Cloning and Characterization of a cDNA for 1-Aminocyclopropane-1-carboxylate Oxidase from Papaya Fruit

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A full-length complementary DNA (cDNA) clone encoding a putative 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) from papaya was amplified by polymerase chain reaction technique from cDNAs synthesized from messenger RNA. Nucleotide sequence analysis of this cDNA clone revealed that it comprised a complete open reading frame coding for 310 amino acid residues. The deduced amino acid sequence showed high identity (72−80%) with the sequence of ACC oxidase from other plant species. No transit peptide was found. The 12 residues (P-5, A-27, G-32, H-39, H-177, D-179, L-195, Q-196, G-218, H-234, R-244, and S-246) are conserved as they are among all enzymes that require ferrous ion and ascorbate for activity. These suggest that the papaya cDNA clone encodes a cytosolic ACC oxidase. Furthermore, the coding region of ACC oxidase cDNA from papaya was introduced into an expression vector, pET-20b(+), and transformed into *Escherichia coli* BL21(DE3). A 0.45 mL enzyme crude extract from 5 mL culture in a typical assay produced 42 ppm of ethylene. A 38 kDa ACC oxidase protein was detected as the distinctive protein by Coomassie blue staining of SDS−PAGE, and western blot immunoanalysis confirmed the results of Coomassie blue staining. These indicate that this ACC oxidase cDNA clone can express active ACC oxidase enzyme in the *E. coli* system.

Keywords: *1-Aminocyclopropane-1-carboxylate oxidase; ACC oxidase; papaya (Carica papaya L. cv. Tainong 2)*

INTRODUCTION

The role of the plant hormone ethylene has been established as the critical metabolite that initiates fruit ripening (Brady and Speirs, 1991; Yang and Dong, 1993; Yang and Hoffman, 1984). 1-Aminocyclopropane-1-carboxylate oxidase (ACC oxidase) catalyzes the final oxidation step of ACC to ethylene in higher plants (Yang and Dong, 1993; Yang and Hoffman, 1984). There are several homologous complementary DNAs (cDNAs) of ACC oxidase isolated from plants (Dong et al., 1992a; Kim and Yang, 1994; MacDiarmid and Gardner, 1993; Nadeau et al., 1993; Pogson et al., 1995; Tang et al., 1993); however, none of the studies given investigates the papaya.

Papaya is a major economic crop in Taiwan. From the field to market, methods of storage life enhancement and the quality of harvested products are very important. In many cases, postharvest losses are regulated by the plant hormone, ethylene. Ethylene can also cause premature ripening of the fruit, resulting in reduced storage life and poor fruit quality. Our aims are to clone genes involved in ethylene biosynthesis in papaya and to apply antisense to reduce ethylene production by lowering the expression of ethylene biosynthesis genes, ultimately enhancing the storage life and quality of harvested products.

We report in this paper the cDNA sequence and deduced amino acid sequence of ACC oxidase from papaya fruit. In addition, the coding region of ACC oxidase cDNA was introduced into an expression vector, pET-20b(+), and transformed into *Escherichia coli* BL21(DE3). It was demonstrated that this cDNA clone can express active ACC oxidase in the *E. coli* system.

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MATERIALS AND METHODS

Materials. Papaya (*Carica papaya* L. cv. Tainong 2) fruit with color-break appearance was harvested from a local papaya orchard. The fruit was stored frozen at −70 °C until used.

mRNA Preparation and cDNA Synthesis. Thirty grams of frozen papaya tissue mixed with 2 g of glass beads was ground to powder in a ceramic mortar with liquid nitrogen. The sample was dissolved in 80 mL of extraction buffer containing 57.4 g of guanidium−HCl, 0.4 g of sodium sarcosyl, 2 mL of 1 M sodium citrate (pH 7.0), and 560 μL of mercaptoethanol. Total RNA was prepared according to the guanidium−HCl procedure (Chirgwin et al., 1979). The poly(A)$^+$ RNAs were isolated according to oligo-(dT)cellulose chromatography. Double-strand blunted cDNAs were synthesized using a kit (cDNA synthesis module RPN1256) from Amersham (Little Chalfont, Buckinghamshire, England).

