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

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Deming Gong

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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°C 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 environment 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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List of Abbreviations

$A_{280/595 \text{ nm}}$	Absorbance at 280/595 nm
ACC	l-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
⁰ C	Degrees celsius
ca.	Approximately
CNBr	Cyanogen bromide
DEA	Diethanolamine
DEAE	Diethylaminoethyl
DMF	N, N-dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
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
μ g	Microgram
μ l	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
<i>pers. comm.</i>	Personal communication

pH	- Log [H ⁺]
pI	Isoelectric point
PMSF	Phenylmethanesulphonyl fluoride
ppm	Part per million
PVDF	Polyvinylidene 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	Trifluoroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
Tricine	N-Tris(hydroxymethyl) methylycine
Tris	Tris(hydroxymethyl)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

1. Chapter One: Introduction

Overview

The gaseous phytohormone ethylene is considered to play an important regulatory role in many aspects of plant development and in response to numerous biotic and abiotic stimuli. In higher plants, ethylene is synthesised through an ACC-dependent pathway, which has recently been characterised with two committed enzymes in the pathway, ACC synthase and ACC oxidase. ACC oxidase, which catalyses the aerobic oxidation of ACC to ethylene, has been shown to be encoded by a small multigene family, and the differential expression of this gene family has recently been characterised in several plant species.

In the first section of this thesis, two white clover ACC oxidase genes, TR-ACO2 and TR-ACO3, have been heterologously expressed in *E. coli*, the recombinant proteins purified and assayed for ACC oxidase activity. In the second section, the identification, purification to homogeneity and detailed comparative characterisation of two different isoforms of ACC oxidase from white clover is reported. The aim of this section is to obtain biochemical evidence for the expression and regulation of two isoforms of ACC oxidase during leaf maturation and senescence in white clover. These observations are interpreted in terms of whether the regulation of ACC oxidase gene expression during leaf maturation and senescence in white clover is reflected by significant differences in the regulation of specific ACC oxidase isoforms expressed during these leaf developmental stages.

1.1 Introduction to the Ethylene Biosynthetic Pathway

Ethylene, one of the five 'classical' plant hormones (Kende and Zeevart, 1997), is produced in higher plants from the conversion of methionine to S-adenosyl-L-methionine (SAM or AdoMet) and then ACC, the immediate precursor of ethylene biosynthesis (Adams and Yang, 1979). The enzymes involved in this ACC-dependent pathway comprise SAM synthetase (EC 2.5.1.6), ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.4.3) (Figure 1.1). The two committed steps in ethylene biosynthesis are proposed to be catalysed by ACC synthase and ACC oxidase (Poneleit and Dilley, 1993; Fluhr and Mattoo, 1996; Yang and Oetiker, 1998). Besides being oxidised

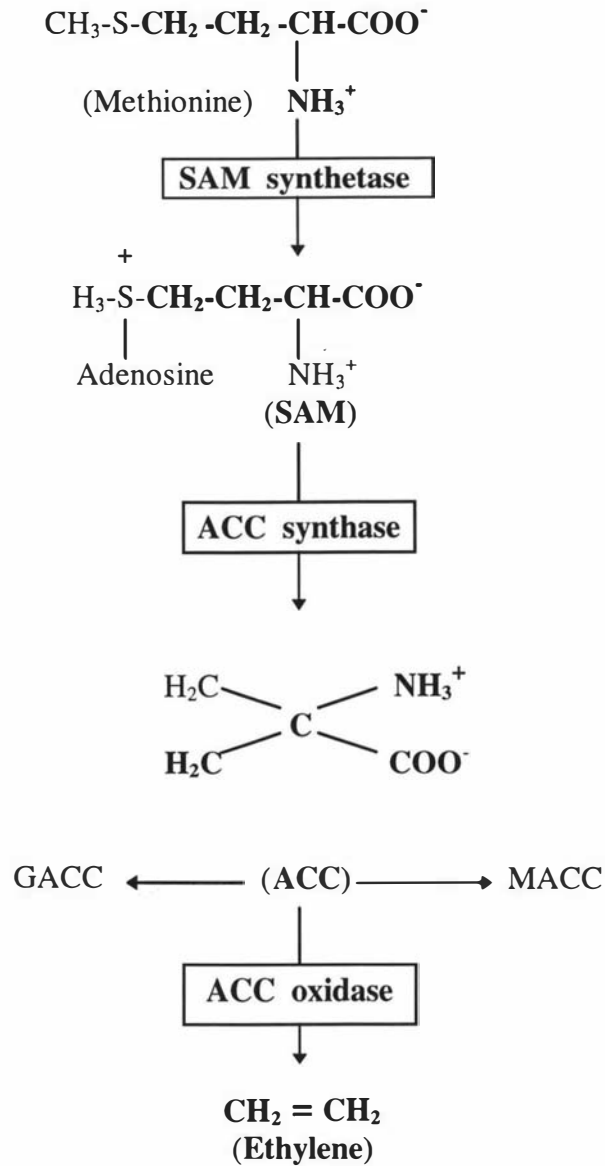


Figure 1.1 Ethylene Biosynthetic Pathway in Higher Plants.
 (modified from Kende, 1993)

GACC, 1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid

MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid

to ethylene, ACC can be irreversibly conjugated to form 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) or 1-(gamma-L-glutamylamino)cyclopropane-1-carboxylic acid (GACC).

1.1.1 SAM Synthetase

The first enzyme in the pathway, SAM synthetase, catalyses methionine to provide SAM. In plant cells, SAM is not only used for ethylene production but also for the biosynthesis of polyamines and transmethylation of proteins, nucleic acids, lipids and carbohydrates (Tabor and Tabor, 1984). Due to the variety of reactions in which SAM synthetase participates, it is not considered a committed enzyme in the pathway although it has been suggested to play an important role in replenishing SAM during the ethylene climacteric in fruit tissues (Whittaker *et al.*, 1997). In a few plants examined so far, SAM synthetase is present in multiple forms and encoded by a small multigene family under developmental and environmental controls (Schroder *et al.*, 1997). Furthermore, the differential expression of these genes has been demonstrated during senescence in carnation flowers (Woodson *et al.*, 1992), fruit ripening in *Actindia chinensis* (Whittaker *et al.*, 1997), in response to treatment of pea ovaries with indole-3-acetic acid (IAA) (Gomez-Gomez and Carrasco, 1998), wounding in tomato fruit tissue (Lincoln *et al.*, 1993) and in salt stressed tomato seedlings (Spartero *et al.*, 1994). Further elucidation of the factors that regulate the expression of these genes may yield mechanisms by which this enzyme does control ethylene biosynthesis.

1.1.2 ACC Synthase

ACC synthase (AdoMet methylthioadenosine-lyase) is a pyridoxal-5'-phosphate-requiring enzyme which catalyses the conversion of the substrate SAM to ACC (Adams and Yang, 1979). ACC synthase is widely considered to be a rate-determining enzyme in the pathway (Kende, 1993; Yang and Dong, 1993). This proposition was initially based on the observation that application of ACC to a variety of plant organs increased ethylene production (Hoffman and Yang, 1980). The level of endogenous ACC was highly correlated to the amount of ethylene produced in plant organs at certain developmental stages, such as in fruit ripening and in response to stress (Yang and

Hoffman, 1984). Additionally, application of aminoethoxyvinylglycine (AVG), an ACC synthase inhibitor, reduced both the level of endogenous ACC and concomitant ethylene production (Yang and Hoffman, 1984). The enzyme is present in cytosolic fractions of plant tissues (Imaseki, 1999) and was partially purified before any ACC synthase genes were cloned (Kende, 1993). However, progress with purification was slow because of the very low abundance and instability of the enzyme (Yang and Oetiker, 1998). Since the first partial purification and characterisation of this enzyme from mesocarp of cucumber fruit (Nakajima and Imaseki, 1986) and wounded ripe tomato fruit (Bleecker *et al.*, 1986), it has been partially purified and characterised from fruits of mung bean (Tsai *et al.*, 1988), tomato (Van Der Straeten *et al.*, 1989), apple (Yip *et al.*, 1991) and zucchini (Sato *et al.*, 1991).

Native ACC synthases have been found to be active as monomers in fruits of both wound-induced tomato (Bleecker *et al.*, 1986) and apple (Yip *et al.*, 1991) or as a homodimer in zucchini fruit (Sato *et al.*, 1991). The molecular mass for the monomer was determined to be 48 kDa in apple fruit (Yip *et al.*, 1991) and the subunits of the homodimer from zucchini fruit as 53 kDa (Sato *et al.*, 1991). In many plants, ACC synthase exists as isoenzymes (Imaseki, 1999). Three ACC synthase isoenzymes from wounded tomato fruit had pI values of 5.3, 7 and 9 (Mehta *et al.*, 1988), higher than the pI (5.0) of a partially purified enzyme from zucchini fruit (Sato *et al.*, 1991). The optimal pH of ACC synthase was determined to be pH 8.0 for an enzyme partially purified from mung bean fruit (Tsai *et al.*, 1988; 1991) and pH 9.5 for an enzyme from zucchini fruit (Sato *et al.*, 1991). The enzyme displays a high substrate specificity for AdoMet and affinity to the substrate is high with apparent K_m values for AdoMet ranging from 17 μ M for the partially purified enzyme from cucumber fruit (Sato *et al.*, 1991) to 60 μ M for the partially purified enzyme from zucchini fruit (Nakajima and Imaseki, 1986). ACC synthase is an extremely labile enzyme and the lability is considered to be due to the catalytic-based inactivation of the enzyme by its substrate (Casas *et al.*, 1993). In terms of abundance, the level of ACC synthase protein was estimated to be less than 0.0001% of total soluble protein in ripening tomato pericarp (Bleecker *et al.*, 1986).

Highly divergent ACC synthase gene families and their differential expression patterns have now been demonstrated in several plant species. Different members of the gene families have been cloned from tomato (nine; Vogel *et al.*, 1998), mung bean (seven; Yu *et al.*, 1998), *Arabidopsis thaliana* (five; Liang *et al.*, 1992), potato (three; Destefano-Beltran *et al.*, 1995), cucumber (three; Mathooko *et al.*, 1998; Shiomi *et al.*, 1998), carnation (three; Park *et al.*, 1992; Jones and Woodson, 1999) and pea (two; Peck and Kende, 1995). In *Arabidopsis thaliana*, at least one ACC synthase has been identified as a pseudogene (Liang *et al.*, 1992). The individual members of ACC synthase have been found to be differentially expressed during ripening of tomato fruit (Nakatsuka, *et al.*, 1997) and natural and pollination-induced senescence of carnation flowers (ten Have and Woltering, 1997; Jones and Woodson, 1999). The differential expression has also been found in response to IAA and ethylene treatment of carnation flowers (Jones and Woodson, 1999), wounding in zucchini (Nakijima *et al.*, 1990) and tomato (Yang and Oeticker, 1998), IAA treatment of *Arabidopsis thaliana* (Liang *et al.*, 1992) and mung bean (Kim *et al.*, 1997; Yu *et al.*, 1998), differences in photoperiod and low temperature in *Stellaria longipes* (Kathiresan *et al.*, 1998) and high level carbon dioxide stress in cucumber fruit (Mathooko *et al.*, 1998). In addition, distinct expression of the ACC synthase genes has also been reported in response to flooding in tomato seedlings (Shiu *et al.*, 1998) and during pathogenic infection in both tomato plants (Spanu *et al.*, 1993) and tomato cells in suspension culture (Spanu *et al.*, 1993; Oetiker *et al.*, 1997). More recently, an IAA-inducible ACC synthase clone of mung bean, VR-ACS6, has been isolated and its promoter activity demonstrated in transgenic tobacco (Yoon *et al.*, 1999).

1.2 ACC Oxidase

1.2.1 Evidence for *in vivo* Conversion of ACC to Ethylene

Direct evidence for the conversion of ACC to ethylene *in vivo* came from ACC-feeding experiments. When labelled ACC was fed to plant organs, except preclimateric fruits and flowers, a significant increase in ethylene production was observed (Cameron *et al.*, 1979; Lurssen *et al.*, 1979). This dependence of ethylene production *in vivo* on ACC concentration has been studied in detail in some plant tissues. For example, in pea epicotyls an apparent K_m for the conversion of internal ACC to ethylene was determined

to be 66 μM (McKeon and Yang, 1984) indicating the high affinity for ACC of the ethylene production system *in vivo*. Furthermore, the system converting ACC to ethylene *in vivo* was observed to be stereospecific for different isomers of 1-amino-2-ethyl-cyclopropane-1-carboxylic acid (AEC) (Hoffman *et al.*, 1982) and display a strong preference for only the [1R, 2S]-diastereoisomer which was converted to 1-butene.

1.2.2 Discovery of the Ethylene-Forming Enzyme

Although ethylene-forming activity (conversion of added ACC to ethylene) *in vivo* was observed from a number of plant organs, no equivalent activity *in vitro* after tissue homogenisation could be obtained. The discovery of the ethylene-forming enzyme (EFE) began when Slater *et al.* (1985), using differential cloning technique, identified 19 non-homologous groups of tomato ripening-related clones. One of these clones, pTOM13 (now LE-ACO1), was found to show high homology to a mRNA which accumulated prior to the wounding-induced ethylene peak in unripe fruit and leaf tissue of tomato (Smith *et al.*, 1986). Subsequently, pTOM13 was shown to be induced by ethylene treatment in mature green tomato fruit (Maunder *et al.*, 1987).

Based on the observation of greatly reduced ethylene production by tomato plants transformed with pTOM13 in the antisense orientation, Hamilton *et al.* (1990) suggested that this gene was related to one of the enzymes involved in ethylene biosynthesis. Since the molecular mass of a partially purified ACC synthase from zucchini fruit (53 kDa) (Sato *et al.*, 1991) was much higher than that of the protein encoded by pTOM13 (35 kDa; Smith *et al.*, 1986), speculation increased that pTOM13 could encode EFE. To confirm this speculation, an activity assay *in vivo* was performed on leaf discs from both wild type and pTOM13-antisense transformed plants. An ethylene-forming ability was found to be reduced in the antisense plants in a gene dosage-dependent manner (Hamilton *et al.*, 1990). Further, a full length clone of pTOM13 (pRC13) was observed to confer EFE activity when the cDNA was expressed heterologously in yeast (Hamilton *et al.*, 1991).

The next major advance in the discovery of the EFE was the observation that the translated amino acid sequence from pTOM13 was found to display a 58 % similarity to

a flavanone 3-hydroxylase (F3H, EC 1.14.11.9) from *Antirrhinum majus* which is a member of the 2-oxoglutarate-dependent dioxygenases (Prescott, 1993). This enzyme is known to be a non-heme iron-containing protein requiring anaerobic conditions during extraction and ferrous iron (Fe^{2+}) and a reducing agent (ascorbate) for maximal activity *in vitro* (Britsch and Grisebach, 1986). By incorporating these components into the reaction mixture *in vitro*, Ververidis and John (1991) were the first to demonstrate authentic EFE activity *in vitro* from the soluble phase of melon fruits.

Both EFE activities *in vitro* and *in vivo* were then observed to show a parallel increase in preclimacteric apple fruits treated with ethylene (Fernandez-Maculet and Yang, 1992), indicating that the activity *in vitro* was representative of the activity *in vivo*. By analysing the stereospecificity of the reaction (Ververidis and John, 1991; Fernandez-Maculet and Yang, 1992; Kuai and Dilley, 1992), the stoichiometry of the EFE catalysed reaction was determined (Dong *et al.*, 1992) (Figure 1.2) and EFE was renamed ACC oxidase.

In this reaction, ACC oxidase catalyses the oxidation of ACC and ascorbate to ethylene, carbon dioxide, dehydroascorbate and cyanide. The cyanide produced can be toxic to plant tissue if it accumulates to high levels, and so it is converted to β -cyanoalnine and asparagine. Ascorbate serves as a co-substrate, being concurrently oxidised into dehydroascorbate in equimolar amounts to the ethylene produced.

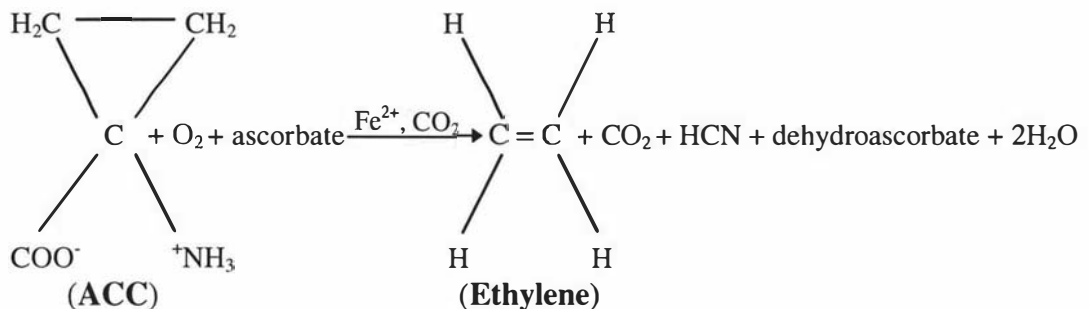


Figure 1.2 Stoichiometry of ACC Oxidation to Ethylene Catalysed by the ACC Oxidase.

1.2.3 Purification and Characterisation of ACC Oxidase

In recent years, ACC oxidases have been isolated, partially purified or purified to homogeneity and characterised from several plant species. Some of the key features of these are summarised in Table 1.1.

Since the first successful extraction from melon fruit (Ververidis and John, 1991), ACC oxidase activity *in vitro* has now been demonstrated in fruits of apple (Dong *et al.*, 1992; Fernandez-Maculeit and Yang, 1992; Kuai and Dilley, 1992; Dilley *et al.*, 1993; Dupille *et al.*, 1993; Pirrung *et al.*, 1993; Poneleit and Dilley, 1993), avocado (McGarvey and Christoffersen, 1992), kiwifruit (MacDiarmid and Gardner, 1993), winter squash (Hyodo *et al.*, 1993), pear (Vioque and Castellano, 1994; Kato and Hyodo, 1999), banana (Moya-Leon and John, 1995), tomato (English *et al.*, 1995), cherimoya (Escribano *et al.*, 1996), citrus peel (Dupille and Zacarias, 1996) and papaya (Dunkley and Golden, 1998). Enzyme activity *in vitro* has also been found in senescing carnation flowers (Nijenhuis-de Vries *et al.*, 1994; Kosugi *et al.*, 1997), leaf tissues of both white clover (Butcher, 1997; Hunter *et al.*, 1999) and barley (Kruzmane and Ievinsh, 1999) and in pine needles (Kruzmane and Ievinsh, 1999). However, the enzyme has been purified to near homogeneity or homogeneity only from fruits of apple (Dong *et al.*, 1992; Dupille *et al.*, 1993; Pirrung *et al.*, 1993), banana (Moya-Leon and John, 1995), papaya (Dunkley and Golden, 1998) and pear (Kato and Hyodo, 1999). So far, no ACC oxidase has been purified to homogeneity from any vegetative plant tissue.

Both native and apparent molecular masses of ACC oxidase determined by gel filtration chromatography and SDS-PAGE analysis, respectively, have been determined from several plant species. The native molecular mass of the partially purified enzyme from melon fruit was determined to be 41 kDa (Smith *et al.*, 1992), higher than both the enzyme from tomato (35 kDa) encoded by pTOM13 (Hamilton *et al.*, 1990) and flavanone 3-hydroxylase (34 kDa) (Britsch and Grisebach, 1986) but similar to other plant 2-oxoglutarate-dependent dioxygenases reported to range from 41 kDa to 45 kDa (Smith *et al.*, 1990). In apple fruits, the apparent molecular masses of the purified enzymes ranged from 35 kDa (Dong *et al.*, 1992) to 40 kDa (Dupille *et al.*, 1993; Pirrung *et al.*, 1993); the native molecular mass was determined to

Table 1.1 Summary of Some Properties of ACC Oxidases Purified from Different Plant Species.

