Characterization of ACC Oxidase from the Leaves of *Malus domestica* Borkh. (Apple)

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ABSTRACT

The expression, accumulation and kinetic properties of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO), the enzyme which catalyses the final step in the ACC-dependent pathway of ethylene biosynthesis in plants, is examined.

The investigation is divided into three sections: (i) identification of two ACO genes in apple leaf tissue, designated *MD-ACO2* and *MD-ACO3*, (ii) kinetic analyses of each of the three isoforms of ACO in apple (MD-ACO1, MD-ACO2 and MD-ACO3) expressed in *E. coli*, and (iii) temporal and developmental expression *in vivo* of each of the ACO genes and accumulation of the corresponding gene products in leaf and fruit tissue.

The coding regions of putative ACO gene transcripts were generated from leaf tissue using RT-PCR. Sequence alignments and interrogation of the expressed sequence tags (ESTs) database indicated that the entire open reading frame (ORF) sequences were encoded by two distinct genes, and these are designated *MD-ACO2* and *MD-ACO3*. A third *ACO* gene had been identified in apple by other research workers previously, and designated *MD-ACO1*. Differences are obvious in the number of base-pairs (bp) constituting the entire ORF of *MD-ACO1* (942 bp), *MD-ACO2* (990 bp) and *MD-ACO3* (966 bp). *MD-ACO1* and *MD-ACO2* share a close nucleotide sequence identity of 93.9 % in the ORF but diverge in the 3′ untranslated regions (3′-UTR) (69.5 %). In contrast, *MD-ACO3* shares a lower sequence identity with both *MD-ACO1* (78.5 %) and *MD-ACO2* (77.8 %) in the ORF, and in the 3′-UTR (*MD-ACO1*, 68.4 %; *MD-ACO2*, 71 %). A comparison of the gene structures show that the endonuclease restriction sites are unique to each individual *MD-ACO* sequence. Genomic Southern analysis, using probes spanning the 3′-UTR and the 3′-end of the coding region confirmed that *MD-ACO3* is encoded by a distinct gene. However, while the distinction between *MD-ACO1* and *MD-ACO2* is not as definitive, different gene expression patterns adds credence to their distinctiveness. Each of the three deduced amino acid sequences contain all of the residues hitherto reported to be necessary for maximal ACO activity.

Expression of *MD-ACO1*, *MD-ACO2* and *MD-ACO3* as fusion proteins in *E. coli* was induced using isopropyl-β-$_D$-thiogalactopyranoside (IPTG), the recombinant proteins purified by nickel-nitrilotriacetic acid (NiNTA) affinity chromatography and the products had predicted masses determined from the nucleotide sequences, including the His-tag of 38.53 kDa (MD-ACO1), 40.39 kDa (MD-ACO2) and 39.3 kDa (MD-ACO3). Polyclonal antibodies were raised against the *MD-ACO3* fusion in rabbit for western blot analysis. Antibodies had been raised previously

against recombinant MD-ACO1, and while it was considered likely the MD-ACO2 would be recognized by the MD-ACO1 antibodies, MD-ACO2 does not appear to be recognized *in vivo* by the antibody.

Analyses of the kinetic properties of the three apple ACOs was undertaken. Apparent Michaelis constants (K_m) of 89.39 μM (MD-ACO1), 401.03 μM (MD-ACO2) and 244.5 μM (MD-ACO3) have been determined which suggests differences in the affinity of each enzyme for the substrate ACC. Maximum velocity (V_{max}) was determined for MD-ACO1 (15.15 nmol), MD-ACO2 (12.94 nmol) and MD-ACO3 (18.94 nmol). The catalytic constant (K_{cat}) was determined for MD-ACO1 (6.6×10^{-2}), MD-ACO2 (3.44×10^{-2}) and for MD-ACO3 (9.14×10^{-2}), with K_{cat}/K_m ($\mu M\ s^{-1}$) values of 7.38×10^{-4} $\mu M\ s^{-1}$ (MD-ACO1), 0.86×10^{-4} $M\ s^{-1}$ (MD-ACO2) and 3.8×10^{-4} $\mu M\ s^{-1}$ (MD-ACO3). The optimal pH for MD-ACO1 was 7.0 - 7.5, MD-ACO2 7.5 - 8.0 and MD-ACO3 7.0 - 8.0. All three isoforms exhibited absolute requirements for the co-substrate ascorbate *in vitro* with optimal activity at 30 mM. Similarly, ferrous iron ($FeSO_4.7H_20$; of 15 – 25 μM) and sodium bicarbonate ($NaHCO_3$; of 30 mM) were required for optimal activity, and were the same for all isoforms. No significant difference in thermostability was found in this study between the MD-ACO isoforms at the P = 0.05 level. However, the activities of the enzyme differed significantly between temperatures over time.

In vivo expression of each of the ACO genes in leaf tissue determined using RT-PCR and cDNA Southern analysis reveal that both *MD-ACO2* and *MD-ACO3* are expressed in young leaf tissue and in mature leaf tissue. While *MD-ACO3* is expressed predominantly in young leaf tissue, *MD-ACO2* (in common with *MD-ACO1*) is expressed predominantly in mature fruit tissue. None of the *MD-ACO*s were observed to be senescence associated genes (SAG). MD-ACO3 protein accumulated predominantly in young leaf tissue and less intensely in both mature leaf tissue and young fruit tissue, while MD-ACO1 accumulated only in mature fruit tissue. For the developmental studies, samples were collected at approximately 11 am in this study. *MD-ACO2* and *MD-ACO3* were also expressed in leaf tissue collected over a 24 h period in the spring and also in the autumn. For both genes transcripts accumulated in the presence of fruit but tended to disappear in the absence of fruit.