Subcloning and DNA Sequence Analysis. One microgram of blunted cDNAs was ligated with 30 pmol of Marathon cDNA adaptor (Clontech, Palo Alto, CA) at 22 °C for 4 h. AO-1 primer (5′ GCT TGT GAG AAC TGG GGT TT 3′) was synthesized according to the sequence of an apple ACC oxidase (Dong et al., 1992). Using 0.1 μg of the ligated cDNA as a templete, 10 pmol of Clontech adaptor primer and 10 pmol of AO-1 primer were added. One 0.9 kbp DNA (3′-RACE: 3′-cDNA end) was amplified by using the PCR technique. The 0.9 kbp fragment was subcloned into pGEM-T (Promega, Madison, WI) using JM109 as a host. Nucleotide sequence was determined in both directions by the dideoxy technique using Sequenase (United States Biochemical, Cleveland, OH). An AO-3 primer (5′ CCC TTG CTC GCC ATT ATT TCC 3′) was synthesized according to the determined 0.9 kbp DNA

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   1          AAAAACTTTATTTACAAACAAACCCAGAAAAAGTTTCGACGATTTCTTCCCGAGAAATTCTTTCTGCAGAG

  72  ATG GAG AAC TTC CCA GTC ATC AAC ATG GAG GGT CTC AAC GGT GAG GAG AGA GCT GCT ACC
   1   M   E   N   F   P   V   I   N   M   E   G   L   N   G   E   E   R   A   A   T

 132  ATG AAG AAG ATT GAA GAT GCT TGT GAG AAC TGG GGT TTC TTT GAG CTG GTG AAT CAT GGG
  21   M   K   K   I   E   D   A   C   E   N   W   G   F   F   E   L   V   N   H   G

 192  ATC CCA ATT GAG CTG CTG GAC ACT GTC GAA AGA TTG ACA AAA GGG CAC TAC AGA AAA TGC
  41   I   P   I   E   L   L   D   T   V   E   R   L   T   K   G   H   Y   R   K   C

 252  ATG GAG CAG AGA TTC AAG GAA ATA ATG GCG AGC AAG GGC TTA GAT GGT ATC CAA ACA GAG
  61   M   E   Q   R   F   K   E   I   M   A   S   K   G   L   D   G   I   Q   T   E

 312  GTC ACT GAT ATG GAC TGG GAA AGC ACC TTT TTC AAC TGC CAT CTC CCT GAG CCT AAC ATA
  81   V   T   D   M   D   W   E   S   T   F   F   N   C   H   L   P   E   P   N   I

 372  GCT GAG ATT CCA GAT CTC GAC GAT GAA TAC AGG AAA GTG ATG AAA GAA TTT GCT CTG AAA
 101   A   E   I   P   D   L   D   D   E   Y   R   K   V   M   K   E   F   A   L   K

 432  CTG GAG AAA ATA GCA GAG GAG CTT CTT GAT TTG TTA TGC GAG AAT CTC GGG CTG GAA AAA
 121   L   E   K   I   A   E   E   L   L   D   L   L   C   E   N   L   G   L   E   K

 492  GGG TAT TTG AAA AAA GCA TTT ACG TGG TCA AGA GGT CCA ACT TTC GGC ACC AAA GTC AGC
 141   G   Y   L   K   K   A   F   T   W   S   R   G   P   T   F   G   T   K   V   S

 552  AAC TAC CCT CCA TGC CCT AAA CCA AAC TTG ATC AAA GGG CTC CGG GCA CAC ACC GAC GCC
 161   N   Y   P   P   C   P   K   P   N   L   I   K   G   L   R   A   H   T   D   A

 612  GGC GGC ATC ATC TTG CTC TTC CAG GAC GAC AAA GTC AGC GGC CTC CAA CTC CTC AAA GAC
 181   G   G   I   I   L   L   F   Q   D   D   K   V   S   G   L   Q   L   L   K   D