Plant Source	Purity (fold)	Purification Steps	Molecular Mass		pI	pH Optimum	K _m for ACC (μM)	Reference
			Native	Denatured				
<i>Fruit</i>								
Melon	Partially purified (4)	(NH ₄) ₂ SO ₄ precipitation Mono Q anion exchange	41	-	-	7.5	60.0	Smith <i>et al.</i> (1992)
Apple	Partially purified (10)	Polyethylene glycol (PEG) fractionation Calcium phosphate gel adsorption	-	-	-	7.2-7.6	6.4	Kuai and Dilley (1992)
Apple	Near homogeneity (180)	DEAE Sepharose anion exchange Phenyl Sepharose hydrophobic interaction Sephadex G-150 gel filtration	39	35	-	-	-	Dong <i>et al.</i> (1992)
Apple	Homogeneity (170)	(NH ₄) ₂ SO ₄ precipitation Phenyl Sepharose hydrophobic interaction DEAE Memsep 1000 anion exchange Superose 12 gel filtration Mono Q anion exchange	40	40	-	7.4	20	Dupille <i>et al.</i> (1993)
Apple	Homogeneity (21)	(NH ₄) ₂ SO ₄ precipitation Butyl Toyopearl 650 M hydrophobic interaction DEAE Toyopearl anion exchange Mono P chromatofocusing Superdex 75 gel filtration	-	40	-	-	12	Pirung <i>et al.</i> (1993)

Table 1.1 Continued

Plant Source	Purity (fold)	Purification Steps	Molecular Mass		pI	pH Optimum	K _m for ACC (μM)	Reference
			Native	Denatured				
Banana	Homogeneity (145)	PEG-(NH ₄) ₂ SO ₄ precipitation Phenyl Sepharose hydrophobic interaction Mono Q anion exchange Mono P chromatofocusing Superdex 75 gel filtration	40	36	4.9	-	56	Moya-Leonand John (1995)
Cherimoya	Partially purified (41)	(NH ₄) ₂ SO ₄ precipitation Mono Q anion exchange Mono P chromatofocusing Superdex 75 gel filtration	62-66	35	4.35	7.4	82	Escribano <i>et al.</i> (1996)
Papaya	Homogeneity (81)	(NH ₄) ₂ SO ₄ precipitation Sephadex G100-200 gel filtration DEAE Sephadex anion exchange	-	27.5	-	7.4	37	Dunkley and Golden (1998)
<i>Flower</i>								
Carnation	Partially purified (43)	(NH ₄) ₂ SO ₄ precipitation HiTrap DEAE Sepharose anion exchange TSK gel G3000 SWXL gel filtration	35	36	-	7-7.5	111-125	Kosugi <i>et al.</i> (1997)

be 40 kDa (Dupille *et al.*, 1993; Pirrung *et al.*, 1993) and a mean molecular mass of 35.332 ± 5 kDa was determined for the highly purified enzyme with electrospray mass spectrometry (EMS; Pirrung *et al.*, 1993). In banana fruit, the native and apparent molecular mass of the purified enzyme were found to be 40 kDa and 36 kDa, respectively (Moya-Leon and John, 1995). An apparent molecular mass of 37 kDa was determined for the enzyme from kiwifruit (Xu *et al.*, 1998) which was the same size observed for the recombinant enzyme from kiwifruit expressed in *E. coli*. In addition, the native and apparent molecular mass of the partially purified enzyme from senescing carnation flowers were estimated to be 35 kDa and 36 kDa, respectively (Kosugi *et al.*, 1997). An apparent molecular mass (34.8 kDa) of the crude enzyme from sunflower roots was observed to be similar to that deduced from the nucleotide sequence of ACCO1, a root-specific ACC oxidase gene (Liu *et al.*, 1997). All above findings indicate that these ACC oxidases are active as monomers with native molecular masses varying from 35 kDa to 41 kDa. However, a higher native molecular mass of 62 kDa to 66 kDa, with an apparent subunit molecular mass of 35 kDa, has been determined for a partially purified enzyme from cherimoya fruit, indicating that the enzyme could be active as a dimer (Escribano *et al.*, 1996). Recently, an unusually low apparent molecular mass (27.5 kDa) of a purified ACC oxidase from papaya fruit has been reported (Dunkley and Golden, 1998) whereas an active recombinant ACC oxidase protein from papaya fruit expressed in *E. coli* had an apparent molecular mass of 38.0 kDa (Lin *et al.*, 1997). The data suggest that either the ACC oxidase protein behaves anomalously on SDS-PAGE or is truncated during expression in fruit tissue. Alternatively, the ACC oxidase cDNA from papaya fruit expressed in *E. coli* may not be the same gene as that codes for the 27.5 kDa enzyme purified from the fruit tissue.

In terms of amino acid sequencing from purified ACC oxidases, no sequence was obtained from the purified apple protein by Edman methods (Dong *et al.*, 1992) and the purified ACC oxidase from apple fruit was not accessible to N-terminus sequencing (Dupille *et al.*, 1993), suggesting that its N-terminus is blocked. EMS showed that the molecular mass ($35,332 \pm 5$ amu) was approximately 50 amu higher than that predicted from the cDNA sequence, and the blocking group at the N-terminus of a purified apple ACC oxidase was identified as N-acetyl group (Pirrung *et al.*, 1993). A purified apple

ACC oxidase was cleaved with cyanogen bromide (CNBr) and the amino acid sequence of one of the peptide fragments matched that predicted from an apple ACC oxidase cDNA pA12 (Dong *et al.*, 1992). Similarly, the sequence of one of the tryptic peptides of the purified apple protein had a strong homology to that predicted from pTOM13 (Dupille *et al.*, 1993). A relatively high percentage of Val (10.4 %) was found in the purified ACC oxidase from papaya fruit (Dunkley and Golden, 1998). The majority of Val residues was considered to be embedded in the inner portion of the molecule while the more polar residues (Asp, Glu, Arg, Lys and His) occupied the outer surface of the molecule. This amino acid composition was similar to that from apple fruit (Castiglione *et al.*, 1996). The presence of Cys residues in the papaya ACC oxidase molecule suggests the existence of intradisulphide bonds (Dunkley and Golden, 1998).

ACC oxidase has been shown to be an acidic protein in some fruits. The partially purified enzyme from banana fruits had a pI of 4.90 (Moya-Leon and John, 1995) and 4.35 from cherimoya (Escribano *et al.*, 1996) determined by chromatofocusing on a Mono P column. In tomato, pIs of 4.90, 5.09 and 6.14 have been predicted for the ACC oxidase proteins encoded by the three genomic clones of ACC oxidase (Bouzayen *et al.*, 1993) and pI values of 4.9, 5.6 and 5.0 were estimated for the tomato ACC oxidase isoforms coded for by the cDNAs LE-ACO1, LE-ACO2 and LE-ACO3, respectively (Bidonde *et al.*, 1998). Using denaturing isoelectric focusing (IEF), Bidonde *et al.* (1998) observed that one of the three isoforms of tomato ACC oxidase expressed in yeast, LE-ACO2, had a pI of 6.8 whereas both LE-ACO1 and LE-ACO3 gave the same pI of 6.2.

The optimal pH for ACC oxidase activity *in vitro* determined from crude and purified enzyme preparations ranged from pH 6.8 in apple fruit (Poneleit and Dilley, 1993) and sunflower seedlings (Finlayson *et al.*, 1997) to pH 7.5-8.0 for avocado fruit (McGarvey and Christoffersen, 1992). The pH optima have been determined at various carbon dioxide or sodium bicarbonate levels (Kuai and Dilley, 1992; Smith *et al.*, 1992; Dupille *et al.*, 1993; Escribano *et al.*, 1996; Kosugi *et al.*, 1997; Bidonde *et al.*, 1998; Dunkley and Golden, 1998; Hunter, 1998; Kruzmane and Ievinsh, 1999).

1.2.3.1 Kinetic Properties of ACC Oxidase

Although a number of plant enzymes, such as IAA oxidase, peroxidase, lipoxygenase and hydrogen peroxide-generating oxidase (Yang and Hoffman, 1984) are capable of oxidising ACC to ethylene in the presence of various cofactors, their K_m values for ACC were very high relative to the observed levels of ACC *in vivo* (McKeon *et al.*, 1995). Based on the dependence of the ethylene production rate upon the internal ACC concentration, the high affinity of ACC oxidase for its substrate ACC ($K_m = 66 \mu\text{M}$) was first demonstrated *in vivo* in pea epicotyl segments (McKeon and Yang, 1984).

All ACC oxidases from different plant species were found to display Michaelis-Menten kinetics for the substrate ACC. Apparent K_m values for ACC have been determined to range from $6.4 \mu\text{M}$ in the absence of carbon dioxide (Kuai and Dilley, 1992) or $20 \mu\text{M}$ in the presence of a saturating carbon dioxide level (20 %) (Poneleit and Dilley, 1993) for the partially purified enzyme in apple fruits to $435 \mu\text{M}$ for the crude enzyme in winter squash fruits (Hyodo *et al.*, 1993), indicating that the enzyme has a high affinity for ACC. For the same plant species and tissue, the enzyme in a crude extract had a higher K_m for ACC when compared with the purified and partially purified enzyme. For instance, the K_m for ACC of the partially purified enzyme from carnation flowers (Kosugi *et al.*, 1997) ranged from $111 \mu\text{M}$ to $125 \mu\text{M}$ when compared with $425 \mu\text{M}$ determined for the crude enzyme (Nijenhuis De Vries *et al.*, 1994). In addition, the K_m value for ACC is affected by the presence or absence of carbon dioxide or bicarbonate in the reaction mixture. For example, when bicarbonate was omitted from the assay mixture, the K_m for ACC of the purified enzyme from banana fruit decreased from $194 \mu\text{M}$ to $82 \mu\text{M}$ (Escribano *et al.*, 1996). The pH of the reaction mixture also affects the K_m for ACC. For example, the K_m values for ACC of the crude enzyme from leaves and roots of corn ($275 \mu\text{M}$ and $667 \mu\text{M}$, respectively) and sunflower ($161 \mu\text{M}$ and $331 \mu\text{M}$, respectively) at pH 6.5 changed to $212 \mu\text{M}$ and $219 \mu\text{M}$ for corn and $178 \mu\text{M}$ and $191 \mu\text{M}$ for sunflower, respectively at pH 6.8 (the optimal pH) (Finlayson *et al.*, 1997).

The K_m for another substrate, dioxygen, of the partially purified ACC oxidase from apple fruit was found to be 0.4 % (v/v) O_2 in the presence of 1 mM ACC (Kuai and Dilley, 1992). This result is similar to the 0.3 % (v/v) O_2 determined over a range of 1

mM to 10 mM ACC *in vivo* (Yip *et al.*, 1988) and 0.2 % (v/v) for ethylene production by apple tissue (Burg, 1973), but lower than the 2.2 % (v/v) for the enzyme from banana tissue slices when corrected for a dioxygen gradient in the tissue (Banks, 1985). The response to dioxygen of the crude enzyme extracted from both roots and leaves of corn and sunflower seedlings was found to show typical hyperbolic kinetics (Finlayson *et al.*, 1997). The K_m for O₂ was *ca.* 4-fold higher for the leaf enzyme (2.68 %;v/v) when compared to 0.66 % (v/v) observed for the enzyme from corn roots. The K_m for O₂ of an ACC oxidase from pear fruit has recently been determined to be 0.53 % (v/v; Kato and Hyodo, 1999). In addition, ACC concentration in apple fruit was observed to affect the K_m for O₂ with the K_m being 0.3 % (v/v) O₂ determined at 10 mM ACC, but 6.2 % (v/v) O₂ in the absence of ACC (Yip *et al.*, 1988). In addition to the substrates ACC and dioxygen, ACC oxidases from a variety of plant species have been shown to require ascorbate as co-substrate and ferrous iron and carbon dioxide or bicarbonate as essential cofactors.

In the absence of ascorbate in the reaction mixture, no ACC oxidase activity *in vitro* was observed in the partially purified enzymes from fruits of banana (Moya-Leon and John, 1995), cherimoya (Escribano *et al.*, 1996) and pear (Vioque and Castellano, 1998). Increasing ascorbate concentrations stimulated enzyme activity with an apparent K_m for ascorbate of 2.9 mM and 6.5 mM for the partially purified ACC oxidase from fruits of cherimoya (Escribano *et al.*, 1996) and banana (Moya-Leon and John, 1995), respectively. The optimal concentration of ascorbate for the enzyme was determined to range from 3 mM for three isoforms of tomato ACC oxidase expressed in yeast (Bidonde *et al.*, 1998) to 20 mM for the partially purified enzyme from carnation flowers (Kosugi *et al.*, 1997). In addition, supraoptimal concentrations have been shown to reduce enzyme activity *in vitro*. For example, when the concentration of ascorbate was increased from the optimal 10 mM to 100 mM, the activity of a partially purified enzyme from zucchini fruit was reduced by 80 % (Hyodo *et al.*, 1993). No other highly purified member of the 2-oxoacid-dependent dioxygenases family requires ascorbate for catalysis but it is often added to optimise substrate conversion *in vitro* (Zhang *et al.*, 1997).

ACC oxidase activity *in vitro* requires CO₂ as an essential cofactor and activator in common with other plant 2-oxoacid-dependent dioxygenases (Dong *et al.*, 1992; Dilley *et al.*, 1993; Fernandez-Maculet *et al.*, 1993; Poneleit and Dilley, 1993; Prescott and John, 1996; John, 1997). CO₂ is also known to stimulate the conversion of ACC to ethylene *in vivo* (Kao and Yang, 1982; McRae *et al.*, 1983). Direct evidence for CO₂ activation on ACC oxidase activity *in vitro* first came from Dong *et al.* (1992) and Dilley *et al.* (1993) who found that activity *in vitro* of the purified enzyme from apple fruit was completely abolished or low in the absence of CO₂. The half-maximal concentration of CO₂ for the partially purified enzyme from apple fruit was determined to be 0.68 % (v/v) (Poneleit and Dilley, 1993). Undissociated CO₂ rather than bicarbonate was proposed to be the compound responsible for the activation (Dong *et al.*, 1992; Dilley *et al.*, 1993). The optimal concentration of sodium bicarbonate for the partially purified enzyme was reported to be *ca.* 10 mM in fruits of banana (Moya-Leon and John, 1995) and cherimoya (Escribano *et al.*, 1996), similar to those found in other fruits (McGarvey and Christoffersen, 1992; Smith *et al.*, 1992; Fernandez-Maculet *et al.*, 1993; Pirrung *et al.*, 1993; Smith and John, 1993). Sodium bicarbonate was also essential for the activity of the partially purified enzyme from carnation flowers although no optimal sodium bicarbonate concentration was found up to 50 mM (Kosugi *et al.*, 1997).

Carbon dioxide has been found to increase the K_m values for the substrates ACC and dioxygen and co-substrate sodium ascorbate (Fernandez-Maculet *et al.*, 1993; Poneleit and Dilley, 1993; Smith and John 1993; Finlayson and Reid, 1994). For example, the K_m values for ACC of the enzyme partially purified from apple fruit was observed to be 2 μM, 7 μM and 20 μM at ambient CO₂ (*ca.* 0.035 %, v/v), 5 % (v/v) and 20 % (v/v) CO₂ levels, respectively (Poneleit and Dilley, 1993). Increasing CO₂ levels also caused the pH optimum to shift from pH 7.0 to pH 6.5 for ACC oxidase from sunflower leaves (Finlayson and Reid, 1994) and from pH 7.4 to pH 6.7 for the partially purified enzyme from apple fruit (Mizutani *et al.*, 1995).

ACC oxidase is an iron-requiring enzyme and is classified as a member of a family of non-heme iron containing proteins that include plant and bacterial oxidative enzymes

(Pirrung *et al.*, 1993). It has high sequence similarity with several members of the ferrous iron dependent oxygenase/oxidase family (Hamilton *et al.*, 1990; Prescott, 1993; Roach *et al.*, 1995). The requirement of Fe^{2+} for activity *in vitro* has been considered to be due to the loss of Fe^{2+} from the enzyme during extraction (Escribano *et al.*, 1996). Maximum activity of the partially purified enzyme was observed with a concentration range from 20 μM for the enzyme partially purified from senescing carnation flowers (Kogusi *et al.*, 1997) to 50 μM for an ACC oxidase partially purified from cherimoya fruit (Escribano *et al.*, 1996). Supraoptimal concentrations of ferrous iron reduce ACC oxidase activity. For example, the activity of the enzyme from winter squash was inhibited by 50 % when the Fe^{2+} concentration was increased from 20 μM to 500 μM (Hyodo *et al.*, 1993).

In recent years, there have also been some studies on inhibition of ACC oxidase activity *in vitro*. Nijenhuis De Vries *et al.* (1994) reported that the enzyme from senescing carnation flowers had a K_i of 5 mM for α -aminoisobutyrate (AIB, an analog of ACC) which was 10-fold higher than the apparent K_m for ACC (425 μM). The K_i value was similar to those reported for fruits of apple (5.7 mM; Fernandez-Maculet and Yang, 1992) and pear (4.2 mM; Vioque and Castellano, 1994) but higher than those for avocado fruit (0.3 mM; McGarvey and Christofferson, 1992) and chick-pea seeds (0.5 mM; Munoz De Rueda *et al.*, 1995). A cyclobutane ACC analog, 1-aminocyclobutane-1-carboxylate (ACBC), strongly inhibited the activity *in vitro* of the partially purified enzyme from senescing carnation flowers (Kosugi *et al.*, 1997). It acted as a strong competitive inhibitor with the K_i of 20 μM to 31 μM which was only approximately one-fifth of the K_m value (125 μM) for ACC. It was suggested that ACBC could bind to the active site of the enzyme (Kosugi *et al.*, 1997).

In addition, Co^{2+} was reported to be 50 % inhibitory at a concentration of 25 μM to the enzyme partially purified from melon fruit (Ververidis and John, 1991) and 46 % inhibitory for the enzyme partially purified from apple fruit at 10 μM (Kuai and Dilley, 1992); a similar value found in the enzyme from carnation flowers (Nijenhuis-de Vries, 1994). Pirrung *et al.* (1995) examined the inhibition of a purified ACC oxidase from apple fruit with different amino acid hydroximates and suggested that they acted through

both chelation and hydrophobic interactions at the active site. The most potent inhibitor, ACC-hydroxamate, had a K_i similar to the K_m for the substrate ACC. Although ACC oxidase did not require 2-oxoglutarate, it was inhibited by 2-oxoacids (Itturriagagoita-Bueno *et al.*, 1996). Chelating agents, such as tropolones, were also found to inhibit ACC oxidase activity *in vitro* which was recovered by the addition of Fe^{2+} in the assay mixture (Mizutani *et al.*, 1995). Thus, the inhibition of ACC oxidase activity and ethylene production was attributed to the Fe^{2+} chelating ability of these tropolones.

1.2.3.2 Mechanism of Catalysis of ACC Oxidase

It has been suggested that ACC oxidase contains critical His residues at the active site because of fully conserved His residues observed among a number of enzymes sequenced (Christoffersen *et al.*, 1993; Kadyrzhanova *et al.*, 1997). Inactivation experiments using diethylpyrocarbonate (DEPC) (Zhang *et al.*, 1995) presented evidence for the presence of His residues at the active site of an ACC oxidase from tomato fruit expressed in *E. coli*. By using site-directed mutagenesis, Lay *et al.* (1996) showed with an ACC oxidase from kiwifruit that substitutions of Lys¹⁷² by either Ala or Cys or that of Gly¹³⁷ by Pro led to no great loss of activity in crude extracts of *E. coli* cells expressing the mutant ACC oxidase protein. However, substitutions of Asp¹⁷⁹, His¹⁷⁷ and Lys¹⁵⁸ resulted in a complete loss of enzyme activity. Shaw *et al.* (1996) and Kadyrzhanova *et al.* (1997) also concluded that His¹⁷⁷, Asp¹⁷⁹ and His²³⁴ were essential for catalytic activity of an ACC oxidase from apple fruit and suggested that they acted as the putative ligands for Fe^{2+} . A putative CO₂ binding site, Lys¹⁷², played no role in the catalytic activity of the enzyme from kiwifruit (Lay *et al.*, 1996). Recently, Tayeh *et al.* (1999) presented kinetic and mutagenic evidence for the role of His residues in an ACC oxidase from tomato fruit; His¹⁷⁷, His²¹¹ and His²³⁴ may serve as ligands for Fe^{2+} at the active site iron of the enzyme and/or play structural or catalytic role.

Activation of ACC oxidase by one of its products, CO₂, was hypothesised to be in a manner similar to the CO₂-dependence of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) activity (Lorimer and Mizioroko, 1980; Lorimer, 1983) and phosphotriesterase (Hong *et al.*, 1995). It is known that Rubisco activation by its

substrate, CO₂, involves carbamate formation at a specific Lys residue of the enzyme although the molecular basis for the feedback activation by CO₂ is not yet understood. The CO₂ activation of ACC oxidase may also involve the formation of a carbamate complex between CO₂ and ACC oxidase (Dong *et al.*, 1992; Dilley *et al.*, 1993; Fernandez-Maculet *et al.*, 1993; Yang *et al.*, 1993) at a unique Lys residue of the enzyme (Dilley *et al.*, 1993; Poneleit and Dilley, 1993; Lay *et al.*, 1996). The direct evidence for an interaction between CO₂ and the enzyme rather than with ACC was presented by Fernandez-Maculet *et al.* (1993). However, Lay *et al.* (1996) found that Lys¹⁷², a putative carbon dioxide binding site, played no role in the catalytic activity of the enzyme and suggested that the carbamate was probably formed at Arg³⁰⁰.

