PURIFICATION AND CHARACTERIZATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE AND A RIPENING RELATED PROTEIN IN APPLE FRUIT (*MALUS DOMESTICA* BORKH)

By

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE AND A RIPENING RELATED PROTEIN IN APPLE FRUIT (*MALUS DOMESTICA* BORKH)

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I. ACC Oxidase was extracted, partially purified and characterized from Golden Delicious apple fruit cortical tissue. The presence of 5% PVPP was necessary in the extraction medium to obtain an active enzyme from apple tissue. Extractable apple ACC oxidase activity correlated well with the climacteric of ethylene production. Polyethylene glycol fractionation followed by calcium phosphate gel adsorption resulted ca. 10-fold purification. Apple ACC oxidase has a K_m for ACC of 6.4 μM, K_m for O_2 of 0.4% and pH optimum of 7.2. The enzyme was stereospecific for substrate. Apple ACC oxidase required both ascorbate and Fe^{2+} as cofactors. The optimum ascorbate concentration was 1-2 mM and the optimum Fe^{2+} was 100 μM. The enzyme was strongly inhibited by $CuCl_2$, $ZnCl_2$, $CoCl_2$ (K_i = 0.2 μM), and $AgNO_3$ at 1 μM. The enzyme was also inhibited by 2-aminoisobutyrate (K_i = 388 μM), n-propylgallate (K_i = 77 μM), 5,5'-dithio-bis(nitrobenzoate) (DTNB), but not by Triton X-100 (0.3%, v/v) or CO (10%, v/v). The inhibition of n-propylgallate on the enzyme activity could be reversed by including more Fe^{2+} in the assay medium, indicating that n-propylgallate probably

acted as a chelating agent for Fe^{2+} in the enzyme reaction, rather than a free radical scavenger. The molecular mass of the enzyme was estimated to be 44 KD by gel filtration.

II. Proteins were extracted from cortical tissue of preclimacteric and postclimacteric apple fruits and analyzed by two-dimensional PAGE. A protein (PAp5) was found to accumulate in postclimacteric apple fruit. PAp5 had a molecular weight of 36.5 KD and isoelectric point 5.3. Polyclonal antibody was raised against the protein. Western blot analysis indicated that PAp5 was absent from apple prior to the onset of the ethylene climacteric but increased markedly as the climacteric developed. The <u>in vitro</u> translation of poly (A)+ RNA from apple at six stages of ripening and immunoprecipitation with PAp5 antisera showed that translatable mRNA for PAp5 increased during ripening.

To my wife, Chunfeng, and my family

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figures Page

SECTION I

SECTION II

SECTION III

LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
Ado-Met	S-adenosyl methionine
AEC	1-amino-2-ethylcyclopropane-1-carboxylic acid
AIB	2-aminoisobutyrate
AVG	aminoethoxyvinylglycine
CCCP	carbonyl cyanide m-chlorophenylhydrazone
DNP	2,4-dinitrophenol
DTNB	5,5'-dithio-bis(2-nitrobenzoate)
DTT	dithiothreitol
EDTA	ethylenediaminetetraactate
EFE	ethylene-forming enzyme
HRP	horse radish peroxidase
IAA	indole-3-acetic acid
IgG	Immunoglobulin G
PAGE	polyacrylamide gel electrophoresis
PAp5	Protein No. 5
PG	polygalacturonase
PVPP	polyvinylpolypyrrolidone
SDS	sodium dodecyl sulfate

LITERATURE REVIEW

The Biochemistry of Fruit Ripening

Blackman and Parija (1928) developed the first theory on the mechanism of fruit ripening. They suggested that the ripening events were consequences of a breakdown in the resistance that kept cellular compartments contained. The theory remained basically unchanged until the 60s, when evidence showing the biochemical changes during fruit ripening started to accumulate. For example, during the ripening of avocado, there were large increments in the biosynthesis of protein (Richmond and Biale, 1966) and nucleic acid (Richmond and Biale, 1967) and the inhibition of protein or nucleic acid synthesis prevented normal ripening (Sacher, 1973). Frenkel and Dilley (1968) indicated that protein synthesis was required for normal ripening of pears and the proteins synthesized early in the ripening process were, in fact, enzymes required for ripening. Brady (1976) showed that most of the increment in protein synthesis early in the climacteric rise of banana fruit resulted in an increase in turnover and the replacement of preexisting species of protein.

During the past decade, an enormous amount of evidence has accumulated suggesting that alterations in gene expression play an important role in the process of fruit ripening. A study of polyribosome profiles has shown that the climacteric peak of ethylene production is preceded by a burst in mRNA synthesis and ribosome production (Drouet et al., 1983). Christoffersen et al (1982) isolated the mRNA populations from

avocado at four stages of ripening and analyzed the in vitro translation products of the mRNAs by two dimensional gel electrophoresis. Three mRNAs were found to increase dramatically with the climacteric rise in respiration and ethylene production. A cDNA library was also constructed from poly(A)+ RNA of ripe avocado fruit (Christoffersen et al., 1984). Colony hybridization identified a number of ripening specific clones. In tomato, at least six abundant mRNAs that are present in immature green tomatoes decline in quantity during maturation and ripening but several other mRNAs either appear or increase greatly during ripening (Grierson et al., 1985). Slater et al., (1985) prepared a cDNA library from polyA-containing RNA from ripe tomato fruit and screened the library by differential hybridization, 146 ripening-related clones were found. The study of the changes in mRNA and protein during ripening in apple fruit indicated that the level of at least six mRNAs were found to increase, while one mRNA decreased; and the level of at least three proteins increased with ripening (Lay-Yee et al., 1990).

Several enzymes have also been identified to be closely related with fruit ripening. These enzymes are: 1) ACC synthase and ACC oxidase (Bufler, G., 1984; 1986); 2) ß-galactosidase in apple fruits (Bartley, 1976); 3) cellulase in avocados (Tucker & Laties, 1984); 4) invertase (Iki et al., 1978) and polygalacturonase in tomatoes (Brady et al., 1982; Tucker & Grierson, 1982).

Christoffersen et al., (1984) showed that the polypeptide for cellulase appeared during the climacteric rise in respiration and ethylene production associated with avocado ripening and its increase correlated with the increase in cellulase enzyme activity. Tucker & Laties (1984) identified, by immunoprecipitation, a 53 KD polypeptide of the in vitro translated system as the precursor of cellulase and found that the mRNA encoding the enzyme was induced during ripening. A cellulase cDNA clone was isolated (Christoffersen et al., 1984). Blot analysis of RNA with this clone as the probe revealed that there was at least 50-fold increase of cellulase mRNA during avocado fruit ripening (Christoffersen et al., 1984).

Polygalacturonase activity and its protein level increases during tomato fruit ripening (Brady et al., 1982; Brady et al., 1983). There are three structurally related isoforms in ripe tomato fruit (PG I, PG II, and PG converter) (Ali & Brady, 1982; Pressey, 1984). These three isoenzyme forms arise from post translational modification of the same polypeptide (Ali & Brady, 1982; Moshrefi & Luh, 1983). Grierson et al (1985) described the precipitation of an in vitro translated polypeptide of 48 KD from ripe tomato, using antisera against purified PG, and the polypeptide was not detectable in unripe tomato. Slater et al (1985) identified a cDNA clone which hybrid-selected a mRNA encoding a 48 KD polypeptide, which was precipitated by PG antisera. Dot

hybridization of mRNA from ripe and unripe fruits with the cDNA indicated that there was a large increase in the amount of PG mRNA during ripening (Slater et al., 1985). Other workers showed similar results, except that PG precursor was found to be a 54 KD instead of a 48 KD polypeptide (Biggs et al., 1986; Sato et al., 1984; 1985).

PG has long been thought to be the key determinant of softening of tomato fruits (Huber, 1983). However, recent studies indicated that PG is not the sole determinant of tomato fruit softening (Giovannoni et al., 1989; Smith et al., 1988). Giovannoni et al., (1989) expressed PG gene in a tomato mutant, rin, which is inhibited in many aspects of fruit ripening including PG production, softening, color development, and ethylene production. In the presence of propylene, they induced PG production as well as polyuronide degradation in the transgenic fruits, but no softening was observed. Smith et al., (1988) introduced a construct designed to express PG antisense RNA into wild type tomato plants and were able to inhibit PG production of the transgenic tomato fruits. But they did not observe any difference in flesh firmness changes during ripening between the control and the transgenic fruits.

The Physiology, Biochemistry and Molecular Biology of Ethylene Biosynthesis Enzymes

Ethylene is a plant hormone which controls many aspects of plant growth and development (Abeles, 1973). The biosynthesis of ethylene is induced during several developmental stages, including seed germination, fruit ripening, leaf and flower senescence and abscission (Abeles, 1973), and by a number of external stimuli such as wounding, auxin treatment, chilling, and drought (Yang & Hoffman, 1984).

Considerable progress has been made in the elucidation of the regulation of ethylene biosynthesis since the discovery that Ado-Met was the likely intermediate in ethylene biosynthesis (Adams & Yang, 1977) and that ACC was the immediate precursor of ethylene (Adams & Yang, 1979; Lürsen et al., 1979). Much of the work has been focused on the purification and characterization (Kende, 1989) of ACC synthase, which catalyses the conversion of Ado-Met to ACC, and ACC oxidase, which catalyses the conversion of ACC to ethylene; and the cloning of the genes coding for these two enzymes (Van Der Straeten et al.,1989; 1990; Sato et al., 1989; 1991; Dong et al., 1991a; 1991b; Nakajima et al., 1990; Wang & Woodson, 1991; Holdsworth et al., 1987; McGarvey et al., 1990).

ACC Synthase

Boller et al., (1979) identified ACC synthase activity in homogenate from pericarp tissue of tomato fruits soon after

ACC was found to be the immediate precursor of ethylene formation. They showed that Ado-Met was the substrate for ACC synthase with a Km = 13 μM, that enzyme activity was low in green tomatoes but increased substantially during ripening, and that AVG inhibited the enzyme activity. Later, Yu et al., (1979) found the enzyme required pyridoxal phosphate (PLP) for maximal activity. The proposed reaction mechanism involves the formation of a Schiff base between the PLP coenzyme and Ado-Met (Yu et al., 1979). ACC synthase was induced by factors that stimulated ethylene biosynthesis (Yang & Hoffman, 1984).

Studies of Kende and Boller (1981) and Yoshii and Imaseki (1982) indicated that not only de novo synthesis but also inactivation of ACC synthase played an important role in the regulation of ethylene synthesis. In vitro inactivation of ACC synthase by its own substrate was reported in tomatoes (Boller, 1985), IAA treated mung bean hypocotyls (Satoh & Esashi, 1986), winter squash (Nakajima & Imaseki, 1986), and apple (Yip et al., 1991). Satoh and Yang (1988) demonstrated that when a partially purified tomato ACC synthase was incubated with Ado(3,4-^{14}C) Met and then subjected to SDS-PAGE, one band with a M.W. of 50 KD was found. The band was believed to be ACC synthase based on the M.W. and on the observation that it was bound to a monoclonal antibody against ACC synthase prepared by Bleecker et al., (1986). They later showed that ACC synthase was also radioactively labeled by

Ado(carboxyl-^{14}C)Met but not by Ado(methyl-^{14}C)Met, suggesting that the 2-aminobutyric acid portion of Ado-Met is linked to ACC synthase during the autoinactivation process. Recently, Yip et al (1990) labeled purified apple and tomato ACC synthase with Ado(^{14}C)Met and NaB^3H_4, and did a trypsin digestion. HPLC separation of the trypsin digest yielded one radioactive peptide. Sequence analysis of this peptide revealed that it was the same lysine residue that binds the PLP and also covalently links to the 2-aminobutyric acid portion of Ado-Met during the inactivation of the enzyme by Ado-Met.

Because of its low abundance, progress in the purification of ACC synthase was slow (Kende, 1989). Recently, the enzyme has been reported to be purified and characterized from tomato (Bleecker et al., 1986; Van Der Straeten et al., 1989), winter squash (Nakagawa et al., 1988; Nakajima et al., 1988), zucchini squash (Sato et al., 1991), apple (Yip et al., 1991), and mung bean hypocotyl (Tsai et al., 1988). cDNAs encoding ACC synthase have been cloned and sequenced in tomato (Van Der Straeten et al., 1990; Olson et al., 1991), winter squash (Nakajima et al., 1990), zucchini squash (Sato et al., 1991; Sato & Theologis, 1989), and apple (Dong et al., 1991a).

ACC synthase was first purified from tomato (Bleecker et al., 1986) and was identified as a protein of 50 KD. Conventional and HPLC gel filtration indicated that ACC

synthase from tomato had a molecular mass of 55 to 57 KD (Bleecker et al., 1986; Acaster & Kende, 1983; Yang, 1980). Privalle and Graham (1987) labeled ACC synthase from tomato pericarp tissue with NaB^3H_4 and found that the radioactivity was associated with a 50 KD polypeptide on SDS-PAGE. Satoh and Yang (1988) demonstrated the same result. In contrast, Van Der Straeten et al., (1989) indicated that ACC synthase was associated with a 45 KD polypeptide. The in vitro translation product of ACC synthase in tomato was a 56 KD polypeptide (Edelman & Kende, 1990), which was undetectable in the green fruits but was induced by ripening and by wounding.

Although White and Kende (1990) did not find isomers of ACC synthase either by SDS-PAGE or electrofocusing of the native enzyme, Van Der Straeten et al., (1990) and Olson et al., (1991) cloned and sequenced two different cDNAs encoding ACC synthase in tomato. The two cDNA shared 68% homology of derived amino acid sequences (Olson et al., 1991). The molecular mass of the derived proteins of the two cDNAs were 54.7 and 53.5 KD, corresponding to 485 and 476 amino acid residues, respectively (Olson et al., 1991). The level of mRNA encoding both isoforms increased during ripening (Olson et al., 1991; Van Der Straeten et al., 1990), but wounding caused an increase in only one of them (Olson et al., 1991). Blot analysis of genomic DNA digested with restriction enzymes confirmed that the two isomers of ACC synthase were encoded by different genes (Olson et al., 1991). Olson et al., (1991)

compared the sequence of the two cDNAs with that of active site dodecapeptide of tomato ACC synthase described by Yip et al., (1990) and concluded that there were at least three isoforms of ACC synthase in tomato fruits.

The ACC synthase purified from zucchini squash (Sato et al., 1991) was a dimer of two identical subunits of approximately 46 KD each. The subunit existed in vivo as a 55 KD species similar to the size of the in vitro translation product (Sato et al., 1991). Sequence analysis of cDNA encoding the ACC synthase revealed that the coding region spanned 493 amino acid residues, corresponding to a 55,779 dalton polypeptide (Sato et al., 1991). The active sequence was the same as that of one of the tomato cDNA (Sato et al., 1991; Van Der Straeten et al., 1990; Olson et al., 1991).

The size of purified ACC synthase from winter squash was estimated to be 50 KD (Nakajima et al., 1988). However, SDS-PAGE fluorograms of an in vitro translation product and the in vivo labeled enzyme showed that the size of the enzyme subunit was 58 KD (Nakajima et al., 1988). The enzyme was probably degraded to a 50 KD polypeptide after transcription (Nakajima et al., 1988). It was suggested that the partial degradation of the enzyme occurred at the carboxyl end, judging from the hydropathy plot of the amino acid sequence (Nakajima et al., 1990). The cDNA clone encoding the enzyme containing an open reading frame encoding 493 amino acids, corresponding to a 55.9 KD polypeptide (Nakajima et al., 1990). Northern blot

analysis showed that the ACC synthase gene was activated by tissue wounding, and its expression was repressed by ethylene (Nakajima et al., 1990).

The ACC synthase purified from apple fruits was a single polypeptide enzyme with a molecular mass of 48 KD (Yip et al., 1991; Dong et al., 1991a; Dong et al., 1991b). Unlike tomato, winter squash and zucchini squash, ACC synthase obtained by in vitro translation was similar in size to the mature protein (Dong et al., 1991a), indicating that ACC synthase in ripe fruit undergoes minor, if any, post transcriptional proteolytic processing. The cDNA for the ACC synthase was estimated to encode 463 amino acid residues (Dong et al., 1991a).

The deduced amino acid sequences of ACC synthase from various sources showed a high degree of conservation (Dong et al., 1991a), with a 52%, 53% and 62% sequence identity between apple and tomato enzymes, apple and winter squash enzymes, and tomato and winter squash enzymes, respectively. The active site of ACC synthase of tomato, apple, winter squash, and zucchini squash is highly conserved, being the same or having only one amino acid difference (Dong et al., 1991a; Olson et al., 1991; Nakajima et al., 1990; Sato et al., 1991; Yip et al., 1990; Van Der Straeten et al., 1990). In spite of the fact that ACC synthase from different species showed high degree of homology, they seemed to be quite different immunologically. The antibody raised against ACC synthase

from one species does not cross react with the enzyme from another species, and vice versa (Yip et al., 1990). Moreover, wound-induced ACC synthase of tomato does not cross react with antibodies againt the ripening-related ACC synthase.

Oeller et al., (1991) showed that tomato fruit ripening was inhibited by expression of antisense RNA to ACC synthase and application of exogenous ethylene or propylene could induce the normal ripening of the tomato fruit.

