Purification and Properties of the Apple Fruit Ethylene-Forming Enzyme†

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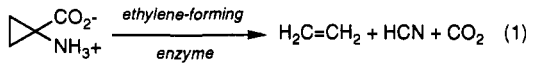
ABSTRACT: The enzyme that oxidatively converts 1-aminocyclopropanecarboxylic acid (ACC) to ethylene, a key plant growth hormone, has been classified, on the basis of a comparison of homologous protein sequences (derived from the cDNA sequences), as a member of a family of non-heme iron proteins that includes plant and bacterial oxidative enzymes. This knowledge has facilitated the purification of the relatively abundant ethylene-forming enzyme to homogeneity from apple tissue. The properties of the enzyme are consistent with two other recent reports that describes its purification by different protocols, lending credence to the assertion that the key protein has been isolated. New characterizations of the protein have been conducted. Electrospray mass spectrometry shows that its molecular weight (35 331.8 ± 5 amu) is ~50 amu higher than that predicted from the cDNA sequence, identifying the blocking group at the N-terminus as acetyl. The enzyme is activated by bicarbonate at low concentration but is inhibited at high concentration, with the maximum activation occurring at 5 mM. The iron concentration leading to half-maximal activity is 1 μM. The enzyme self-inactivates during turnover. The availability of the purified enzyme will permit definitive studies of the mechanism by which ethylene is produced and provide opportunities to discover molecules that inhibit the process.

The characteristics of the macromolecule that conducts the oxidative conversion of 1-aminocyclopropanecarboxylic acid (ACC) to ethylene (eq 1) have been of interest since the original report that ACC is the immediate precursor of this plant growth hormone (Adams & Yang, 1979). However, because



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over a decade of effort had failed to provide an authentic cell-free ethylene-forming enzyme (EFE), the lore developed that the enzyme is membrane-bound and does not survive breakage of the cell wall. Consequently, the many mechanistic studies of ethylene production (Liu et al., 1984; Pirrung, 1983, 1987; Pirrung & Trinks, 1989; Pirrung & McGeehan, 1983, 1985, 1986; Adlington et al., 1983; Baldwin et al., 1985, 1988a,b) have been limited to biosynthetic experiments in which only substrates and products can be inferred; many facts remain unknown. The sequence of the cDNA for the ethylene-forming enzyme (EFE) from tomato (Spanu et al., 1991; Hamilton et al., 1991), as first noted by Hamilton et al. (1990), has homology to a flavanone-3β-hydroxylase enzyme (Britsch, 1990) believed to be a non-heme iron protein (Hedden, 1992). The known lability of such enzymes suggests a possible reason for the earlier difficulties in obtaining valid EFE activity. Cell-free extracts have subsequently been obtained from a variety of fruit tissue (Ververidis & John, 1991; McGarvey & Christoffersen, 1992; Fernandez-Maculet & Yang, 1992). The apple cDNA has recently been cloned and it is closely related to the tomato sequence (Dong et al., 1992a). Recent reports of the purification of the protein from apple fruit (Dupille et al., 1992; Dong et al., 1992b) prompt us to communicate our results concerning the production of homogeneous ethylene-forming enzyme. Our experiments support the results of the foregoing reports as well as provide new, key information concerning the enzyme's molecular composition.