Site-directed mutagenesis has recently been used to examine the mechanism by which CO₂ activates ACC oxidase (Charng *et al.*, 1996; John *et al.*, 1997; Kadyrzhanova *et al.*, 1997; 1998). It has been shown that carbamylation of one of the seven conserved Lys residues is not likely as the mechanism of CO₂ activation. They reported that the Lys residues, Lys¹⁵⁸, Lys¹⁵⁹, Lys²³⁰, Lys²⁹² and Lys²⁹⁶, were not candidates for carbamylation. Kadyrzhanova *et al.* (1998) found that there were eight highly conserved Lys residues (Lys⁷², Lys¹⁴⁴, Lys¹⁵⁸, Lys¹⁷², Lys¹⁹⁹, Lys²³⁰, Lys²⁹² and Lys²⁹⁶) among 38 known or putative ACC oxidases. However, mutants at each of these eight Lys residues produced in *E. coli* were found to be activated by CO₂ (Kadyrzhanova *et al.*, 1998), indicating that none of these Lys residues were the carbamylation target for CO₂ activation. One residue in the C-terminal region, Arg²⁹⁹, was found to be essential for enzyme activity and CO₂ activation; the mutants Arg²⁹⁹ His, Arg²⁹⁹ Leu and Arg²⁹⁹ Glu were only 1 %, 0.5 % and 0 %, respectively, as active in wild type while none were activated by CO₂. Their experiments also suggest that the C-terminus of the ACC oxidase protein contains residues for CO₂ activation probably by direct interaction with the iron centre. Recently, it has been proposed that HCO₃⁻ (not CO₂) binds directly to Arg¹⁷⁵. In this way, HCO₃⁻ serves to position Fe²⁺ into its binding site (His¹⁷⁷, Asp¹⁷⁹ and His²³⁴) (Professor David Dilley, Michigan State University, USA, *pers. comm.*).

His residues involved in ferrous iron binding by ACC oxidases have been indicated by chemical modification (Zhang *et al.*, 1995; Lawrence *et al.*, 1996). Metal-catalysed

oxidation (MCO) and mutagenesis studies on the iron binding site of ACC oxidase (Zhang *et al.*, 1997) showed that His¹⁷⁷, Asp¹⁷⁹ and His²³⁴ acted as metal ligands during catalysis and that it was possible to replace at least one of the iron binding ligands of ACC oxidase and still retain some activity. Two His and one Asp residues (His¹⁷⁷, His²³⁴ and Asp¹⁷⁹) were considered to be essential ligands for iron while His³⁹ and two Glu residues (Glu¹⁸⁸ and Glu²⁹⁴) were not essential (Kadyrzhanova *et al.*, 1998). The essential role of these three putative Fe²⁺ ligand residues for ACC oxidase activity was also shown by site-directed mutagenesis of the protein and subsequent expression in *E. coli* (Kadyrzhanova *et al.*, 1997).

The lability of ACC oxidase activity *in vitro* has been observed from a number of plant species including fruits of apple (Dupille *et al.*, 1993; Pirrung *et al.*, 1993), cherimoya (Escribano *et al.*, 1996) and papaya (Dunkley and Golden, 1998), senescing carnation flowers (Kosugi *et al.*, 1997) and chick pea seeds (Munoz De Rueda *et al.*, 1995). In particular, ACC oxidase extracted from senescing carnation flowers lost approximately 50 % of the initial activity after storage at 4°C for only 8 h, precluding the further purification for the enzyme. The instability of the ACC oxidase from apple fruit was believed to be due to either the aerobic auto-oxidation of ascorbate catalysed by Fe²⁺ and/or the denaturation of the enzyme by Fe³⁺ generated by oxidation of Fe²⁺ (Dupille *et al.*, 1993). The addition of PA, an inhibitor of metallo-proteases, into the enzyme extract from apple fruit stabilised ACC oxidase activity for at least 18 h at 4°C, probably by preventing these processes through the chelation of Fe²⁺ (Dupille *et al.*, 1993).

The catalytic lability of the enzyme has recently been studied (Smith *et al.*, 1994; Barlow *et al.*, 1997; Zhang *et al.*, 1997). Smith *et al.* (1994), who first observed the auto-inactivation of the enzyme extracted from melon fruit under catalytic conditions, determined that it had a half-life of around 14 min under optimised catalytic conditions. There have been several studies on the mechanism of ACC oxidase inactivation. Smith *et al.* (1994) reported that the inactivation of the purified recombinant ACC oxidase from tomato fruit was the consequence of an intermediate generated during catalytic turnover. However, in the absence of ACC, another distinct ascorbate-dependent inactivation was found to be due to hydrogen peroxide attack on the enzyme. This

inactivation could be partially prevented by the addition of catalase. A detailed study by Barlow *et al.* (1997) suggested that at least three distinct mechanisms for the inactivation of ACC oxidase occur: 1) partial unfolding of the catalytically active conformation (non-oxidative inactivation), 2) oxidative damage from hydrogen peroxide which is catalase protectable and 3) oxidative damage to the active site region which is non-catalase protectable. In addition, Vioque and Castellano (1998) proposed that the inactivation of the ACC oxidase from pear fruit was not due to end-product (ethylene, dehydroascorbic acid and cyanic) inhibition but to the formation of a catalytically inactive enzyme-ACC-Fe²⁺-ascorbate complex. Functional alteration through metal-catalysed oxidation is proposed to be responsible for this inactivation (Ramassamy *et al.*, 1998).

1.2.4 Subcellular Localisation of ACC Oxidase

The subcellular compartment in which the ACC oxidase resides has been debated for many years. Different subcellular locations have been shown by a number of experiments. Before 1990, it was thought that this enzyme was membrane-bound (Yang and Hoffman, 1984). Since 1990, this issue has been re-examined by a number of researchers who have used a variety of techniques. A cytosolic localisation was initially indicated by the recovery of ACC oxidase activity *in vitro* in the soluble phase of fruits of melon (Ververidis and John, 1991) and avocado (McGarvey and Christoffersen, 1992). In avocado fruit, ACC oxidase activity was retained in a 100,000 x g supernatant which had a pH optimum of 7.5 to 8; the proposed pH of the cytoplasm. Using suspension-cultured cells of tomato in concert with cell-fractionation techniques followed by immunoblot analysis with a monoclonal antibody, Reinhardt *et al.* (1994) found that most of ACC oxidase antigen was localised to the cytosol and was absent from the vacuole. The lack of activity of this enzyme from avocado fruit below pH 6 (McGarvey and Christoffersen, 1992) precludes the possibility of the vacuole localisation of this enzyme. When the cell wall was digested in the preparation of protoplasts, these cells still retained ACC oxidase activity and the ACC oxidase antigen (Reinhardt *et al.*, 1994) indicating the absence of extracellular site for the enzyme from tomato. These findings are consistent with the absence in many ACC oxidase cDNA clones of an N-terminal consensus signal sequence required for the secretion of the

enzyme *via* the endoplasmic system (Balague *et al.*, 1993; Christofferson *et al.*, 1993). Analysis of the deduced amino acid sequences from three cDNAs of ACC oxidase (LE-ACO1, 2 and 3) from tomato also shows no obvious signal peptide that might allow these proteins to cross the plasma membrane (Bouzayen *et al.*, 1993). Also, there is no transit peptide in a full length cDNA of an ACC oxidase from papaya fruit, suggesting that ACC oxidase is a cytosolic enzyme (Lin *et al.*, 1997).

Using a membrane-impermeable protein-binding reagent, 2,4,6-trinitrobenzenesulfonic acid (TNBS), however, Latche *et al.* (1993) observed that extracellularly located (apoplastic) ACC oxidase activity increased dramatically during ripening of melon fruit. This observation was confirmed by Rombaldi *et al.* (1994) in thin sections of ripening tomato fruit using immunocytological techniques. By a combination of cell fractionation and immunocytological methods with intact protoplasts, Ramassamy *et al.* (1998) further demonstrated that an ACC oxidase from apple fruit was localised at the external face of the plasma membrane (plasmalemma). In addition, both iron and ascorbate were present in the apoplasm (Longnecker and Welch, 1990; Luwe *et al.*, 1993), as were two other ascorbate-utilising enzymes, ascorbate oxidase (Esaka *et al.*, 1989) and peroxidase (Takahama and Oniki, 1992). With an increase in the number of ACC oxidase proteins purified and with the production of antibodies, more information on the localisation of the enzyme may be forthcoming in the future.

1.3 Molecular and Biochemical Evidence for the Presence of ACC Oxidase Isoforms in Plants

Due to the importance of ethylene for plant growth and development, plants may have evolved distinct ACC oxidase isoforms to ensure that ACC oxidase will always be available for catalysing ethylene production under various physiological, developmental and environmental conditions. Indeed in recent years, there have been more and more molecular and biochemical evidence for the presence of isoforms of ACC oxidase which are differentially expressed during plant development and in response to various stimuli.

Since pTOM13 was identified as the first ACC oxidase gene using an antisense gene approach in tomato fruit (Hamilton *et al.*, 1990) in concert with heterologous expression in yeast and *Xenopus* oocytes (Hamilton *et al.*, 1991; Spanu *et al.*, 1991), a number of ACC oxidase genes have now been cloned and characterised from a variety of plant species. ACC oxidase was thought to be expressed in a constitutive manner in plant tissue because of the fact that most plant tissues can convert ACC to ethylene. However, recently more and more studies on differential expression patterns of ACC oxidase suggest that this enzyme is a regulated step in ethylene production during plant development and in response to environmental cues. The transcriptional regulation of the enzyme plays an important role in ethylene biosynthesis during fruit ripening, senescence, in response to wounding (Grierson *et al.*, 1998) and in vegetative tissues (Jin *et al.*, 1999). Further, ACC oxidase and ACC synthase together contribute to positive feedback loop wherein ethylene treatment leads to increased ethylene production (Kende and Zeevaart, 1997).

1.3.1 Sequence Homology and Divergence of ACC Oxidase Multigene Families

By screening genomic libraries, multiple ACC oxidase genes have now been identified and characterised from a number of plant species. These include four members in tomato (Bouzayen *et al.*, 1993; Barry *et al.*, 1996; Nakatsuka *et al.*, 1998), four in petunia flowers (Tang *et al.*, 1993), three in melon (Lasserre *et al.*, 1996) and three in tobacco (Kim *et al.*, 1998). By screening cDNA libraries, three in sunflower seedlings (Liu *et al.*, 1997), two in banana fruit (Huang and Do, 1998), two in mung bean hypocotyl (Kim and Yang, 1994; Jin *et al.*, 1999), two in broccoli floral tissue (Pogson *et al.*, 1995) and one in apricot fruit (Mbeguie-A-Mbeguie *et al.*, 1999) have been isolated and characterised. Shiomi *et al.* (1998) cloned two ACC oxidase homologs from cucumber fruit using the reverse transcriptase-polymerase chain reaction (RT-PCR) and both 5'- and 3'-rapid amplification of cDNA ends (RACE) PCR. Recently, by a combination of RT-PCR and 3'-RACE, three ACC oxidase genes, designated TR-ACO1, TR-ACO2 and TR-ACO3, have been isolated and characterised during leaf ontogeny in white clover (Hunter *et al.*, 1999).

Sequence comparison between ACC oxidase genes indicates a high nucleotide sequence homology both within the same family and between families from different plant species (Lasserre *et al.*, 1996). When deduced amino acid sequences were compared, the three cDNAs of ACC oxidase from tomato (LE-ACO1, LE-ACO2 and LE-ACO3) showed up to 88 % to 90 % identity (Barry *et al.*, 1996) and the four cDNAs of ACC oxidase from petunia flowers were 88 % to 94 % identical (Lasserre *et al.*, 1996). However, the four ACC oxidase cDNAs of petunia flowers displayed only 42 % to 43 % homology in their 5'-untranslated regions (UTRs) and 50 % to 57 % homology in their 3'-UTRs (Tang *et al.*, 1994). The three ACC oxidase cDNAs from tomato also had only 58 % to 65 % similarity in their 5'-UTRs and 44 % to 52 % homology in their 3'-UTRs, respectively (Barry *et al.*, 1996).

Two ACC oxidase genes from peach fruit, ppACO1 and ppACO2, displayed 68 % identity in the deduced amino acid sequences (Bonghic *et al.*, 1998) while ppACO2 showed a higher similarity with one of the three melon ACC oxidase genes, CM-ACO3, than ppACO1. Two ACC oxidase cDNA clones from cucumber fruit, CS-ACO1 and CS-ACO2, were 73 % identical to each other at the amino acid level and 66 % homologous at the nucleotide level (Shiomi *et al.*, 1998). Furthermore, both cDNAs had all the conserved amino acids common to all members of the Fe(II) ascorbate family of dioxygenases (Lasserre *et al.*, 1996). CS-ACO1 was highly homologous to CM-ACO1 isolated by Lasserre *et al.* (1996) with a homology of 96 % at the nucleotide and 97 % identity at the amino acid levels, respectively, while CS-ACO2 was 74 % similar to other ACC oxidases at both levels (Shiomi *et al.*, 1998). Similarly, three cDNA sequences of ACC oxidase from white clover leaves had 75 % to 84 % homology within the reading frame but only 55 % to 61 % similarity was found in their 3'-UTRs (Hunter *et al.*, 1999). In concert with these reports which show ACC oxidase is a small multigene family, more and more studies now reveal that ACC oxidases are differentially expressed during plant development and in response to various stimuli.

1.3.2 Differential Expression and Regulation of ACC Oxidase Genes during Plant Development

1.3.2.1 Temporal Expression and Regulation

A number of fruits produce ethylene during ripening (Yang and Hoffman, 1984). This production of ethylene was found to be due to synthesis *de novo* of both ACC synthase and ACC oxidase transcripts (Hamilton *et al.*, 1990; Rottmann *et al.*, 1991). The requirement for ethylene biosynthesis for fruit to ripen normally was demonstrated using transgenic technology (Picton *et al.*, 1993). ACC oxidase transcript levels have been found to increase greatly during ripening of climacteric fruits (Holdsworth *et al.*, 1987; Hamilton *et al.*, 1990; Ross *et al.*, 1992; Balague *et al.*, 1993; Dillely *et al.*, 1995; Lasserre *et al.*, 1996; Huang and Do, 1998), during flower and leaf senescence (Woodson *et al.*, 1992; Tang *et al.*, 1994; John *et al.*, 1995; Pogson *et al.*, 1995; Hunter *et al.*, 1999) and following pollination in orchid (O'Neill *et al.*, 1993) and geranium flowers (Clark *et al.*, 1997).

Three distinct ACC oxidase genes from tomato have been shown to be differentially expressed during fruit development and ripening (Barry *et al.*, 1996). The basal level of the LE-ACO1 transcript was detectable in green tomato fruit, increased dramatically at the breaker stage as the fruit began to ripen and peaked in abundance in orange fruit. After this, expression declined to a level observed in the breaker fruit and remained at that lower level until the fruit was fully ripe. No LE-ACO2 expression was observed in the fruit at any developmental stage. The LE-ACO3 transcript appeared only transiently at the breaker stage and then declined markedly. This transient appearance of LE-ACO3 may indicate the existence of negative feedback regulation of this gene (Nakatsuka *et al.*, 1998). A fourth tomato ACC oxidase gene (LE-ACO4), with low sequence similarity compared to all other three ACC oxidase genes (Barry *et al.*, 1996), has recently been observed to be expressed upon commencement of ripening (Nakatsuka *et al.*, 1998).

Two ACC oxidase genes, CM-ACO1 and PH-ACO1, from melon and petunia fruit, respectively, were induced during fruit ripening (Lasserre *et al.*, 1996). A sequence coding banana ACC oxidase was shown by northern analysis to be present at a low level

in immature (preclimateric) banana fruit but increases dramatically during ripening (Lopez-Gomez *et al.*, 1997). ACC oxidase transcripts increased significantly both prior to and during climateric ethylene production in kiwifruit (Whittaker *et al.*, 1997). The expression of an ACC oxidase gene from passion fruit, PE-ACO1, was found to be differentially regulated; the level of PE-ACO1 mRNA increased in both arils and seeds as fruit ripen from immature green through to the purple stage (Mita *et al.*, 1998). Similarly, ACC oxidase mRNAs were found to be up-regulated during the ripening of three apple cultivars (Atkinson *et al.*, 1998). Recently, a full length ACC oxidase cDNA, Pa-ACO, isolated from a cDNA library from ripe apricot fruit, has been observed to be transcriptionally regulated (Mbeguie-A-Mbeguie *et al.*, 1999). Wilkinson *et al.* (1995) identified five mRNAs with increased expression during ripening of a non-climateric strawberry fruit although no relationship exists between ethylene production and non-climateric fruit ripening. One ACC oxidase gene, *acaco-1*, has also been found to be induced in the ripening of pineapple, another non-climateric fruit (Cazzonelli *et al.*, 1998).

In senescing carnation flowers, Woodson *et al.* (1992) observed that the abundance of ACC oxidase mRNAs increased concomitantly with the ethylene climacteric. In petunia flowers, Tang *et al.* (1994) demonstrated that expression of one of the ACC oxidase genes, ACO1, led to an increase in ACC oxidase activity during flower senescence and that this gene was expressed in other floral organs following ethylene treatment. Both ACO3 and ACO4 were specifically expressed in pistils during flower development. *In situ* hybridisation revealed that ACC oxidase mRNA was specifically localised to the secretory cells of the stigma and the connective tissue of the receptacle. Similarly, one of two broccoli ACC oxidase cDNAs (ACC ox1 and ACC ox2) from senescing floret tissue, ACC ox1, was observed at low levels in whole florets at the time of harvest but increased dramatically in abundance after harvest (Pogson *et al.*, 1995). However, transcripts corresponding to ACC ox2 were found exclusively within the reproductive structures and were absent at harvest.

In addition, *caaco1*, one of the two chick-pea ACC oxidase genes, was found to remain constant during the initial stages of embryogenesis before decrease at the later stages

(Gomez-Jimenez *et al.*, 1998). However, during germination, *caaco1* mRNA levels peaked at 24 h which is consistent with the maximal ACC oxidase activity *in vitro* and in ethylene production. Therefore, the authors propose that ACC oxidase is regulated at the translational level during embryogenesis but at the transcriptional level during germination.

1.3.2.2 Spatial Expression and Regulation

Differential spatial expression of ACC oxidase genes has been observed from several plant species. Using ribonuclease protection assays (RPA) with gene-specific probes, Barry *et al.* (1996) found that all three tomato ACC oxidase genes were expressed during flower development with spatially regulated patterns; one of the three, LE-ACO1, was predominantly expressed in the petals and stigma and style whereas LE-ACO2 was expressed only in tissues associated with the anther cone. LE-ACO3, however, was expressed in all floral parts examined except for the sepals. One of the melon ACC oxidase genes, CM-ACO1, was expressed in leaves, flowers, roots and etiolated hypocotyls while both CM-ACO2 and CM-ACO3 were predominantly present in etiolated hypocotyls and flowers, respectively. Similarly, Liu *et al.* (1997) found that ACCO1 was a predominant homologue in roots of sunflower whereas ACCO2 was the major homologue in both hypocotyls and leaves. In contrast, ACCO3 was constitutively expressed in all these organs. In the same plant, Finlayson *et al.* (1997) presented biochemical evidence for the existence of organ-specific isoenzymes in leaves and roots. Therefore, it could be speculated that the isoenzyme in roots is encoded for by ACCO1 while ACCO2 encoded for the isoenzyme expressed in leaves, and the two putative isoforms possibly operate through different mechanisms in response to CO₂ levels. All three tobacco ACC oxidase clones, pNG-ACO1, 2 and 3, also displayed differential spatial expression patterns; pNG-ACO2 mRNA was expressed mainly in stems whereas both pNG-ACO1 and 3 were expressed predominantly in roots (Kim *et al.*, 1998). Recently, one of the melon ACC oxidase genes, CM-ACO1, has been observed to accumulate in the mesocarp and placenta of two cultivars at commercial harvest maturity whereas CM-ACO2 was almost constitutively expressed (Shiomi *et al.*, 1999).

In addition, one of two peach ACC oxidase genes, ppACO1, has been shown to be constitutively expressed in the fruit abscission zone and surrounding tissues and in mesocarp through fruit development (Bonghric *et al.*, 1998). The level of one passion fruit ACC oxidase gene, PE-ACO1, was observed to be lower in the seed when compared with the aril (Mita *et al.*, 1998). Not only have ACC oxidase genes been shown to be developmentally expressed and regulated, their expression is also influenced by a number of biotic and abiotic factors.