ACC Oxidase(Ethylene-forming Enzyme; EFE)

Due to the early difficulties in extracting ACC oxidase from plants, much of the work on the characterization of the enzyme was done using intact tissue, protoplasts, or vacuoles isolated from protoplasts (Yang & Hoffman, 1984). The enzyme was found to be dependent on oxygen right after ACC was identified as the immediate precursor of ethylene (Adams & Yang, 1979). Early studies by Burg (1973) indicated that an apparent Km for O_2 of 0.2% for the ethylene production by apple fruit tissue. Yip et al., (1988) found that, in apple fruit tissue, Km for O_2 was 6.2% in the absence of added ACC and was 0.3% at a saturating level of ACC. The apparent Km for O_2 was 1% in flower tissue of morning glory (Konze et al., 1980). Banks (1985) reported an apparent Km for O_2 of 2.2% for banana tissue slices when corrected for an O_2 gradient in the tissue. The dependence of ethylene production on ACC levels has been studied in a number of tissues. Based on the internal concentration of ACC, the apparent Km for ACC was

estimated to be 66 μM in pea epicotyls (McKeon & Yang, 1984), 34 μM in mung bean hypocotyls (Yip et al., 1988), 120 μM in etiolated wheat leaves (Yip et al., 1988), 8.1 μM in apple fruit (Yip et al., 1988) and 61 μM in isolated pea leaf vacuoles (Guy & Kende, 1984).

Numerous factors have been shown to inhibit the conversion of ACC to ethylene (Yang & Hoffman, 1984), including heavy metal ions such as Co^{2+} and Ag^{+} (Apelbaum et al., 1981a; Yu & Yang, 1979; Bradford et al., 1982; Satoh & Esashi, 1983), uncouplers such as DNP and CCCP (Yu et al., 1980; Apelbaum et al., 1981b), membrane disrupting agents such as Triton X-100 and osmotic shock (Apelbaum et al., 1981a; Mitchell et al., 1988; Mayne & Kende, 1986), free radical scavengers such as n-propylgallate (Apelbaum et al., 1981b; Satoh & Esashi, 1983), ACC analogues such as 2-aminoisobutyrate (AIB) (Satoh & Esashi, 1980; 1982; 1983; Liu et al., 1984), and high temperature (Yu et al., 1980). Satoh and Esashi (1983) compared the inhibitory effect of AIB on ethylene production with that of n-propylgallate and Co^{2+} and found that only AIB was effective in causing the accumulation of endogenous free ACC in the tissue and that the degree of inhibition of ethylene production by AIB decreased with increasing concentration of pre-loaded ACC, while the inhibition by n-propylgallate and Co^{2+} changed little. Kinetic analyses showed that AIB competitively inhibited the conversion of pre-loaded ACC to ethylene; n-propylgallate and

Co^{2+} inhibition was general.

Soon after ACC was identified as the immediate precursor of ethylene (Adams & Yang, 1979), Konze and Kende (1979) reported an enzyme extract from etiolated pea seedling capable of converting ACC to ethylene. Many similar enzyme systems were reported later on, including a carnation microsomal system (Mayak et al., 1981), pea microsomal system (McRae et al., 1982), IAA-oxidase (Vioque et al., 1981), peroxidase (Rohwer & Mader, 1981), lipoxygenase (Bousquet & Thimann, 1984; Wang & Yang, 1987), and a cell free system from barley root (Nilsen et al., 1988).

Ethyl substitution of each of the four methylene hydrogens of ACC results in four stereoisomers of AEC whose absolute configurations are: (1R, 2R), (1S, 2S), (1R, 2S) and (1S, 2R) (Hoffman et al., 1982). Hoffman et al., (1982) examined the conversion of these four stereoisomers of AEC to 1-butene by postclimacteric apple and etiolated mungbean hypocotyls and found that only (1R, 2S)-AEC was effectively converted to 1-butene. McKeon and Yang (1984) compared the stereospecificity, with regard to the conversion of AEC isomers to 1-butene, by pea epicotyls and the pea epicotyl extract of Konze and Kende (1979). They found that only (1R, 2S)-AEC was preferentially converted to 1-butene in pea epicotyls. This conversion was inhibited by ACC, indicating that 1-butene formation from (1R, 2S)-AEC and ethylene formation from ACC were catalyzed by the same enzyme.

Furthermore, pea epicotyls effectively converted ACC to ethylene with a low Km (66 μM). In contrast, the pea epicotyl extract catalyzed the conversion of AEC isomers to 1-butene without stereodiscrimination and converted ACC to ethylene with a high Km (389 mM). The work was extended by Pirrung (1983; 1986), Venis (1984), Guy and Kende (1984), and Wang and Yang (1987) who demonstrated that none of the *in vitro* enzyme systems above resembled the *in vivo* enzyme in terms of stereospecificity and affinity for ACC. Therefore, these enzymic systems are not the physiological systems responsible for the conversion of ACC to ethylene *in vivo*. The conversion of ACC to ethylene has been attributed to non-enzymatic, chemical reaction between ACC and free radical products generated by these enzymes, whereas the physiological enzyme catalyzes directly the oxidation of ACC to ethylene (Yang & Hoffman, 1984).

The authentic *in vitro* ACC oxidase activity was reported first by Guy and Kende (1984), who observed that vacuoles isolated from protoplasts accounted for 80% of the protoplast ethylene production. Unlike the other *in vitro* enzyme systems, the vacuoles resembled the *in vivo* ACC oxidase activity in that they stereochemically discriminated between AEC isomers and having a high affinity for ACC (Km = 61 μM). The *in vitro* activity was shown to be dependent on the membrane integrity (Mayne & Kende, 1986). These observations were confirmed by Porter et al., (1986) who also found that

the protoplasts had lost much of the ACC oxidase activity compared with its parent tissue.

Important progress was made recently by Ververidis and John (1991), who demonstrated complete recovery of ACC oxidase activity from melon fruit in the soluble enzyme fraction. The ACC oxidase activity had a apparent Km of 85 μM and stereospecificity was demonstrated. They also showed that the ACC oxidase activity did not depend on membrane integrity but required ascorbic acid and ferrous ion as cofactors. Their work was confirmed by other groups (Kuai & Dilley, 1992; Fernández-Maculet & Yang, 1992). It was suggested that the dependence of *in vitro* ACC oxidase activity of vacuoles prepared by Guy and Kende (1984) on membrane integrity was probably due to the leaking of the cofactors essential for the enzyme activity (Fernández-Maculet & Yang, 1992). The isolation and further purification of this enzyme should help in characterizing the reaction mechanism catalyzed by this enzyme and studying the regulation of the enzyme *in vivo*.

cDNAs for ACC oxidase have been cloned for tomato (pTOM 13; Holdsworth et al., 1987), avocado (pAVOe3; McGarvey et al., 1990) and carnation (pSR 120; Wang & Woodson, 1991). The identity of the tomato cDNA clone was confirmed by Hamilton et al., (1990), who showed that antisense RNA for pTOM 13 reduced ethylene synthesis in a gene dosage-dependent manner. Tomato pTOM 13 encodes for a 33.5 KD polypeptide (Holdsworth et al., 1987). Avocado clone pAVOe3 DNA contains a 320 amino

acid open reading frame, corresponding to a polypeptide of 36,230 dalton with a pI 4.94 (McGarvey et al., 1990). Carnation clone pSR120 DNA has a 321 amino acid open reading frame, corresponding to a polypeptide of 36,819 daltons with a pI 6.43 (Wang & Woodson, 1991). The DNA sequence homology between pTOM 13 and pAVOe3 is 72% and the derived protein sequence of pAVOe3 shares 76% identity with that of pTOM 13 (McGarvey et al., 1990). Carnation pSR120 has 64% and 68% of DNA sequence homology and 77% and 73% amino acid identity with pTOM 13 and pAVOe3, respectively (Wang & Woodson, 1991)

Recently, Hamilton et al., (1991) directly sequenced pTOM 13 homologous mRNA from tomato fruit and found that the sequence was identical to pTOM 13 between nucleotides 172 and 274 in all but two positions, where an additional C and G were present. The absence of the two nucleotides from pTOM 13 was assumed to be a cloning artifact (Hamilton et al., 1991). Inclusion of these nucleotides resulted in 60 additional bases to the 5' end of the open reading frame. The corrected pTOM 13 was constructed and expressed in yeast and authentic ACC oxidase activity was demonstrated (Hamilton et al., 1991).

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SECTION I

EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE FROM APPLE (*MALUS DOMESTICA* BORKH.) FRUIT.

ABSTRACT

ACC oxidase (ethylene-forming enzyme - EFE) was extracted, partially purified and characterized from Golden Delicious apple fruit cortical tissue. The presence of 5% (w/v) polyvinylpolypyrrolidone (PVPP) was necessary in the extraction medium to obtain an active enzyme from apple tissue. The specific activity was 40 to 100 nl ethylene hr^{-1} mg^{-1} protein in the crude extract; polyethylene glycol fractionation followed by calcium phosphate gel adsorption resulted in ca. 10-fold purification. Enzyme activity was retained at -20°C when the enzyme was stored with 30% glycerol. Ascorbate and Fe^{2+} were not essential in the extraction medium for apples. Apple ACC oxidase has a K_m for ACC of 6.4 μM, K_m for O_2 of 0.4% and a pH optimum of 7.2 in Tris-Mes buffer and 7.6 in phosphate buffer. The enzyme was stereospecific for substrate; it converted (±)allocoronamic acid to 1-butene while (±)coronamic acid was 10-fold less effective as a substrate. When the likely substrate availability <u>in vivo</u> is considered, the characteristics of apple ACC oxidase are consistent with those from a range of other tissues and isolated pea leaf vacuoles, all of which also contain the enzyme.

INTRODUCTION

Unequivocal *in vitro* demonstration of the enzyme that converts 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene in plants has been problematic. This led to the assumption (Guy and Kende, 1984) that the enzyme was membrane bound and required some structural or compartmental association to be active. Ververidis and John (1991) recently demonstrated complete recovery of ACC oxidase (EFE) activity from melon fruit (*Cucumis melo*). *C. melo* ACC oxidase has an apparent K_m for ACC of 85 μM and stereospecificity for substrate was demonstrated. Prior attempts by other researchers to obtain bonafide enzyme activity of ACC oxidase in plant cell extracts gave only artifactual results; the cell-free systems exhibited apparent K_m values of 10 mM or more for ACC (Yang and Hoffman, 1984; Nilsen et al., 1988). Moreover, some cell-free systems (Vinkler and Apelbaum, 1983) lacked stereospecificity for substrate as shown by Venis (1984) and Pirrung (1983) or were contaminated with non-specific free-radical-producing enzymes (Diolez et al. 1986; Konze and Kwiatkowski, 1981, Pirrung, 1986). Ethylene formation from ACC by putative ACC oxidase has been shown by intact protoplasts (Bouzayen et al., 1990) isolated vacuoles (Guy and Kende, 1984) and in several intact tissues (Hoffman et al., 1982; Yip et al., 1988). Following the lead of Ververidis and John (1991) we have confirmed their results

with C. melo, extended this to C. pepo and herein report extraction, partial purification and preliminary characterization of ACC oxidase from apple fruit.

MATERIALS AND METHODS

Plant Materials

Apples (Malus domestica Borkh. cv Golden Delicious) employed as the enzyme source were stored in air at 1°C for several months and left at ca. 20°C for two days before extraction. C. melo cv. Honeyrock and C. pepo cv. Zucchini were obtained from a local grocer.

Extraction and Partial Purification of Apple ACC Oxidase

Cortical tissue (130 g) was ground in liquid N_2 using an electrically driven mortar and pestle. The ground powder was added to 200 ml of extraction buffer containing 0.1 M Tris.HCl (pH 7.7), 30 mM sodium ascorbate and 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). After the slurry thawed completely, it was filtered through two layers of Miracloth and centrifuged at 30,000 x g for 20 min. The pH of the resulting supernatant solution (pH 7.7) was adjusted to 5.5 with 2N acetic acid and solid polyethyleneglycol 4000 (PEG) was added (0.05 g/ml extract) and centrifuged at 15,000 x g for 5 min. Additional PEG was added to the supernatant solution (0.35 g/ml of original extract) and centrifuged at 15,000 x g for 5 min. The resulting pellet was suspended in 50 ml of 0.1 M glycylglycine (pH 7.0) with the aid of a ground

glass tube and pestle. The enzyme suspension was clarified by centrifuging at 15,000 x g for 5 min. The pH of the supernatant solution was adjusted to 5.5 with 2N acetic acid and 2.5 ml of calcium phosphate gel (10 mg solids per ml) was added. The mixture was stirred for 5 min., centrifuged at 15,000 x g for 5 min. and the pellet was discarded. Five ml of calcium phosphate gel was added to the supernatant solution, mixed for 5 min. and the gel was pelleted by centrifuging at 15,000 x g 5 min. and the supernatant was discarded. The enzyme was eluted from the gel pellet with 20 ml of 0.1 M phosphate buffer (pH 7.0). Enzyme activity was fully retained following storage at -20°C when held in 30% (v/v) glycerol.

Enzyme Assay In Vitro and In Vivo

The reactions were performed as described by Ververidis and John (1991). Each 25 ml vial contained a total volume of 2.7 ml of reaction mixture (0.05 M Tris.HCl, 10% (v/v) glycerol, 30 mM sodium ascorbate, 0.1 mM $FeSO_4$ and 1 mM ACC (Sigma), pH 7.2). The vials were gently shaken for 1 hr and ethylene which accumulated in the headspace was determined by gas chromatography. The reaction mixture for tissue discs contained 0.8 M mannitol and 1mM ACC with one gm. of freshly cut tissue in each vial.

Partially purified enzyme (post calcium phosphate step) was used for the determinations of the apparent K_m for O_2 and ACC and for determining the pH optimum. Oxygen levels from 0.1 to 20% (v/v) were obtained by flushing each vial with

nitrogen and injecting varying amounts of air. The vials were equilibrated for 1 hr and the reaction was initiated by adding 0.2 ml of enzyme solution. Oxygen concentrations were confirmed by analysis of 0.5 ml gas samples employing a paramagnetic O_2 analyzer as described in Jobling et al., (1991). Ethylene was determined in 1 ml gas samples taken immediately after sampling for O_2 analyses.

Ethylene and 1-butene were determined using a Carle Series 100 AGC gas chromatograph (FID detector) employing an activated alumina column and He as the carrier gas. Ethylene and 1-butene were quantified using 1 μl l^{-1} standards. Protein content was determined as described by Bradford (1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The results of Ververidis and John (1991) were confirmed by demonstrating results similar to theirs with *Cucumis melo* (cv. Ogen) using cv. Honeyrock; ACC oxidase activity was found in extracts from *C. pepo* cv. Zucchini (Table 1). Using the same extraction protocol, only a small amount of total ACC oxidase activity from apple fruit tissue was recovered. By including 5% (w/v) polyvinylpolypyrrolidone (PVPP) in the initial extraction medium, high activity of ACC oxidase was obtained from apple fruit based on the *in vivo* conversion rate of ACC by tissue discs. It is presumed that PVPP complexed polyphenols which are abundant in apple fruit and are

Table 1. Requirements for extraction of ACC oxidase activity from Golden Delicious apples, Honeyrock, and Zucchini squash.

Extraction medium	Ethylene production relative to tissue disc (%)		
	Apple	Melon[z]	Squash[z]
1. [0.1 M Tris(pH 7.7), 30 mM sodium ascorbate][y]	10	85	112
2. (1)[x] + 5% PVPP	65		
3. (2) + anaerobic extraction	64		
4. (2) + Fe^{2+} (0.1 mM)	63		
5. (2) - sodium ascorbate	48		
6. (6) + 2-mercaptoethanol (0.2% v/v)	10		
7. (6) + dithiothreitol (5 mM)	12		

[z]ACC oxidase activity of melon tissue discs was 59.1 ± 3.0 nl ethylene hr^{-1} gFW^{-1} and for squash tissue discs the activity was 9.60 ± 0.34 nl ethylene hr^{-1} gFW^{-1}. For apple tissue discs ACC oxidase activity was 208 nl ethylene hr^{-1} gFW^{-1}. Values are relative to the respective <u>in vivo</u> ethylene production by tissue discs on an equivalent FW basis.

[y]Extraction medium of Ververidis and John (1991). Cortical tissue ground to powder with liquid N_2 then thawed in the extraction medium; the filtered and centrifuged extract was employed as a crude enzyme extract to assay the enzyme.

[x]Numbers in parentheses refer to the extraction media numbers; e.g. medium 2 is medium (1) plus PVPP, medium 3 is medium (2) plus N_2 purging, etc.

problematic to recovering numerous enzyme activities once the fruit cells are disrupted (Hulme et al. 1964). The presence of ascorbate in the initial extraction medium resulted in improving the recovery of the enzyme activity by ca. 25% in the presence of PVPP (medium 2 vs. medium 5); including Fe^{2+} and employing anaerobic media were not necessary to extract ACC oxidase activity from apple fruit (Table 1). Using 2-mercaptoethanol or dithiothreitol for sulfhydryl group protection in the Ververidis and John (1991) extraction medium without PVPP did not provide protection against enzyme inactivation during extraction.

