requires membrane integrity are im-

portant steps toward understanding the biochemical mechanism of ethylene formation from ACC.

LITERATURE CITED

1. Acaster MA, Kende H (1983) Properties and partial purification of 1-aminocyclopropane-1-carboxylate synthase. *Plant Physiol* **72**: 139-145
2. Adams DO, Yang SF (1977) Methionine metabolism in apple tissue. Implication of *S*-adenosylmethionine as an intermediate in the conversion of methionine to ethylene. *Plant Physiol* **60**: 892-896
3. Adams DO, Yang SF (1979) Ethylene biosynthesis: Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc Natl Acad Sci USA* **76**: 170-174
4. Bleeker AB (1987) The regulation of ethylene biosynthesis in plants; properties of ACC synthase. *In* DD Randall, RE Sharp, AJ Novacky, DG Blevins, eds, *Current Topics in Plant Biochemistry and Physiology*, Vol 6. University of Missouri, Columbia, pp 15-24
5. Bleeker AB, Robinson G, Kende H (1988) Studies on the regulation of 1-aminocyclopropane-1-carboxylate synthase in tomato using monoclonal antibodies. *Planta* **173**: 385-390
6. Bleeker AB, Kenyon WH, Somerville SC, Kende H (1986) Use of monoclonal antibodies in the purification and characterization of 1-aminocyclopropane-1-carboxylate synthase, an enzyme in ethylene biosynthesis. *Proc Natl Acad Sci USA* **83**: 7755-7759
8. Boller T, Herner RC, Kende H (1979) Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. *Planta* **145**: 293-303
9. Guy M, Kende H (1984) Conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene by isolated vacuoles of *Pisum sativum* L. *Planta* **160**: 281-287
10. Hoffman NE, Yang SF, Ichihara A, Sakamura S (1982) Stereospecific conversion of 1-aminocyclopropanecarboxylic acid to ethylene by plant tissues. Conversion of stereoisomers of 1-amino-2-ethylcyclopropanecarboxylic acid to 1-butene. *Plant Physiol* **70**: 195-199
11. Imaseki H, Nakajima N, Todoka I (1988) Biosynthesis of ethylene and its regulation in plants. *In* GL Steffens, TS Rumsey, eds, *Biomechanisms Regulating Growth and Development*. Kluwer, Dordrecht, pp 205-227
12. Konze JR, Kende H (1979) Interactions of methionine and selenomethionine with methionine adenosyltransferase and ethylene-generating systems. *Plant Physiol* **63**: 507-510
13. Lürssen K, Naumann K, Schröder R (1979) 1-Aminocyclopropane-1-carboxylic acid—an intermediate of the ethylene biosynthesis in higher plants. *Z Pflanzenphysiol* **92**: 285-294
14. Mayne RG, Kende H (1986) Ethylene biosynthesis in isolated vacuoles of *Vicia faba* L.—requirement for membrane integrity. *Planta* **167**: 159-165
15. Mehta AM, Jordan RL, Anderson JD, Mattoo AK (1988) Identification of a unique isoform of 1-aminocyclopropane-1-carboxylic acid synthase by monoclonal antibody. *Proc Natl Acad Sci USA* **85**: 8810-8814
16. Mehta AM, Mattoo AK, Jordan R, Sloger M, Anderson JD (1987) ACC synthase from tomato fruit: identification, general occurrence and developmental regulation using monoclonal antibodies (abstract 687). *Plant Physiol* **83**: S-114
17. Nakajima N, Imaseki H (1986) Purification and properties of 1-aminocyclopropane-1-carboxylate synthase of mesocarp of *Cucurbita maxima* Duch. fruits. *Plant Cell Physiol* **27**: 969-980
18. Nakajima N, Nakagawa N, Imaseki H (1988) Molecular size of wound-induced 1-aminocyclopropane-1-carboxylate synthase from *Cucurbita maxima* Duch. and change of translatable mRNA of the enzyme after wounding. *Plant Cell Physiol* **29**: 989-998
19. Privalle LS, Graham JS (1987) Radiolabeling of a wound-inducible pyridoxal phosphate-utilizing enzyme: evidence for its

- identification as ACC synthase. Arch Biochem Biophys **253**: 333-340
20. **Satoh S, Yang SF** (1988) S-Adenosylmethionine-dependent inactivation and radiolabeling of 1-aminocyclopropane-1-carboxylate synthase isolated from tomato fruits. Plant Physiol **88**: 109-114
 21. **Tsai D-S, Arteca RN, Bachman JM, Phillips AT** (1988) Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyls. Arch Biochem Biophys **264**: 632-640
 22. **Venis MA** (1984) Cell-free ethylene-forming systems lack stereochemical fidelity. Planta **162**: 85-88
 23. **Yang SF** (1980) Regulation of ethylene biosynthesis. HortSci **15**: 238-243
 24. **Yang SF, Hoffman NE** (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol **35**: 155-189
 25. **Yu Y-B, Yang SF** (1979) Auxin-induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. Plant Physiol **64**: 1074-1077
 26. **Yu Y-B, Adams DO, Yang SF** (1979) 1-Aminocyclopropanecarboxylate synthase, a key enzyme in ethylene biosynthesis. Arch Biochem Biophys **198**: 280-286