1.3.3 Differential Expression and Regulation of ACC Oxidase Genes in Response to Different Cues

1.3.3.1 Plant Hormones

Ethylene has been shown to stimulate ACC oxidase activity in both vegetative (Chalutz *et al.*, 1984; Kim and Yang, 1994; Kwak and Lee, 1997) and reproductive tissues (Bufler, 1986; Dominguez and Vendrell, 1993; McKeon *et al.*, 1995; Guillen *et al.*, 1998). The accumulation of ACC oxidase transcripts associated with this stimulation (Drory *et al.*, 1993; Nadeau *et al.*, 1993; Avni *et al.*, 1994; Kim and Yang, 1994; Tang *et al.*, 1994; Peck and Kende, 1995; Barry *et al.*, 1996; Kim *et al.*, 1997) indicates positive transcriptional regulation.

A low level of ACC oxidase activity in mature green tomato fruit increased substantially in response to ethylene treatment in a dose and time-dependent manner. This increase was inhibited by an ethylene inhibitor, 2,5-norbornadiene (NBD) (Liu *et al.*, 1985). Using a new inhibitor of ethylene action, 1-methylcyclopropene (MCP; Sisler *et al.*, 1995), Nakatsuka *et al.* (1997) showed that the expression and regulation of one of the tomato ACC oxidase genes, LE-ACO1, was under a positive feedback control mechanism even at developmental stages with high rates of ethylene evolution. Ethylene was found to enhance the expression of the ACC ox2 in broccoli (Pogson *et al.*, 1995) and the tomato LE-ACO1 transcript (Barry *et al.*, 1996; Nakatsuka *et al.*, 1997). Ethylene treatment also increased the abundance of ACC oxidase transcripts in the corollas of petunia flowers (Tang *et al.*, 1994), etiolated pea seedlings (Peck and Kende, 1995), mung bean hypocotyls (Kim and Yang, 1994; Kim *et al.*, 1997), banana fruit (Lopez-Gomez *et al.*, 1997) and leaves of tobacco (Kim *et al.*, 1998). Exposing passion

fruit to ethylene (20 $\mu\text{L/L}$) for 24 h significantly increased the level of PE-ACO1 expression in both arils and seeds. However, the ethylene-induced expression was found to be inhibited when exposing the fruit to ethylene (20 $\mu\text{L/L}$) and NBD (5000 $\mu\text{L/L}$) (Mita *et al.*, 1998). Furthermore, only a very low concentration of ethylene (0.1 $\mu\text{L/L}$) was needed to induce ACC oxidase transcripts in intact leaves of tobacco treated for 24 h (Kim *et al.*, 1998). Recently, accumulation of melon CM-ACO1 mRNA in two cultivars has been observed to be greatly stimulated by ethylene (Shiomi *et al.*, 1999). Exogenous ethylene treatment (20 $\mu\text{L/L}$) has also been found to induce an increase in the level of an ACC oxidase transcript, VR-ACO1, from mung bean hypocotyl. The induction of VR-ACO1 by ethylene is under both transcriptional and posttranscriptional controls in hypocotyls whereas it is mainly at the transcriptional level in roots (Jin *et al.*, 1999).

An increase of ethylene production in etiolated pea seedlings by IAA was observed to be due to the induction of an ACC oxidase gene transcript (Peck and Kende, 1995). IAA treatment also induced the expression of one of the two ACC oxidase genes from cucumber fruit, CS-ACO1 (Shiomi *et al.*, 1998), and this expression paralleled ethylene production in response to IAA treatment. In contrast, CS-ACO2 was constitutively expressed and was not affected by IAA treatment. In addition, both the pVR-ACO1 gene transcript in mung bean (Kim and Yang, 1994) and the expression of the LE-ACO1 GUS-promoter fusion in tomato (Blume and Grierson, 1997) were observed to increase in response to exogenous methyl jasmonate.

1.3.3.2 Wounding

Wounding leaf tissue has been shown to increase transcription of the ripening-related ACC oxidase in fruits of peach (Callahan *et al.*, 1992), melon (Lasserre *et al.*, 1996), tomato (Barry *et al.*, 1996) and banana (Dominguez and Vendrell, 1993; Lopez-Gomez *et al.*, 1997). Liu *et al.* (1997) also observed an increase of ACC oxidase mRNA levels upon wounding hypocotyls of sunflower seedlings. Using MCP, Bouquin *et al.* (1997) found the wounding-induced increase of the melon ACC oxidase transcript to be independent of ethylene, and distinct wounding and ethylene response motifs in the promoter region have been identified. Similarly, one of the two cucumber ACC oxidase

genes, CS-ACO1, was induced by wounding whereas CS-ACO2 was constitutively expressed and was not affected by wounding (Shiomi *et al.*, 1998). These authors suggest that both genes are involved in the regulation of ethylene biosynthesis at the transcriptional level.

1.3.3.3 High Temperature and Pathogen Infection

In heat-treated (44°C for 24 h in the dark) carnation flowers, the ethylene climacteric occurred 24 h later than that in control flowers (maintained at 23°C). Maximum ethylene production decreased from 44 nl/g/h to 31 nl/g/h in heat-treated flowers. The accumulation of one ACC oxidase gene, DCACO1, was also found to be delayed by 24 h in the heat-treated flowers (Verlinden and Woodson, 1998). The accumulation of two of the three tobacco ACC oxidase gene transcripts, NG-ACO1 and NG-ACO3, was found to increase markedly in tobacco leaves infected with the tobacco mosaic virus (TMV) (Kim *et al.*, 1998). In tomato, LE-ACO1 was observed to be the main ACC oxidase gene expressed in response to infection of tomato fruit by *Colletotrichum gloeosporioides*, and there was no detectable expression of both LE-ACO2 and LE-ACO3 (Cooper *et al.*, 1998). In addition, the CM-ACO1 and CM-ACO3 transcripts in leaves of melon were found to increase in response to salt and drought stress (Guis *et al.*, 1997).

Both the cloning and molecular characterisation of ACC oxidase genes in the plant species examined thus far suggest that the enzyme is encoded by a small multigene family and that these genes are regulated at both the transcriptional and posttranscriptional levels in higher plants. The differential expression and regulation of the ACC oxidase multigene families in plants suggests the occurrence of different isoforms of this enzyme with different enzymatic properties (K_m , pI, etc.) which regulate ethylene biosynthesis during plant development and in response to various stimuli. The occurrence of such isoforms of ACC oxidase will allow for the optimal response of these plants to produce ethylene by operating through a number of regulatory networks.

1.3.4 Biochemical Evidence for the Occurrence of ACC Oxidase Isoforms

Two ACC oxidase activities (designated EFE1 and EFE2) were first observed upon ammonium sulphate fractionation of a crude extract of avocado fruit (McGarvey and Christoffersen, 1992). EFE1 had a relatively low K_m (32 μ M) for ACC with optimal pH being 7.5 to 8.0 and optimal temperature 25°C to 30°C. Subsequently, two temperature optima were observed in the enzyme extracts from fruits of pear (28°C and 38°C; Vioque and Castellano, 1994), citrus peel (35°C and 45°C; Dupille and Zacarias, 1996) and chick-pea seeds (27.5°C and 35°C; Munoz De Ruesa *et al.*, 1995). These results suggest that multiple forms of ACC oxidase may be present in these plant tissues.

In terms of whole plant studies, Finlayson *et al.* (1997) reported that roots and leaves of both corn and sunflower seedlings possessed organ-specific ACC oxidase activities which display a different pH dependence and K_m (affinity) for ACC, dioxygen, ascorbate and carbon dioxide. They hypothesised that ACC oxidase may exist as organ-specific isoenzymes which reflect the environmental and physiological status of each organ. However, this work was performed on crude enzyme extracts and therefore, it is possible that there are interactions between tissue-specific modifying protein (s) and ACC oxidase. Butcher (1997) also hypothesised that more than one ACC oxidase may be present in leaves of white clover based on the different responses to ACC of the crude enzyme extracted from leaves at different developmental stages. Recently, three isoforms of tomato ACC oxidase expressed in yeast were found to display differences in the apparent K_m for ACC, pI and specific activity (Bidonde *et al.*, 1998). However, no ACC oxidase isoforms have been extracted and purified from tomato plant tissues.

1.4 Differential Expression of ACC Oxidase during Leaf Maturation and Senescence in Plants

Ethylene production was observed to increase during senescence in leaf discs of tobacco and sugar beet (Aharoni *et al.*, 1979; Aharoni and Lieberman, 1979), in detached leaves of Chinese tree (Morgan and Durham, 1980) as well as in tobacco (Alejar *et al.*, 1988) and cotton (Morgan *et al.*, 1992). More recently, changes in ACC oxidase gene

expression during leaf maturation and senescence has been investigated in several plant species. The expression of ACC oxidase in senescing leaves has been demonstrated by delayed leaf senescence in tomato plants transformed with antisense ACC oxidase gene constructs (Picton *et al.*, 1993; John *et al.*, 1995). Two of the three melon ACC oxidase genes, CM-ACO1 and CM-ACO3, were expressed differentially during leaf maturation and senescence (Lasserre *et al.*, 1996). CM-ACO1 expression was lowest in young green leaves before increasing to the highest level at the onset of senescence followed by a decline during the later stages of senescence. Expression of CM-ACO3 was found to be highest in young green leaves before declining to its lowest level in the most senescent stage. In tomato (Barry *et al.*, 1996), the abundance of the LE-ACO1 transcript dramatically increased (up to 27-fold) at the onset of leaf senescence before declining slightly at the most advanced stage. LE-ACO3 was observed to be expressed only transiently and the level accumulated was only approximately 50 % of the LE-ACO1 gene message. No LE-ACO2 gene expression was detected in leaves at any developmental stage by RPA assay. Two of the three tobacco full length ACC oxidase clones, pNG-ACO1 and pNG-ACO3, have been observed to accumulate during leaf senescence while pNG-ACO2 was constitutively present in leaf tissue (Kim *et al.*, 1998). These studies have provided the molecular evidence for the presence of ACC oxidase isoforms during leaf maturation and senescence in plants but information on enzyme isoforms expressed during leaf maturation and senescence is still lacking.

1.5 Ethylene Biosynthesis during Leaf Maturation and Senescence in White Clover

White clover (*Trifolium repens* L.) is the plant used in this study from which to extract, purify and characterise isoforms of ACC oxidase during leaf maturation and senescence. White clover has a typical stoloniferous growth habit (Thomas, 1987). Butcher (1997) and Hunter *et al.* (1999) utilised the sequential manner of white clover leaf development as a model system to study ethylene biosynthesis during leaf maturation and senescence. It has been shown that leaf senescence of white clover is related to an increase in ethylene production. Endogenous levels of ACC have also been observed to increase and precede that of ethylene evolution (Butcher *et al.*, 1996; Butcher, 1997; Hunter *et al.*, 1999). Hunter *et al.* (1999) have recently demonstrated that two ACC oxidase

transcripts (TR-ACO2 and TR-ACO3) were differentially expressed during leaf maturation and senescence. Levels of TR-ACO2 expression were high in developing and mature green leaves and declined to the lowest levels in the most senescent leaves. Expression of TR-ACO3 was found to be very low in mature green leaves but this increased prior to the rise in ethylene production in senescing leaves. However, ACC oxidase activity *in vitro* (in crude extracts) was observed to be highest in mature green leaves which unexpectedly declines as the leaves senesce. The results suggest the likely presence of ACC oxidase isoforms in leaf tissue of white clover. Currently, the ACC oxidase activity *in vitro* and antibody recognition data match the expression of TR-ACO2 only. The discrepancy between TR-ACO3 gene expression and no apparent corresponding enzyme activity *in vitro* needs to be investigated further. This study, therefore, focuses on investigating the function of ACC oxidase during leaf maturation and senescence in white clover. Two approaches are used. The first is to express the two cDNAs TR-ACO2 (expressed in mature green leaf tissue) and TR-ACO3 (expressed in senescent leaf tissue) in *E. coli* to characterise biochemically the gene products. The second is to extract, purify and characterise ACC oxidase isoforms from both mature green and senescent leaves of white clover before comparing their biochemical properties.

1.6 Aims of the Thesis

The main aims for this dissertation are:

- To investigate whether two white clover ACC oxidase transcripts, TR-ACO2 and TR-ACO3, can be translated into active ACC oxidase enzymes in *E. coli*.
- To extract, identify and purify different isoforms of ACC oxidase *in vitro* from leaf tissues at different stages of development in white clover (mature green and senescent leaves).
- To determine the physicochemical characteristics and kinetic properties of the purified isoforms of this enzyme.

2. Chapter Two: Materials and Methods

2.1 Expression and Purification of ACC Oxidases in *E. coli*

2.1.1 Chemicals

Where possible, molecular biology grade or analytical grade chemicals and reagents supplied by either Sigma or BDH (Table 2.1) were used in this part of the study. Water used was purified by reverse-osmosis (RO) followed by a microfiltration system containing ion exchange, solvent exchange, organic and inorganic removal cartridges (Milli-Q, Millipore Corp., Bedford, Massachusetts, USA). All solutions were made with the Milli-Q water and sterilised by autoclaving at 103 kPa for at least 20 min or by filtering through a sterile 0.22 μm nitrocellulose filter (Millipore, Bedford, Massachusetts, USA). All solutions were stored at 4°C or room temperature unless otherwise stated. The pH was measured using a digital pH meter (model TS-2, Science and Technology (NZ) Ltd.).

2.1.2 Growth and Storage Conditions of *E. coli*

Materials

- Luria-Bertani (LB) broth: 1.0 % (w/v) bactotryptone, 0.5 % (w/v) bacto yeast extract (Difco Laboratories Ltd., Surrey, UK) and 1.0 % (w/v) NaCl. The components were dissolved in water and pH adjusted to 7.0 prior to autoclaving
- Agar (GIBCO BRL)

Table 2.1 Manufacturers' Addresses of Commonly Used Chemicals.

Company	Full Name	Address
Sigma	Sigma Chemical Company	St. Louis, MO, USA
Pharmacia	Pharmacia Biotech	Uppsala, Sweden
BIO-RAD	BIO-RAD Laboratories	Hercules, CA, USA
BDH	British Drug Houses	Poole, Dorset, UK
Boehringer Mannheim	Boehringer Mannheim Biochemica	Mannheim, Germany
GIBCO BRL	Life Technologies GIBCO BRL	Gathersbury, MD, USA

- Ampicillin (Amp¹⁰⁰) (Sigma): 100 mg/ml stock solution filter-sterilised using a 0.22 µm nitrocellulose filter.
- 100 % (v/v) glycerol

Single colonies of an *E. coli* strain TB-1 (Life Technologies), transformed with the partial reading frame of either TR-ACO2 or TR-ACO3 (Hunter *et al.*, 1999) cloned, as an inframe translational fusion, into the *E. coli* expression vector, pPROEX-1 (Figure 2.1), were obtained from Dr. Donald Hunter, Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand. The *E. coli* TB-1 strain was maintained on solid LB Amp¹⁰⁰ (1.5 % (w/v) agar in LB broth containing 100 µg/ml ampicillin) and subcultured every six weeks. To store an *E. coli* colony for a longer time, a 0.8 ml aliquot from a 10 ml overnight culture (Section 2.1.3) was added to 0.2 ml of pre-sterilised 100 % (v/v) glycerol, snap frozen in liquid nitrogen and stored at -80°C. Fresh *E. coli* cultures were revived from the frozen stocks every three months by plating an aliquot of the frozen stock onto fresh LB Amp¹⁰⁰ agar plates.

2.1.3 Expression of ACC Oxidases in *E. coli*

Materials

- MSE Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, Manor Royal, Sussex, England)
- Spectrophotometer (Ultraspec 3000, Pharmacia Biotech)
- LB broth (Section 2.1.2)
- 100 mM IPTG stock (stored at -20°C)
- Amp¹⁰⁰ (Section 2.1.2)
- Cell lysis buffer: 50 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, containing 25 % (v/v) glycerol, 2 mM Dithiothreitol (DTT) and 1.0 mg/ml of lysozyme (Boehringer Mannheim)

A single colony of *E. coli* strain TB-1, transformed with TR-ACO2 or TR-ACO3, was grown in 10 ml LB broth supplemented with 100 µg/ml ampicillin at 37°C overnight. A 0.5 ml aliquot of the overnight culture was then used to inoculate 10 ml of a fresh LB broth, and this was incubated until the absorbance at 595 nm ($A_{595 \text{ nm}}$) of the culture reached 0.6 (moderate density). Induction of foreign protein expression involved the addition of IPTG from a 100 mM stock to a final concentration of 0.6 mM to the culture, which was incubated for the appropriate time interval at 27°C or 37°C. In other experiments, a series of IPTG concentrations (0, 0.3, 0.6, 0.9 and 1.2 mM) was also added and cells incubated at 27°C for 5h after the addition of IPTG. At the end of each induction period, cells were collected by centrifugation at 20,800 x g for 15 min at 4°C. The supernatant was discarded and the cell pellets stored at -20°C overnight.

Cell extracts were prepared from the cell pellets by resuspension in approximately four volumes (v/w) of the cell lysis buffers containing 0.5 %, 0.1 %, 0.05 % and 0 % of SDS or Triton X-100 (w/v; v/v). The suspension (kept on ice) was then sonicated with a MSE Soniprep 150 Ultrasonic Disintegrator for four periods of 15 seconds, with a 30 second interval between each period. The sonicator was fitted with 3 mm diameter probe and used at an amplitude of 14 microns. The resulting cellular debris from sonication was pelleted by centrifugation at 20,800 x g for 20 min at 4°C. The proteins in the supernatant were then separated using SDS-PAGE (Section 2.2.11).

To remove detergents used in the lysis buffers, the solubilised protein preparations were passed through a Sephadex G-25 gel filtration column (Section 2.2.5) pre-equilibrated with lysis buffer with no detergent added.

2.1.4 Purification of His-Tagged Fusion Proteins by Affinity Chromatography

Materials

- Concentrator (YM-10 membrane, WR Grace and Co., Danvers, MA, USA)
- Lysis buffer: 50 mM Tris-HCl, pH 7.5, containing 25 % (v/v) glycerol, 2 mM DTT, 1.0 mg/ml lysozyme, 0.5 mM phenylmethanesulfonyl fluoride (PMSF, from 50 mM stock dissolved in 100 % (v/v) iso-propanol) and 100 mM PA (from 1 M stock

dissolved in 100 % (v/v) methanol)

- Ni-NTA resin (GIBCO BRL)
- Buffers A, B and C (pH 8.5) (refer to Table 2.2)
- MgCl₂

Table 2.2 Formulation of Buffers A, B and C used in the Affinity Chromatography.

Component	Concentration (mM)		
	Buffer A	Buffer B	Buffer C
Tris-HCl	20	20	20
KCl	100	1	100
Imidazole	20	-	100
2-mercaptoethanol	10	10	10
PMSF	0.5	0.5	0.5
PA	10	10	10
Glycerol	10 % (v/v)	10 % (v/v)	10 % (v/v)

Induced His-tagged proteins were purified by metal-chelate affinity chromatography essentially according to the instructions supplied with the pPROEXTM-1 Vector Protein Expression System (Life Technologies).

An aliquot (20 ml) of overnight culture (Section 2.1.3) was used to inoculate 2 L of fresh LB broth and incubation at 37°C continued until an A_{595 nm} of 0.6 was obtained. IPTG was then added to the culture at the final concentration of 0.6 mM and the culture was grown at 27°C for a further 5 h. Cell pellets were resuspended in the lysis buffer containing 0.5 % (w/v) SDS and the mixture was incubated for 40 min at 4°C with gentle stirring. MgCl₂ was added to the lysate to a final concentration of 10 mM followed by incubation for a further 20 min. The lysate was then sonicated for four periods of 15 seconds with 30 second intervals. Unbroken cells and cell debris were removed by centrifugation at 30,000 x g for 20 min at 4°C. The resulting supernatant

(protein sample) was then gently loaded onto the top of a 2 ml Ni-NTA column pre-equilibrated with 10 column volumes of Buffer A at a flow rate of 0.5 ml/min. The column was then washed with 20 column volumes of Buffer A, two column volumes of Buffer B and then two column volumes of Buffer A. Protein absorbed to the column was eluted with 10 column volumes of Buffer C at a flow rate of 0.5 ml/min. The fusion proteins in the fractions eluted from the column were identified by protein assay (Section 2.2.10). Fractions containing TR-ACO2 or TR-ACO3 fusion proteins were then pooled and protein content assayed again (Section 2.2.10). Proteins in the purified samples were separated by SDS-PAGE analysis (Section 2.2.11). After use, the Ni-NTA resin was washed and recharged immediately according to the Manufacturer's instructions.