 672  GGC AAA TGG GTT GAT GTT CCA CCA ATG CGC CAC TCC ATT GTC GTC AAC CTC GGC GAC CAA
 201   G   K   W   V   D   V   P   P   M   R   H   S   I   V   V   N   L   G   D   Q

 732  CTC GAG GTG ATT ACC AAC GGG AAA TAC AAG AGC GTG GAG CAC AGA GTG GTG GCA CAA ACC
 221   L   E   V   I   T   N   G   K   Y   K   S   V   E   H   R   V   V   A   Q   T

 792  GAC GGG ACG AGG ATG TCG ATA GCT TCT TTC TAC AAC CCC GGA AGC GAC GCG GTG ATT AGT
 241   D   G   T   R   M   S   I   A   S   F   Y   N   P   G   S   D   A   V   I   S

 852  CCC GCG GAA TTA TTG GTG GAG AAA GAA ACA GAG GAG AAG AAA ACA GCG TAC CCG AAA TTC
 261   P   A   E   L   L   V   E   K   E   T   E   E   K   K   T   A   Y   P   K   F

 912  GTG TTC GAG GAT TAC ATG AAG CTG TAT GCT GGG TTG AAG TTT CAG GCC AAG GAG CCG AGA
 281   V   F   E   D   Y   M   K   L   Y   A   G   L   K   F   Q   A   K   E   P   R

 972  TTT GAT GCC ATG AAA GCG GCC GCA AAA GCT TAAATCTTAAACCGGGGCGGGCATCAAATTCACTACTTG
 301   F   D   A   M   K   A   A   A   K   A   *

1041  GGCTTGATTTAGAAAAATATAGTTATAAAAAAGATGGAAGTGAAAGAGGAGATATTTAATTGGGTATTTTATGGTGAAG

1120  AATTAAGTAAAGAGTAAAAAGGATTGTTGGAGTGTGGGAAAACGGTGTCGTATATAGAGTTATTGTTTGTAGACAGGAA

1199  C
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Figure 1. Nucleotide sequence of papaya ACC oxidase cDNA and the deduced amino acid sequence. Numbers to the left refer to nucleotide and amino acid residues. Promoter sequence, consensus sequence of the translation start site, and polyadenylation signal are underlined. The asterisk denotes the stop signal.

Table 1. Comparison (Percent Identity, Percent Similarity) of Amino Acid Sequences for ACC Oxidase of Papaya Fruit and Other Plant Species

file name	EMBL no.	genus species	% identity	% similarity	English name	strain, cultivar, or variety name
papaya	L76283	*Carica papaya*	–	–	papaya	Tainong 2
petacola	L21976	*Petunia hybrida*	80.064	88.746		V/R
hnnacc	L29405	*Helianthus annuus*	79.545	87.338	sunflower	Dahlgren
actaccoxi	M97961	*Actinidia deliciosa*	78.778	88.746	kiwifruit	Hayward
maurrp	M81794	*Malus sylvestris*	76.452	86.774	apple	
vru06046	U06046	*Vigna radiata*	76.129	86.129	mung bean	
dinacca	L35152	*Dianthus caryophyllus*	72.903	83.226		red sim
boaccox2	X81629	*Brassica oleracea*	71.987	86.319	broccoli	
bnaaccox	L27664	*Brassica napus*	71.569	85.948		Bridger
boaccox1	X81628	*Brassica oleracea*	70.588	84.967	broccoli	
doraccoxid	L07912	*Doritaenopsis* sp.	72.848	82.781	orchid	Hausermann's Red Bird Cardinal
dorcaroxi	L37103	*Doritaenopsis* sp.	71.613	82.258	orchid	Hausermann's Red Bird

sequence. Using the blunted cDNA with adaptor as a template, one 0.3 kbp DNA (5′-RACE: 5′-cDNA end) was amplified by using PCR with adaptor primer and an AO-3 primer. The 0.3 kbp DNA was subcloned and sequenced. Sequence analysis revealed that 5′-RACE and 3′-RACE cover the full-length ACC oxidase cDNA (1.2 kbp) (Sanger et al., 1977). Using the 0.3 kbp DNA (5′ RACE) and the 0.9 kbp DNA (3′-RACE) as templates, one fused full-length cDNA of ACC oxidase was created by using the PCR technique (Accession no. L76283).