These results show that *MD-ACO1*, *MD-ACO2* and *MD-ACO3* are differentially expressed, and that MD-ACO3 is encoded by a gene distinct from MD-ACO1 and MD-ACO2. MD-ACO1 and MD-ACO2 are either allelic forms of the same gene or closely clustered. Although there is some variation in kinetic properties which may reflect different physiological environments, they do not vary greatly.

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LIST OF ABBREVIATIONS

A_{595}	Absorbance at 595 λ nm
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC Oxidase
ACS	ACC Synthase
AEC	1-amino-2-ethyl-cyclopropane-1-carboxylic acid
AdoMet	S-adenosyl-L-methionine
AEC	1-amino-2-ethyl-cyclopropane-1-carboxylic acid
Amp^{100}	Ampicillin (100 mg mL^{-1})
amu	Atomic mass unit
ANS	Anthocyanidin synthase
APS	Ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BLAST	Basic Logical Alignment Search Tool
Borax	di-Sodium tetraborate decahydrate
bp	Base-pair
BSA	Bovine serum albumin
°C	Degrees Celsius
ca.	*circa* (approximately)
CAPS	3-(Cyclohexylamino)propanesulfonic acid
CBB	Coomassie Brilliant Blue
cv.	Cultivar
Ci	1 Curie = 3.7×10^{10} disintegrations per second
CTAB	Hexadecyltrimethylammonium bromide
DAFB	Days after full bloom
cDNA	DNA complimentary to a RNA, synthesized from RNA by reverse transcription *in vitro*
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DAOCS	Deacetoxycephalosporin C synthase
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DMF	N,N-dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
DSBH	Double stranded beta helix (jellyroll)
dNTP	2'-deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
DW	Dry weight
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra acetic acid
EFE	Ethylene forming enzyme
EIN	Ethylene insensitive
EST	Expressed Sequenced Tag
ExPASy	Expert protein analysis system: proteomics server of the Swiss Institute of Bioinformatics (SIB)
FID	Flame ionization detector
FPLC	Fast protein liquid chromatography
FW	Fresh weight
GACC	1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid
g	Acceleration due to gravity (9.8 m/s^2)
g	Gram
GC	Gas Chromatography
GCG	Gene Computer Group
GST	Glutathione S-Transferase
GUS	*E. coli* β-glucuronidase
h	Hour
His-tag	Histidine tagged
IgG	Immunoglobulin G
IEC	Internal ethylene concentration
IPTG	Isopropyl-β-$_D$-thiogalactopyranoside ($C_9H_{18}O_5S$)
IPNS	Isopenicillin N synthase
Kb	Kilo base-pairs
kDa	Kilo Daltons
K_i	Inhibition constant
K_m	The Michaelis constant (substrate needed to occupy 50 % of the active sites)
L	Litre
LB	Luria-Bertani (media or broth)
Log	Logarithm
M	Molar (moles per litre)

MACC	1-(malonylamino) cyclopropane-1-carboxylic acid
MCS	Multiple cloning site
MD-ACO	*Malus domestic* ACC Oxidase
mol	mole (amount of a substance, Avogadro's number)
μmol	Micromole
mg	Milligram
μg	Microgram
mL	Millilitre
μL	Microlitre
Milli-Q water	Water purified by a Milli-Q ion exchange column
min	Minute
MOPS	Sodium [3-(*N*-Morpholino)]propanesulphonate
Mr	Relative molecular mass (g mol^{-1})
n	Number of replicate
NBT	*p*-nitro blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
ng	Nanograms
Ni-NTA	Nickel-nitrilotriacetic acid
nL	Nanolitres
nmol	Nanomole
2-ODD	2-oxogluturate dependent dioxygenase
OD_{595}	Optical Density at 595 λ nm
ORF	Open reading frame
PA	1,10-phenanthroline
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
PBS-T	Phosphate buffered saline containing 0.5 % (v/v) Tween-20
PCR	Polymerase chain reaction
Pers. Comm.	Personal communication
pH	-Log (H^+)
pI	Isoelectric point
pmol	Picomole
pn	Net photosynthesis (carbon assimilation measurements)
ppm	Parts per million
PVDF	Polyvinylidine difluoride
PVP-40	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone

3´RACE	Three prime-rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
RO	Reverse osmosis
RT-PCR	Reverse Transcriptase-polymerase chain reaction
s	Second
SAM	S-adenosyl-$_L$-methionine
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSPE	Saline, sodium phosphate, and EDTA buffer
TEMED	*N*, *N*, *N*', *N*'-tetramethylethylenediamine
T_m	Melting temperature (in molecular biology: the temperature at which DNA strands separate in preparation for annealing)
TEV	Tobacco etch virus
Tris	Tris (hydroxymethyl) aminomethane
Triton X-100	Octylphenoxy polyethoxyethanol
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Unit (commercial enzymes are in U μL^{-1}, where unit is based on enzyme activity)
UTR	Untranslated region
UV	Ultra violet light
V	Volt (m^2 kg $s^{-3}A^{-1}$)
V_{max}	Maximum velocity
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X-Gal	5-Bromo-4-chloro-3-indolyl β-$_D$-galactopyranoside

AMINO ACID ABBREVIATIONS

Amino Acid (AA)	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V