Stereospecificity for Substrate

ACC oxidase was partially purified by polyethylene glycol precipitation followed by calcium phosphate gel adsorption (Table 2). Partially purified apple ACC oxidase was employed to determine stereospecificity of the enzyme for substrate. These were (±)allocoronamic and (±)coronamic acid isomeric homologs of ACC (kindly supplied to us by H. Kende and by M. Venis) were converted unequally to 1-butene with the (±)allocoronamic acid being the preferred configuration of 1-amino-2-ethylcyclopropane-1-carboxylic acid [(1S,2R)-AEC] by a ratio of ca. 10:1 vs (±)coronamic acid (Table 3). This is accepted evidence of equivalence between *in vivo* and *in vitro* conversion of ACC to ethylene (Hoffman et al., 1982; Pirrung, 1983; Adlington et al., 1983; Venis, 1984). Apple cortical discs showed the same stereospecificity for the AEC isomers as

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Table 2. Partial purification of ACC oxidase from Golden Delicious apple fruits[z]

step	Enzyme activity[y]	Protein (mg)	Specific activity[x]	Recovery (%)
Crude extract	5010	124	40.4	100
PEG precipitate	5236	37.4	140	105
Calcium phosphate gel eluate	3888	9.6	405	78

[z]130 g of fruit cortical tissue
[y](nl ethylene hr^{-1})
[x](nl ethylene hr^{-1} mg^{-1} protein)

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Table 3. Stereospecific conversion of racemic mixtures (±) coronamic acid and (±)allocoronamic acid by apple ACC oxidase to 1-butene.

Enzyme source[z]	Substrate	Olefin production[x]	
		1-butene formed	ethylene formed
Enzyme extract[y]	(±)allocoronamic acid (1R,2S)-and (1S,2R)-AEC	433.4 ± 2.5	-
"	(±)coronamic acid (1S,2S)-and (1R,2R)-AEC	47.4 ± 6.5	-
"	1-aminocyclopropane-1-carboxylic acid	-	958.0 ± 5.4
Fruit discs	(±)allocoronamic acid	54.3 ± 1.7	-
"	(±)coronamic acid	6.04 ± 1.7	-
Crude extract	1-aminocyclopropane-1-carboxylic acid		100 ± 11.0

[z]Activity for enzyme extract expressed on mg protein basis and for fruit discs on gFW basis.
[y]A partially purified enzyme was employed (after elution from calcium phosphate gel).
[x](nl olefin hr^{-1} mg $protein^{-1}$ or gFW^{-1})

the partially purified enzyme thus proving authenticity of ACC oxidase as the enzyme.

Kinetic Properties of Apple ACC Oxidase

The K_m for ACC and O_2 and the pH optimum were determined with partially purified (sp. act ca. 1000 nl ethylene hr^{-1} mg^{-1} protein) apple ACC oxidase. The apparent K_m for ACC was 6.4 μM (Fig. 1) determined at pH 7.2 and varied from 5.8 to as high as 8 μM in three independent determinations. This compares to a K_m of 85 μM for the enzyme from C. melo (Ververidis and John, 1991) and a K_m of 8.1 μM for apple fruit tissue and 34 μM for mung bean hypocotyl tissue as determined in vivo by Yip et al. (1988). The apparent K_m for ACC of isolated pea leaf vacuoles was 61 μM (Guy and Kende, 1984 and that of the enzyme in etiolated wheat leaf tissue was reported to be 120 μM (Yip, et al., 1988). It is reasonable and probable that the apparent K_m values for ACC reported for ACC oxidase employing intact tissues or isolated vacuoles are higher than our value of 6.4 μM for the extracted enzyme because of uncertainties in knowing the actual ACC concentration at the enzyme active site in the in vivo studies. Alternatively, the specific assay conditions of pH, ionic strength and buffer employed for the in vivo determinations differed from our assay conditions with the partially purified enzyme. The difference in K_m values for the C. melo of 85 μM as determined in vitro by Ververidis and John (1991) and our value for K_m of 6.4 μM ACC for the apple

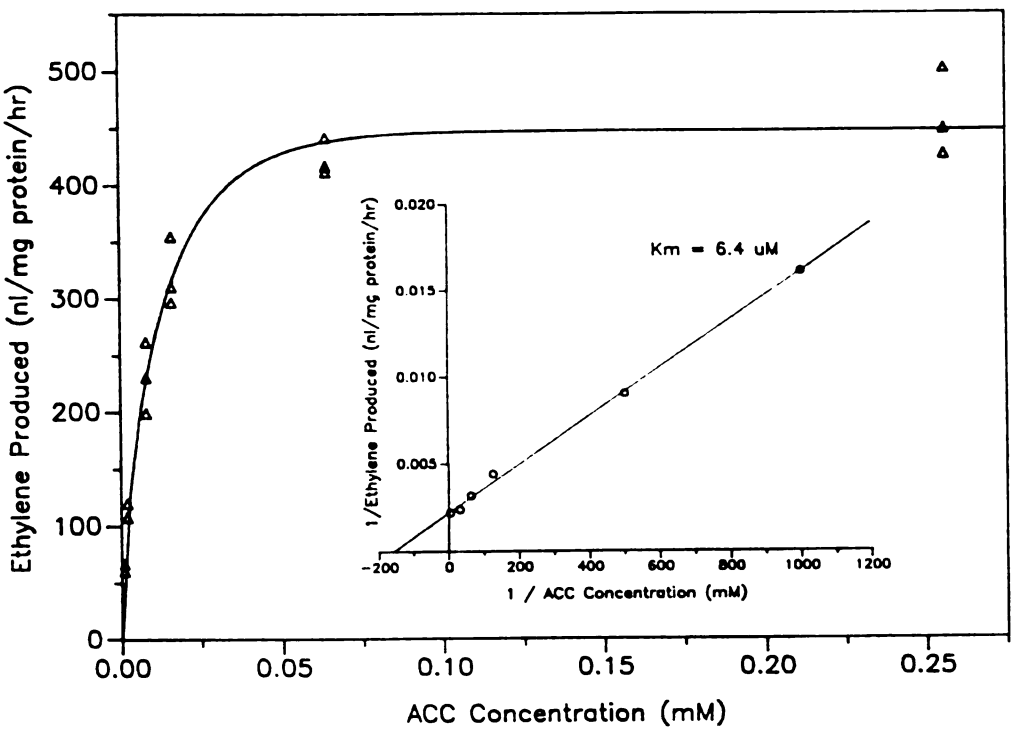

Fig. 1. Ethylene production vs. ACC cocentration for partially purified ACC oxidase from apples (Sp. Act. 958 nl ethylene hr^{-1} mg $protein^{-1}$). Inset is the Lineweaver-Burk plot yielding an apparent K_m of 6.4% determined at pH 7.2 in Tris buffer at 20 °C.

fruit enzyme may reflect a true difference because the assay conditions were very similar.

The apparent K_m for O_2 of the apple ACC oxidase was determined to be 0.4% O_2 (Fig. 2). Early studies by Burg (1973) indicated an apparent K_m for O_2 of 0.2% for ethylene production by apple tissue. Banks (1985) reported an apparent K_m for O_2 of 2.2% for banana tissue slices when corrected for an O_2 gradient in the tissue. The concentration of ACC in apple tissue is known to affect the value of the K_m for O_2; in the absence of added ACC the K_m was 6.2% O_2 and for a saturating level of 10 mM ACC the K_m was 0.3% O_2 (Yip et al., 1988). This is consistent with the ACC oxidase being a bi-substrate enzyme (ACC and O_2) as pointed out by Yip et al., (1988). Our value of K_m for O_2 of 0.4% determined at 1 mM ACC agrees closely with that of 0.3% O_2 determined by Yip et al., (1988) at 1 to 10 mM ACC *in vivo*.

The pH Optimum for Apple ACC Oxidase

The pH optimum for apple ACC oxidase was found to be 7.2 with Tris-Mes buffer and pH 7.6 with phosphate buffer (Fig. 3). The relative activity with phosphate buffer was slightly lower than that observed with Tris-Mes. We are not aware of any other report of pH optimum determined for the enzyme. However, pH 7.2 was pH employed by Ververidis and John (1991) in their report on ACC oxidase from *C. melo*.

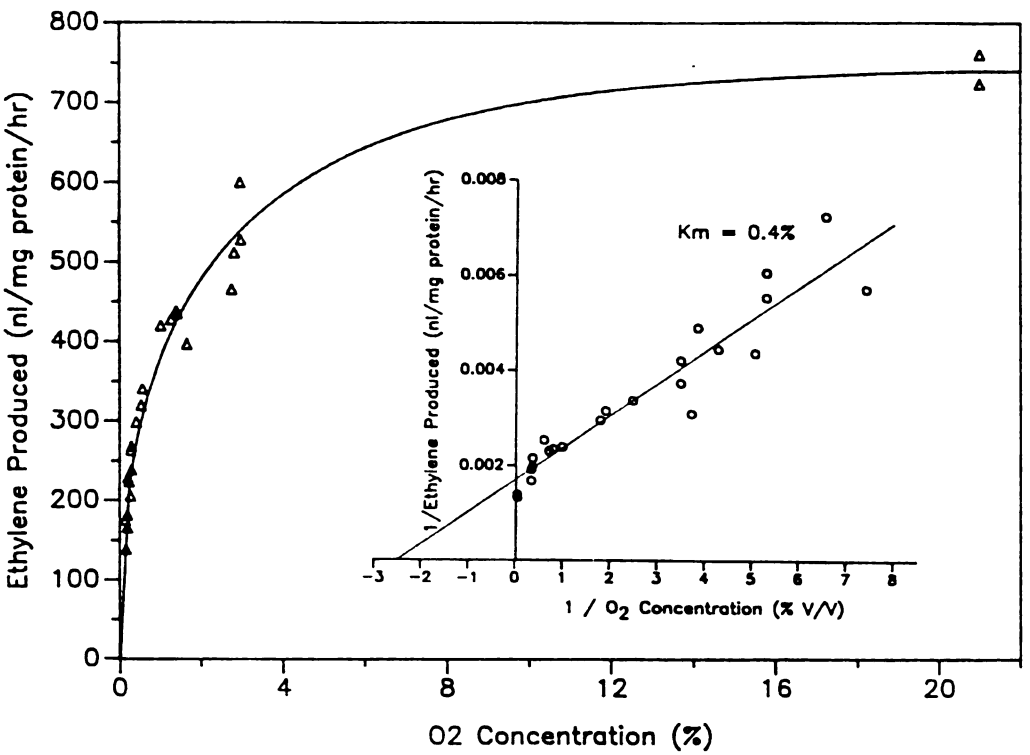

Fig. 2. Ethylene production vs. oxygen concentration for partially purified ACC oxidase from apples. Inset is Lineweaver-Burk plot yielding an apparent K_m of 0.4% (v/v) oxygen determined at pH 7.2 with 1 mM ACC at 20 °C.

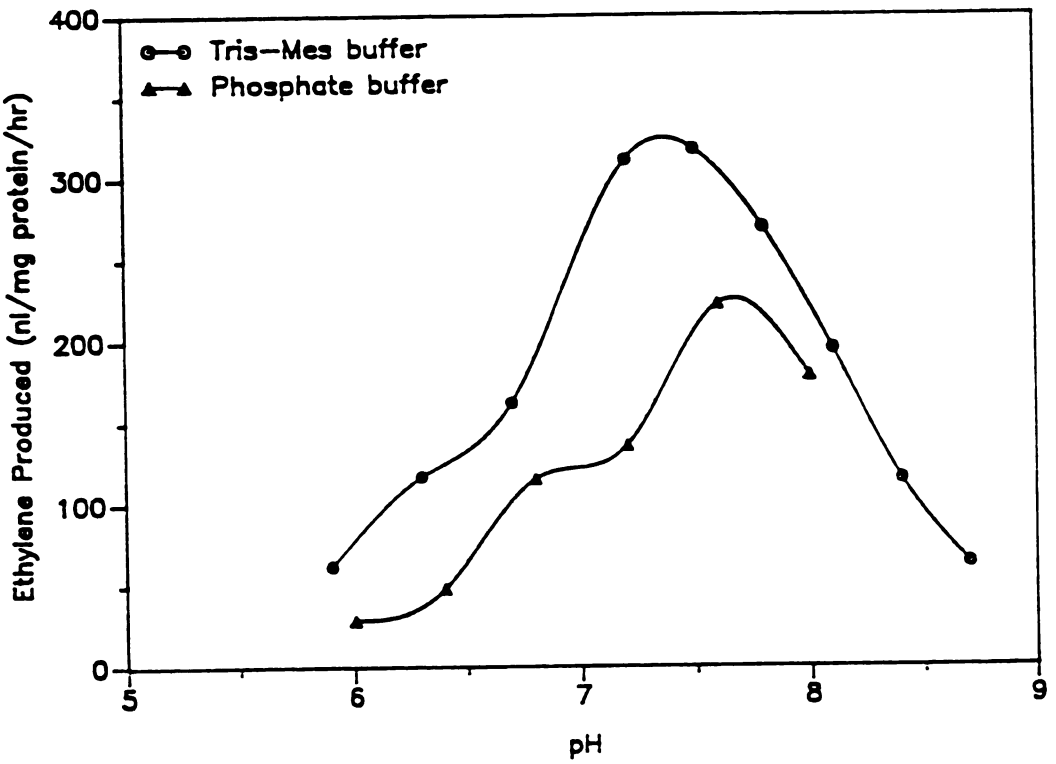

Fig. 3. Ethylene production vs. pH for partially purified ACC oxidase from apple fruit in the presence of 1 mM ACC at 20 °C. (○), Tris between pH 7 and pH 8.7 and Mes (●), between pH 5.9 and pH 6.7; (▲) Phosphate buffer.

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SECTION II

FURTHER CHARACTERIZATION OF ACC OXIDASE FROM APPLE (*MALUS DOMESTICA* BORKH.) FRUIT

ABSTRACT

The apple ACC oxidase required both ascorbate and Fe^{2+} as cofactors. The optimum ascorbate concentration was 1-2 mM and the optimum Fe^{2+} concentration was 100 μM. The enzyme was strongly inhibited by $CuCl_2$, $ZnCl_2$, $CoCl_2$, and $AgNO_3$ at a concentration as low as 1 μM. The enzyme was also inhibited by n-propylgallate and 5,5'-dithio-bis(nitrobenzoate) (DTNB), but not by Triton X-100 (0.3%) or CO (10%). The inhibition of enzyme activity by n-propylgallate was reversed by increasing the Fe^{2+} concentration in the assay medium. This indicates that n-propylgallate probably acts as an inhibitor as a chelating agent for Fe^{2+} in the enzyme reaction, rather than a free radical scavenger. The inhibition of the enzyme activity by DTNB suggests that the -SH groups of the enzyme might have important roles in the enzyme reaction. Kinetic studies of the inhibition of $CoCl_2$, n-propylgallate and 2-aminoisobutyrate (AIB) showed that AIB inhibited the enzyme activity in a competitive manner with respect to ACC with a K_i of 388 μM, whereas $CoCl_2$ and n-propylgallate inhibited the enzyme activity in a noncompetitive manner with a K_i of 0.2 μM and 77 μM, respectively. Extractable apple ACC oxidase activity correlated well with the climacteric of ethylene production. The molecular mass of the enzyme was estimated to be 44 KD by gel filtration.

INTRODUCTION

Ethylene, the simplest plant hormone which regulates many aspects of plant growth and development, is synthesized from methionine via Ado-Met and ACC (Yang & Hoffman, 1984). The conversion of ACC to ethylene is catalyzed by the enzyme ACC oxidase. ACC oxidase has been isolated recently by Ververidis and John (1991) from melon fruit (Cucumis melo). The enzyme was found to resemble the in vivo ACC oxidase in terms of stereospecificity towards AEC isomers and affinity for ACC. The melon fruit ACC oxidase had a Km of 85 μM for ACC and the enzyme was not dependent on membrane integrity for its activity as suggested before (Mayne & Kende, 1986; Kende, 1989), instead, it required ascorbate and Fe^{2+} as cofactors (Ververidis & John, 1991). Their results were confirmed and extended by Kuai & Dilley (1992) and Fernández & Yang (1992) for apple fruits. The apple ACC oxidase has a Km for ACC of 6.4 μM, Km for O_2 of 0.4%, and pH optimum 7.2 (Kuai & Dilley, 1992).

Early studies with intact tissues, protoplasts and vacuoles isolated from protoplasts indicated that the conversion of ACC to ethylene was inhibited by heavy metal ions (Apelbaum et al., 1981; Yu & Yang, 1979; Bradford et al., 1982; Satoh & Esashi, 1983), Triton X-100 (Apelbaum et al., 1981; Michell et al., 1988; Mayne & Kende, 1986), free radical scavengers (Apelbaum et al., 1981; Satoh & Esashi, 1983), and

AIB (Satoh & Esashi, 1980; 1982; 1983; Liu et al., 1984).

In the present study, the cofactor requirements for apple ACC oxidase activity, the inhibition of heavy metal ions, Triton X-100, n-propylgallate, AIB, etc. on the enzyme activity were investigated using a partially purified enzyme. The molecular mass of the enzyme was also determined by Sephadex G-75 gel filtration.

MATERIALS AND METHODS

Plant Materials

Apples (Malus domestica Borkh. cv Golden Delicious) for studying ACC oxidase activity vs ripening were harvested from the Horticulture Research Center of Michigan State University. The apples used elsewhere and other fruits were obtained from local suppliers.