Recombinant DNA Preparation and Transformation. With 50 ng of ACC oxidase cDNA as a template, 10 pmol of each of 5′-primer and 3′-primer was added (5′-primer, 5′ CAT

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              papaya     MENFPVI NMEGL---NG EERAATMKKI EDACENWGFF ELVNHGIPIE LLDTVERLTK   54
            petaco1a     MEN..I. SLDKV---.. V......EM. K.........  ........R. VM....KM..   54
              hnnacc     MAN.... ...N.---.. S..GV..E.. N.........  ........HD ...K..KM..   54
           actaccoxi     MEA.... D..K.---.. ....P..E.. K.........  .......SH. .M........   54
              maurrp     MAT...V DLSLV---.. ......LE.. N.........  ......MST. ......KM..   54
            vru06046     MAN...V D.GK.---.T ...GTA.EM. K.........  .......S.. .M....K...   54
             dinacca  MANIVN..I. D..K.NNY.. V..SLVLDQ. K...H.....  QV...SLSH. .M.K...M..   60
            boaccox2  MEKNIK...V DLSK.---.. ...DQ..ALV D...Q.....  ..L.....YD .M.NI..M..   57
          bnaaccoxi   MEKNIK.... DLSK.---.. ...DQ..AL. D...Q.....  ..L.....YD .M.NI..M..   57
            boaccox1  MEKNIK...V DLSK.---I. ...DQ..AL. N.........  .I....L.HD .M.N..KM..   57
         doraccoxid               ..L.---Q. SQ.P.A.ALL R.......LY  ..L....SH. .MNR..TVN.   46
          dorcaroxi    MESGS.... ...L.---Q. SQ.P.A.ALL R.........  ..L....SH. .MNR..AVN.   56

              papaya  GHYRKCMEQR FKEIMASKGL DGIQT-EVTD MDWESTFFNC HLPEPNIAEI PDLDDEYRKV  113
            petaco1a  ...K......  ...LV...A. E.V.A-....  ........LK ...IS..S.V ....E...E.  113
              hnnacc  D..K......  ...MV.A.A. E.VKA-....  ........LR .R.TS..S.. ...V....EL  113
           actaccoxi  E..N......  .K.MV.T... EAV.S-.IN. L.......LR ...VS..S.. ...EQDH..A  113
              maurrp  D..K.T....  ...MV.A... .DV.S-.IH. L.......LR ...SS..S.. ...EE....T  113
            vru06046  E..K.T....  ...MV.N... ESV.S-.IN. L.......LR ...VS.VS.N T...QD...I  113
             dinacca  E..K.FR..K  ..DMVQT... VSAES-Q.N. I......YLR .R.TS..S.V .....Q...L  119
            boaccox2  E..K.F...K  ...MLR..N. .TLE.-..EN V.......LH ...QT.LYD. ..MS....AA  116
          bnaaccoxi   E..KQF..HK  ...MLR.... .TLE.-..E. I.......LH ...QT.LYD. .NMSEQ..TA  116
            boaccox1  E..KIS...K  .NDMLK.... ENLER-..E. V......YLR ...QS.LYD. ..MS....TA  116
         doraccoxid   E...RFR...  ...F-...T. .TVENV.PEN L.......LR ...TS..SQ. .....DC.ST  105
          dorcaroxi   E...RFR...  ...F-...T. .SVENVDPDN L.......LR ...TS..SQ. .....DC.AT  115