2.1.5 Activity Assay of TR-ACO2 and TR-ACO3 Recombinant Proteins

Materials

- Catalase (Sigma)
- Bovine serum albumin (BSA) (Fraction V, standard grade, Serva Feinbiochemica, Heidelberg, Germany)

The concentrated fusion protein preparations were exchanged immediately (Section 2.2.6.3.2) into enzyme activity assay buffer (Section 2.2.8) until pH 7.5 was reached. Catalase (500 µg/ml) and BSA (100 µg/ml) were added to the reaction mixture and ACC oxidase activity assayed as described in Section 2.2.8.

2.2 Purification and Characterisation of ACC Oxidase from Leaves of White Clover

2.2.1 Chemicals

Unless otherwise stated, chemicals and reagents used in this section were of analytical grade supplied mainly by either Sigma or BDH (Table 2.1). Water used in all purification steps was Milli-Q water (Section 2.1.1). Solutions and buffers were made using the water unless otherwise stated and stored at either 4⁰C or room temperature as required.

2.2.2 Growth of White Clover Plants and Sampling of Leaves

2.2.2.1 Plant Materials and Growth Conditions

White clover (*Trifolium repens* L., Figure 2.2) cultivar Grasslands Challenge, genotype 10F (AgResearch Grasslands, Palmerston North, New Zealand) was grown in a greenhouse in horticultural grade bark/peat/pumice (Dalton Nursery Mix, Tauranga, New Zealand) mixed in a ratio of 50 : 30 : 20 with the addition of nutrients (Table 2.3) at the Plant Growth Unit, Massey University, Palmerston North, New Zealand. The greenhouse was temperature-controlled to provide a minimum temperature of 12°C (night time) and 18°C (day time) with venting at 25°C. Plants were automatically irrigated for 5 min at 10 am and 5 pm using a timer-controlled mist watering system (Automation Services Ltd., Auckland, New Zealand). Aphids and white fly were controlled by spraying with the insecticide Attack[®] (Crop Care Holdings Ltd., Richmond, Nelson, New Zealand) and blackspot and mite controlled by Benlate (Du Pont de Nemours and Co., In., Wilmington, Delaware, USA).

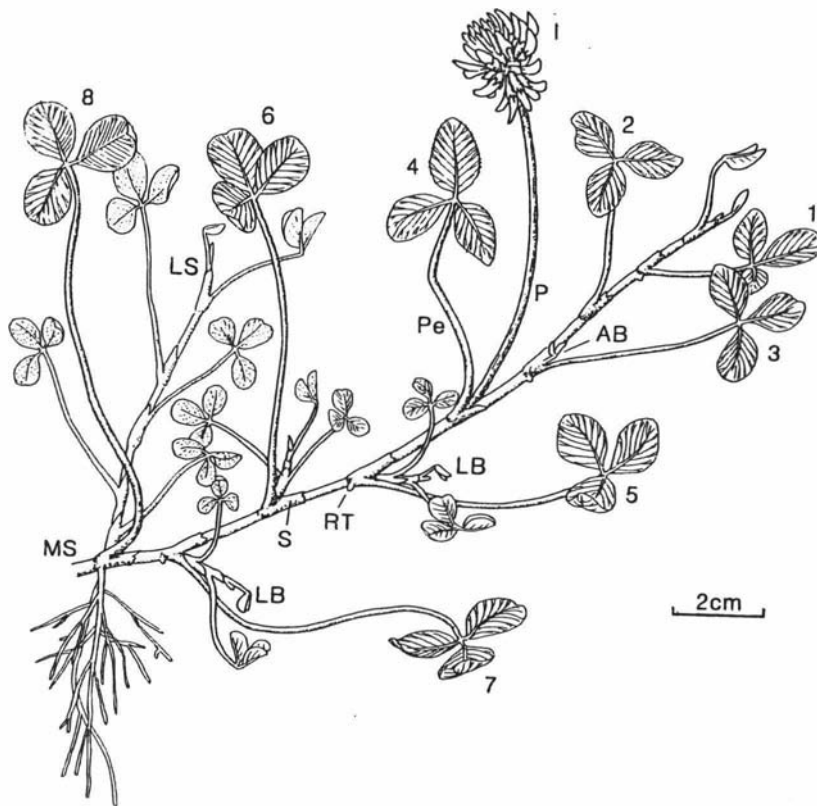


Figure 2.2 A Typical Mature Stolon of White Clover Plant.
(from Thomas, 1987)

AB = axillary bud, I = inflorescence, LB = lateral branches, LS = lateral stolon,
MS = main stolon, P = peduncle, Pe = petiole, RT = root primordium.

Table 2.3 Nutrients Added to Long Term Horticultural Grade**Bark Base** (Dalton, Mt. Manganui, New Zealand).

Ingredient	Concentration (g/L)	Supplier
Dolomite	3.0	Hodder Toley, Palmerston North, New Zealand
Agricultural lime	3.0	Hodder Toley, Palmerston North, New Zealand
Iron sulphate	0.5	Hodder Toley, Palmerston North, New Zealand
Osmocote	50.0	Carran Agencies, Hamilton, New Zealand

2.2.2.2 Propagation of Stock Plants and Initiation of the Plant Growth Model System

To maintain stock plants, apical cuttings with three to four leaves were grown in trays filled with the nutrient-horticultural bark as described in Section 2.2.2.1. During the course of experiments, approximately 10 trays (50 cm x 30 cm) of stock plants were continuously maintained with new apical cuttings used to obtain fresh stock plants.

To obtain the model growth system of single stolons (Butcher, 1997; Hunter, 1998), apical cuttings with the first four to five nodes and one to two major roots included were taken from the stock plants. All leaves except the youngest fully emerged leaf were then excised at the junction of the petiole and stolon. The apical cuttings were then planted along the central line of each tray with six plants per tray and the trays maintained in the greenhouse. Stolons were tied down by plastic coated wire to ensure that they grew straight and all axillary shoots and buds, flowers and unhealthy leaves were routinely (at weekly intervals) removed using sharp scissors. Stolons were allowed to grow until leaves at different developmental stages from initiation through immature green to mature green to senescence were obtained (usually at least 10 weeks).

2.2.2.3 Sampling of Leaves

Healthy, young (immature) green leaves (nodes 1 to 3) and mature green leaves (nodes 4 to 9) were taken from both stock plants and the model growth system plants while senescent leaves (nodes 12 to 16) were sampled only from the model growth system plants. Leaves 4 to 9 and leaves 12 to 16 were designated mature green and senescent leaves, respectively as determined both visually and by quantification of chlorophyll loss (Hunter *et al.*, 1999). Five to six days before sampling, the stolons were pruned and underwent no further manipulation until harvesting. Leaves were excised with sharp scissors at the junction of the lamina and petiole, placed as pooled leaves into 50 ml Nunc™ centrifuge tubes (Nalge Nunc International, Naperville, USA), snap frozen immediately in liquid nitrogen and were either used immediately after harvesting or stored in these tubes at -80°C until use.

2.2.2.4 Wounding Treatment for Mature Green Leaves

Trays of stock plants were equilibrated for 24 h in a constant temperature (20°C) room as were three 9-L polystyrene containers containing moistened paper towels. Mature green leaves were then excised from the stock plants, wounded by making approximately 4 mm wide parallel excisions along the lamina and immediately placed onto the moistened paper towels. The wounded leaves were incubated at 20°C for 3 h and 6 h, respectively, then snap-frozen in liquid nitrogen and stored at -80°C until use.

2.2.3 Extraction of ACC Oxidase

A summary of the various sequential steps in the purification scheme used in this thesis is shown in Figure 2.3. All solutions and equipment coming into contact with the plant extract were pre-chilled and all centrifugation and other steps were performed on ice or at 4°C. ACC oxidase extraction from white clover leaf tissues was carried out using a procedure modified from Kuai and Dilley (1992) in apple fruit and McGarvey and Christoffersen (1992) in avocado fruit.

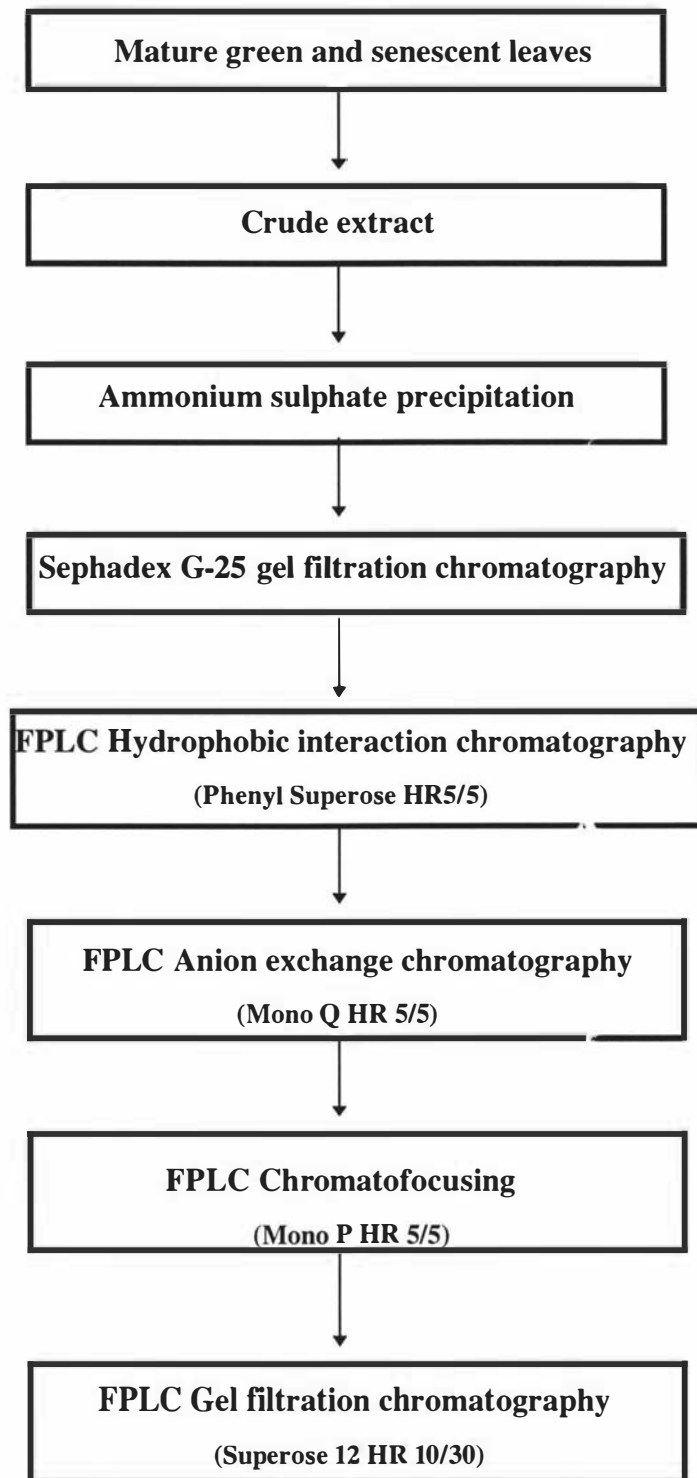


Figure 2.3 Diagram Summarising the Purification Procedure of ACC Oxidase from Mature Green and Senescent Leaves.

Materials

- Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA, USA)
- Suprafuge 22 (Heraeus Sepatech GmbH, Am Kalkberg, Germany)
- Sephadex G-25^R resin (Pharmacia Biotech)
- 1 M Tris-HCl, pH 7.5 (kept at 4°C)
- 1 M sodium ascorbate (freshly made, pH 5.9 to 6.0, adjusted with saturated NaOH solution)
- 1 M DTT (stored aliquoted at -20°C for one month)
- 100 % (v/v) glycerol
- 1 mM PA stock (stored at -20°C)

Approximately 300 g of leaf tissue was frozen in liquid nitrogen and ground quickly to a fine powder (no visible granular material) with a large pre-cooled mortar and pestle. The ground powder was added to three volumes (v/w) of ice-cold extraction buffer consisting of 100 mM Tris-HCl (pH 7.5, at 4°C), 10 % (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT and 10 µM PA (both DTT and PA were added to the buffer immediately prior to use) and incubated on ice with gentle stirring for 40 min. The homogenate was then filtered through double layers of Miracloth with gentle squeezing and centrifuged at 26,000 × g for 30 min at 4°C. The resulting supernatant ('crude extract') was used for further purification.

2.2.4 Ammonium Sulphate Salt Precipitation

Salt precipitation of proteins by increasing ionic strength and decreasing solubility of protein ('salting-out' effect) utilises the hydrophobic property of protein (Harris, 1989). It can reduce the total volume of the protein preparation as well as aiding purification. In this study, total protein in the crude extract was fractionated using between 30 % and 90 % saturated ammonium sulphate.

Materials

- Ammonium sulphate
- Resuspension buffer: 50 mM Tris-HCl, pH 7.5 (at 4°C), containing 10 % (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT and 10 µM PA

After the volume of the resulting crude extract (Section 2.2.3) was measured, the crude extract was transferred to a clean pre-cooled beaker and fine ammonium sulphate powder added slowly to make 30 % (w/v, 164 g/L) saturation. The extract was incubated on ice with stirring (used a magnetic follower and stirrer) for 40 min, and then centrifuged at 26,000 x g for 20 min at 4°C to pellet nucleic acid and residual cellular debris. The supernatant was then transferred to a clean pre-cooled beaker and ammonium sulphate powder was added slowly to make the solution 90 % (w/v, 402 g/L) saturated. The extract was incubated on ice with stirring for approximately 1.5 h until all ammonium sulphate was dissolved and the precipitated proteins collected by centrifugation at 26,000 x g for 30 min at 4°C. The supernatant was discarded and the protein precipitate finally resuspended in a minimal volume of pre-cooled resuspension buffer (approximately 30 ml).

2.2.5 Sephadex G-25 Gel Filtration Chromatography

Sephadex G-25 gel filtration chromatography was performed using a method modified from that described by Neal and Florini (1973).

Materials

- Syringe barrel (Becton-Dickerson, Lincoln Park, NJ, USA)
- Sephadex^R G-25 resin (Pharmacia Biotech)
- 15 ml Microsep or Centriprep-10 centrifugal concentrators (10 kDa cut-off, NANOSEPTM, Pall Filtron Corporation)
- GF-A glass microfibre filter paper (Whatman International, Maidstone, England)

Columns were routinely prepared using 50 ml syringe barrels. Glass fibre discs were cut and placed in the bottom of the barrels and the barrels were filled with a slurry of clean Sephadex G-25 resin. The protein suspensions from Section 2.1.3 or Section 2.2.4 (approximately 25 % (v/v) of total column volume) was carefully loaded onto the column pre-equilibrated either with the lysis buffer with no detergent added (Section 2.1.3) or the resuspension buffer (Section 2.2.4) and allowed to pass through the column at 4°C. Approximately one column volume of the resuspension buffer was used

to elute the protein at a flow rate of 4 ml/min. The eluted protein was collected as 2 ml fractions and detection of protein carried out using the Bradford method (Section 2.2.10.1).

Fractions containing eluted protein were pooled and concentrated using Microsep or Centriprep-10 centrifugal concentrators. This was undertaken by centrifuging protein samples at 3,000 x g for several hours at 4°C, and protein content quantified (Section 2.2.10.1) until the appropriate concentration was obtained. The concentrated protein samples were then aliquotted into either 1.5 ml-microtubes (for samples to be loaded onto the Phenyl Superose HR 5/5 column) or 15 ml-plastic tubes (for samples to be loaded onto the Phenyl Sepharose HiLoad 26/10 column or Ni-NTA column) and kept at -20°C until use.

2.2.5.1 Cleaning of Sephadex G-25 Resin

Once used, the Sephadex G-25 resin was immediately placed into a clean beaker, washed with 0.2 N NaOH solution followed by Milli-Q water several times until clean and then stored at 4°C until use.

To retain enzyme activity as long as possible, all steps including protein extraction, ammonium sulphate precipitation, Sephadex G-25 gel filtration chromatography and concentration of protein preparations were performed within one day, and the concentrate stored at -20°C overnight for further purification next day.

2.2.6 Fast Protein Liquid Chromatography (FPLC)

Buffers and solutions used in all FPLC steps were filtered through a 0.20 µm hydrophilic polypropylene membrane filter (GelmanSciences, Ann Arbor, Michigan, USA) immediately prior to use. Protein samples were either filtered through a 0.22 µm nitrocellulose filter (Section 2.1.1) or centrifuged at 10,000 x g for 20 min at 4°C immediately prior to being loaded onto each column. All FPLC buffers contained only 5.0 % (v/v) rather than the commonly used 10 % (v/v) glycerol to reduce the back pressure of the columns.

2.2.6.1 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) separates proteins on the basis of their different surface hydrophobic interactions with the matrix which results from the non-polar external amino acid side chains on the protein surface (Roe, 1989). The hydrophobicity of the matrix of the column is due to either alkyl or aryl functional groups that are covalently linked to an inert matrix such as Superose or Sepharose. The binding of protein molecules to these groups requires the presence of high concentrations of salt. Salt ions decrease the availability of water molecules to the protein, removing ordered water from hydrophobic patches on the protein surface and therefore increasing the hydrophobic interaction between the hydrophobic patches on the protein and the groups on the resin. Proteins are eluted by decreasing the salt concentration in the buffer.

In this thesis, HIC was used as the first FPLC step in the ACC oxidase purification. The columns used were Phenyl Superose and Phenyl Sepharose (Table 2.4), connected to a Pharmacia FPLC system equipped with a single path UV-1 monitor, chart recorder and fraction collector (Pharmacia Biotech).

Table 2.4 Supports used in FPLC.

Chromatographic Method	Trade Name (Manufacturer)	Matrix Base
Hydrophobic interaction	Phenyl Superose HR5/5 (Pharmacia Biotech)	Cross-linked agarose
	Phenyl Sepharose High Performance HiLoad 26/10 (Pharmacia Biotech)	Cross-linked agarose
Anion exchange	Mono Q HR 5/5 (Pharmacia Biotech)	Beaded hydrophilic polyether resin
	Resources Q HR 5/5 (Pharmacia Biotech)	Beaded hydrophilic polyether resin
Chromatofocusing	Mono P HR 5/5 (Pharmacia Biotech)	Beaded hydrophilic polyether resin
Gel filtration	Superose 12 HR 10/30 (Pharmacia Biotech)	Cross-linked agarose

Materials

- Phenyl Superose HR 5/5 prepacked column (Pharmacia Biotech)
- Phenyl Sepharose High Performance HiLoad 26/10 prepacked column (Pharmacia Biotech)
- Centriprep-10 centrifugal concentrator (molecular mass cut-off: 10 kDa, Amicon Inc., Beverly, MA, USA)
- Buffer A: 50 mM Tris-HCl, pH 7.5, containing 5.0 % (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT, 10 μ M PA and 2.0 M ammonium sulphate (FPLC grade)
- Buffer B: Buffer A with ammonium sulphate omitted

A Phenyl Superose HR 5/5 column (bed volume 1 ml) or a Phenyl Sepharose HiLoad 26/10 column (bed volume 53 to 58 ml) was equilibrated with Buffer A (kept chilled on ice). Approximately 9.5 mg (for the Phenyl Superose HR5/5 column) or 200 mg (for the Phenyl Sepharose HiLoad 26/10 column) of total protein extract was adjusted to 2.0 M ammonium sulphate by dissolving the appropriate amount of ammonium sulphate in the protein extract and then loaded onto each column. For the Phenyl Sepharose column, *ca.* 8 ml of the protein solution was injected two to three times onto the column with an 8 ml-loop, allowing the column to equilibrate between each injection. Bound protein was eluted automatically using a linear reverse gradient of ammonium sulphate from 100 % Buffer A : 0 % Buffer B (kept chilled on ice) to 0 % Buffer A : 100 % Buffer B at a flow rate of 0.5 ml/min for Phenyl Superose and 5 ml/min for the Phenyl Sepharose column. Fractions (0.8 ml from the Phenyl Superose column or 16 ml from the Phenyl Sepharose column) were collected using an automatic fraction collector in tubes placed on ice, and samples (0.2 ml) taken from all fractions were assayed for ACC oxidase activity *in vitro* (Section 2.2.8) as soon as possible. ACC oxidase purity was assessed by western analysis using TR-ACO1, TR-ACO2 and TR-ACO3 polyclonal antibodies as appropriate (Section 2.2.12), and protein content in specific fractions determined using the Bradford method (Section 2.2.10.1). Protein elution profiles were monitored spectrophotometrically during chromatography at 280 nm. The most active fractions were pooled and concentrated using a Centriprep-10 centrifugal concentrator (Section 2.2.5) to an appropriate volume.