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MATERIALS AND METHODS

Purification of the Ethylene-Forming Enzyme. Cortical tissue of *Malus pumila* (Golden Delicious) was ground to a powder under liquid nitrogen and stored at −80 °C. The frozen tissue (100 g) was combined with 5% (w/v) insoluble poly(vinylpyrrolidone) powder and then with 200 mL of 100 mM potassium phosphate (pH 7.8), 200 mM sucrose, and 3 mM dithiothreitol and allowed to thaw at 4 °C for 30 min with occasional stirring. The suspension was then filtered through two layers of Miracloth and four layers of gauze and centrifuged at 11000*g* for 30 min. The resulting supernatant was referred to as crude extract. EFE was precipitated from the crude extract with 50–80% saturation of $(NH_4)_2SO_4$ and pelleted at 11000*g* for 30 min. This precipitate was resuspended using a syringe with a 25 gauge needle in 3.0 mL of 40 mM Tris-HCl (pH 7.8), 200 mM sucrose, 2 mM dithiothreitol, and 30% (v/v) glycerol (buffer A), adjusted to 21% saturation with solid $(NH_4)_2SO_4$. This suspension was loaded onto a butyl–Toyopearl 650M (Toyo Soda Manufacturing Co.) column (1.5 × 25 cm) previously equilibrated with 21% $(NH_4)_2SO_4$ in buffer A. The column was eluted at 1 mL min^{-1} with a stepwise gradient of 50 mL each of 21%, 14%, 7%, and 0% saturated $(NH_4)_2SO_4$ in buffer A, and the active fractions were combined and concentrated to approximately 0.5 mL using a Centricon 30 (Amicon) at 5000*g*. This concentrated effluent was then loaded onto a DEAE–Toyopearl (Toyo Soda Manufacturing Co.) column (0.7 × 13.5 cm) previously equilibrated with 50 mM NaCl in buffer A. The column was flushed with 10 mL of 50 mM NaCl in buffer A, and the protein eluted at 0.2 mL min^{-1} with a stepwise gradient of 3 mL each of 75, 100, 125, 150, 175, and 200 mM NaCl in buffer A. The active fractions were combined and concentrated to approximately 0.3 mL using a Centricon 30 (Amicon) at 5000*g*. Denaturing gel electrophoresis (Laemmli, 1970) against protein standards was used to determine the M_r of the enzyme. EFE (5 μg per lane) was lyophilized and resuspended in 0.02 mL of sample buffer [25 mM Tris-HCl (pH 6.8), 40% glycerol, 8% SDS, and 0.4% bromophenol blue], heated to 100 °C, and cooled to room temperature for

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F3H      178  HTDPGTITLLLQD...257  VVNLGDHGHFLSNGRFKNADHQA
H6H      236  HIDIGFVTILLQD...254  VVNLGLTLKVITNEKFEGSIHRV

pHTOM5   157  HTDAGGIILLFQD...194  VVNLGDQLEVITNGKYKSVLHRV
pAE12    177  HSDAGGIILLFQD...214  VINLGDQIEVITNGKYKSVMHRV

DAOCS    184  HYDLSTLTLVHQT...224  VVFCGAVGTLAIGGKVKAPKHRV
IPNS     214  HEDVSLITVLYQS...252  LINCGSYMAHITDDYYPAPIHRV
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FIGURE 1: Deduced protein sequence comparison between the ethylene-forming enzyme and other known non-heme iron proteins.

loading. The minigels were stained with 0.125% Coomassie Blue. Electrospray mass spectrometry was performed using a quadrupole mass analyzer on highly purified samples of the EFE in 40 mM NH_4OAc (pH 7.8) buffer that were obtained by repeated Centricon spins.

Activity Assay. Approximately 0.005–0.5 mg of protein in a 500-μL volume was added to 2.5 mL of assay buffer [50 mM Tris-HCl (pH 7.2), 10% (v/v) glycerol, 0.1 mM $FeSO_4$, 10 mM ascorbate, 1 mM ACC, and 1 mM 2-oxoglutarate] in 25-mL Erlenmeyer flasks. The vials were sealed with serum caps and incubated for 1 h at 23 °C shaking gently. The headspace (1.0 mL) was analyzed by gas chromatography on a Varian 3400 GC equipped with a flame ionization detector and an 80% Porapak N/20% Porapak Q column. Ethylene production was quantitated by comparison with a 97.7 ppm ethylene gas mixture in helium (Alltech Associates). A unit is defined as 1 nL h^{-1}.