              papaya  MKEFALKLEK IAEELLDLLC ENLGLEKGYL KKAFTWSRG- -PTFGTKVSN YPPCPKPNLI  171
            petaco1a  .RD..KR...  L.........  ..........  .N..YG.K.- -.N.......  .......D..  171
              hnnacc  ..D..G....  L.........  ..........  ....YG.K.- -.N.......  .....T.D..  171
           actaccoxi  .....E....  L..Q......  ..V.......  ....YG.K.- -.........  .....R.E..  171
              maurrp  .....VE...  L..K......  ..........  ..V.YG.K.- -.N.......  .......D..  171
            vru06046  ..Q..EE...  L..H......  ..........  ..V.YG.K.- -.N.......  .....T.D..  171
             dinacca  .....AQI..  LS.Q......  ..........  .N..YGAN.- -.........  .......D..  177
            boaccox2  ..D.GKR..N  L.........  ..........  ..V.SGTK.- -.........  .......EM.  174
          bnaaccoxi   ..D.GKR...  L.........  ..........  ..V.RGTK.- -.........  .....N.EM.  174
            boaccox1  ..D.GKR..N  L..D......  ..........  ..V.HGTK.- -.........  ..A....EM.  174
         doraccoxid   ......E..N  L..R......  .D........  ..V.CGGSDG L.........  .......E..  165
          dorcaroxi   .....RE...  L..R......  .D........  .RV.CGGSDG L.........  .......D..  175

              papaya  KGLRAHTDAG GIILLFQDDK VSGLQLLKDG KWVDVPPMRH SIVVNLGDQL EVITNGKYKS  231
            petaco1a  ..........  ..........  ..........  Q.I.......  ..........  ..........  231
              hnnacc  ..........  ..........  ..........  E.I.......  ...I.....I  ..........  231
           actaccoxi  ..........  ........N.  ..........  E.I.....K.  ...I.I....  ..........  231
              maurrp  ......S...  ..........  ..........  E.......H.  ...I.....I  ..........  231
            vru06046  ..........  ..........  .........D  Q.I.......  ...I......  ..........  231
             dinacca  ..........  ..........  ..........  H.......K.  ..........  ..........  237
            boaccox2  ..........  .L........  ..........  V......LK.  ...I......  ..........  234
          bnaaccoxi   ..........  .L........  ..........  D......LK.  ...I......  ......W...  234
            boaccox1  ..........  ..........  ..........  D.I....LN.  ...I......  ......R...  234
         doraccoxid   ..........  ..........  ..........  E.I....V..  .....I....  ..........  225
          dorcaroxi   ..........  ..........  ..........  E.IE...L.Y  .....I....  ..........  235

              papaya  VEHRVVAQTD GTRMSIASFY NPGSDAVISP AELLVEKET- ---EEKKTAY PKFVFEDYMK  287
            petaco1a  .M...I..K. .A...L....  ........Y. .PA.....A- ---..N.QV. .....D....  287
              hnnacc  .M...I....  ..........  ...N....Y. .PT.L...P- ---T..EQS. .....D....  287
           actaccoxi  .M...I..P. .N........  .......MY. .PA..D..E- ---DQQ.QV. ..........  287
              maurrp  .M...I..S.  ..........  ...N.SF...  .PAVL..K-- ---T.DAPT. .....D....  286
            vru06046  .M...I....  .....L....  ...D......  .PA..-..S- ---D.TSQV. .....N....  286
             dinacca  .M...I....  .N........  ........Y.  .PT.....-- ---...CR..  .........N  292
            boaccox2  IM...MT.KE  .N........  ......E...  .PS....DS- ---D.----.  .S...D....  286
          bnaaccoxi   IM...MT.KE  .N........  ......E...  .HS..D..S- ----.----.  .S...D....  285
            boaccox1  .M....T.KE  .N........  ......E...  .SS.AC...- ----.----.  .S...D....  285
         doraccoxid   .L........  .N........  ........F.  .PA.....AE EKE.K..EI.  .....Q...N  285
          dorcaroxi   .L........  .N........  ........F.  .PA.....AE --E....EI.  .....Q...N  293