2.2.6.1.1 Column Cleaning

A) Phenyl Superose HR 5/5 Column

Materials

- 40 % (v/v) acetic acid
- 100 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid (TFA)
- 0.1 M NaOH

a) Regular cleaning

The column was washed with 100 % Buffer B (Section 2.2.6.1) immediately after each separation until the base-line was stable, and then equilibrated with at least 10 column volumes of 20 % (v/v) acetonitrile solution before storage at room temperature (20°C).

b) Rigorous cleaning

Before cleaning, the top filter (Filter HR5) was changed by following the instructions supplied with the product (Pharmacia Biotech) and the pressure limit control was set at 2.0 MPa. Then each wash step below (at least 10 column volumes of each solution) was carried out in sequence until the base-line became stable. After washing, the column was equilibrated with at least 10 column volumes of 20 % (v/v) acetonitrile before storage.

- Wash with distilled water
- Wash with 40 % acetic acid
- Wash with distilled water
- Wash with 100 % acetonitrile containing 0.1 % (v/v) TFA
- Wash with distilled water
- Wash with 0.1 M NaOH

B) Phenyl Sepharose HiLoad 26/10 Column

Materials

- 1 M NaOH
- 30 % (v/v) and 20 % (v/v) acetonitrile

The column was cleaned by passing at least four column volumes of 1 M NaOH through the column at a flow rate of 2 ml/min to remove most proteins non-specifically adsorbed to the gel and at least four column volumes of 30 % (v/v) acetonitrile to remove hydrophobic proteins, lipoproteins and lipids. After cleaning, the column was immediately equilibrated with two column volumes of filtered water and at least two column volumes of 20 % (v/v) acetonitrile at a flow rate of 4 ml/min before storage.

2.2.6.2 Anion Exchange Chromatography

Ion exchange chromatography separates proteins on the basis of the net overall charge on the surface of protein molecules at a given pH due largely to the side chains of acidic and basic amino acids. The functional ligands on the matrix interact with proteins mainly by electrostatic attraction. Separation of proteins is achieved by their difference in equilibrium distribution between a buffered mobile phase and a stationary phase consisting of a matrix to which charged inorganic groups are attached (Roe, 1989). The bound proteins are eluted by increasing the ionic strength.

Materials

- Mono Q HR 5/5 (1 ml) and Resources Q HR 5/5 (1 ml) prepacked column (Pharmacia Biotech, Table 2.4)
- Buffer A: 50 mM Tris-HCl, pH 7.5, containing 5.0 % (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT and 10 μ M PA
- Buffer B: Buffer A, pH 7.5, containing 1.0 M NaCl (FPLC grade)

A 1 ml Mono Q HR 5/5 or Resources Q HR 5/5 strong anion exchange column was pre-equilibrated with Buffer A (kept chilled on ice). The pooled and concentrated active fractions from hydrophobic column chromatography (Section 2.2.6.1) were loaded onto the column. Bound proteins were eluted using a linear increasing gradient of sodium chloride concentration from 100 % Buffer A : 0 % Buffer B (kept chilled on ice) to 0 % Buffer A : 100 % Buffer B at a flow rate of 0.5 ml/min. Fractions (1.2 ml) were automatically collected in tubes placed on ice, and samples (0. ml) taken from eluted fractions were assayed for ACC oxidase activity (Section 2.2.8) as soon as possible. Enzyme purity was assessed by western blotting using the TR-ACO2 antibody

(Section 2.2.12). Fractions containing the highest ACC oxidase activity after anion exchange were pooled, concentrated, desalted (Section 2.2.6.3.2) and used for further purification or enzyme kinetic experiments.

2.2.6.2.1 Column Cleaning

Materials

- 2 M NaCl
- 2 M NaOH
- 75 % (v/v) acetic acid

The column was cleaned by performing the following steps in sequence (0.5 column volume of each solution) at a reversed flow rate of 0.25 ml/min. After cleaning, the column was equilibrated with at least 10 column volumes of 20 % (v/v) acetonitrile before storage.

- 2 M NaCl solution injected and then rinsed with Milli-Q water
- 2 M NaOH solution injected and then rinsed with Milli-Q water
- 75 % (v/v) acetic acid injected and then rinsed with Milli-Q water

2.2.6.3 Chromatofocusing

Chromatofocusing separates biomolecules on the basis of the pI value. This method can be considered as an extension of isoelectric focusing and ion exchange chromatography (Roe, 1989). In chromatofocusing, a pH gradient is formed by equilibrating the column with start buffer and eluting with a Polybuffer with a lower pH.

Materials

- Mono P HR 5/5 (1 ml) pre-packed column (Pharmacia Biotech, Table 2.4)
- Polybuffer 74 (sterile filtered solution, pH 4.0, Pharmacia Biotech)
- 25 mM imidazole-HCl, pH 7.5, containing 5.0 % (v/v) glycerol, 15 mM sodium ascorbate, 2 mM DTT and 10 μ M PA
- pH meter (Model 3020, Jenway, Germany)

- pH electrode (Schottgerate, Hofheim, Germany)

A 1 ml Mono P HR5/5 column was pre-equilibrated (Section 2.2.6.3.1) with 25 mM imidazole-HCl (pH 7.5, kept on ice), containing 5.0 % (v/v) glycerol, 15 mM sodium ascorbate, 2 mM DTT and 10 μ M PA. The desalted and buffer exchanged (Section 2.2.6.3.2) pooled active fractions from the Mono Q column were loaded onto this column. A pH gradient from pH 7.5 to 4.0 was generated in the column during the passage of a solution of Polybuffer 74 (pH 4.0, kept on ice) containing 15 mM sodium ascorbate, 2 mM DTT and 10 μ M PA. Bound proteins were eluted with the Polybuffer 74 at a flow rate of 0.5 ml/min and 0.8 ml-fractions were collected in tubes placed on ice. Each fraction was subjected to pH measurement, enzyme activity assay (Section 2.2.8) and western analysis using the TR-ACO2 antibody (Section 2.2.12).

The pI for each isoform was determined by pH measurement and enzyme activity assay for each fraction eluted from the chromatofocusing column. The pH measurement in the fraction with highest enzyme activity was determined to be the pI value of each isoform.

2.2.6.3.1 Column Equilibration

Materials

- 5 M NaOH
- 25 mM imidazole-HCl buffer, pH 7.5, containing 5.0 % (v/v) glycerol, 15 mM sodium ascorbate, 2 mM DTT and 10 μ M PA

One ml of 5 M NaOH solution was injected into the column, and then the column was equilibrated with 25 mM imidazole-HCl buffer (start buffer) at a flow rate of 0.5 ml/min until the pH of the eluate was the same as the start buffer (pH 7.5). Then a blank run (no protein sample loaded) was performed by eluting the column with Polybuffer 74 and the pH gradient monitored in 0.8 ml fractions.

2.2.6.3.2 Desalting and Buffer Exchange

Materials

- Macrosep concentrator (NANOSEP™, Pall Filtron Corporation)
- 25 mM imidazole-HCl buffer, pH 7.5, containing 5.0 % (v/v) glycerol, 15 mM sodium ascorbate, 2 mM DTT and 10 μM PA

The protein sample from anion exchange chromatography or fusion protein preparation (Section 2.1.5) was concentrated by centrifuging at 3,000 x g at 4°C to 1/10th of the original volume. The concentrated sample was reconstituted back to the original volume with the 25 mM imidazole-HCl (pH 7.5) or enzyme activity assay buffer (pH 7.5; Section 2.2.8). This concentration and dilution procedure was repeated at least six times.

2.2.6.3.3 Column Cleaning

Materials

- As for Mono Q column chromatography (Section 2.2.6.2.1)
- 0.5 M Na₂SO₄
- 20 % (v/v) ethanol

The cleaning procedure for the Mono P column was similar to that for Mono Q column (Section 2.2.6.2.1). Before storing the column for a long period, the column was washed through sequentially with 5 ml 0.5 M Na₂SO₄, 10 ml water and 10 ml 20 % (v/v) ethanol.

2.2.6.4 Gel Filtration Chromatography

Gel filtration or gel permeation or size exclusion chromatography separates different biomolecules based on their stoke radius which depends on the native molecular mass and overall shape of the protein (Preneta, 1989). Protein mixtures are subjected to a matrix of evenly sized beads that contain a number of pores at limited size. The larger molecules are excluded from the pores and are eluted first from the column, the medium molecules have access to a portion of the pore and move down the column at a rate

depending on their size. The smallest molecules are able to penetrate the pores and therefore are the last to be eluted from the column. The buffer usually contains a low concentration of salt to minimise interaction between the matrix and protein. A long column with a small diameter operating with a low flow rate gives better resolution of a mixture of proteins than a short column with a big diameter. The volume of the protein sample to be applied should be between 0.1 % to 1.0 % of the bed volume of the column. In this thesis, this chromatography was used as the final purification step. The column used was a Superose 12 HR10/30 column (Table 2.4) which has a 24.0 ml bed volume, 10 mm x 300 - 310 mm bed dimension, a 10 μ m particle size and a separation range from 1 to 300 kDa.

Materials

- Superose 12 HR 10/30 column (Pharmacia Biotech)
- Buffer A: 50 mM Tris-HCl, pH 7.5, containing 5.0 % (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT, 10 μ M PA and 150 mM NaCl (FPLC grade)
- Molecular Mass Protein Marker kit (MW-GF-200, Sigma) (Table 2.5)

Table 2.5 Molecular Masses of Protein Standards.

Protein Marker	Molecular Mass (kDa)
Blue Dextran 2000	2,000
β -amylase	200
Alcohol dehydrogenase	150
Bovine serum albumin	66
Carbonic anhydrase	29
Cytochrome C	12.4

In this chromatography, interaction of packing materials with protein and protein-protein interactions, including ion, hydrophobic and other non-specific interaction could affect the separation. To minimise these effects, different ionic strength buffers (50, 150 and 250 mM NaCl in Buffer A) were tested in preliminary experiments with 150 mM

NaCl in Buffer A resulting in good separation. Therefore, a Superose 12 HR 10/30 column (25 ml) was pre-equilibrated with at least 50 ml Buffer A containing 150 mM NaCl (pH 7.5) (kept on ice) at a flow rate of 0.2 or 0.3 ml/min. The concentrated protein preparation (220 μ l to 240 μ l) from the Mono P column was applied onto the column. Protein was eluted using the same buffer (kept on ice) at a flow rate of 0.3 ml/min and fractions (0.66 ml) were collected in tubes placed on ice. Each fraction was assayed for enzyme activity (Section 2.2.8) and enzyme purity assessed by western blotting using the TR-ACO2 antibody (Section 2.2.12).

Gel filtration chromatography is an alternative to SDS-PAGE analysis for the determination of molecular mass of protein. The elution volumes of globular proteins are mainly determined by their hydrodynamic radius which is related to the molecular mass. Therefore, the elution volume is an approximately linear function of the logarithm of molecular mass (M_r) ($\text{Log}(M_r)$). In this study, the native molecular mass of each active isoform was determined using size-exclusion chromatography on the Superose 12 HR 10/30 column.

To determine the native molecular mass of each isoform, protein markers of different sizes (Table 2.5) were chromatographed separately through the column and the elution volume (V_e) of each protein determined by its absorbance at 280 nm ($A_{280 \text{ nm}}$). The protein markers were dissolved in Buffer A at following concentration: bovine serum albumin (10 mg/ml), alcohol dehydrogenase (5 mg/ml), β -amylase (4 mg/ml), carbonic anhydrase (3 mg/ml) and cytochrome C (2 mg/ml). One hundred μ l of the protein marker solution was applied separately to the column. Column chromatography was carried out as described above, and V_e determined by measuring the volume of eluate collected from the point of sample application to the centre of the eluate peak.

Blue Dextran 2000 (*ca.* 2,000 kDa) was used to determine the void/exclusion volume (V_o). Two hundred μ g of the Blue Dextran was dissolved in 100 μ l Buffer A, applied to the Superose 12 column and eluted with the same flow rate as the protein markers. Fractions (0.66 ml) were collected and the elution of Blue Dextran monitored at 280 nm. The V_o was determined by measuring the volume of eluate from the point of sample

application to the centre of the Blue Dextran peak. An estimate of the molecular weight of the native isoform was determined from a standard curve constructed using $\text{Log}(M_r)$ values for the calibrated marker proteins against a ratio of V_e to V_o (V_e/V_o).

2.2.6.4.1 Column Cleaning

Materials

- 50 % (v/v) acetic acid
- 20 % (v/v) ethanol
- 0.1 M NaOH

The pressure limit control of the column was set at 2.0 MPa, then the column was washed in the following sequence (one column volume of each solution) at a low flow rate of 0.1 ml/min until base-line was stable.

- 50 % (v/v) acetic acid
- Milli-Q water
- 20 % (v/v) ethanol
- 0.1 M NaOH
- Milli-Q water and then 3 x 200 μl injections of 50 % (v/v) acetic acid.

After cleaning, the column was equilibrated with at least 10 column volumes of 20 % (v/v) acetonitrile before storage.

2.2.7 Affinity Chromatography

2.2.7.1 Isolation of Immunoglobulin (IgG) from Serum

Materials

- UV-visible recording spectrophotometer (UV-160A, Shimadzu Corporation, Tokyo, Japan)
- Diethylaminoethyl (DEAE) Sephacel resin (Pharmacia Biotech)
- Immune rabbit serum (TR-ACO2 antibody, Section 2.2.12.2)
- 50 mM sodium phosphate containing 250 mM NaCl (1 x PBS salt buffer, pH 7.4)

- Saturated ammonium sulphate solution: 80 g ammonium sulphate powder was added to 100 ml Milli-Q water, dissolved by heating and then cooled on ice (crystallised) for approximately 1 h (stored at 4°C).
- 70 mM sodium phosphate buffer (pH 6.3)

Five ml immune serum was first mixed with 5 ml 1 x PBSalt buffer, 10 ml saturated ammonium sulphate solution was then added slowly into the mixture and stirred for 30 min at room temperature. The mixture was centrifuged at 10,000 x g for 15 min at 4°C, the pellet resuspended in 5 ml of 70 mM sodium phosphate buffer (pH 6.3) and then dialysed against 2 L of 70 mM sodium phosphate buffer (pH 6.3) for 24 h at 4°C with six changes of the buffer.

The DEAE Sephacel column (bed volume 2 ml) was first equilibrated with 70 mM sodium phosphate buffer (pH 6.3) at room temperature before the dialysed serum protein sample was applied onto the column and then eluted with the same buffer. Fractions (0.5 ml) were collected and the $A_{280\text{ nm}}$ measured by UV-visible recording spectrophotometer. Fractions with an $A_{280\text{ nm}}$ of 0.40 or higher were pooled, the $A_{280\text{ nm}}$ re-measured and protein concentration determined as described in Section 2.2.10.2.

The IgG was eluted as a single asymmetric peak (Figure 2.4), and impurities were bound and retarded by the ion exchanger. Fractions (5 to 15) with high $A_{280\text{ nm}}$ were pooled (total volume 4.2 ml) and all other fractions discarded. Since the $A_{280\text{ nm}}$ of fractions 9 to 13 were above 2.0, the pool was diluted 10-fold and then the $A_{280\text{ nm}}$ remeasured. The diluted solution gave an $A_{280\text{ nm}}$ of 0.210 and the corresponding concentration of IgG was calculated as 0.294 mg/ml (Section 2.2.10.2). Therefore, the pooled fractions had a protein concentration of 2.94 mg/ml and a total of 12.4 mg IgG from the original 5 ml serum.

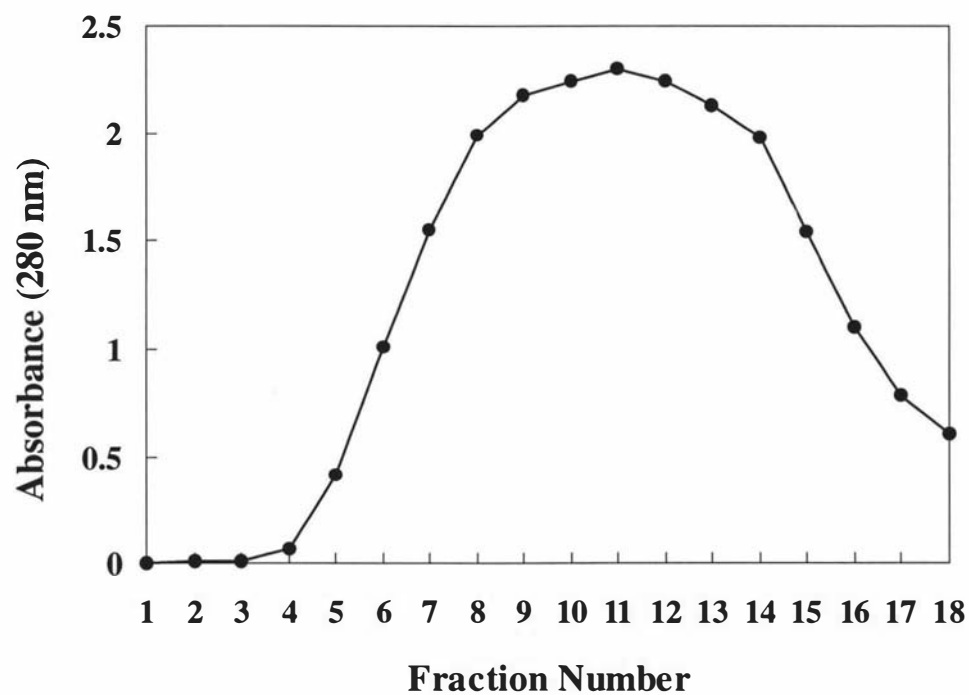


Figure 2.4 Elution of IgG from a DEAE Sephacel Column using 70 mM Sodium Phosphate buffer (pH 6.3).

2.2.7.2 Immunoaffinity Chromatography

Materials

- Dialysis membrane: Visking, Size 5, 12 kDa to 14 kDa cut-off (Medicell International Ltd), prepared by boiling in Milli-Q water for 10 min and then washing several times with Milli-Q water or prepared dialysis tubing (GIBCO BRL)
- GF-A glass microfibre filter paper (Section 2.2.5)
- Purified IgG (2.2.7.1)
- CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals Ltd., UK) (stored at 4°C)
- Coupling buffer: 100 mM NaHCO₃ (pH 8.3) containing 500 mM NaCl
- 1 x PBSalt buffer (pH 7.4) (Section 2.2.6.5.1)
- 1 mM HCl
- 100 mM Tris-HCl (pH 8.0)
- 100 mM sodium acetate-HCl (pH 4.0)
- 200 mM glycine-HCl (pH 2.5)
- 50 mM diethanolamine (DEA) (pH 11.0)

In this study, immunoaffinity chromatography was carried out essentially according to the method described by McManus and Osborne (1990) and Hill *et al.* (1989).

a) Preparing the gel for coupling TR-ACO 2 antibody

One g of freeze-dried CNBr-activated Sepharose 4B powder was suspended in 1 mM HCl by gently stirring with a spatula to ensure that all particles were dispersed. The gel was allowed to swell at room temperature for 15 min and then placed into a 15-ml syringe barrel with a microfibre glass filter paper in the bottom. The gel was washed with approximately 200 ml of 1 mM HCl and care was taken not to allow the gel to dry.

b) Coupling the antibody

The purified IgG (4.2 ml, 12.4 mg IgG) was dissolved in the coupling buffer (5 ml coupling solution/g dried powder). The coupling solution containing the IgG was gently mixed with the gel by end-over-end shaking overnight at 4°C. After the mixture was transferred to a sintered glass funnel, it was washed with 10 gel volumes of coupling buffer to remove excess ligand and then allowed to stand for 2 h in 100 mM Tris-HCl

buffer. The product was then washed with four cycles of five gel volumes of 100 mM sodium acetate-HCl buffer (pH 4.0) containing 500 mM NaCl followed by 100 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl. The gel was washed with a further three cycles of five gel volumes of 200 mM glycine-HCl (pH 2.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 100 mM NaCl followed by 50 mM DEA (pH 11.0) containing 10 mM EDTA and 100 mM NaCl. Then the column was equilibrated with 1 x PBSalt buffer (pH 7.4) at room temperature and stored at 4°C until further use.

c) Sample loading and column washing

Both native and denatured (by the addition of 1.0 % (w/v) SDS in the resuspension buffer) protein extracts, recovered from ammonium sulphate precipitation and Sephadex G-25 gel filtration chromatography from mature green leaf tissues (Sections 2.2.3, 2.2.4 and 2.2.5), were dialysed against 1 x PBSalt buffer (pH 7.4) for 24 h at 4°C with five to six changes of the buffer. The dialysate was centrifuged at 10,000 x g for 20 min at 4°C immediately prior to loading onto the column and the sample was then incubated at room temperature. The flow rate was adjusted to be as slow as possible to allow all antigen (ACC oxidase protein) molecules to pass through the column to saturate all available antibody binding sites. The eluate from the column was collected and re-loaded onto the column for further four times. To remove all non-specifically bound proteins prior to elution, the affinity column with the antigen bound was washed by passing at least 20 gel volumes of 1 x PBSalt buffer (pH 7.4) through the column several times until the $A_{280\text{ nm}}$ of the eluate reached zero.

d) Elution

To elute the bound ACC oxidase protein from the immunoadsorbent, the affinity column was washed twice with two gel volumes of 200 mM glycine-HCl buffer (pH 2.5) containing 10 mM EDTA and 100 mM NaCl, then 50 mM DEA (pH 11.0) containing 10 mM EDTA and 100 mM NaCl and then 1x PBSalt buffer (pH 7.4). The eluate (approximately 5.0 ml) from each buffer wash was collected and the $A_{280\text{ nm}}$ measured. The eluates with higher $A_{280\text{ nm}}$ were then pooled, concentrated and assayed for protein concentration (Section 2.2.10.2). Proteins eluted from the gel were subjected to SDS-

PAGE analysis (Section 2.2.11) and western analysis using the TR-ACO2 antibody (Section 2.2.12).

e) Column regeneration

The affinity medium was regenerated for re-use by washing the gel with three cycles of three column volumes of 100 mM Tris-HCl (pH 8.5) containing 500 mM NaCl, and then 100 mM sodium acetate (pH 4.5) containing 500 mM NaCl followed by re-equilibration in 1x PBSalt buffer (pH 7.4).