RESULTS

Using the GCG program (Devereux et al., 1984), a search of GenBank was conducted for proteins homologous to the translation products of the EFE cDNAs pHTOM5 (tomato) and pAE12 (apple). Several related enzymes were found that have regions of high homology, and several residues within the regions are fully conserved, including histidines (indicated by arrows in Figure 1) that are believed to bind an essential catalytic iron. This family of non-heme iron proteins, which is covered in greater detail in the Discussion section, includes an oxidase and a dioxygenase involved in the biosynthesis of the penicillin and cephalosporin antibiotics and dioxygenases involved in two biosynthetic pathways in plants (Figure 2). The analysis of sequence homology clearly places the tomato and apple EFEs in this non-heme iron protein family. Because

Table I

fraction	total protein (mg)	activity (units)	specific activity (units/mg)	yield (%)	x-fold purification
crude	27	2025	75	100	
50–80% ppt	6.9	1228	178	60.6	2.4
butyl–Toyopearl	0.19	220	1159	10.9	15.5
DEAE–Toyopearl	0.04	64	1595	3.2	21.3

one of these, H6H, was purified to homogeneity from *Hyoscyamus* roots (Yamada et al., 1990) and sequenced in order to obtain the cDNA clone, this work provided a model for our purification of the EFE from cell-free extracts of apple fruit. Apple tissue is directly frozen in liquid nitrogen; significant activity is obtained from a storable, crude powder. A four-step extraction, precipitation, and chromatographic purification sequence provides pure EFE (Table I), as evidenced in the SDS–PAGE analyses in Figure 3. The enzyme is surprisingly abundant; it is present at significant levels even in the crude extract, and over the course of several experiments, only a 20–30-fold purification factor has been necessary to reach homogeneity. The validation of the preparation is based on its stereoselective processing of allocoronamic but not coronamic acid, stereoisomeric alkylated ACC analogues which are also differentiated by the *in vivo* ethylene-forming enzyme. Some kinetic properties of the enzyme were determined: the Michaelis constant for ACC is 12 ± 4 μM, and that for iron is 1 μM. The k_{cat} is (2.5 ± 0.2) × 10^{-3} s^{-1} under standard assay conditions (*vide infra*).

Dong et al. (1992b) have reported that the enzyme is activated by CO_2, that the activation saturates at 4% CO_2 in the gase phase, and that half-maximal enzyme activity is observed at 0.5% CO_2. They state that this concentration of CO_2 in equilibrium with solution corresponds to a bicarbonate concentration of 0.15 mM. Inclusion of bicarbonate directly in the assay buffer offers greater operational simplicity and results in a specific activity of our purified EFE of 18 nmol mg^{-1} min^{-1}, which is approximately the value reported by Dong et al. (1992b) for their preparation. The concentration dependence of the activation of the enzyme by bicarbonate is not simple, however. As shown in Figure 4, it follows saturation behavior up to 5 mM but is inhibitory at higher concentrations.

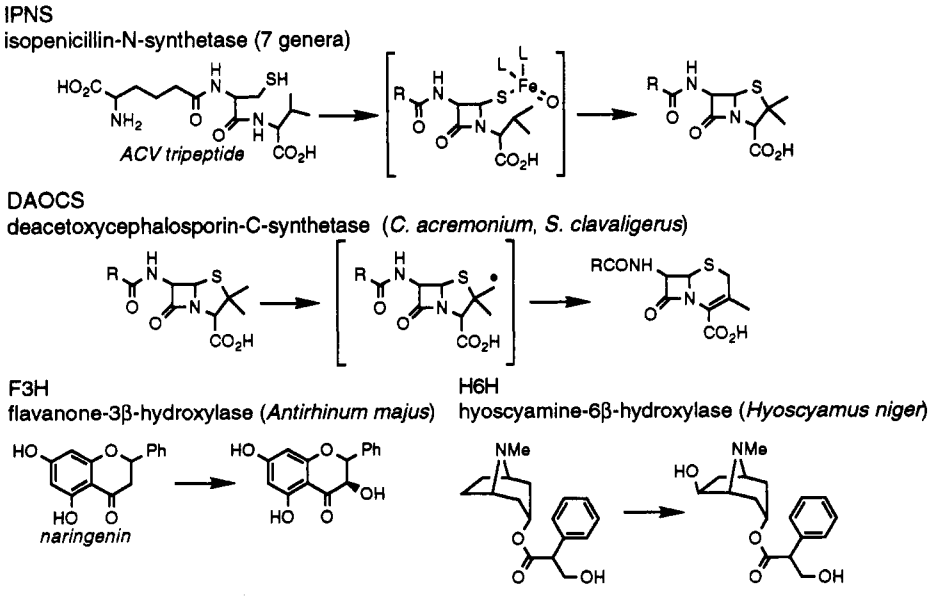

FIGURE 2: Biosynthetic transformations mediated by non-heme iron proteins related to the ethylene-forming enzyme.