Enzyme Assay In Vivo and In Vitro

To study ACC oxidase activity from different fruits and apple ACC oxidase vs. ripening, enzyme activity was determined as described before with some modifications (Kuai & Dilley, 1992). In vivo activity of ACC oxidase was determined by incubating one gram of slices (0.2 mm thick) of fruit tissue in 3 ml of assay medium containing 0.05 M Tris, pH 7.2, 10% glycerol and 1 mM ACC and measuring the ethylene produced by sampling the headspace of sealed reaction vials. For enzyme extraction, fruit tissue was pulverized to a powder under liquid nitrogen. ACC oxidase was extracted with 0.2 M Tris

(0.3 M for kiwifruit), pH 7.7, 30 mM ascorbate and 5% insoluble polyvinylpolypyrrolidone (PVPP) (0.5 g tissue per ml of extraction buffer). The slurry was filtered through Miracloth. The *in vitro* activity was determined by incubating 2.5 ml of buffered assay medium (0.05 M Tris, pH 7.2, 10% glycerol, 0.1 mM $FeSO_4$, 1 mM ACC) and 0.5 ml of enzyme filtrate in a 25 ml erlenmeyer flask sealed with a rubber serum bottle stopper.

For enzyme reaction studies, a partially purified enzyme was employed after purification as described before (Kuai & Dilley, 1992). Enzyme desorbed from calcium phosphate gel was used for these studies. The assay medium consisted of 0.05 M Tris, pH 7.2, 10% glycerol, 2 mM ascorbate, 0.1 mM $FeSO_4$, and various ACC and inhibitor concentrations.

Determination of Molecular Mass of ACC Oxidase

Bovine serum albumin, cytochrome c, carbonic anhydrase and aprotinin (Sigma) were employed for calibration of a gel filtration column. A Sephadex G-75 column (1.5 x 70 mm, bed volume 105 ml) was equilibrated with a running buffer (0.05 M Tris, pH 7.7, 0.1 M KCl). The column was run at a flow rate of 6 ml/min. Blue dextran was used to measure void volume (V_0) of the column.

RESULTS AND DISCUSSION

Extraction of ACC Oxidase from Various Fruit Sources

Although the recovery of *in vitro* ACC oxidase activity

varied among fruits of various types, the enzyme activity was demonstrated in all the fruits examined (Table 1).

Cofactor Dependence

Both ascorbate and Fe^{2+} are essential for assaying ACC oxidase activity in vitro (Ververidis & John, 1991; Kuai & Dilley, 1992; Fernández & Yang, 1992). Ascorbate and Fe^{2+} were usually used at a concentration of 30 mM and 0.1 mM, respectively, in the assay medium. The dependence of the enzyme activity on ascorbate concentration in the presence of 0.1 mM Fe^{2+} is shown in Fig. 1. The optimum concentration of ascorbate was 1 to 2 mM (Fig. 1) and higher concentrations inhibited enzyme activity. At 30 mM of ascorbate, enzyme activity was only about 50% of the activity found at 1 to 2 mM. The dependence of the enzyme activity on Fe^{2+} concentration in the presence of 2 mM of ascorbate is shown in Fig. 2. The enzyme exhibited high activity at only 2 to 5 μM Fe^{2+} and gradually increased to a maximum at about 100 to 200 μM (Fig.2).

The Effect of Heavy Metal Ions

Co^{2+} has long been shown to be a potent inhibitor of the conversion of ACC to ethylene in vivo (Apelbaum et al., 1981; Yu & Yang, 1979; Bradford et al., 1982; Satoh & Esashi, 1983). In addition to Co^{2+}, Cu^{2+}, Zn^{2+} and Ag^{+} are also potent inhibitors of apple ACC oxidase (Table 2). The concentration of these ions (1 μM) employed was much lower than those previously used in vivo, which was usually several hundred μM.

Table 1 *In vivo* and *in vitro* activity of ACC oxidase from different fruits

Fruit	Ethylene Production (nl/g FW/hr)	ACC Oxidase Activity (nl/g FW/hr)	
		in vivo [z]	*in vitro* [y]
Apple	60.0	128.0 ± 25	69.0 ± 5.0
Avocado	15.0	14.0 ± 1.9	106.0 ± 4.6
Banana	0.67	3.8 ± 1.8	3.5 ± 0.4
Kiwifruit	0.05	12.0 ± 3.2	10.0 ± 0.4
Nectarine	1.7	20.0 ± 7.0	58.0 ± 3.0
Pear	83.0	106.0 ± 17	77.0 ± 2.1
Plum	0.26	1.3 ± 0.06	2.2 ± 0.7
Tomato	5.4	19.0 ± 3.9	23.0 ± 4.6

[z] *In vivo* activity was determined by incubating 1 g slices (0.2 mm thick) in 3 ml of assay medium containing 0.05 M Tris, 10% glycerol and 1 mM ACC.

[y] *In vitro* activity was determined with a crude extract obtained from liquid nitrogen powder extracted with 0.2 M Tris (0.3 M was used for kiwifruit), pH 7.7, 30 mM ascorbate, 5% PVPP.

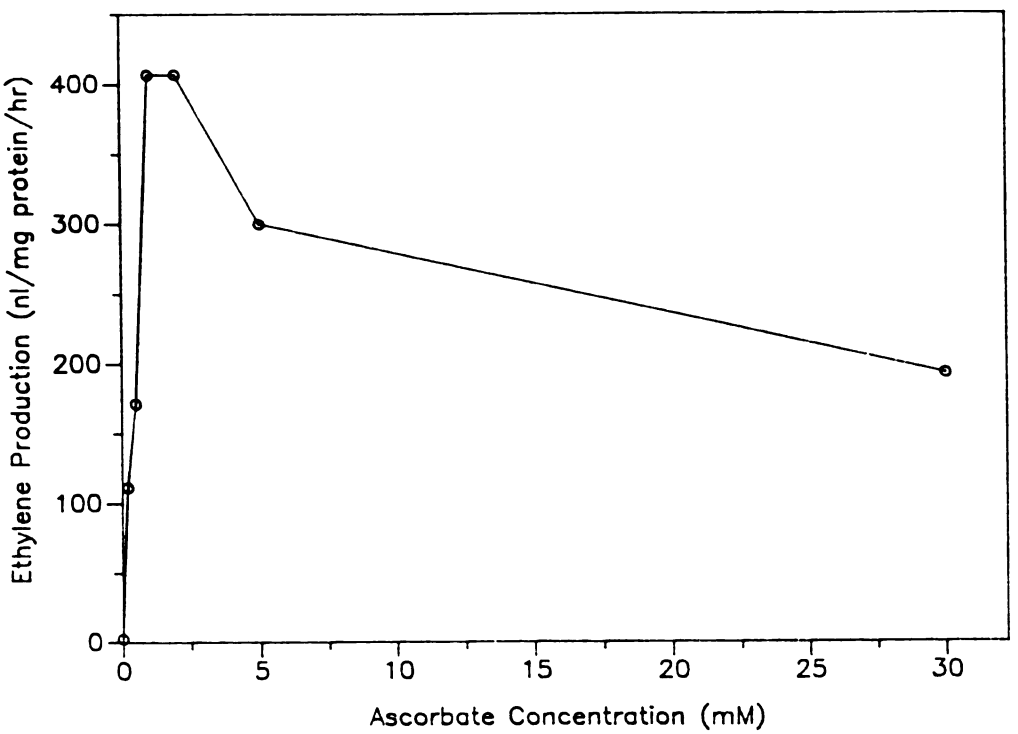

Fig. 1. The dependence of ACC oxidase activity on ascorbate concentration. The enzyme was assayed in the presence of 50 mM Tris, pH 7.2, 0.1 mM Fe^{2+}, 1 mM ACC, 10% glycerol and various concentrations of ascorbate at 20 °C

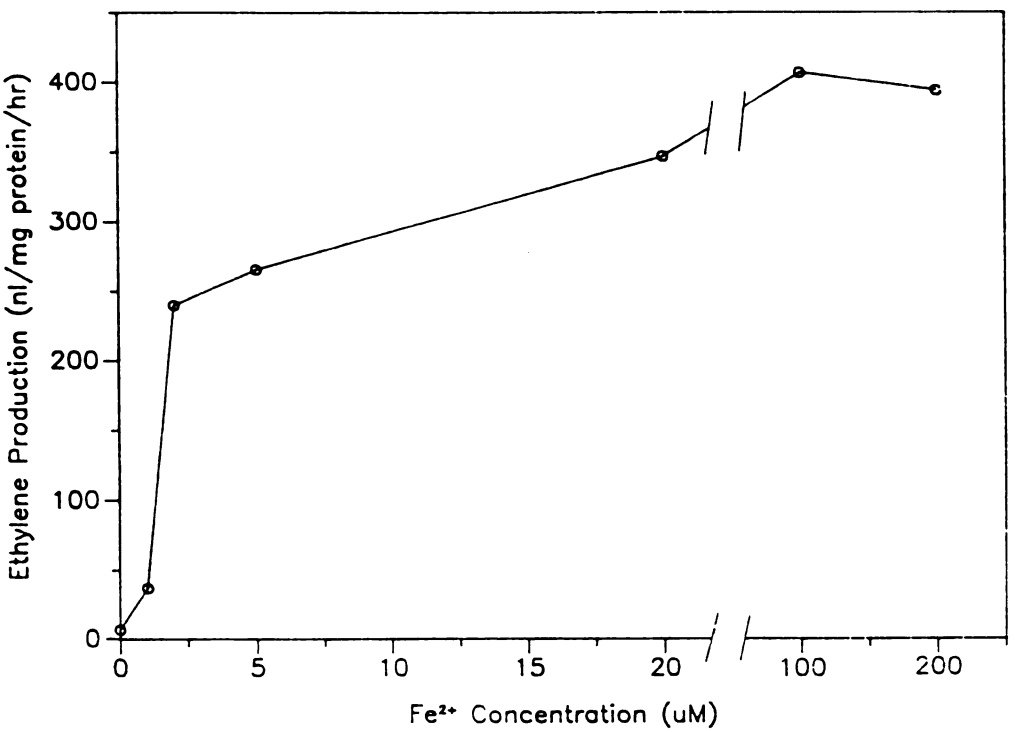

Fig. 2. The dependence of ACC oxidase activity on Fe^{2+} concentration. The enzyme was assayed in the presence of 50 mM Tris, pH 7.2, 2 mM ascorbate, 1 mM ACC, 10% glycerol and various concentrations of Fe^{2+} at 20 °C.

Table 2 Effect of heavy metal ions on activity of ACC oxidase assayed in the presence of 0.1 mM Fe^{2+}

Metal ion	Ethylene production as % of control [z]
$CuCl_2$ 1 μM	21a
$ZnCl_2$ 1 μM	34a
$CoCl_2$ 1 μM	36a
$AgNO_3$ 1 μM	42a
$NiCl_2$ 1 μM	84b
$MnCl_2$ 1 μM	95b
$HgCl_2$ 1 μM	97b

[z]The control assay medium consisted of 0.05 M Tris, pH 7.2, 10% glycerol, 2 mM ascorbate, 0.1 mM Fe^{2+}, 1 mM ACC and enzyme in a total volume of 3 ml and yielded 221 nl ethylene/mg protein/hr.

It is interesting to note that Ag^+ was a strong inhibitor of ACC oxidase. It has been well documented that Ag^+ inhibits ethylene action and autocatalytic ethylene production of climacteric fruits; and Ag^+ has been used commercially in cut carnations to extend their vase-life (Hobson, et al., 1984; Saltveit et al., 1978; Beyer, 1976; Veen & Van de Geijn, 1978). These results suggest that the inhibition of autocatalytic ethylene production by Ag^+ could be due to both the inhibition of ethylene action and of ACC oxidase activity.

The Fe^{2+} requirement for ACC oxidase catalyzed oxidation of ACC to ethylene may be as an Fe^{2+}-enzyme complex. There are numerous examples of Fe^{2+} being bound to histidine and cysteine ligands in oxidases in which an O_2 Fe^{2+} ternary complex is formed as exemplified by prolylhydroxylase (Haneuske-Abel & Günzler, 1982). The inhibition of ACC oxidase by Co^{2+} may be due to replacement of Fe^{2+} by Co^{2+} at the enzyme active center.

The Effect of Other Inhibitors on ACC Oxidase Activity

Inhibition of apple ACC oxidase activity by metal ion chelating agents was observed (Table 3). This suggests that a certain Fe^{2+}-complex may be involved in the enzyme reaction.

Many studies using intact tissue or vacuoles isolated from protoplasts indicated n-propylgallate as a strong inhibitor of the conversion of ACC to ethylene (Apelbaum et al., 1981; Satoh & Esashi, 1983). It was suggested that n-propylgallate inhibited the reaction because of its free

Table 3 The effect of selected compounds on ACC oxidase activity

Assay medium		Ethylene production as % of control [z]
complete		100
complete plus EDTA (0.1 mM)		61
complete plus n-propylgallate	+ 0.1 mM $FeSO_4$	4
	+ 1.0 mM $FeSO_4$	73
complete plus DTNB (0.1 mM)	-DTT	35
	+1.0 mM DTT	93
complete plus Triton x-100 (0.3%, v/v)		100
complete plus CO (10%, v/v)		100

[z]the control assay medium consisted of 0.05 M Tris, pH 7.2, 10% glycerol, 2 mM ascorbate, 0.1 mM Fe^{2+}, 1 mM ACC and enzyme in a total volume of 3 ml.

radical scavenger property (Yang & Hoffman, 1984). Studies using partially purified ACC oxidase revealed that n-propylgallate did inhibit the ACC oxidase activity, but the inhibition effect could be reversed by including more Fe^{2+} in the assay medium (Table 3), suggesting that n-propylgallate probably acted as a chelating reagent for Fe^{2+} and suppressed the reaction in a similar manner as EDTA, rather than a free radical scavenger.

SH-groups in the enzyme may play an important role in the enzyme activity, because the enzyme activity was inhibited by 5,5'-dithio-bis(2-nitrobenzoate) (DTNB) (Table 3). The enzyme could be partially protected by adding DTT in the assay buffer (Table 3).

Triton x-100 was found to inhibit the conversion of ACC to ethylene by intact tissue or vacuoles isolated from protoplasts, which led to the conclusion that ACC oxidase activity was dependent on membrane integrity (Apelbaum et al., 1981; Mayne & Kende, 1986). Triton X-100 did not inhibit activity of the enzyme in vitro (Table 3). This is consistent with the observations of Ververidis and John (1991) with C. melo ACC oxidase. The inhibition of Triton X-100 on the conversion of ACC to ethylene by intact tissues or vacuoles could be due to disruption of compartmentation and leaking of the cofactors required for enzyme activity.

CO has been shown to inhibit the conversion of ACC to ethylene by microsomal membranes isolated from etiolated wheat

shoots and it was suggested that cytochrome P-450-mediated monooxygenase reactions might be intimately involved in the conversion of ACC to ethylene in wheat seedling (Kraus, et al., 1992). CO did not inhibit ACC oxidase activity (Table 3), suggesting that cytochrome P-450 is not involved in the conversion of ACC to ethylene.

Thermostability of ACC Oxidase

The enzyme showed good thermostability up to 40 °C above which it greatly lost its activity. About 5% residual activity was retained following incubation at 63 °C or above for 5 min (Fig. 3).

Substrate Specificity

Early studies showed that cyclopropanecarboxylic acid and cyclopropylamine were not effective inhibitors of ACC oxidase in vivo (Yang & Hoffman, 1984). These substances could not substitute for ACC as substrates for the enzyme (Table 4), suggesting that both the amino and the carboxyl groups are important for the substrate to bind the enzyme.

Molecular Mass Calibration

The molecular mass of ACC oxidase was estimated to be 33-36 KD from the derived amino acid sequence of cDNAs encoding the enzyme (Holdsworth et al., 1987; McGarvey et al., 1990; Wang & Woodson, 1991). The molecular mass of apple ACC oxidase was estimated by Sephadex G-75 gel filtration to be 44 KD (Fig. 4).

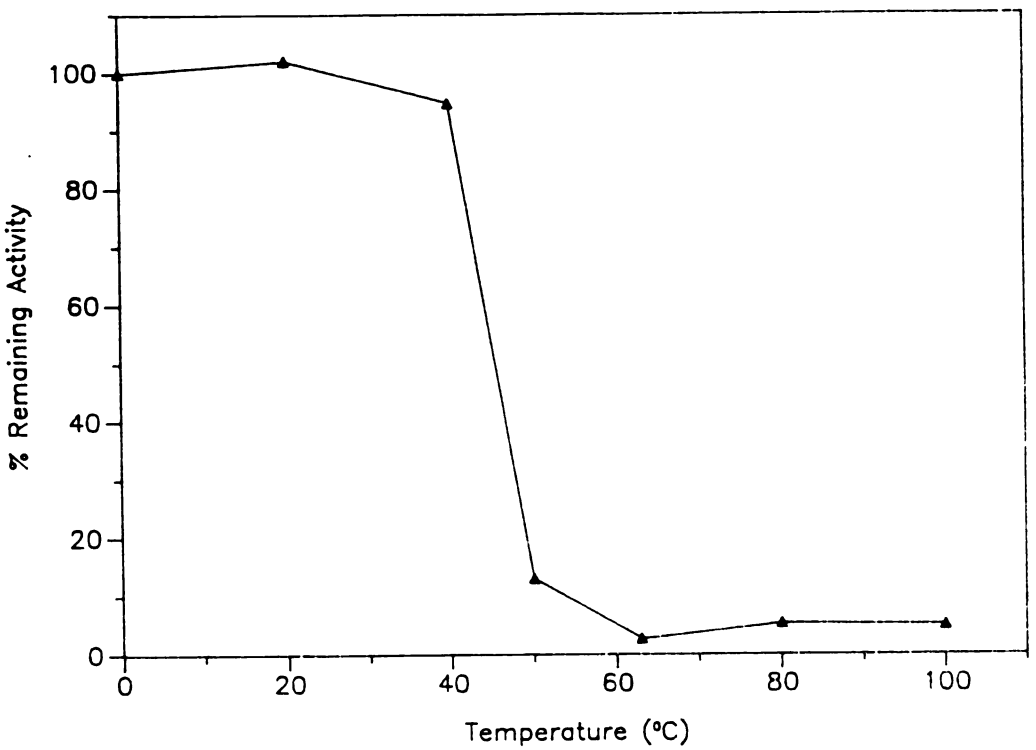

Fig. 3. The thermostability of ACC oxidase. The starting enzyme preparation had a specific activity of 335 nl ethylene hr^{-1} mg $protein^{-1}$. The enzyme was incubated at designated temperature for 5 min and assayed as described in Materials and Methods.