2.2.8 Assay of ACC Oxidase Enzyme Activity *in vitro*

ACC oxidase activity *in vitro* was determined by a modified method from Hunter (1998). The assay measures the enzyme activity by quantifying ethylene produced from the substrate ACC in the presence of co-substrate sodium ascorbate and the cofactors NaHCO₃ and FeSO₄.

Materials

- Heating blocks (Grant Instruments (Cambridge) Ltd., Herts, England)
- 1 ml syringes (Becton Dickinson) and needles (Precision Glide 25 G1(0.5 mm x 25 mm), Becton Dickinson, Rutherford, NJ, USA)
- Orbital shaker (Watson Victor Ltd., New Zealand)
- 4.5 ml Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA)
- Buffer A (2 x stock): 100 mM Tris-HCl, pH 7.5, containing 20 % (v/v) glycerol (stored at 4°C)
- 1 M DTT stock (stored at -20°C)
- 40 mM ACC (stored at -20°C)
- 0.667 mM FeSO₄·7 H₂O (made fresh)
- 1 M sodium ascorbate (made fresh, pH 5.9 to 6.0)
- 1 M NaHCO₃ (made fresh)
- 1 mM PA stock (stored at -20°C)
- 50 mM PMSF stock

A standard reaction mixture was made for the appropriate numbers of sample. For 20 assays, the composition of reaction mixture was shown in Table 2.6. After all components were mixed (ACC was added to the mixture just prior to assay), the pH of the mixture was re-adjusted to 7.5 with HCl. The final concentrations of the components in the reaction mixture (after the enzyme preparation is added) are also shown in Table 2.6. To assay ACC oxidase enzyme activity, 0.8 ml aliquots (in triplicate) of the reaction mixture were pipetted into 4.5 ml Vacutainer tubes and then put into a heating block at 30°C on an orbital shaker for 7 min. Triplicate 0.2 ml enzyme preparations were aliquotted into microfuge tubes and equilibrated in another heating block for 1 min at 30°C. To perform the reaction, 0.2 ml of the pre-equilibrated enzyme samples were mixed with 0.8 ml of the pre-equilibrated reaction mixture. The Vacutainer tubes were then sealed immediately and shaken at 200 rpm for 20 min within which a linear enzyme activity was observed. After which, 1 ml gas samples were withdrawn with an air tight syringe from the head space of the reaction tubes and stored with the needle tip embedded in rubber until injection into either a Photo Vac 10S 50 (Section 2.2.9.1) or Varian 3400 Gas Chromatograph (Section 2.2.9.2) for ethylene analysis.

To determine the stability of ACC oxidase activity *in vitro*, the enzyme extract from mature green leaves after Sephadex G-25 column chromatography was maintained at 20°C for 0 to 4 h or at 4°C for 0 to 12 h and the enzyme activity *in vitro* assayed as described above. To determine the effect of two protease inhibitors on the ACC oxidase activity *in vitro*, PA or PMSF was added into 0.2 ml senescent leaf enzyme extract recovered from Sephadex G-25 column at the final concentrations of 10 µM and 1 mM, respectively. The treated enzyme samples and control (containing no added protease inhibitor) were incubated at 4°C for 0 to 14 h under aerobic conditions, and then enzyme activity assayed as described above.

Table 2.6 Formulation of Standard Reaction Mixture for Assay of ACC Oxidase Activity *in vitro*.

Component	Final Concentration (mM)	Amount for 20 Assays (ml)
Buffer A (2 x):	-	10
Tris-HCl	50	-
Glycerol	10 % (v/v)	-
1M DTT	2	0.04
40 mM ACC	1	0.5
0.667 mM FeSO ₄ ·7 H ₂ O	0.02	0.6
1M sodium ascorbate	30	0.6
1M NaHCO ₃	30	0.6
Water	-	3.66

2.2.8.1 Determination of pH Optimum

Partially purified isoform preparations (after Mono Q anion exchange chromatography step) were used to determine pH optimum, apparent K_m and V_{max} values for ACC and optimal requirements for co-substrate and cofactors. For the determination of the pH optimum, the 50 mM Tris-HCl buffer in the activity assay mixture was replaced by 50 mM sodium acetate-HCl (for pH 4.0, 4.5, 5.0, 5.5 and 6.0), 50 mM 3-[N-Morpholino]propanesulphonate (MOPS)-NaOH (for pH 6.5, 7 and 7.5), 50 mM Tris-HCl (for pH 7.0, 7.5, 8.0, 8.25, 8.5, 8.75 and 9.0) and 100 mM phosphate (K₃PO₄-H₃PO₄) buffer (for pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0). The pH of each solution was further checked once all components of the reaction mixture had been added and adjusted if necessary. The enzyme activity of each isoform preparation over the pH range was assayed as described above. All buffers were stored at 4°C and pH checked prior to being used.

2.2.8.2 Determination of K_m and V_{max}

The values of apparent K_m and V_{max} for the substrate ACC were determined by triplicate measurements of initial velocity for different ACC concentrations. The initial velocity data against ACC concentrations were graphed using a computer programme ENZYLOT (Walker, 1997). The saturating ACC concentration for each isoform was determined from the graph. Eadie-Hofstee regression was used to fit the data in a defined concentration range to a straight line and K_m and V_{max} values were determined from the regression equation. The range of ACC concentrations used in this thesis was determined from preliminary experiments in which different ranges of concentrations of ACC were tried initially. For isoform MGI, the concentrations of ACC were 0, 0.02, 0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 6, 8, 10 mM, respectively. In preliminary experiments, the ACC concentrations gave a very low ACC oxidase activity for isoform SEII and so for SEII the concentrations of ACC were increased to 0, 0.08, 0.2, 0.4, 0.8, 2, 4, 6, 8, 10 mM, respectively. The activity assay was performed at the optimal pH for each isoform as described previously.

2.2.8.3 Optimal Requirements for Co-substrate and Cofactors

The optimal requirements of each isoform for co-substrate and cofactors were determined at their optimal pHs and saturating ACC concentrations. The different concentrations used in this study for sodium ascorbate were 0, 1, 2, 4, 8, 12, 16, 20, 24, 32 and 40 mM, respectively; for sodium bicarbonate were 0, 1, 2.5, 5, 10, 15, 20, 25, 30, 35 and 40 mM, respectively; for $FeSO_4$ were 8, 12, 16, 20, 24, 28, 32, 36, 40 and 44 μM , respectively. Activity assays were carried out as described above with the various concentrations of sodium ascorbate, sodium bicarbonate or $FeSO_4$ substituting as appropriate.

2.2.8.4 Determination of Relative Abundance of Enzyme Isoforms

To analyse the relative abundance of two ACC oxidase isoforms, the partially purified protein preparations, recovered from the Phenyl Superose column from 9 mg protein extracts of mature green and senescent leaves, were subjected to activity assay at the optimal pH and saturating ACC concentration for each isoform as described above.

2.2.9 Ethylene Analysis

Materials

- Photo VacTM 10S 50 (Photo VacTM, Markham, Ontario, Canada)
- Varian 3400 Gas Chromatograph (Varian Institute Group, Walnut Creek Division, 2700 Mitchell Drive Walnut Creek, California, USA)
- Hewlett Packard 3390A Integrator (Hewlett Packard Company, Avondale, PA, USA).
- Ethylene standard (0.101 ppm in nitrogen, BOC Gases (New Zealand) Ltd.)

2.2.9.1 Measurement of Ethylene by Gas Chromatography using a Photo Vac 10S 50

The concentration of ethylene produced by ACC oxidase was analysed using a portable Photo VacTM 10S 50 equipped with a photoionisation detector and an external peak integrator (Hewlett Packard 3390A). An external carrier gas (clean dry air at 40 psi) was passed through a column of PurafilTM (Papworth Engineering, Cambridge, New Zealand) before entering the Photo Vac. An α -standard calibration gas of 0.101 ppm ethylene in nitrogen was used to calibrate the Photo Vac and recalibrated with the standard injected at least three times until a stable consistent ethylene peak was observed. The peak retention time for ethylene was observed to be between 0.78 and 0.80 min, and peak height was used for ethylene quantification. Samples (1 ml) for ethylene measurement were injected into the instrument and quantified as above.

2.2.9.2 Measurement of Ethylene by Gas Chromatography using a Varian 3400 Gas Chromatograph

The concentration of ethylene in gas sample produced by the enzyme reaction was also analysed using a Varian 3400 Gas Chromatograph equipped with an 80 % Porapak N/20 % Porapak Q column and a flame ionisation detector. At least 1 h before use, the instrument was set up as follows: Column 120°C, Injector 110°C, Auxiliary 120°C and Detector 130°C.

The carrier gas used was nitrogen with a column flow rate of 1.5 ml/min. The ethylene standard used and calibration were the same as above. Peak retention time for ethylene was observed to be between 0.70 and 0.72 min.

The concentration of ethylene was measured by Photo Vac10S 50 and Varian 3400 Gas Chromatograph as ppm ethylene in 1 ml mixed gas from the headspace of the reaction containers. The following equation was used to calculate the number of moles produced by the reaction:

$$n \text{ (mol)} = PV/RT \times \text{ppm} \times 10^{-6} \text{ (Hunter, 1998)}$$

Where: n = total number of moles

P = pressure (101325 Pa)

V = volume of head space

R = universal gas constant (8.314)

T = temperature (298 K)

In this study, the unit of ACC oxidase activity is expressed as nmol ethylene or pmol ethylene/min or h/mg protein.

2.2.10 Quantification of Protein Concentration

2.2.10.1 Bradford Method

The protein concentration in fractions during purification was measured using the Bradford microassay methods described by Bradford (1976) and Zor and Selinger (1996) with BSA as standard protein.

Materials

- Anthos HT II plate reader (Anthos Labtech Instruments, Salzburg, Austria)
- Microtitre plate (Nunc, A/S, Roskilde, Denmark)
- Protein assay reagent (BIO-RAD)
- BSA (1 mg/10 ml stock, stored at -20°C)

Samples for protein measurement were diluted as appropriate with water and 4 or 8 μ l of the diluted sample added to 156 or 152 μ l H₂O, respectively in the microtitre plate. After which, 40 μ l of the protein assay reagent was added and mixed well. After standing for 5 min, the absorbance at 595 nm ($A_{595 \text{ nm}}$) was measured using an Anthos HT II plate reader. The protein concentration of sample was determined using the protein standard curve prepared from the BSA concentrations used (Figure 2.5). Only absorbances within the linear region of the standard curve were used to calculate the protein concentration of samples.

2.2.10.2 UV Method

Material

- UV-visible recording spectrophotometer (UV-160A, Shimadzu Corporation, Tokyo, Japan)

The UV method was used to determine the IgG concentration, protein content in all fractions eluted from the immunoaffinity column and the UV absorbance of different components in FPLC buffers. The IgG concentration was determined by measuring the $A_{280 \text{ nm}}$, and then calculated by the following formula: $A_{280 \text{ nm}} 1.4 = 1.0 \text{ mg/ml}$ (McManus and Osborne, 1990).

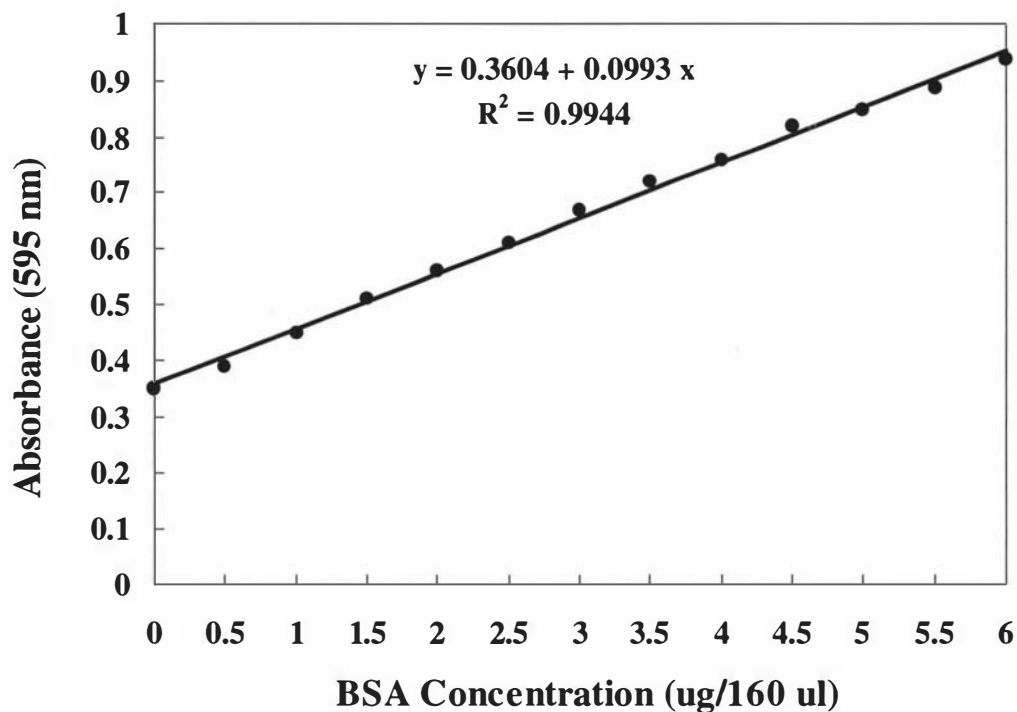


Figure 2.5 A Typical Protein Standard Curve used to Estimate Protein Concentration of Samples for the BIO-RAD Protein Microassay Procedure.

2.2.11 Electrophoresis of Protein

2.2.11.1 Linear Slab Gel SDS-PAGE

Denaturing, discontinuous electrophoresis against standard protein markers was used to separate different proteins during purification and determine the apparent molecular weights of the purified isoforms. Electrophoresis was performed in 15 % (w/v) polyacrylamide gels in the presence of 0.1 % (w/v) SDS essentially according to the method of Laemmli (1970) on a vertical mini-gel electrophoresis apparatus.

Materials

- Mini-PROTEAN® II cell (BIO-RAD)
- Power pack (either Pharmacia Biotech or BIO-RAD)
- Prestained molecular mass standards (low range, BIO-RAD, Table 2.7)
- 40 % (w/v) acrylamide-bis stock solution (BIO-RAD, stored at 4°C)
- 10 % (w/v) ammonium persulphate (APS) (Univar, Auburn, NSW, Australia)
- N, N, N', N-tetramethylethylenediamine (TEMED) (Riedel de Haen ag seelze, Hannover, Germany, stored at 4°C)
- 2 x resolving (separating) gel buffer (Table 2.8) (pH 8.8, stored at 4°C)
- 2 x stacking gel buffer (Table 2.8) (pH 6.8, stored at 4°C)
- 5 x SDS running buffer (Table 2.8) (pH *ca.* 8.3, stored at room temperature)
- 2 x SDS gel loading buffer: 24 mM Tris-HCl (pH 6.8), containing 20 % (v/v) glycerol, 5.0 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol and 0.04 % (w/v) bromophenol blue (stored at -20°C).
- Water-saturated isobutanol

A separating (resolving) gel solution (15.0 %) was made by mixing the components in the order as shown in Table 2.9. The solution was then transferred between two glass plates of a gel sandwich until the top level was approximately 1 cm below where the bottom of a well-forming comb sits. Water-saturated isobutanol was immediately layered onto the gel surface to protect from oxidation and the gel allowed to sit for at least 25 min. During this time, a stacking gel solution (4.0 %) was made up as shown in Table 2.9.

Table 2.7 Summary of the Range of Molecular Masses of Prestained Range SDS-PAGE Standards used in this Thesis.

Protein Markers	Calibrated Molecular Masses (kDa)				
	a	b	c	d	e
Phosphorylase B	104.0	103.0	104.0	111.0	111.0
Bovine serum albumin	80.0	77.0	82.0	84.0	77.0
Ovalbumin	46.9	48.0	48.3	53.2	48.2
Carbonic anhydrase	33.5	34.2	33.4	34.9	33.8
Soybean trypsin inhibitor	28.3	28.4	28.3	28.7	28.6
Lysozyme	19.8	20.5	19.4	20.5	20.5

Table 2.8 Formulation of Mini-Gel Buffer Solutions.

Component	2 x Separating Buffer	2 x Stacking Buffer	5 x SDS Running Buffer
Tris	45.0 g	6.0 g	15.0 g
Glycine	-	-	72.0 g
SDS	1.0 g	0.4 g	5.0 g
Water	400 ml	150 ml	900 ml
pH (with HCl)	8.8	6.8	8.3
Total volume ¹	500 ml	200 ml	1000 ml

¹ made up to the volume with water

Table 2.9 Separating and Stacking Gel Solutions for Using in SDS-PAGE.

Order of Addition	Components	Separating Gel (ml)	Stacking Gel (ml)
1.	40 % acrylamide-bis stock	4.0	1.0
2.	2 x Separating gel buffer (pH 8.8) (Table 2.8)	5.0	-
2.	2 x Stacking gel buffer (pH6.8) (Table 2.8)	-	5.0
3.	Distilled water	1.0	4.0
4.	10 % (w/v) APS	0.1	0.1
5.	TEMED (added immediately prior to loading)	0.01	0.01

After polymerisation of the separating gel, the layer of water-saturated isobutanol was discarded and the remaining liquid removed with 3 MM paper. The stacking gel solution was then poured on top of the separating gel and a well-forming comb placed in gently. After approximately 30 min, the gel sandwich apparatus was clamped in the Mini-PROTEANI II[®] cell, SDS running buffer poured into the chamber until the top of glass plates was covered with the buffer and then the comb removed carefully.

Protein samples containing the identical protein amount (generally 5 μ l to 12 μ l per lane) were resuspended in the same volume of 2 x SDS gel loading buffer, mixed well and placed in a boiling water bath for 5 min. Imidazole containing protein samples eluted from the Ni-NTA affinity column were heated at 37°C for 10 min instead of boiling. After both denaturation treatments, samples were centrifuged at 10,000 x g for 1 min and allowed to cool to room temperature for loading. One or two lanes were routinely reserved for loading an aliquot (6 μ l) of prestained molecular mass markers (Table 2.7). All gels were run at a constant voltage of 200 V for 90 min at room temperature.

2.2.11.2 Detection of Protein in Gels and Drying of Gels

Materials

- Coomassie Brilliant Blue (CBB) stain solution: 0.1 % (w/v) Coomassie Brilliant Blue R-250, 40 % (v/v) methanol and 10 % (v/v) acetic acid
- Destain solution: 30 % (v/v) ethanol
- Modified stain: 0.2 % (w/v) Coomassie Brilliant Blue R-250, 20 % (v/v) methanol and 0.5 % (v/v) acetic acid
- Modified destain: 30 % (v/v) methanol

After electrophoresis, the separated protein bands were detected by CBB staining. Gels were immersed in the CBB stain for 30 min with gentle shaking followed by destain solution until protein bands became visible. For protein bands used for amino acid sequencing, the bands in gels were stained with a modified stain for 20 min and destained with modified destain (Rosenfeld *et al.*, 1992) until protein bands became visible. The gels were usually photographed immediately, and then air dried by placing between two sheets of Gel Air Cellophane Support (BIO-RAD) at room temperature.