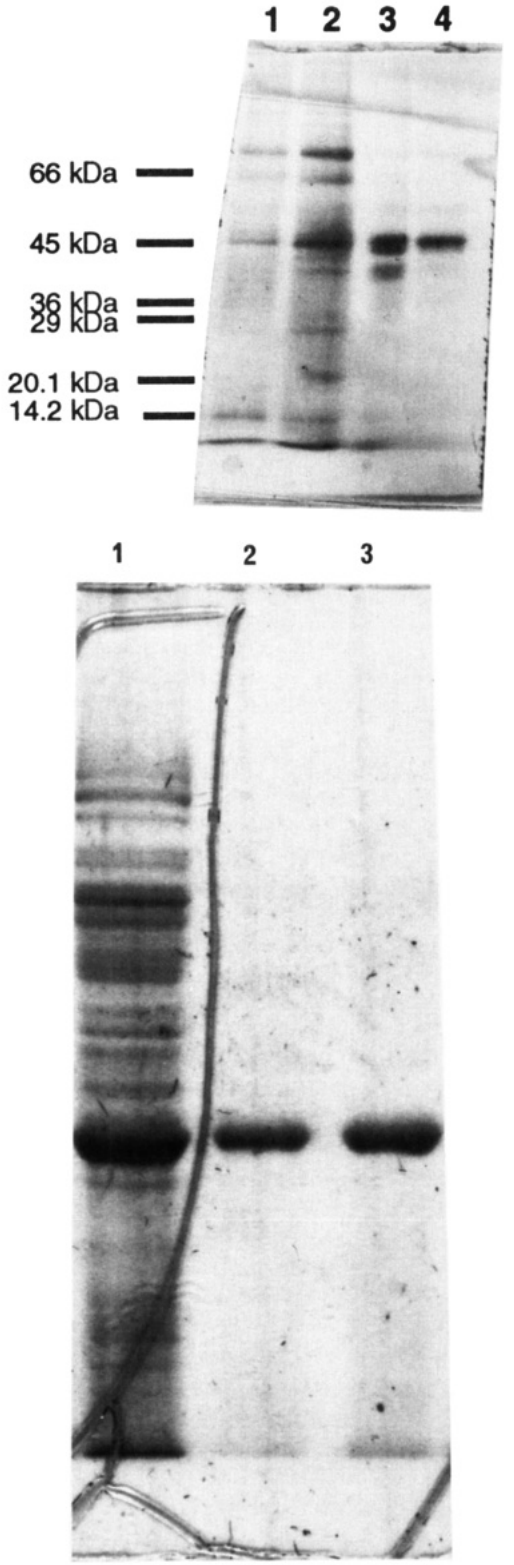

FIGURE 3: SDS–PAGE analysis of the ethylene-forming enzyme (EFE) on mingels, 5 μg of protein per lane. (a, top) Fractions obtained during the purification of the ethylene-forming enzyme. The molecular mass standards included glyceraldehyde-3-phosphate dehydrogenase, 36 kDa, and ovalbumin, 45 kDa. Lane 1: Crude. Lane 2: $(NH_4)_2SO_4$ ppt. Lane 3: butyl–Toyopearl. Lane 4: DEAE–Toyopearl. (b, bottom) Lane 1: Crude. Lanes 2 and 3: Fraction submitted for mass spectrometry.

The meager catalytic properties of the enzyme, which turns over once every few minutes, are puzzling; its true activity is higher because the activity is time-dependent, and the time resolution of current assays is too low. As shown in Figure 5, the enzyme loses activity during turnover with a half-life of ~2 h. It is difficult to measure the initial velocity at very low percent conversion to obtain the true k_{cat}. Much more sensitive assays for the products of this enzyme must be developed in order to overcome this problem.