Table 4 Substrate specificity of ACC oxidase

Substrate (1 mM)	Ethylene Production (nl/mg protein/hr) [z]
1-aminocyclopropane-1-carboxylic acid	335
cyclopropylcarboxylic acid	0
cyclopropylamine	0

[z]Enzyme was assayed in the presence of 50 mM Tris, pH 7.2, 10% glycerol, 2 mM ascorbate, 0.1 mM Fe^{2+}, 1 mM substrate and enzyme in a total volume of 3 ml.

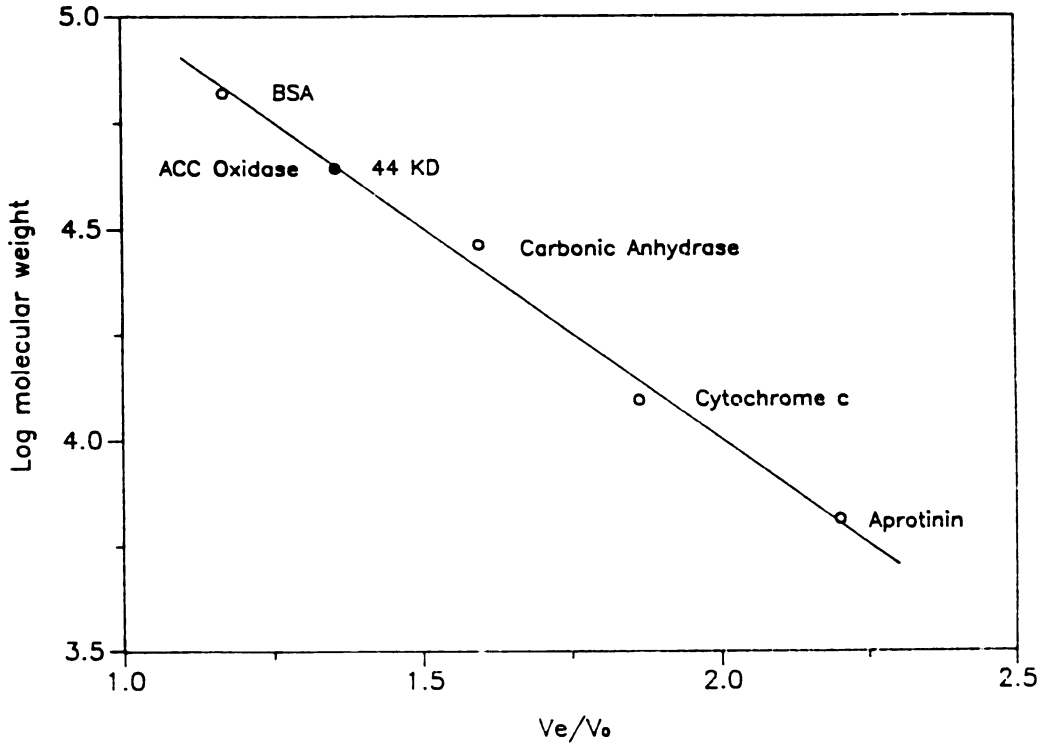

Fig. 4. The molecular weight calibration of ACC oxidase of apple fruit.

The Inhibition Kinetics of Co^{2+}, N-propylgallate and 2-Aminoisobutyric Acid

Co^{2+} was found to be a potent inhibitor of ACC oxidase over a range of ACC and Co^{2+} concentrations (Fig. 5). The double reciprocal plot of the data in Fig. 5 yielded a common intercept with the control on the abscissa, suggesting Co^{2+} inhibited the enzyme activity in a noncompetitive manner with respect to ACC (Fig. 6). The K_i for Co^{2+} obtained from Fig. 7 is 0.2 μM.

N-propylgallate inhibition kinetics are shown in Figures 8 & 9, indicating noncompetitive inhibition and a K_i of 77 μM (Fig. 10).

Inhibition by 2-aminoisobutyric acid (AIB) (Fig. 11) was found to be competitive with respect to ACC (Fig. 12) with a K_i of 388 μM (Fig. 13).

The kinetic analyses for Co^{2+}, n-propylgallate and AIB for the apple enzyme are consistent with the earlier studies on the inhibition by these inhibitors on the ethylene production of cotyledonary segments of cocklebur seed (Satoh & Esashi, 1983).

The Change of ACC Oxidase Activity During Apple Fruit Ripening

During the course of apple fruit ripening, the *in vivo* ACC oxidase activity measured with fruit discs increased markedly (Bufler, 1986); extractable ACC oxidase activity shows a similar trend over the course of ripening (Fig. 14).

In summary, the apple ACC oxidase required both ascorbate

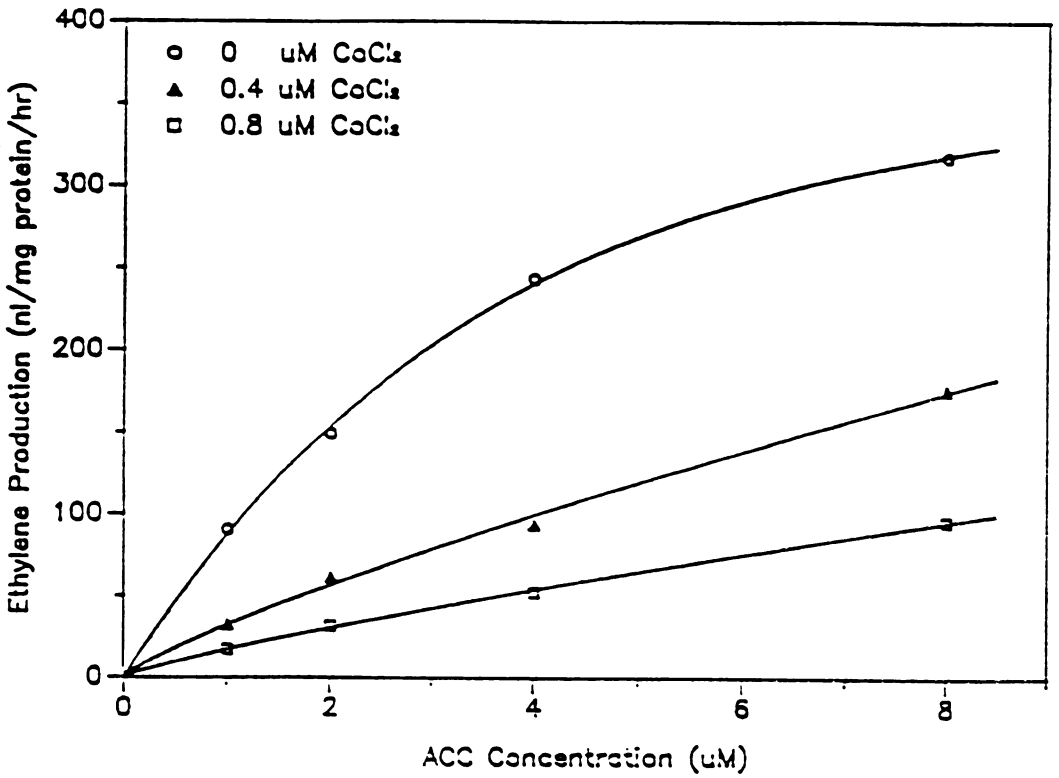

Fig. 5. The inhibition of ACC oxidase by $CoCl_2$. The enzyme assayed in the presence of 50 mM Tris, pH 7.2, 2 mM ascorbate, 0.1 mM Fe^{2+}, 10% glycerol and various concentrations of inhibitor and ACC at 20 °C.

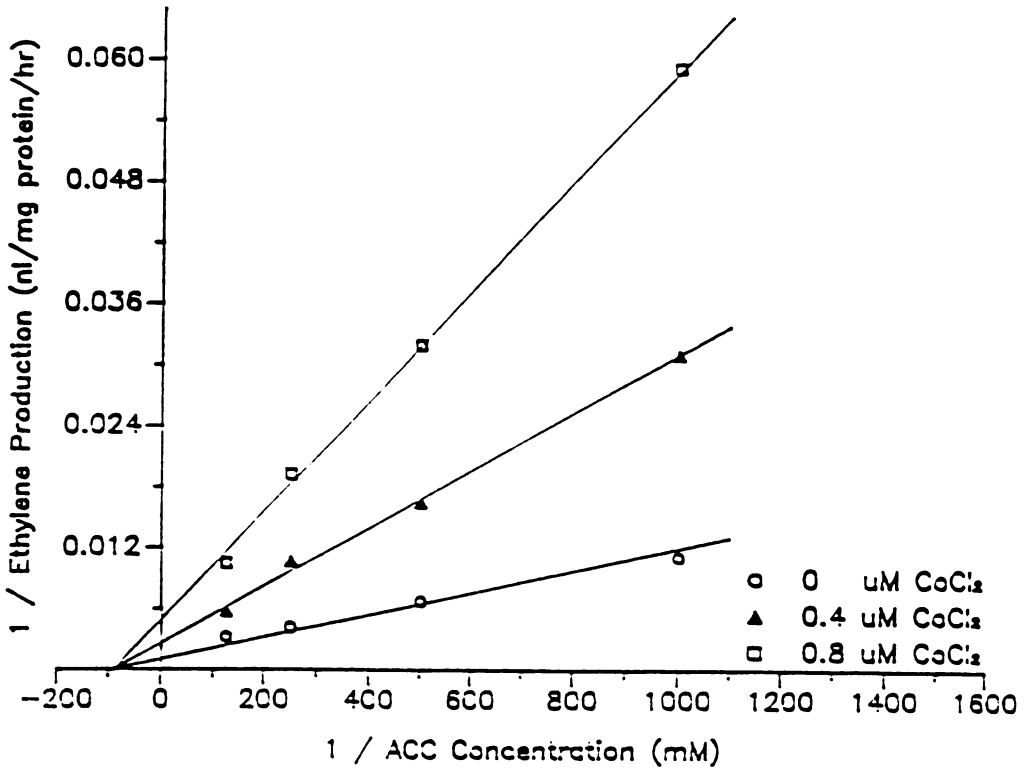

Fig. 6. Double reciprocal plot of Fig. 5.

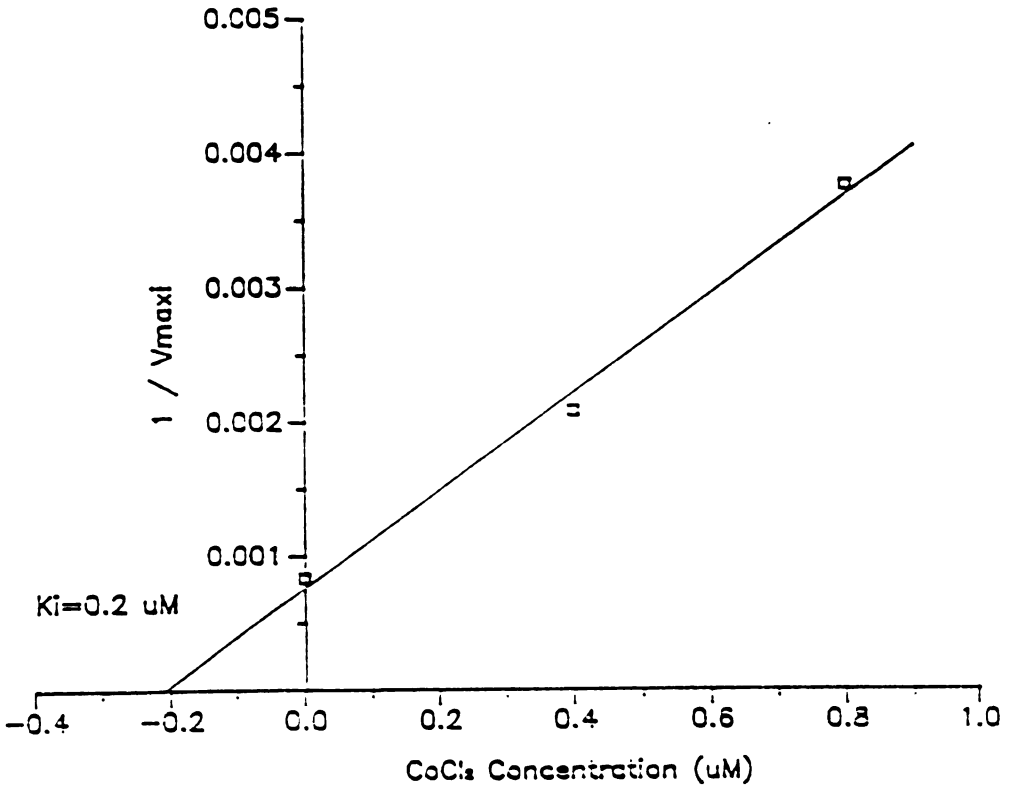

Fig. 7. 1/Vmaxi vs. $CoCl_2$ concentration. Vmax at three concentrations of $CoCl_2$ (0, 0.4 and 0.8 μM) was obtained from Fig. 6.

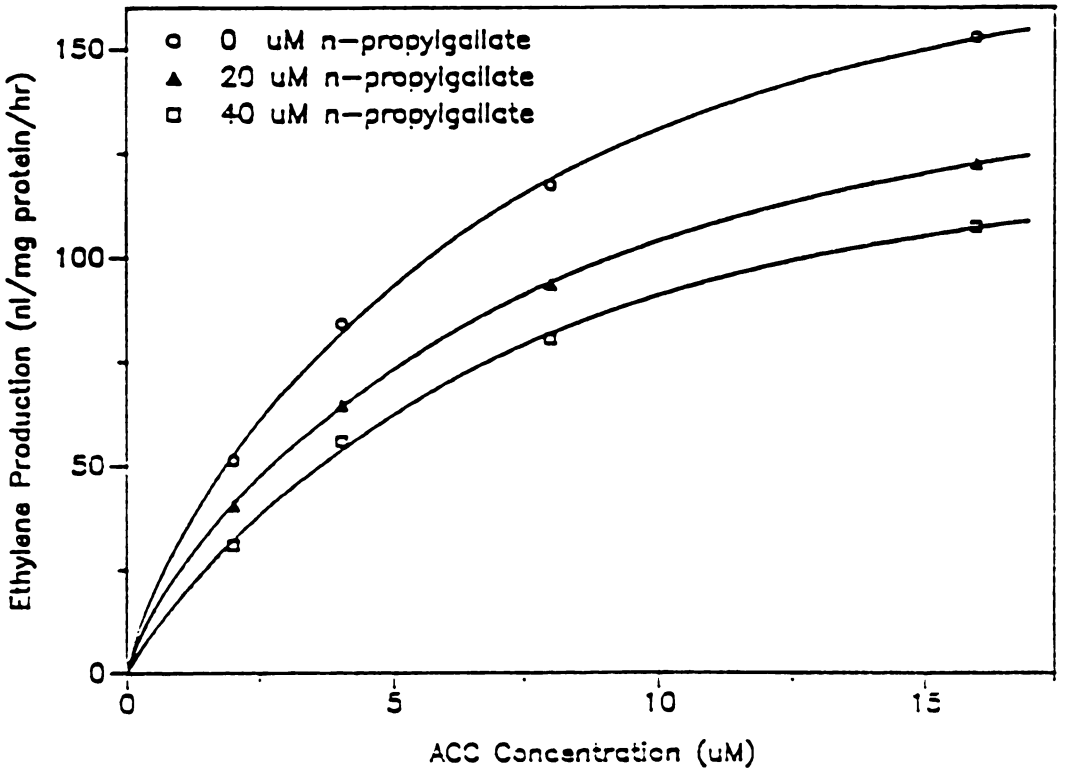

Fig. 8. The inhibition of ACC oxidase by n-propylgallate. The enzyme was assayed in the presence of 50 mM Tris, pH 7.2, 2 mM ascorbate, 0.1 mM Fe^{2+}, 10% glycerol and various concentrations of inhibitor and ACC at 20 °C.