              papaya  LYAGLKFQAK EPRFDAMK-A AAKA                                          310
            petaco1a  ..........  ....E...-.  METDVKMDPI ATV                              319
              hnnacc  ..........  ....E...-S  S                                           307
           actaccoxi  ..........  ....E...-.  MEN.VNLGPI ATI                             319
              maurrp  ..S.......  ....E...-.  KESTPVATA                                 314
            vru06046  ..........  ....E...-.  V-SSVDVGAI ATV                             317
             dinacca  ..LK....E.  ....E...-.  METTGPIPTA                                321
            boaccox2  ....V...P.  ....E...NV  ..-TTDLNPV ATVETF                         321
          bnaaccoxi   ..S.V...P.  ....E...N.  ..VTTDVNPV ATEETF                         321
            boaccox1  ....V...P.  ....E...N.  N.V-TELNPT AAVETF                         320
         doraccoxid   ..IRK..E..  ....E...-S  MEIVMSSQPI PTA                           317
          dorcaroxi   ..IRK..E..  ....E...-S  MEIVMSSQPI PTA                           325
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Figure 2. Optimal alignment of ACC oxidases from several plant species. Papaya, this study; petacola, *Petunia hybrida* (Tang et al., 1993); hnnacc, sunflower (unpublished); actaccoxi, kiwifruit (MacDiarmid and Gardner, 1993); maurrp, apple (Dong et al., 1992); vru06046, mung bean (Kim and Yang, 1994); dinacca, *Dianthus caryophyllus* (unpublished); boaccox2, broccoli (Pogson et al., 1995); bnaaccox, *Brassica napus* (unpublished); boaccox1, broccoli (Pogson et al., 1995); doraccoxid, orchid (Nadeau et al., 1993). Numbers refer to amino acid residues of each species. A dot refers to identities with papaya. A dash denotes deletion. Amino acid residues that are conserved for enzymes requiring ferrous ion and ascorbate are indicated with circles.

GCC ATG GAG AAC TTC CCA GTC ATC AA 3′; 3′-primer, 5′ GGA ATT CCA AGC TTT TGC GGC CGC TTT CAT GG 3′). A 0.93 kbp DNA fragment amplified by the PCR technique was ligated with pGEM-T (Promega) and transformed into *E. coli* JM109 host. A positive clone was selected by hybridization with ^{32}P-labeled ACC oxidase cDNA as probe, and plasmid DNA was prepared; appropriate plasmid DNA was digested with *Nco*I and *Eco*RI and then run on 0.8% agarose. A 0.93 kbp insert DNA containing *Nco*I and *Eco*RI sites was recovered and ligated with pET-20b(+) (pretreated with *Nco*I and *Eco*RI) from Novagen (Madison, WI). The recombinant gene was transformed into *E. coli* BL21(DE3). Transformed clone was selected by hybridization with ^{32}P-labeled ACC oxidase cDNA as probe.

Culture and Enzyme Extraction. The transformed *E. coli* were grown at 37 °C in 5 mL of Luria Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 0.9. The culture temperature was reduced to 25 °C for 30 min, and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 25 °C for 5 h at 120 rpm, and then the bacterial cells were harvested by centrifugation at 800*g* for 15 min and washed with 10 mM Tris buffer (pH 7.5) containing 150 mM NaCl. The cell pellet was frozen at −20 °C until use. The bacterial cells were suspended in 0.6 mL of 250 mM MOPS buffer (pH 7.3) containing 5 mM DTT, 25% (v/v) glycerol, 250 μg/mL catalase, and 250 mM $NaHCO_3$ and then sonicated in an ice bath for 30 s of eight bursts at maximum output. The homogenate was centrifuged at 15000*g* for 15 min. The supernatant fraction contained active ACC oxidase.

Enzyme Assay by the Quantitation of Ethylene Production. ACC oxidase activity was assayed according to a modified method (Dong et al., 1992b). The standard reaction mixture (1 mL) contained 100 mM MOPS (pH 7.3), 10 μM $FeSO_4$, 20 mM sodium ascorbate, 2 mM DTT, 10% (v/v) glycerol, 250 μg/mL catalase (bovine heart), 100 mM $NaHCO_3$, and 1 mM ACC in a sealed 15 mL test tube. The reaction was initiated by the addition of 450 μL of enzyme preparation. After a 10 min incubation with shaking at 30 °C, a 1 mL gas sample was withdrawn with a syringe from the headspace of the tube for quantitation of ethylene production by gas chromatography (Hitachi G-3000).