Dong et al. (1992b) have reported that the predicted (based on the cDNA sequence) and measured M_r of the EFE is 35 kDa. Because repeated attempts to confirm these values by denaturing gel electrophoresis analysis against standards suggested an M_r of ~40 kDa (Figure 3a), a highly purified sample (Figure 3b) of our homogeneous protein was subjected to electrospray mass spectrometry (Chait & Kent, 1992) using a quadrupole mass analyzer. A mean molecular mass for the EFE of 35 331.8 ± 5 amu is calculated from the masses of 11 ions carrying charges of 26–37 (Figure 6). The predicted average molecular mass based on the pAE12 sequence minus Met1 is 35 279.3 amu. It is widely observed that eukaryotic proteins lack an initiator methionine, which is removed by a specific methionine amino-terminal peptidase (MAP)(Yoshida & Lin, 1972; Flinta et al., 1986; Arfin & Bradshaw, 1988; Kendall & Bradshaw, 1992). The observed value still leaves a mass of ~52.5 amu unexplained. The failure of Dong et al. (1992b) to obtain a sequence of the EFE by Edman methods suggested that the protein is blocked at the amino terminus. The *N*-acetyl group usually observed when an initiator methionine is removed (Wold, 1981; Tsunasawa & Sakiyama, 1984) increases the M_r by 42 amu. Until higher precision data can be obtained on peptides derived from the EFE, acetyl is the most reasonable assignment for the amino-terminal blocking group.

DISCUSSION

The evidence from the sequence homology analysis presented above suggests that the EFE is a member of a small family of oxygenase proteins. Isopenicillin-N-synthase (IPNS) is the best studied member of the family. It removes four hydrogen atoms from the ACV tripeptide to directly produce isopenicillin N and two water molecules (Abraham et al., 1981). The mechanism of oxygen activation by this enzyme is unclear. It is believed that the iron is ligated by three histidine residues and perhaps an aspartate and that the thiol of the tripeptide is bound to the iron in the catalytically significant complex (Ming et al., 1990; Scott et al., 1992; Chen et al., 1989). Deacetoxycephalosporin C synthetase (DAOCS) is not an overt oxygenase (Baldwin et al., 1987a). No oxygen atoms are introduced into the substrate, but the ring expansion introduces an unsaturation, so that the product does lie two oxidation states higher than the starting material. A variety of experiments have suggested that the enzyme generates an alkyl radical at the substrate methyl group (Baldwin et al., 1987b, 1991) and that this might occur via hydrogen atom abstraction by an iron–oxo. The *C. acremonium* enzyme is bifunctional, further hydroxylating deacetoxycephalosporin C. It is reasonable that this enzyme uses the iron–oxo for both steps. DAOCS decarboxylates 1 mol of α-ketoglutarate in conducting each of the ring expansion and hydroxylation steps, thereby accounting for the oxidizing equivalents of the 2 mol of oxygen consumed. Many non-heme iron dioxygenase enzymes use α-ketoglutarate as cosubstrate, converting it to succinate and CO_2 while introducing an oxygen atom into the substrate. The well-known mammalian prolyl hydroxylase is one example of an α-ketoglutarate-dependent dioxygenase enzyme, but the chicken prolyl hydroxylase sequence has no homology to any of the enzymes in Figure 1. Flavanone-3β-hydroxylase (F3H) (Britsch, 1990) and hyoscyamine-6β-hydroxylase (H6H)