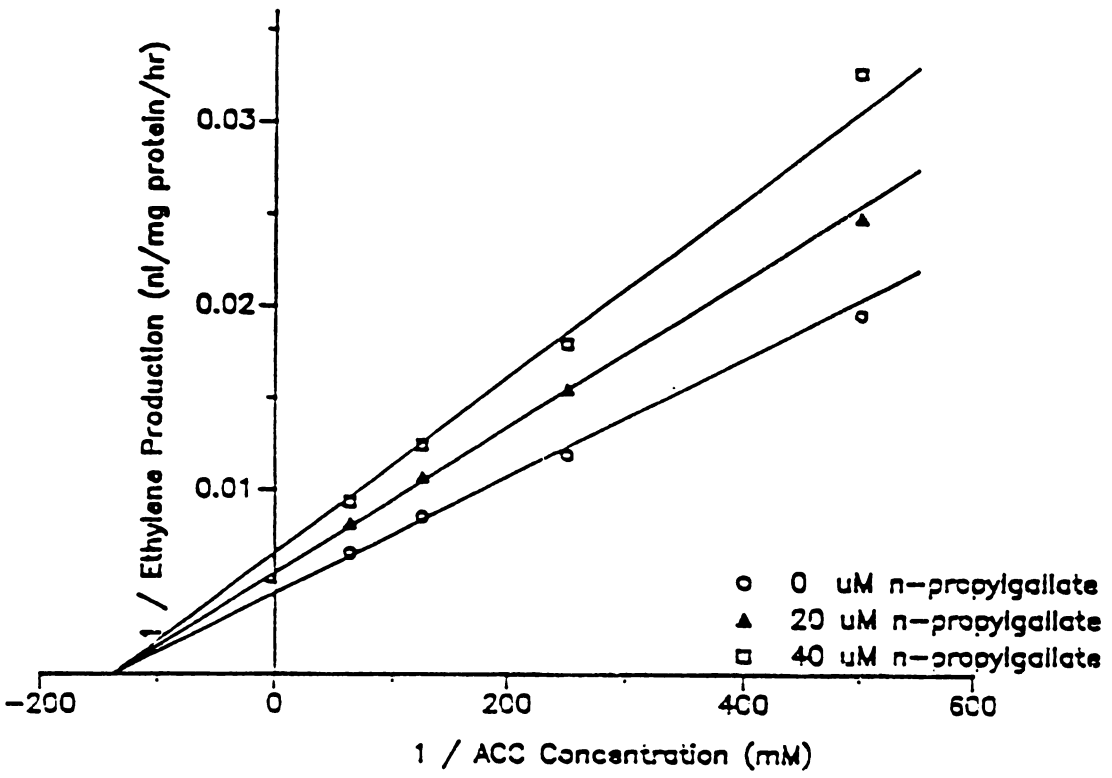

Fig. 9. Double reciprocal plot of Fig. 8.

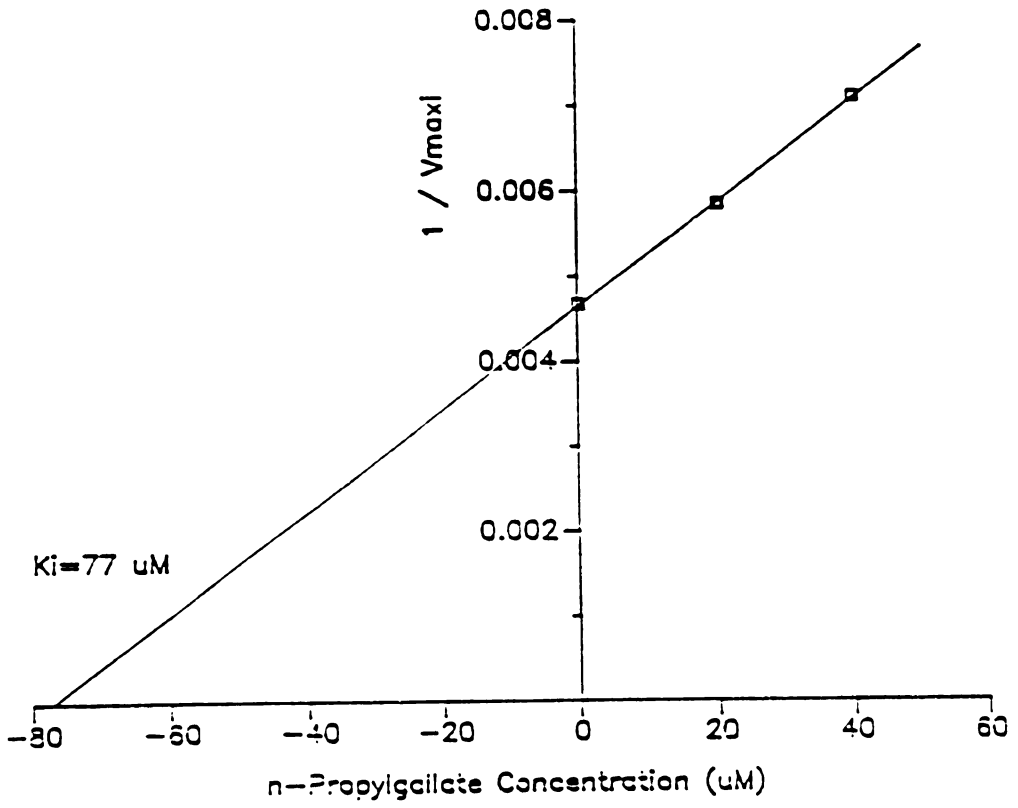

Fig. 10. 1/Vmaxi vs. n-propylgallate concentration. Vmax at three concentrations of n-propylgallate (0, 20 40 μM) was obtained from Fig. 9.

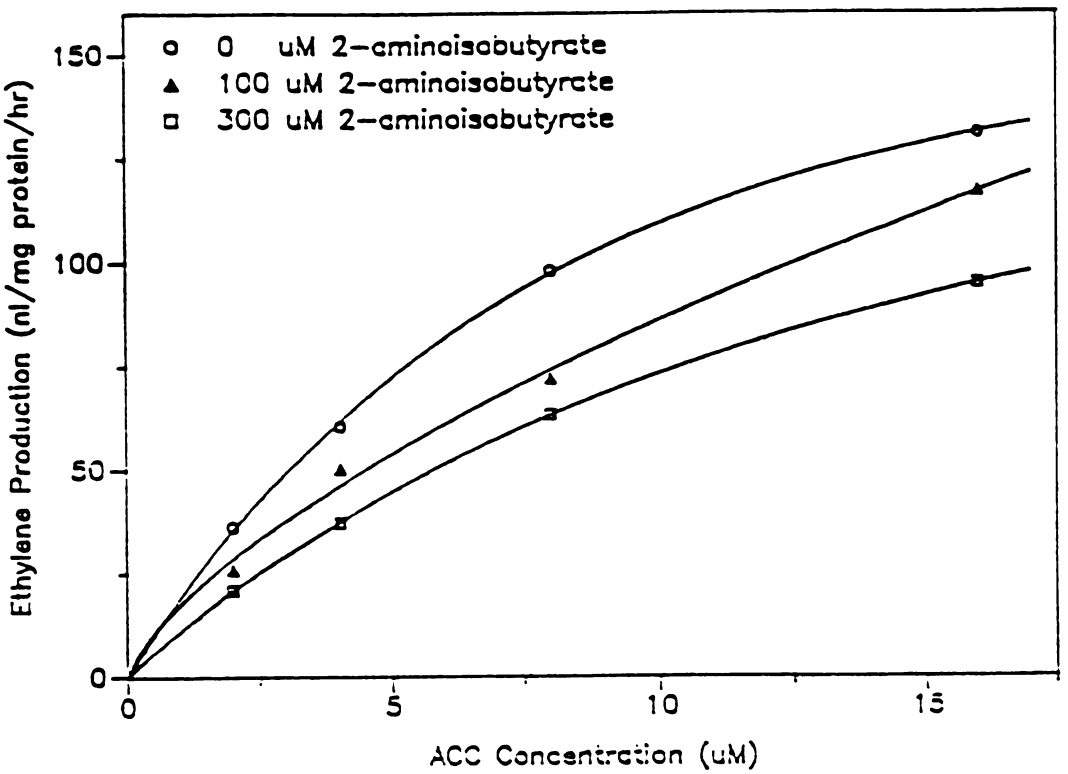

Fig. 11. The inhibition of ACC oxidase activity by 2-aminoisobutyrate. The enzyme was assayed in the presence of 50 mM Tris, pH 7.2, 2 mM ascorbate, 0.1 mM Fe^{2+}, 10% glycerol and various concentrations of inhibitor and ACC at 20 °C.

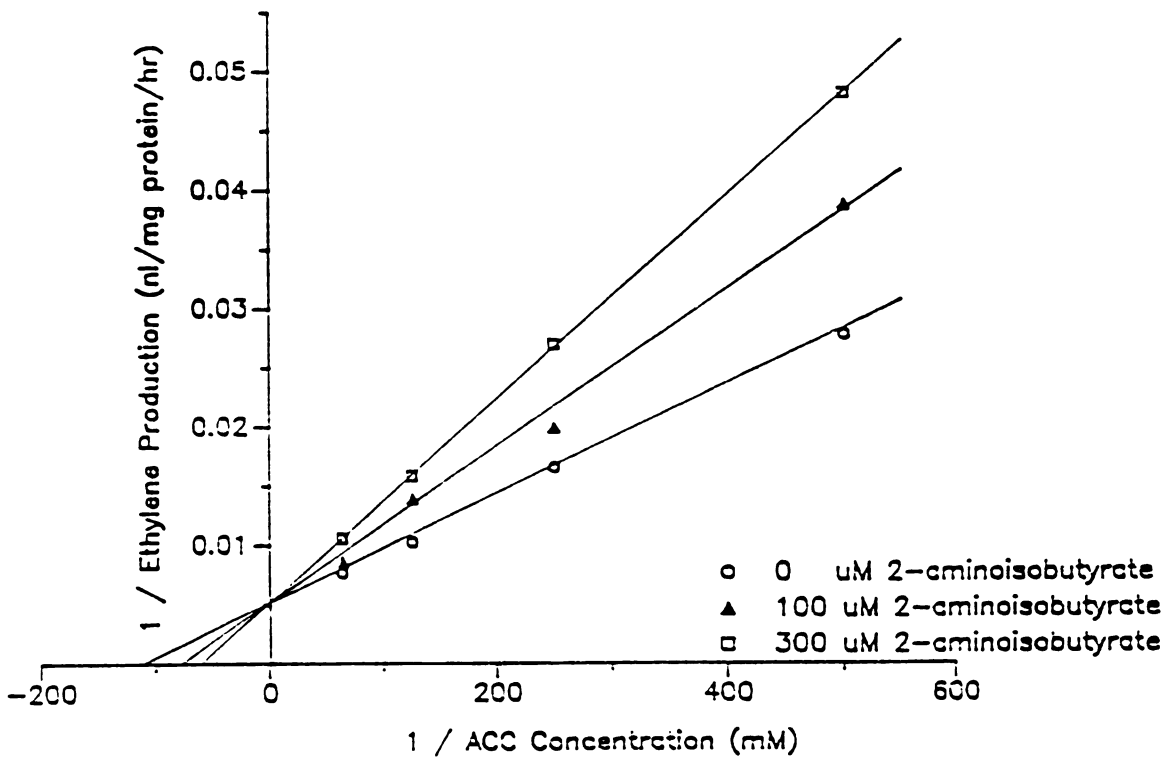

Fig. 12 Double reciprocal plot of Fig. 11.

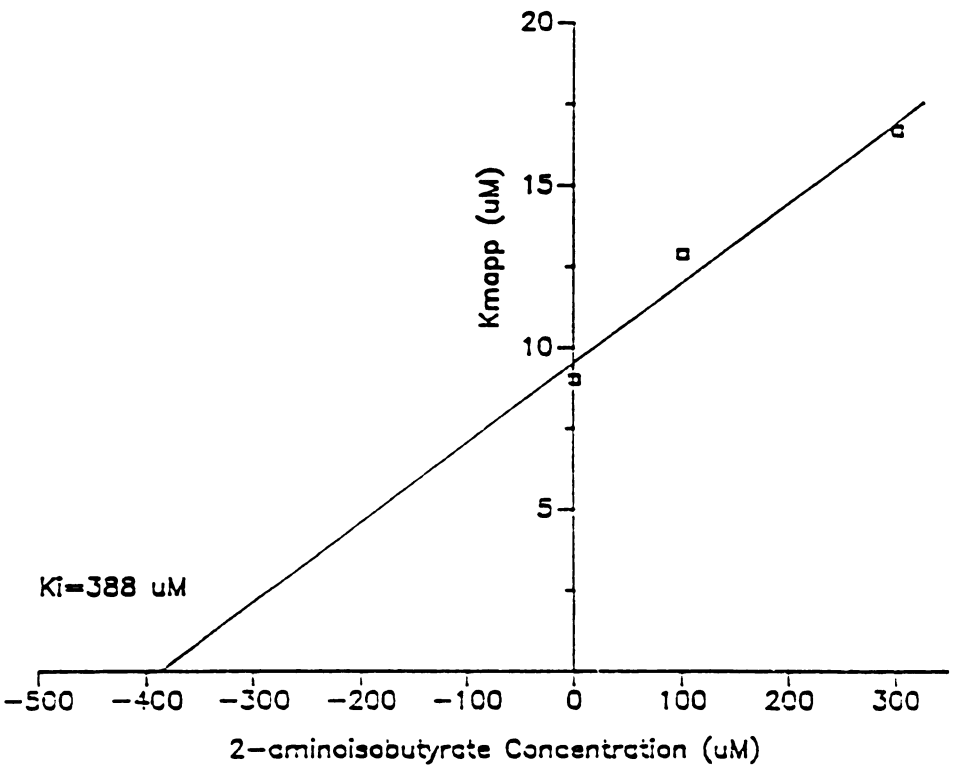

Fig. 13. Kmapp vs. 2-aminoisobutyrate concentration. Km at three concentrations of 2-aminoisobutyrate (0, 100 and 300 μM) was obtained from Fig. 12.

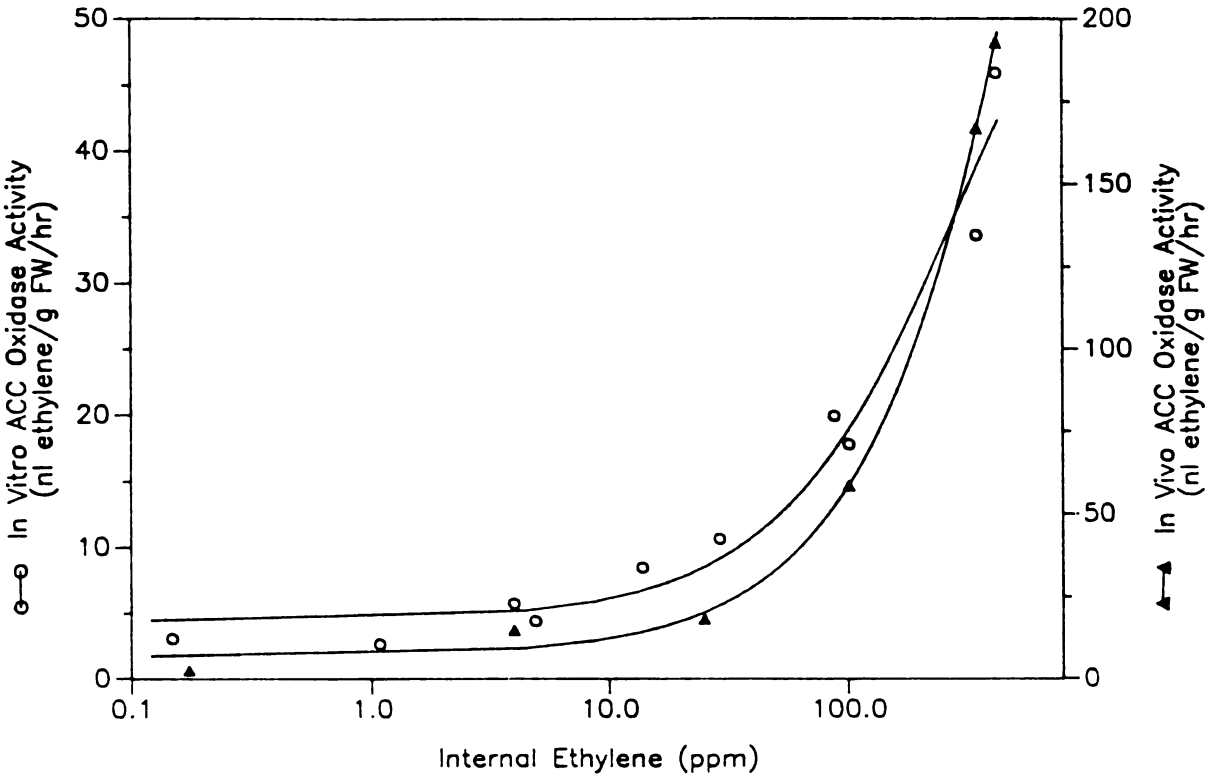

Fig. 14. The <u>in vivo</u> and <u>in vitro</u> ACC oxidase activity of apple fruit vs. internal ethylene concentration. <u>In vivo</u> activity was determined by incubating 1 g slices (0.2 mm) thick in 3 ml of assay medium containing 50 mM Tris, 1 mM ACC and 10% glycerol. <u>In vitro</u> activity was determined with a crude extract obtained from liquid N_2 powder extracted with 0.2 M Tris, pH 7.7, 30 mM ascorbate, 5% PVPP

and Fe^{2+} as cofactors. The optimum concentrations were 1 to 2 mM for ascorbate and 100 μM for Fe^{2+}. The enzyme was strongly inhibited by $CuCl_2$, $ZnCl_2$, $CoCl_2$, and $AgNO_3$. The enzyme was also inhibited by n-propylgallate, DTNB and AIB. N-propygallate probably inhibited the enzyme activity by chelating Fe^{2+}. Kinetic analyses indicated that AIB inhibited ACC oxidase activity in a competitive manner and n-propylgallate and Co^{2+} inhibited the enzyme activity in a noncompetitive manner with respect to ACC. Extractable apple ACC oxidase was found to be correlated with fruit ripening. Apple ACC oxidase was estimated to be a 44 KD polypeptide by Sephadex G-75 gel filtration.

