Characterisation of ACC Oxidase Isoforms during Leaf Maturation and Senescence in White Clover (*Trifolium repens* L.)

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Abstract

One-aminocyclopropane-1-carboxylic acid (ACC) oxidase, the enzyme which catalyses the final step in the ACC-dependent pathway of ethylene biosynthesis in plants, has been studied during leaf maturation and senescence in white clover (*Trifolium repens* L.).

The coding regions from two white clover ACC oxidase genes, designated TR-ACO2 (expressed in mature green leaves) and TR-ACO3 (expressed in senescent leaves), have been expressed in *E. coli* as fusion proteins. The expression of the two proteins has been optimised in terms of induction time with isopropyl-β-D-thiogalactopyranoside (IPTG) and IPTG concentration. The solubility of the fusion proteins was low but lysis buffer containing 0.5 % (w/v) SDS or 0.5 % (v/v) Triton X-100 produced a higher protein yield. The recombinant TR-ACO2 and TR-ACO3 proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and had an apparent molecular mass of 38 kDa. Enzyme activities of the purified TR-ACO2 and TR-ACO3 fusion proteins were 0.34 and 0.23 nmol ethylene/h/mg protein, respectively.

Activity *in vitro* of ACC oxidase, extracted from both mature green and senescent leaf tissues, was observed to be very labile at 20^0C with lower temperature, ascorbate and 1,10-phenanthroline (PA) required to help stabilise the enzyme activity *in vitro* during enzyme extraction and purification.

Three isoforms of ACC oxidase, one from mature green leaves, designated MGI and two from senescent leaves, designated SEI and SEII, have been identified. Two of the three isoforms (MGI and SEII) were purified to homogeneity as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie Brilliant Blue staining and western analysis. The purified isoforms MGI and SEII had specific enzyme activities of 25.2 and 29.8 nmol ethylene/h/ mg protein at pH 7.5 with approximately 100- and 144-fold purification, respectively. During

purification, both isoforms were recognised by an antibody raised against the protein product of TR-ACO2 expressed in *E. coli.*

The native molecular mass of the purified isoforms MGI and SEII was determined to be 37.5 kDa by size exclusion chromatography and molecular masses of MGI and SEII were observed to be 37 kDa and 35 kDa, respectively by SDS-PAGE analysis. The data indicate that both isoforms are active as monomers. Both isoforms were found to be neutral or near neutral proteins with apparent isoelectric points (pIs) of 7.36 for isoform MGI and 7.0 for SEII determined by chromatofocusing. The optimal pHs for MGI and SEII were 7.5 and 8.5, respectively.

The two isoforms also displayed differences in apparent K_m and V_{max} values for the substrate ACC. The K_m values for MGI and SEII were determined to be 39.7 μM and 110.0 μM, respectively. SEII had a higher V_{max} value for ACC than MGI. The data indicate that MGI displays a higher affinity for ACC, SEII requires a higher ACC concentration to achieve the higher enzyme activity and can operate in an enviroment with higher levels of ACC. In addition, both isoforms exhibited absolute requirements for the co-substrate ascorbate and the cofactors bicarbonate and ferrous iron for maximal enzyme activity *in vitro* with different optimal concentrations for ascorbate and ferrous iron. The data suggest that the two ACC oxidase isoforms are differentially regulated by pH and ACC concentration and are activated by different levels of cofactors. The significant differences between the two isoforms (pH optimum and K_m for ACC) may reflect the distinct physiological status of the leaf tissue in which each isoform is active.

These results show that now widely observed transcriptional regulation of the ACC oxidase gene family is also expressed in terms of differential regulation of isoforms of this enzyme in higher plants.

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$A_{280/595\ nm}$	Absorbance at 280/595 nm
ACC	1-aminocyclopropane-1-carboxylic acid
AdoMet	S-adenosyl-L-methionine
AEC	1-amino-2-ethyl-cyclopropane-1-carboxylic acid
Amp^{100}	Ampicillin (100 mg/ml)
APS	Ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
^0C	Degrees celsius
ca.	Approximately
CNBr	Cyanogen bromide
DEA	Diethanolamine
DEAE	Diethylaminoethyl
DMF	N, N-dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene forming enzyme
ELISA	Enzyme-linked immunosorbent assay
EMS	Electrospray mass spectrometry
FPLC	Fast protein liquid chromatography
FW	Fresh weight
g	Gram or acceleration due to gravity (9.8 m/s^2)
h	Hour
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid

HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IEF	Isoelectric focusing
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilodaltons
K_m	Substrate concentration at half maximum reaction rate
L	Litre
Log	Logarithm
LB	Luria-Bertani (media or broth)
M	Molar, moles per litre
MCP	1-methylcyclopropene
Mes	2-(N-morpholino)ethanesulphonic acid
mg	milligram
µg	Microgram
µl	Microlitre
Milli-Q water	Water purified by a Milli-Q ion exchange column
min	Minute
ml	Millilitre
MOPS	3-[N-Morpholino]propanesulphonate
Mr	Relative molecular mass (g/mol)
n	number of replicates
Ni-NTA	Nickel-nitrilotriacetic acid
nmol	Nanomole
nl	Nanolitre
PA	1,10-phenanthroline
PAGE	Polyacrylamide gel electrophoresis
PBSalt	Phosphate buffered saline (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pers. comm.	Personal communication

pH	- Log $[H^+]$
pI	Isoelectric point
PMSF	Phenylmethanesulphonyl fluoride
ppm	Part per million
PVDF	Polyvinylidine difluoride
PVPP	Polyvinyl polypyrrolidone
RACE-PCR	Rapid amplification of cDNA ends-polymerase chain reaction
RO	Reverse osmosis
RPA	Ribonuclease protection assay
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAM	S-adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
TFA	Trifluoracetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
Tricine	N-Tris(hydroxymethyl) methylycine
Tris	Tris(hydroxymethy)aminomethane
Triton X-100	Octylphenoxy polyethoxyethanol
Tween-20	Polyoxyethylenesorbitan monolaurate
UTR	Untranslated region
UV	Ultra violet light
V	Volt (m^2 kg/s^3/A)
V_e	Elution volume
v/v	Volume per volume
V_{max}	Maximum rate of reaction
V_o	Initial (steady-state) reaction velocity or void volume
w/v	Weight per volume
w/w	Weight per weight

Amino Acid Abbreviations

Amino Acid	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