Western Blot Immunoanalysis. The bacterial cells were suspended in 45 μL of 10 mM Tris buffer (pH 6.8); 50 mg of glass beads was added, and the mixture was vortexed for 5 min. Then 45 μL of 2X SDS−PAGE sample buffer [120 mM Tris buffer (pH 6.8), 10% glycerol, 0.8% SDS, 5.76 mM 2-mercaptoethanol, 0.04% bromophenol blue] was added and heated for 5 min before centrifugation at 12000*g* for 10 min. After centrifugation, 10 μL of supernatant was electrophoresed on 15% SDS−PAGE (Laemmli, 1970) and analyzed by western blot immunoanalysis (Bollag and Edelstein, 1991). The primary antibody provided by Dr. Yee-Yung Charng (Institute of Botany, Academia Sinica, Taiwan) was rabbit antisera prepared against the apple ACC oxidase, and the secondary antibody was goat anti-rabbit IgG horseradish peroxidase (IgG-HRP) from Cappel (Durhan, NC).

RESULTS AND DISCUSSION

Figure 1 shows the nucleotide and deduced amino acid sequences of one cDNA clone. Sequence analysis found that the cDNA was full length, comprising a complete open reading frame coding for 310 amino acid residues. There is no transit peptide, which suggests the enzyme is a cytosolic enzyme as reported in other enzymes. The DNA sequence translation start site (AGAGATGG) matches the consensus sequence (AACAATGG) reported for this region in plants (Lücke et al., 1987). There is a strong promoter sequence TTTACA at position 16.

Table 1 shows high identity (72−80%) with the amino acid sequences of ACC oxidase from other plant species. This was done by the program of the University of Wisconsin Genetics Computer Group.

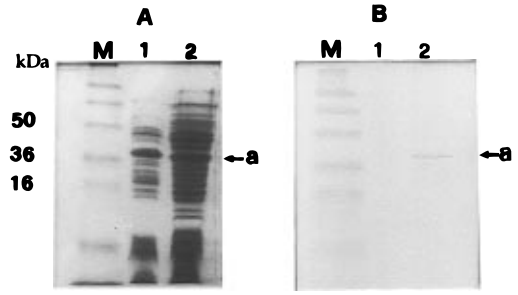

Figure 3. Coomassie blue stained SDS−PAGE and western blot immunoanalysis of the crude extract of recombinant papaya ACC oxidase: (A) staining for protein with Coomassie blue; (B) western blot immunoanalysis; (M) protein molecular weight markers; (lane 1) BL21(DE3) carrying pET-20b(+) as control; (lane 2) BL21(DE3) carrying recombinant papaya ACC oxidase cDNA; ←a denotes 38 kDa ACC oxidase protein.

Figure 2 shows that 12 residues (P-5, A-27, G-32, H-39, H-177, D-179, L-195, Q-196, G-218, H-234, R-244, and S-246 in this study) are conserved among all of the ferrous ion and ascorbate requiring superfamily of enzymes (Tang et al., 1993). ACC oxidase requires ferrous ion and ascorbate for catalytic activity (Dong et al., 1992b). H-177, D-179, and H-234 are the putative amino acid residues involved in chelating ferrous ion from comparison with the structure of isopenicillin *N* synthase, a ferrous ion dependent oxidase that has been analyzed to 2.5 Å resolution by X-ray crystallography (Matsuda et al., 1991; Roach et al., 1995).

ACC oxidase activity assay revealed that 0.45 mL of crude enzyme extract from 5 mL of culture produced 42 ppm of ethylene in 10 min under the assay condition.