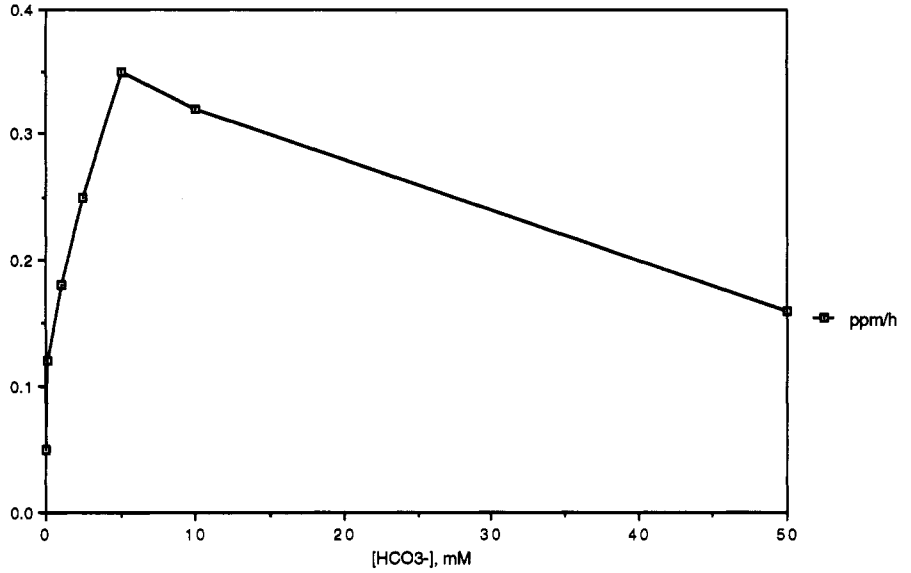

FIGURE 4: Bicarbonate dependence of ethylene-forming enzyme activity.

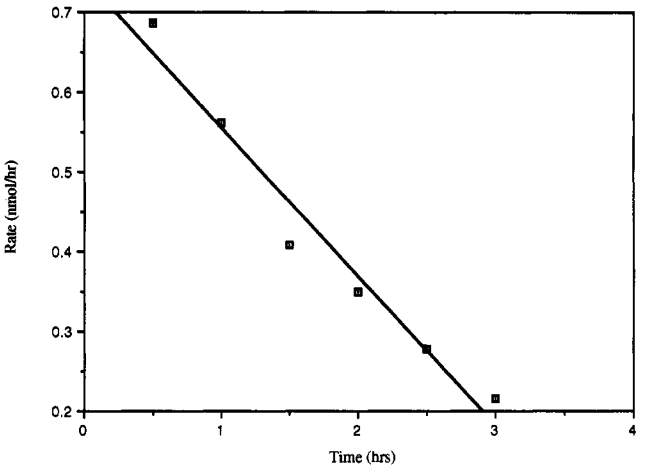

FIGURE 5: Loss of ethylene-forming enzyme activity during turnover.

(Matsuda et al., 1991) both add an oxygen atom to their substrates, presumably *via* an iron–oxo intermediate that has repeatedly been invoked in enzymatic hydroxylations.

The purification herein is easy to conduct and requires less chromatography than the method of Dong et al. (1992b). It avoids the use of Triton, which solubilizes a protein that in our hands cannot be separated from the EFE. The procedure of Dong et al. has produced larger quantities of protein, while our work has been done on a relatively small scale. The purity of our enzyme after the hydrophobic column is likely to be sufficient for many inhibition studies.

Comparisons of the kinetic properties of the EFE with IPNS, the enzyme in this family that has been best studied, are an important prelude to eventually determining the influence of the structure of these enzymes on their function. Two characteristics are quite similar. IPNS activity shows saturation with respect to iron at >10 μM Fe^{+2}, and a fit of the concentration-dependence data to the hyperbolic form of the Michaelis–Menten equation produces an apparent Michaelis constant of 1 μM (Perry et al., 1988), the same value reported here. Secondly, IPNS is inactivated after about 200 substrate turnovers. More detailed studies of the inactivation of the EFE in the presence of substrate and analogues are underway.

There are no motifs such as transmembrane helices (Goldman et al., 1986) in the sequence of the EFE to suggest, as lore has held, that it is an integral membrane protein. An analysis of the protein sequence for other motifs was conducted using the BLOCKS searcher (Henikoff & Henikoff, 1991). Residues 107–134, predicted to be α-helical by both Chou–Fasman (1978) and Garnier–Osguthorpe–Robson (1978) methods, have similarities to cofilin/tropomyosin-type actin-binding domain proteins (Moriyama et al., 1990; Cho et al., 1990), kinesin motor domain proteins (Vallee & Shpetner, 1990), and cereal trypsin/α-amylase inhibitors (Gautier et