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SECTION III

THE STUDY OF A RIPENING-RELATED PROTEIN FROM APPLE (*MALUS DOMESTICA* BORKH.) FRUIT

ABSTRACT

Proteins were extracted from cortical tissue of preclimacteric and postclimacteric apple fruits (*Malus domestica* Borkh. cv. Empire) and analyzed by two-dimensional PAGE. A polypeptide identified as PAp5 was found to accumulate in postclimacteric apple fruit. PAp5 had a molecular weight of 36.5 KD and isoelectric point 5.3. Western blot analysis indicated that PAp5 was absent from apples prior to the onset of the ethylene climacteric but increased markedly as the climacteric developed. *In vitro* translation of poly(A)+ RNA from apples at six stages of ripening (internal ethylene = 0.1, 1.3, 10, 122, 308, 607 ppm, respectively) and immunoprecipitation with PAp5 antisera showed that translatable mRNA encoding PAp5 increased during ripening.

INTRODUCTION

Fruit ripening involves a series of complex biochemical and physiological changes, which lead to the development of color, aroma, flavor and texture of the fruit (Sacher, 1973). During the past decade, evidence has accumulated suggesting that alterations in gene expression play an important role in the process of fruit ripening. For example, analysis of *in vitro* translation products of mRNA extracted from fruits at different ripening stages revealed that new mRNAs were synthesized during fruit ripening (Christoffersen et al., 1982; Grierson et al., 1985; Lay-Yee et al., 1990). The activities of several enzymes such as 1-aminocyclopropane-1-carboxylate synthase, polygalacturonase in tomatoes, cellulase in avocados have been found to be associated with fruit ripening (Bufler, 1984; Brady et al., 1982; Tucker & Laties, 1984;), and there is evidence that the appearance of these enzyme activities is the result of *de novo* synthesis instead of activation (Bleecker et al., 1986; Tucker & Grierson, 1982; Tucker & Laties, 1984). Furthermore, RNA blot analysis with cDNAs encoding the enzymes as probes showed that mRNA coding for these enzymes accumulated markedly during ripening (Olson et al., 1991; Slater et al., 1985; Christoffersen et al., 1984).

Apple fruit ripening is associated with an upsurge in the rate of respiration and ethylene production (Reid et al.,

1973). The activities of ß-galactosidase (Bartley, 1977), ACC synthase (Bufler, 1984) and ACC oxidase (Bufler, 1986) have been shown to increase during apple fruit ripening. Recently, Lay-Yee et al., (1990) found that apple fruit ripening is accompanied by the appearance of at least six mRNAs and the increase of three proteins.

In this report, a ripening-related protein was identified in apple cortical tissues and its expression during ripening was examined.

MATERIALS AND METHODS

Plant Materials

All apple fruits were harvested from the Horticulture Research Center of Michigan State University just prior to the onset of ethylene climacteric (internal ethylene ≤ 0.1 ppm).

Empire apples were used for two dimensional gel electrophoresis studies. Preclimacteric apples were taken at harvest and postclimacteric apples were those stored at 1 °C for a couple of months followed by one week at 20 °C.

Western blot analysis and mRNA extraction: Empire, Golden Delicious and Mutsu apples were used for these experiments. Mutsu apples were used for RNA extraction. The apples after harvest were left at 20 °C to ripen and internal ethylene and flesh firmness were measured as the fruits developed their ethylene climacteric.

Extraction of Proteins for Two-dimensional PAGE

Cortical tissues were taken from both preclimacteric and postclimacteric Empire apples. Tissues were frozen in liquid N_2, freeze-dried and ground into powder. The apple powder was mixed with extraction buffer containing 0.1 M K_2CO_3, 0.2% Triton X-100 and 5 mM DTT (10 ml of extraction buffer/ g apple powder). The slurry was stirred for 30 min and then filtered through Miracloth. The pH of the filtrate was adjusted to 9.5 with 5 M acetic acid and 1 M of magnesium acetate was added to a final concentration of 10 mM Mg^{2+}. The filtrate was then centrifuged at 10,000xg for 10 min. The supernatant was combined, frozen in liquid N_2 and freeze-dried. The dried sample was redissolved in distilled water (1 ml/g apple powder). The final volume was measured and then an equal volume of water saturated phenol was added. The mixture was shaken at room temperature for 10 min, followed by centrifuging at 10,000xg for 10 min. Phenol phase (upper layer) was carefully taken, using a pipette. 4 volumes of 0.1 M NH_4Ac (ammonium acetate) in methanol was added to the phenol extract and the mixture was left at -20 °C overnight. The mixture was centrifuged at 10,000xg for 10 min. The resulting pellet was washed with 2 volumes of 0.1 M NH_4Ac in methanol three times, followed by one wash with acetone. The pellet was dried in air and then dissolved in Farrel's lysis buffer (1975). The sample was clarified by centrifuging at 10,000xg for 10 min.

Two-dimensional Electrophoresis

Two dimensional gel electrophoresis was carried out using the method of O'Farrel (1975) with slight modifications. The first dimension was an isoelectric focusing (IEF) gel (0.3 x 11 cm) containing 3.6% ampholyte mixture (1/3 pH 3-10, 1/3 pH 5-8, 1/3 pH 6.5-9). The second dimension was a 10% SDS-polyacrylamide slab gel (Laemmli, 1970). About 500 μg of proteins was loaded each time. After electrophoresis was completed, the gel was soaked in fixative (acetic acid: methanol: H_2O = 1:5:5) for 30 min, then in Commassie blue stain (0.1% Coomassie brilliant blue R, 7% acetic acid, 50% methanol) overnight, and finally destained in destaining solution (7% acetic acid, 20% methanol, 3% glycerol).

To determine the isoelectric point (pI) of PAp5, the IEF tube gel (first dimension) was cut with a razor blade into a 0.5 cm sections and incubated in 1 ml of distilled water for 30 min after which the pH was measured. The pH coincident with Rf of PAp5 was taken to be the pI.

Purification of PAp5 and Polyclonal Antibody Production

The two-dimensional PAGE of proteins extracted from postclimacteric Empire apples was employed to purify PAp5. Several dozen 2-D gels were done to obtain a few mg of PAp5. PAp5 was cut out from the slab gel with a razor blade after first staining the gel with Commassie blue. The gel pieces containing PAp5 were stored at -20 °C. PAp5 was eluted from the gel pieces electrophoretically in a buffer containing 150 mM Tris, pH 8.8 and 0.14% SDS. The eluted PAp5 was freeze-

dried, redissolved in a small volume of distilled water, and then precipitated by adding 4 volumes of cold (-20 °C) acetone-methanol (1:1).

Antibody production was done in collaboration with NEOGEN Corporation. Four white New Zealand male rabbits were injected with purified PAp5 using an intradermal-intramuscular protocol. The intradermal injections (0.1 mg PAp5 each time) were done on day 0, 7, and 14. The intramuscular injections (0.05 mg PAp5 each time) were done on day 28, 45, and 79. The rabbits were bled out on day 97.

Western Blot Analysis of PAp5

Freeze-dried apple powder was extracted with electrophoresis sample buffer (Laemmli, 1970) (10 ml of sample buffer/g apple powder). The mixture was stirred for 5 min and then boiled for 15 min. The slurry was centrifuged at 30,000 xg for 15 min. The supernatant was filtered through Miracloth. A 10% SDS-polyacrylamide slab gel (1.5 mm thick) was prepared the previous day. 5 μl (about 5 μg protein) of sample was loaded per lane. After electrophoresis, the proteins on the gel were transferred by blotting to a nitrocellulose membrane according to Towbin et al., (1979).

Detection of PAp5 on the nitrocellulose membrane was performed according to instruction manual of Bio-Rad Immuno-Blot Assay Kit with some modifications. The nitrocellulose membrane was soaked in 100 ml of TBS (20 mM Tris, pH 7.5, 500 mM NaCl) containing 3% non-fat dry milk for 4 hours with

gentle shaking. The membrane was incubated with 100 ml TTBS (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20) containing 0.4 ml PAp5 antisera and 3% non-fat dry milk for 1 hour with gentle shaking, followed by rinsing with TTBS three times (5 min each). Then, it was incubated with 100 ml of TTBS containing 20 μl goat anti-rabbit IgG conjugated with HRP (Sigma, Catalog No. A-4914) and 3% non-fat dry milk for 15 min with gentle shaking, followed by washing with TTBS (3 x 5 min). The color was developed by incubating the membrane with 100 ml of substrate (TM Blue precipitating reagent from Transgenic Sciences, Inc., Catalogue No. TM 101)

RNA Extraction, In Vitro Translation and Immunoprecipitation

mRNA was extracted according to Lay-Yee et al (1990), except that guanidine·HCl instead of guanidine isothiocyanate was used and that poly(A)+ RNA was isolated by poly(U)-cellulose column chromatography. Mutsu apples at six stages of ripening were used to extract RNA. Poly(A)+ RNA was translated *in vitro* with rabbit reticulocyte lysate (Promega Biotec.) using the procedures described by the manufacturer. The *in vitro* translation products, radiolabeled with (^{35}S) methionine (approximately 1100 Ci/mmol), were subjected to immunoprecipitation by PAp5 polyclonal antibody according to the procedures described by Firestone and Winguth (1990).

RESULTS AND DISCUSSION

The protocol of protein extraction from apple cortical

tissue used proved to be superior among several protocols tried. Extensive streaking of proteins on two-dimensional electrophoresis gels was observed if proteins were extracted using the other protocols (not shown).

Fig. 1 and 2 shows the two dimensional PAGE analysis of protein populations from preclimacteric and postclimacteric apples. One polypeptide termed PAp5 accumulated extensively in postclimacteric apples (Fig. 2). It was barely detectable in preclimacteric apple fruit (Fig. 1). The molecular weight and isoelectric point was estimated to be 40 KD (Fig. 3) and 5.3 (see Materials and Methods for details). The size and isoelectric point of PAp5 was very close to one of the three ripening related polypeptides of apple fruit reported by Lay-Yee et al (1990).

PAp5 was purified from Empire apple cortical tissue by two-dimensional gel electrophoresis and polyclonal antibody was raised against it. Western blot analysis using PAp5 antisera showed that the level of PAp5 correlated with the development of the ethylene climacteric (Fig. 4 and 5). PAp5 was undetectable in preclimacteric apples, was detected as the climacteric developed, and accumulated extensively as the apples ripened. PAp5 seems to be a very abundant protein in ripe apples. It accounted for about 1% of total proteins estimated from the western blot data. The function of PAp5 is unknown. It is not ACC synthase because ACC synthase has a M.W. of 48 KD (Yip, et al., 1991), whereas PAp5 is a 36.5 KD

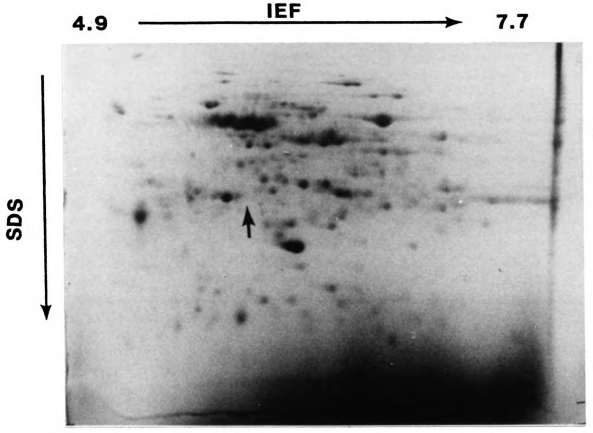

Fig. 1. Two-dimensional PAGE of proteins extracted from cortical tissue of preclimacteric Empire apple fruit (internal ethylene $\leq$ 0.1 ppm). About 500 μg of protein was loaded. The gel was stained with commassie blue.

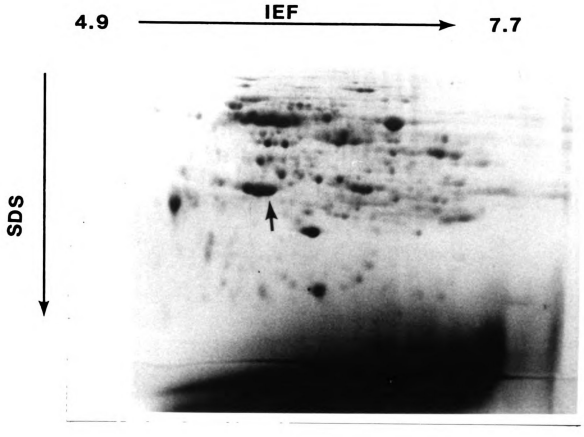

Fig. 2. Two-dimensional PAGE of proteins extracted from cortical tissue of postclimacteric Empire apple fruit (internal ethylene ≥ 300 ppm). About 500 μg of protein was loaded. The gel was stained with commassie blue.

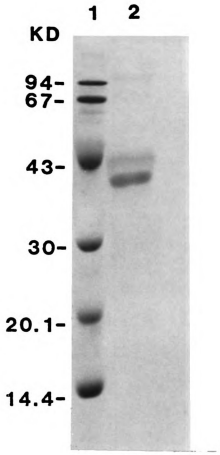

Fig. 3. One-dimensional SDS-PAGE of purified PAp5. Lane 1: molecular weight marker. Lane 2: purified PAp5.

polypeptide. PAp5 IgG was purified by protein A column and an affinity column was made by linking PAp5 IgG to activated agarose. ACC oxidase failed to bind to the immunoaffinity column (not shown). Therefore, PAp5 may not be ACC oxidase either. Western blot analysis of proteins extracted from other cultivars (Mutsu and Golden Delicious) using polyclonal antibody against PAp5 of Empire apples gave similar results (Fig. 6-9), except that the molecular weight of PAp5 in both Golden Delicious and Mutsu fruits is smaller than that in Empire fruit (Fig. 7 & 9).

RNA was extracted from Mutsu apples at six stages of ripening (Table 1). The RNA content of apple cortical tissue was similar to what Lay-Yee reported (1990). Poly(A)+ RNA was isolated by poly(U)-cellulose column chromatography and translated in vitro using a rabbit reticulocyte system with (^{35}S)-methionine. One major radiolabeled polypeptide with a similar molecular weight as PAp5 was immunoprecipitated with PAp5 polyclonal antibody (Fig. 10). The intensity of the radiolabeled polypeptide decreased when pure PAp5 was included in the immunoprecipitation reaction (lanes 7, 8, 9), indicating that pure PAp5 and the radiolabeled polypeptide competed for PAp5 antibody and that the precipitated radiolabeled polypeptide is the precursor of PAp5. The in vitro translated PAp5 increased as the ethylene climacteric developed, indicating that the level of the translatable mRNA for PAp5 increased during ripening.