Figure 3 shows the 38 kDa ACC oxidase protein was detected as the distinctive protein by Coomassie blue staining of SDS−PAGE (panel A, ←a denotes ACC oxidase protein), and western immunoanalysis (panel B, ←a denotes ACC oxidase protein) confirmed the result of Coomassie blue staining.

CONCLUSION

A full-length cDNA clone encoding a putative ACC oxidase from papaya was amplified by PCR. This clone comprised a complete open reading frame coding for 310 amino acid residues. The coding region was introduced into an expression vector, pET-20b(+), and transformed into *E. coli* BL21(DE3). Gene product was confirmed by its enzyme activity to produce ethylene and by western immunoanalysis.

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LITERATURE CITED

Bollag, D. M.; Edelstein, S. J. Immunoblotting. In *Protein Methods*; Wiley-Liss: New York, 1991; pp 181−208.

Brady, C. J.; Speirs, J. Ethylene in fruit ontogeny and abscission. In *The Plant Hormone Ethylene*; Mattoo, A. K., Suttle, J. C., Eds.; CRC Press: Boca Raton, FL, 1991; pp 235−258.

Chirgwin, J. M.; Przybyla, A. E.; MacDonald, R. J.; Rutter, W. J. Isolation of biologically active nucleic acid from sources enriched in ribonuclese. *Biochemistry* **1979**, *18*, 5294−5299.

Dong, J. G.; Olsen, D. B.; Silverstone, A.; Yang, S. F. Sequence of a cDNA coding for a 1-aminocyclopropane-1-carboxylate oxidase homology from apple fruit. *Plant Physiol.* **1992a**, *98*, 1530−1531.

Dong, J. G.; Fernandez-Maculet, J. C.; Yang, S. F. Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from apple fruit. *Proc. Natl. Acad. Sci. U.S.A.* **1992b**, *89*, 9789−9793.

Kim, W. T.; Yang, S. F. Structure and expression of 1-aminocyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. *Planta* **1994**, *194*, 223−229.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lütcke, H. A.; Chow, K. C.; Mickel, F. S.; Moss, K. A.; Kern, H. F.; Scheele, G. A. Selection of AUG initiation codons differs in plants and animals. *EMBO J.* **1987**, *6*, 43−48.

MacDiarmid, C. M.; Gardner, R. C. A cDNA sequence from kiwifruit homologous to 1-aminocyclopropane-1-carboxylic acid oxidase. *Plant Physiol.* **1993**, *101*, 691−692.

Matsuda, J.; Okabe, S.; Hashimoto, T.; Yamada, Y. Molecular cloning of hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, from cultured roots of *Hyoscyamus niger*. *J. Biol. Chem.* **1991**, *266*, 9460−9464.

Nadeau, J. A.; Zhang, X. S.; Nair, H.; O'Neill, S. D. Temporal and spatial regulation of 1-aminocyclopropane-1-carboxylate oxidase in the pollination-induced senescence of orchid flowers. *Plant Physiol.* **1993**, *103*, 31−39.

Pogson, B. J.; Downs, C. G.; Davies, K. M. Differential expression of two 1-aminocyclopropane-1-carboxylic acid oxide genes in broccoli after harvest. *Plant Physiol.* **1995**, *108*, 651−657.

Roach, P. L.; Clifton, I. J.; Fülöp, V.; Harlos, K.; Barton, G. J.; Hajdu, J.; Andersson, I.; Schofield, C. J.; Baldwin, J. E. Crystal structure of isopenicillin *N* synthase is the first from a new structural family of enzymes. *Nature* **1995**, *375*, 700−704.

Sanger, F.; Nicklen, S.; Coulson, A. R. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463−5467.

Tang, X.; Wang, H.; Brandt, A. S.; Woodson, W. R. Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *petunia hybrida*. *Plant Mol. Biol.* **1993**, *23*, 1151−1164.

Yang, S. F.; Dong, J. G. Recent progress in research of ethylene biosynthesis. *Bot. Bull. Acad. Sin.* **1993**, *34*, 89−101.

Yang, S. F.; Hoffman, N. E. Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* **1984**, *35*, 155−189.

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