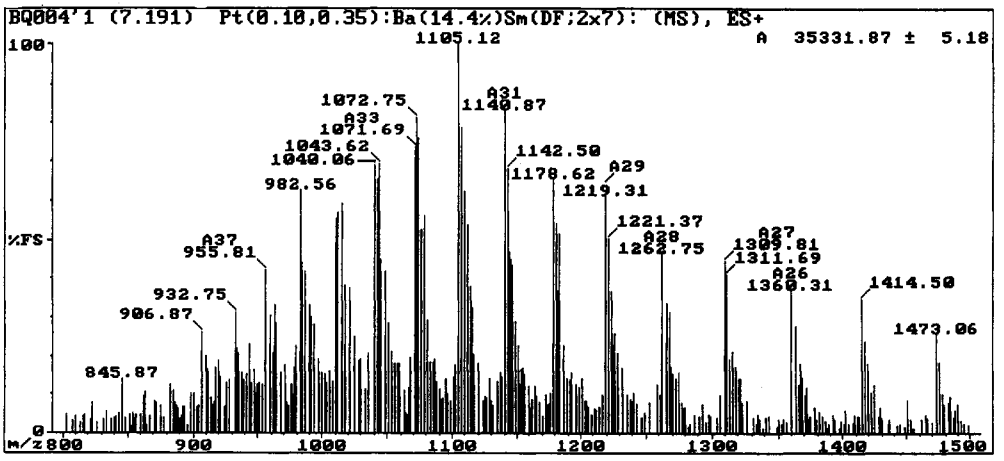

FIGURE 6: Electrospray mass spectrum of the EFE obtained in a 40 mM NH_4OAc solution.

al., 1990). While the EFE is fairly soluble, Dong et al. dissolved it from a protein pellet with Triton, and its purification as reported herein is primarily accomplished by hydrophobic interaction chromatography, suggesting that its hydrophobic properties are also significant. A molecular explanation is that it possesses an amphipathic helix can be inferred on the basis of the hydrophobic amino acids present every 4–5 positions within the highly helical region of the EFE. The kinesin motor proteins and the *Drosophila* segregational protein *ncd* (Endow, 1991), the latter having the highest homology to the EFE, are known to form aggregates *via* a central hydrophobic α-helical domain. These observations account for association of the EFE with hydrophobic surfaces. A possible biological role for this property remains to be established.

The cotranslational N-terminal processing of proteins is of interest due to the relation of the stability of a protein *in vivo* to its amino-terminal residue (N-end rule) (Bachmair et al., 1986). Two major pathways for this processing have been identified (Burstein & Schechter, 1978; Arfin & Bradshaw, 1988). When the initiator methionine is followed by a relatively small residue (Ala, Ser, Gly, Val, or Pro), the methionine is removed (Kendall & Bradshaw, 1992), and for Ala, Ser, and Gly, the amino terminus is acetylated (Boissel et al., 1985). When the adjacent residue is charged, processing does not occur except for type I actins with a Met-Glu or Met-Asp sequence (Rubinstein & Martin, 1983). Known EFE cDNA sequences have been compared (Callahan et al., 1992), and their direct translation products fall into two classes at the N-terminus. The apple and carnation cDNAs code for Met-Ala, while the tomato, peach, and avocado cDNAs code for Met-Glu and Met-Asp. On the basis of the mass spectrometric data, the amino-terminal sequence of the apple EFE is Ac-Ala. Determination of the presence of the initiator methionine and N-acylation is needed for EFEs isolated from the other organisms.

The purification of the ethylene-forming enzyme described herein, in combination with previous assignments of this activity to a protein with about the same molecular weight obtained by different protocols, lends credibility to the assertion that the key protein in ethylene biosynthesis has at last been isolated. The proposition that it is a non-heme iron protein is consistent with its extreme lability and its iron dependence. The simplicity of the purification protocol makes the enzyme readily available for efforts to discover, by screening or by design, compounds that inhibit it. Such materials are desirable for the mechanistic insight they may provide, as well as their practical use as anti-ripening and anti-senescence agents.

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