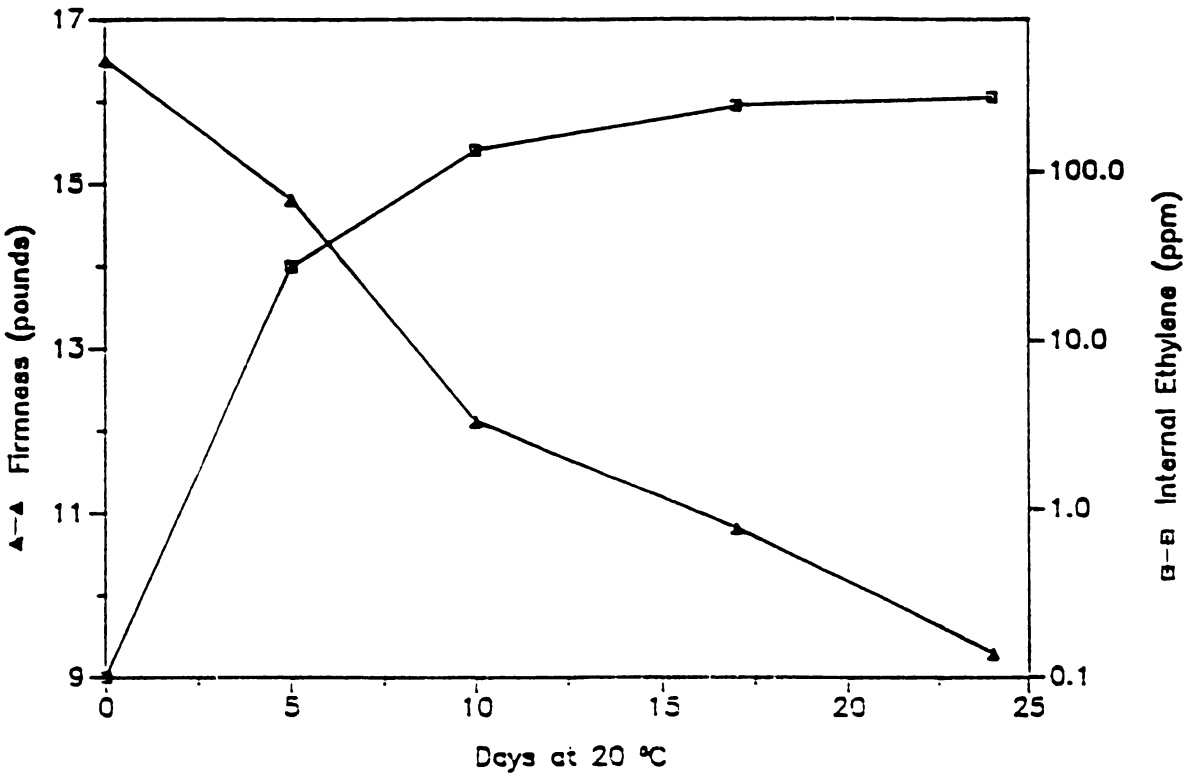

Fig. 4. The changes of internal ethylene and flesh firmness vs. days at 20 °C (Cultivar: Empire)

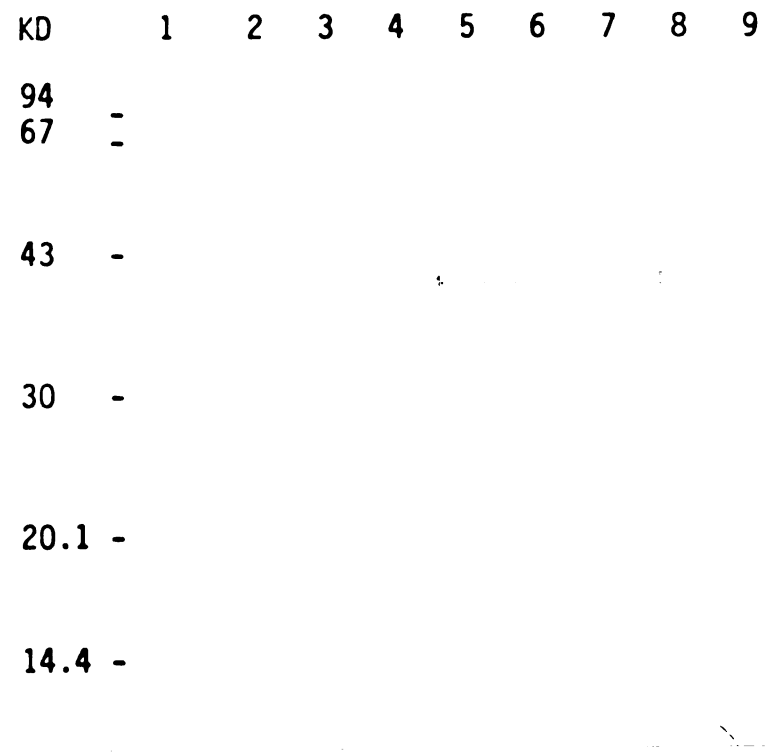

Fig. 5. Western blot analysis of PAp5 (Cultivar: Empire). Proteins were extracted from cortical tissue of fruits at six developmental stages. Lane 1 was the protein sample extracted from immature apples. Lanes 2-6 corresponds to the points in Fig 4 (day 0, 5, 10, 17 and 24, respectively). About 5 μg of protein was loaded in each lane. Lane 7, 8, 9 contain 10, 25, 50 ng of pure PAp5, respectively.

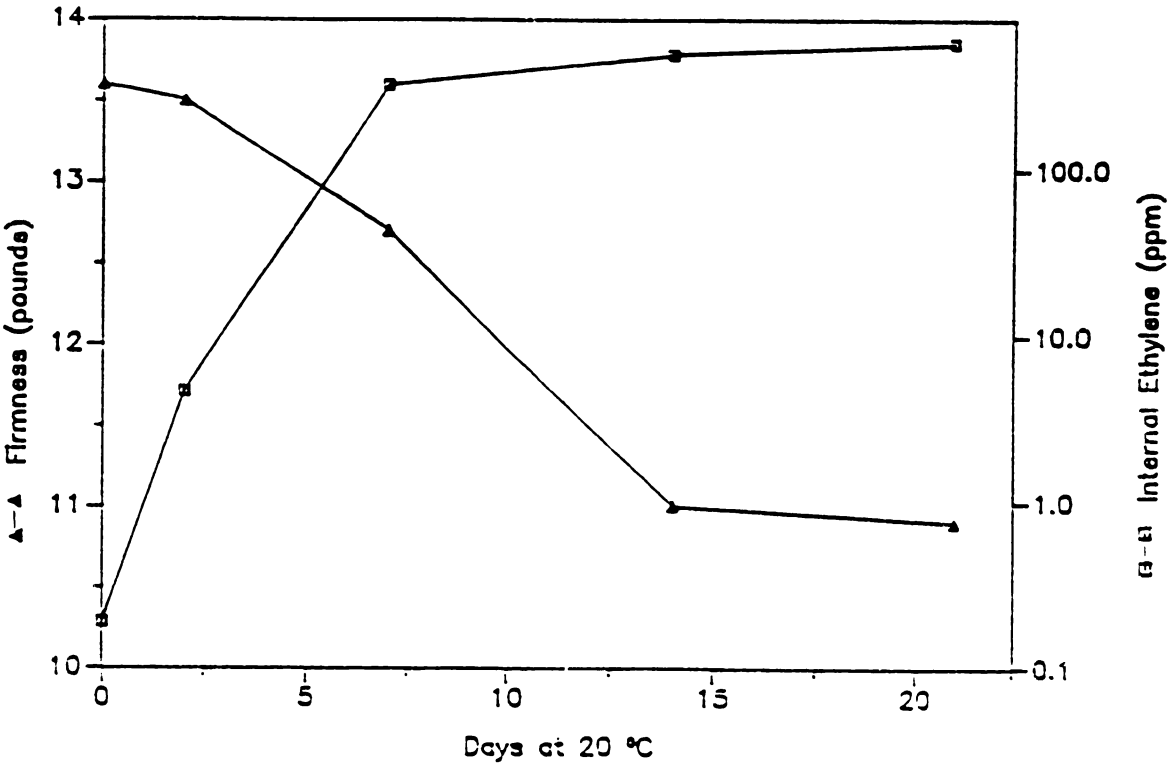

Fig. 6. The changes of internal ethylene and flesh firmness vs. days at 20 °C (Cultivar: Golden Delicious)

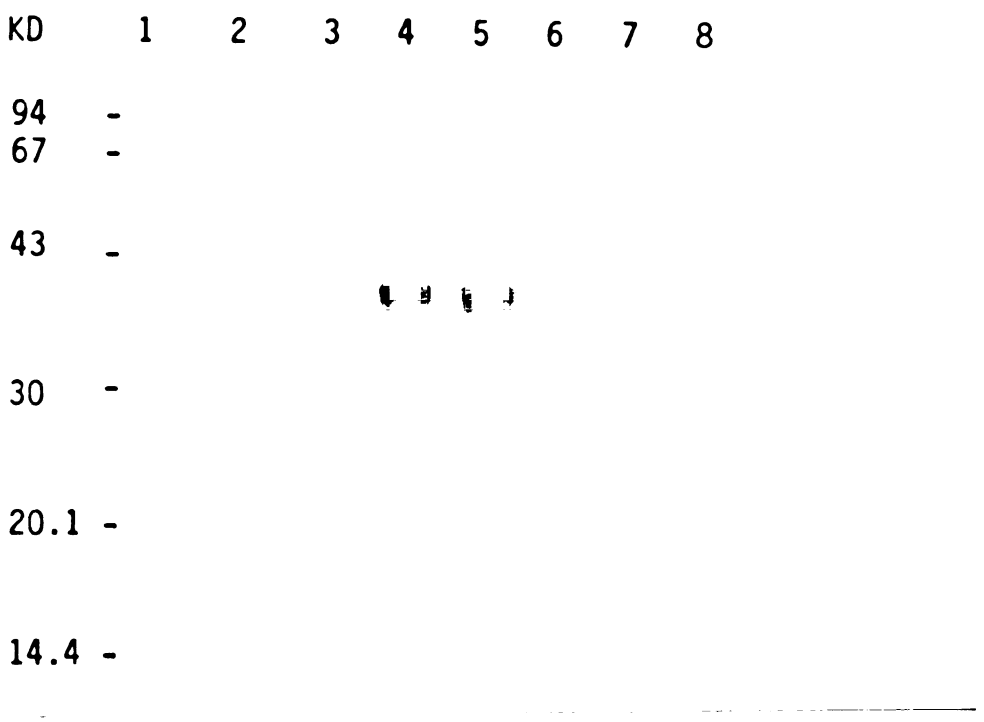

Fig. 7. Western blot analysis of PAp5 (Cultivar: Golden Delicious). Proteins were extracted from cortical tissue of fruits at five developmental stages. Lane 1-5 correspond to the points in Fig. 6 (day 0, 2, 7, 14 and 21, respectively). About 5 μg of protein was loaded each lane. Lane 6, 7, 8 contain 10, 25, 50 ng of pure PAp5.

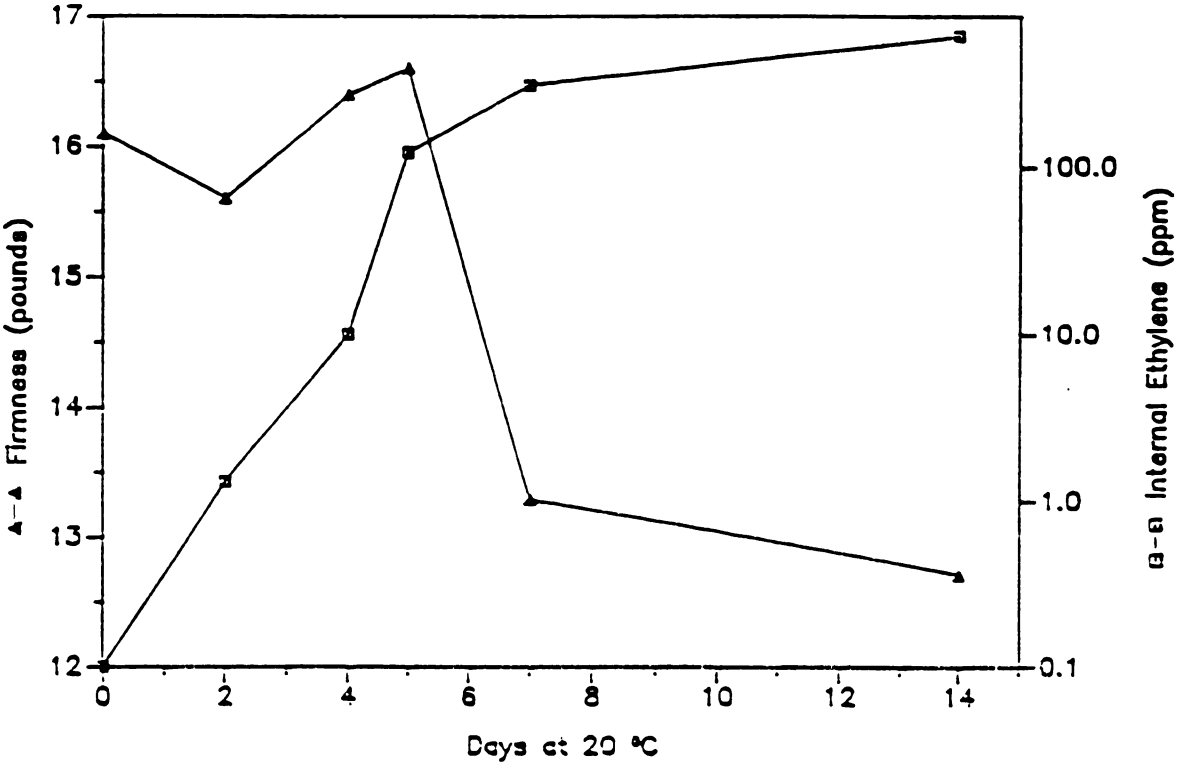

Fig. 8. The changes of internal ethylene and flesh firmness vs. days at 20 °C (Cultivar: Mutsu)

Fig. 9. Western blot analysis of PAp5 (Cultivar: Mutsu). Proteins were extracted from cortical tissue of fruits at six developmental stages. Lane 1-6 correspond to the points in Fig. 8 (day 0, 2, 4, 5, 7 and 14, respectively). About 5 μg of protein was loaded each lane. Lane 7, 8, 9 contain 10, 25, 50 ng of pure PAp5.

Table 1 Extraction of RNA from apple cortical tissue

Stages of Ripening	Internal Ethylene(ppm)	RNA yield (μg/g FW)	A260/A280	A260/A230
I	0.10 ± 0.09	13.2	2.13	1.96
II	1.31 ± 0.69	12.8	2.16	1.96
III	10.3 ± 5.64	12.3	2.13	1.91
IV	122.4 ± 22.5	12.8	2.23	1.96
V	308.1 ± 44.5	13.5	2.19	2.02
VI	607.4 ± 99.8	11.7	2.20	1.99

Fig. 10. Immunoprecipitation of <u>in</u> <u>vitro</u> translated proteins of poly(A)+ RNA extracted from cortical tissue of apple fruits using PAp5 antisera. Lane 1-6 correspond to the six developmental stages in Table 1 starting from I. Lane 7, 8, 9 were the same as lane 6 except that 1 ng, 10 ng, 100 ng of pure PAp5 was included in the immunoprecipitation solution, respectively.

In summary, a protein (PAp5) with a molecular weight of 36.5 KD and isoelectric point 5.3 purified by two-dimensional PAGE was found to be related with apple fruit ripening. Polyclonal antibodies against PAp5 were raised. Western blot analysis indicated PAp5 was absent from apples prior to the onset of the ethylene climacteric but increased markedly as the climacteric developed. Immunoprecipitation of *in vitro* translated products of poly(A)+ RNA with PAp5 antisera showed that translatable mRNA encoding PAp5 increased during ripening.

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CONCLUSIONS AND RECENT PROGRESS

ACC oxidase was extracted, partially purified and characterized from Golden Delicious apple fruit cortical tissue. The presence of 5% (w/v) polyvinylpolypyrrolidone (PVPP) was necessary in the extraction medium to obtain an active enzyme from apple tissue. The specific activity was 40 to 100 nl ethylene hr^{-1} mg^{-1} protein in the crude extract; polyethylene glycol-4000 fractionation followed by calcium phosphate gel adsorption resulted in ca. 10-fold purification.

Apple ACC oxidase required both ascorbate and Fe^{2+} as cofactors. The optimum ascorbate concentration was 1-2 mM and the optimum Fe^{2+} concentration was 100 μM. The failure of early attempts to extract ACC oxidase from plant tissue was probably due to the loss of these cofactors essential for the enzyme activity after tissue was homogenized. Apple ACC oxidase has a K_m for ACC of 6.4 μM, K_m for O_2 of 0.4% and pH optimum of 7.2 in Tris-Mes buffer and 7.6 in phosphate buffer. The enzyme was stereospecific for substrate; it converted (±)allocoronamic acid to 1-butene while (±)coronamic acid was 10-fold less effective as a substrate. Extractable apple ACC oxidase activity correlated well with the climacteric of ethylene production. The characteristics of the extracted enzyme, therefore, resembled those of the <u>in vivo</u> enzyme.

Apple ACC oxidase was strongly inhibited by $CuCl_2$, $ZnCl_2$, $CoCl_2$, and $AgNO_3$ at 1 μM. The enzyme was also inhibited by n-

propylgallate, 5,5'-dithio-bis(nitrobenzoate) (DTNB), but not by Triton X-100 (0.3%) or CO (10%). The inhibition of the enzyme activity was reversed by increasing the Fe^{2+} concentration in the assay medium. This indicates that n-propylgallate probably inhibits as a chelating agent for Fe^{2+} in the enzyme reaction, rather than as a free radical scavenger. The inhibition of the enzyme activity by DTNB suggests that the -SH groups of the enzyme might have important roles in the enzyme reaction. Kinetic studies of the inhibition of $CoCl_2$, n-propylgallate and 2-aminoisobutyrate (AIB) revealed that AIB inhibited the enzyme activity in a competitive manner with a K_i of 388 μM, whereas $CoCl_2$ and n-propylgallate inhibited the enzyme activity in a noncompetitive manner with a K_i of 0.2 and 77 μM, respectively.

ACC oxidase was recently purified from cortical tissue of Golden Delicious apple fruit by a combination of DEAE-sepharose, phenyl-sepharose and HPLC size exclusion chromatography. The molecular weight of the enzyme was estimated by SDS-PAGE to be 35.7 KD. Native isoelectric focusing electrophoresis indicated that the pI of apple ACC oxidase is 5.3. ACC oxidase in ripe apples may account for > 1% of total protein.

A ripening related protein which we termed PAp5 was identified by two-dimensional gel electrophoresis. Proteins were extracted from cortical tissue of preclimacteric and postclimacteric Empire apple fruit and analyzed by two-D PAGE.

PAp5 was found to accumulate in postclimacteric apple fruit. PAp5 had a molecular weight of 36.5 KD and isoelectric point 5.3. Polyclonal antibody was raised against the protein. Western blot analysis indicated that PAp5 was absent from apples prior to the onset of the ethylene climacteric but increased markedly (up to 1% of the total protein) as the climacteric developed. The *in vitro* translation of poly (A)-containing RNA from apples at six stages of ripening (internal ethylene = 0.1, 1.33, 10, 122, 308, 607 ppm, respectively) and immunoprecipitation with PAp5 antisera showed that translatable mRNA encoding PAp5 increased during ripening.

Research conducted in our laboratory subsequent to writing this thesis has indicated that PAp5 is ACC oxidase. This is based on the following:

1) Purified ACC oxidase was recognized by PAp5 antisera on western blot.
2) PAp5 and ACC oxidase have the same pI value and similar molecular weight.
3) Both are ripening-related proteins and accumulate up to 1% or more of total proteins in postclimacteric apple fruit.