2.2.11.3 Determination of Apparent Molecular Mass by SDS-PAGE

For every gel, a lane which contained pre-stained SDS-PAGE standards (BIO-RAD, low range) was loaded alongside the protein samples. The apparent molecular masses of the isoforms were estimated by comparing the mobility of the marker proteins co-electrophoresed with the protein of interest. The distance of migration between the origin and the middle of each of the marker proteins was measured, $\text{Log}(M_r)$ and the distance of migration were used to plot a calibration curve. The size of each unknown protein was determined from the curve. A new curve was plotted for every gel run based on the migration of the SDS-PAGE standards in that gel to ensure precise comparison of different gels.

2.2.12 Western Analysis

Western analysis was carried out essentially by the method of Towbin *et al.* (1979) with modification.

2.2.12.1 Electrophoretic Transfer of Proteins from Gel to PVDF Membrane

Materials

- Mini Trans-Blot[®] Cell and Trans-Blot[®] (BIO-RAD)
- PVDF membrane (0.2 μm pore size, Immobilon-P, Millipore Corporation, Bedford, MA, USA)
- Transfer buffer (25 mM Tris, 190 mM glycine, pH 8.3 and 10 % (v/v) methanol)

After SDS-PAGE (Section 2.2.11.1), proteins separated in gels were electrophoretically transferred from the gel to a PVDF membrane using a Trans-Blot Electrophoretic Transfer Cell. The transfer cassette holder with gel and membrane was assembled as shown in Figure 2.6. The PVDF membrane was soaked in 100 % (v/v) methanol for 2 min prior to being put on top of the gel. To prevent air bubbles from interfering with transfer, the setup of the cassette was performed while immersed in transfer buffer. Transfer was carried out at either a constant 30 V for 1 h at room temperature or 20 V overnight at 4°C. Efficiency of protein transfer was checked by staining the gel with CBB stain after the conclusion of the electrophoretic transfer period.

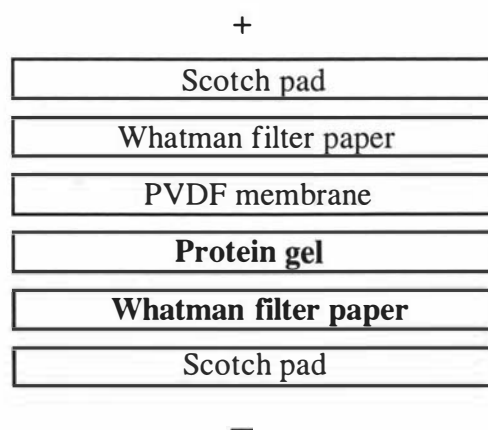


Figure 2.6 A Diagrammatic Setup for Electrophoretic Transfer of Proteins to PVDF Membrane.

2.2.12.2 Immunodetection of Proteins on PVDF Membrane

Materials

- 1 x PBSalt (pH 7.4, Section 2.2.7)
- 1 x PBST (0.05 % (v/v) Tween 20 in 1x PBSalt)
- 0.2 % (w/v) I-block (Tropix, Massachusetts, USA) (0.2 g I-block dissolved in 100 ml 1 x PBST by heating with microwave for 40 seconds)
- Immune serum (TR-ACO1, 2 and 3 antibodies, kindly provided by Dr. Donald Hunter and Sang Dong Yoo, Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand)
- Goat anti-rabbit IgG antibody (Sigma)
- Goat anti-rat IgG antibody (Sigma)
- 150 mM Tris-HCl, pH 9.7
- Alkaline phosphatase substrate solution: 150 mM Tris-HCl, pH 9.7, containing 0.01 % (w/v) 5-bromo-4-chloro-3-indoyl phosphate (BCIP), 0.02 % (w/v) ρ -nitro blue tetrazolium chloride (NBT) and 8 mM $MgCl_2$ (made fresh and stored in dark until use).

After electrophoretic transfer, the membrane was carefully peeled from the gel and placed in a suitable sized clean container, and was blocked in a 0.2 % (w/v) I-block solution for 1 h at room temperature to block unoccupied protein binding sites on the membrane. The blocking solution was then discarded and the membrane rinsed with 1 x PBST. The membrane was then incubated with a solution of either the TR-ACO2 antibody (raised in rabbits) as primary antibody at a dilution of 1 : 2000 (in 1 x PBST) or the TR-ACO1/TR-ACO3 antibodies (raised in rats) as primary antibody at a dilution of 1 : 1000 with gentle shaking at room temperature for 1 h. The antibody solution was then discarded and the membrane washed three times for 30 min with 1 x PBST. The membrane was then incubated in the secondary antibody (either goat anti-rabbit alkaline phosphatase or goat anti-rat alkaline phosphatase) at a dilution of 1 : 5000 (in 1 x PBST) at room temperature for 1 h. The membrane was then washed three times for 30 min in 1 x PBST, twice for 10 min in 150 mM Tris-HCl (pH 9.7), and the membrane was developed in dark with the addition of alkaline phosphatase substrate. When the colour associated with the band of interest had developed, the reaction was stopped

immediately by discarding the substrate solution and washing exhaustively with RO water. The membrane was then photographed as soon as possible.

2.3 Statistical Analysis

Sample means with an estimate of the standard error (SE) have been used in this thesis. Statistical tests were conducted using an Analysis of Variance (ANOVA), carried out using SAS/STAT™ (SAS Institute, Inc., Cary, NC, USA) software. A difference between means at the 5 % ($P \leq 0.05$) was considered significant with a difference at the 1 % level ($P \leq 0.01$) being highly significant.

3. Chapter Three: Results

3.1 Introduction

Two ACC oxidase genes, TR-ACO2 (expressed in mature green leaves) and TR-ACO3 (expressed in senescent leaves), have been shown to be differentially expressed during leaf ontogeny in white clover (Hunter *et al.*, 1999; McManus *et al.*, 1999). These results indicate the presence of ACC oxidase isoforms which may also display differential regulation during leaf maturation and senescence. Two approaches are used to investigate such differential regulation. One is to express TR-ACO2 and TR-ACO3 in *E. coli* and to characterise the enzyme activity that can be reconstituted. The second is to identify and purify any isoforms of ACC oxidase in leaf tissues at different developmental stages and then compare the different physicochemical and kinetic properties of each. In this thesis, both approaches were attempted.

3.2 Expression of ACC Oxidases in *E. coli* and Purification of Recombinant Proteins by Nickel Affinity Chromatography

The translated amino acid sequences from the two ACC oxidase transcripts, TR-ACO2 and TR-ACO3, have been shown to contain amino acid residues that are believed to be important for maximal enzyme activity although neither are full length cDNAs (Hunter, 1998). To investigate whether these two ACC oxidase genes encode functional proteins, the expression of the two ACC oxidase genes in *E. coli* was achieved using the pPROEXTM-1 Protein Expression System. This section describes the expression of these two truncated protein products from both TR-ACO2 and TR-ACO3 in *E. coli*, the purification of the recombinant proteins and enzyme activity assays. Because of the relevance of these reading frames to this thesis, details of the partial amino acid sequences of TR-ACO2 and TR-ACO3 translated from the pPROEXTM-1 plasmid are shown in Figure 3.1.

3.2.1 Expression of ACC Oxidases in *E. coli*

Preliminary experiments performed by Hunter (1998) established that no fusion proteins of both TR-ACO2 and TR-ACO3 were induced in two *E. coli* strains, DH 5 α .

(A)

A²⁷ C E N W G F F E L V N H G I H D L M D T V⁴⁸
 E R L T K E H Y R I C M E Q R F K L V A N K⁷⁰
 G L E A V Q T E V K D M D W E S T F H L R H⁹²
 L P E S N I S E V P D L T D E Y R K A M K E¹¹⁴
 F A L K L E K L A E E L L D L L C E N L G L¹³⁶
 E K G Y L K K A F Y G S K G P T F G T K V A¹⁵⁸
 N Y P P C P K P D L V K G L R A H T D A G G¹⁸⁰
 I I L L F Q D D K V S G L Q L L K D G K W V²⁰²
 D V P P M H H S I V I N L G D Q L E V I T N²²⁴
 G K Y K S V E H R V I A Q S D G T R M S I A²⁴⁶
 S F Y N P G S D A V I Y P A T T L I E E N N²⁶⁸
 E V Y P K F V F E D Y M N L Y A G L K F Q A²⁹²
 K E²⁹⁴

(B)

A²⁷ C Q N W G F F E L V N H G I P H D L M D T⁴⁸
 L E R L T K E H Y R K C M E Q R F K E L V S⁷⁰
 S K G L D A V Q T E V K D M D W E S F H V R⁹²
 H L P E S N I S L P D L S D E R K V M K E F¹¹⁴
 S L R L E K A E E L L D L L C E N L G L E K¹³⁶
 G Y L K K A F Y G S R G P T F G T K V A N Y¹⁵⁸
 P Q C P N P E L V K G L R A H T D A G G I I¹⁸⁰
 L L F Q D D K V S G L Q L L K D D E W I D V²⁰²
 P P M R H S I V V N L G D Q L E V I T N G K²²⁴
 Y K S V E H R V I A Q T N G T R M S I A S F²⁴⁶
 Y N P G S D A V I Y P A P E L L E K E T E E²⁶⁸
 K T N V Y P K F V F E E Y M K I Y A A L K F²⁹²
 Q A K E²⁹⁶

Figure 3.1 Deduced Amino Acid Sequences of the TR-ACO2 (A) and TR-ACO3 (B) Genes (from Hunter, 1998).

The numbers as superscript represent amino acid residue number.

and BL-21 (DE3) after incubation with IPTG, although both strains have been used in the functional expression of the enzyme from other plants (Smith *et al.*, 1994; Zhang *et al.*, 1995; Kadyrzhanova *et al.*, 1997; Lin *et al.*, 1997). However, overproduction of the enzyme was observed when another *E. coli* strain, TB-1, was used and ACC oxidase proteins of the size predicted from translation of each coding frame were obtained (Hunter, 1998). Therefore, this *E. coli* strain was used in this thesis to determine if active enzyme could be reconstituted.

The optimal time of induction with IPTG for the ACC oxidase protein in this strain of *E. coli* has previously been determined by SDS-PAGE analysis to be 3 h at 37°C (Hunter, 1998). In this thesis, the expression of TR-ACO2 and TR-ACO3 was induced with 0.6 mM IPTG at 37°C. After incubation for 3 h, the cells were harvested, pelleted and then resuspended in 2 x SDS-PAGE gel loading buffer. The cell extracts were separated by SDS-PAGE analysis and comparison of induced and uninduced cells showed that both TR-ACO2 and TR-ACO3 were overexpressed (Figure 3.2; TR-ACO3 data not shown) with an intense protein band evident at *ca.* 38.0 kDa. In preliminary experiments performed by Sang Dong Yoo (*pers. comm.*), no ACC oxidase enzyme activity was observed from the crude extract of induced protein products using these conditions. Examination of the supernatant revealed that little protein was being solubilised in the enzyme activity assay buffer. A lower incubation temperature is known to reduce the amount of insoluble heterologous protein produced in *E. coli* by reducing the amount packaged into inclusion bodies. For example, a tomato ACC oxidase protein induced by IPTG at 37°C was observed to be largely insoluble and the ACC oxidase activity of the recombinant protein was very low. By contrast, ACC oxidase induced at 27°C was more soluble and shown to be active (Zhang *et al.*, 1995).

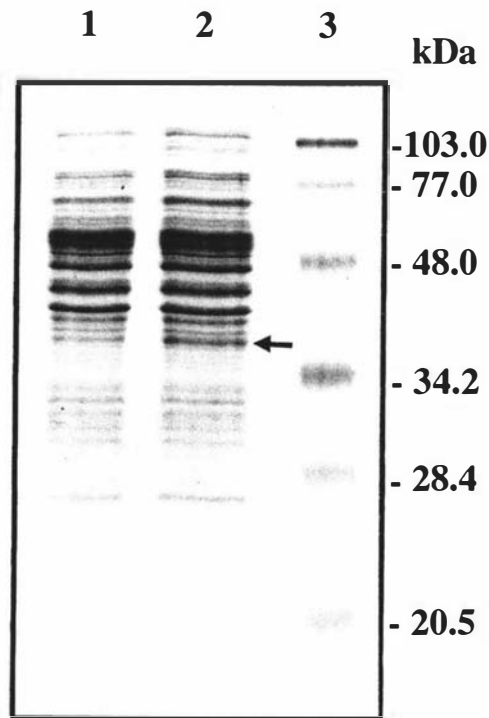


Figure 3.2 SDS-PAGE Analysis of the Expression of TR-ACO2 Recombinant Protein in *E. coli* Strain TB-1.

Lane 1: uninduced cell extract.

Lane 2: induced cell extract.

Lane 3: BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

Cells were grown at 37°C and harvested 3 h after induction with 0.6 mM IPTG.

The position of the recombinant ACC oxidase is indicated by the arrow.

3.2.1.1 Optimisation of Expression of ACC Oxidases in *E. coli*

To examine the expression of ACC oxidase in *E. coli* strain TB-1 and determine the optimal IPTG induction time at 27°C, cultures were grown at 27°C for 2, 3, 4, 5, 6 and 7 h, respectively after the addition of IPTG. The yield of induced protein was monitored by SDS-PAGE analysis. An intense band with an apparent molecular mass of *ca.* 38 kDa was observed and the level of expression after 5 h post-induction was higher than after 2 h to 4 h and did not increase in the 6 h and 7 h samples (Figure 3.3; 2 h data not shown).

The effect of different IPTG concentrations (0, 0.3, 0.6, 0.9 and 1.2 mM) on ACC oxidase induction at 27°C was examined over a 5 h post-induction period at 27°C and protein production monitored by SDS-PAGE analysis. The result showed that very little ACC oxidase protein was expressed without IPTG and increasing concentrations of IPTG increased the production of ACC oxidase protein (data not shown). An IPTG concentration ranging from 0.6 mM to 1.2 mM was found to be optimal for the expression of both TR-ACO2 and TR-ACO3 (data not shown) and so 0.6 mM IPTG was used in all subsequent induction experiments.

3.2.1.2 Effect of Concentrations of SDS and Triton X-100 on the Yield of Extracted ACC Oxidase Protein from *E. coli*

A lysis buffer containing 2.5 % (w/v) SDS has previously been shown to be able to extract fusion ACC oxidase protein from *E. coli* cells (Sang Dong Yoo, *pers. comm.*). To minimise enzyme inactivation by detergents used for lysis, buffers without SDS and with low concentrations of SDS and Triton X-100 were used and the yield of extracted protein compared. The results showed that no SDS in the lysis buffer gave a very low yield of induced proteins (Figure 3.4; TR-ACO2 data not shown), indicating that the solubility of the fusion protein is low even though the incubation temperature after IPTG addition has been lowered to 27°C. However, lysis buffers with 1.5 % (w/v) and 0.5 % (w/v) SDS or 1.5 % (v/v) and 0.5 % (v/v) Triton X-100 gave a higher protein yield (Figure 3.5; 1.5 % SDS (w/v) and Triton X-100 (v/v) data not shown) which was similar to that extracted with the lysis buffer containing 2.5 % (w/v) SDS.

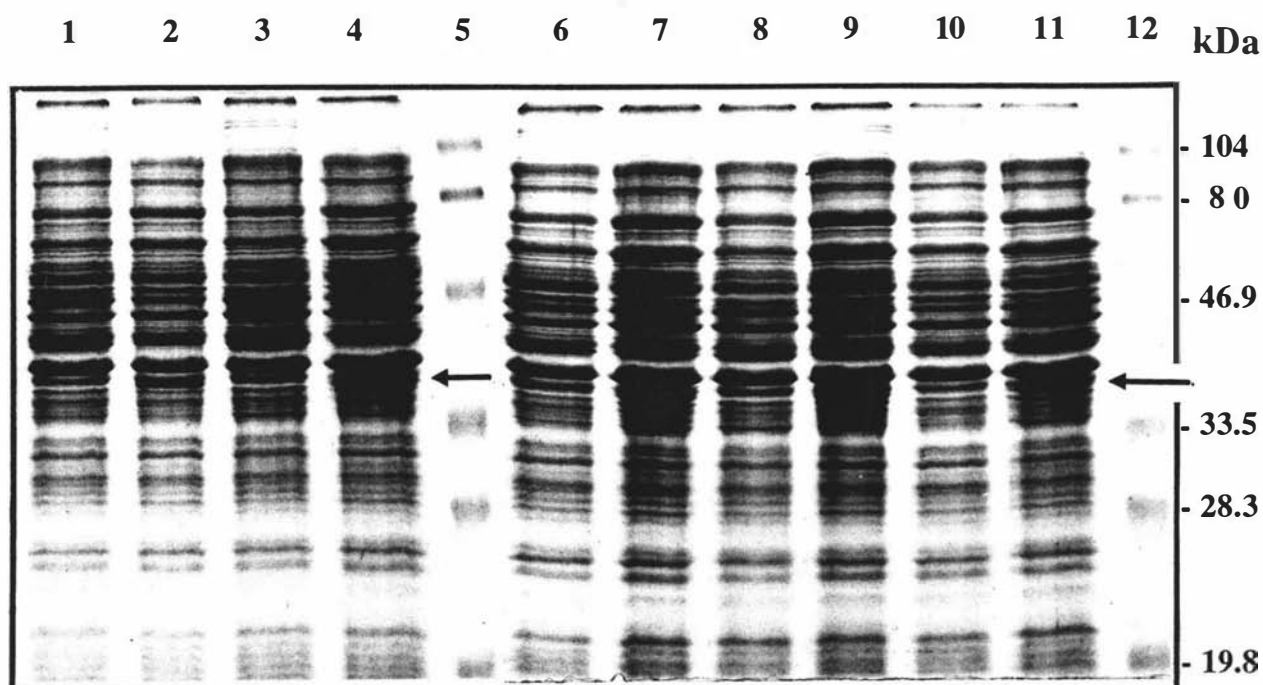


Figure 3.3 The Effect of Different Induction Times after the Induction of IPTG on the Expression of TR-ACO2 Fusion Protein in *E. coli* Strain TB-1 at 27°C.

Lanes 1, 2: uninduced and induced cells, respectively, harvested 3 h after induction.

Lanes 3, 4: uninduced and induced cells, respectively, harvested 4 h after induction.

Lanes 6, 7: uninduced and induced cells, respectively, harvested 5 h after induction.

Lanes 8, 9: uninduced and induced cells, respectively, harvested 6 h after induction.

Lanes 10, 11: uninduced and induced cells, respectively, harvested 7 h after induction.

Lanes 5, 12: BIO-RAD prestained markers (molecular masses are indicated).

The arrow denotes the position of induced TR-ACO2 fusion protein.

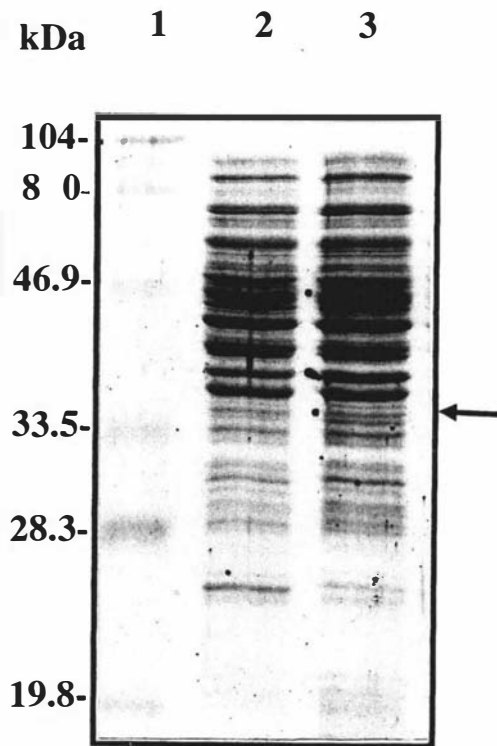


Figure 3.4 SDS-PAGE Analysis of the Expression of TR-ACO3 Fusion Protein in *E. coli* Strain TB-1.

Lane 1: BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

Lanes 2 and 3: soluble fractions (extracted by lysis buffer without SDS) of uninduced and induced cells, respectively.

Cells were grown at 27°C and harvested 5 h after induction with 0.6 mM IPTG.

The arrow denotes the position of induced TR-ACO3 fusion protein.

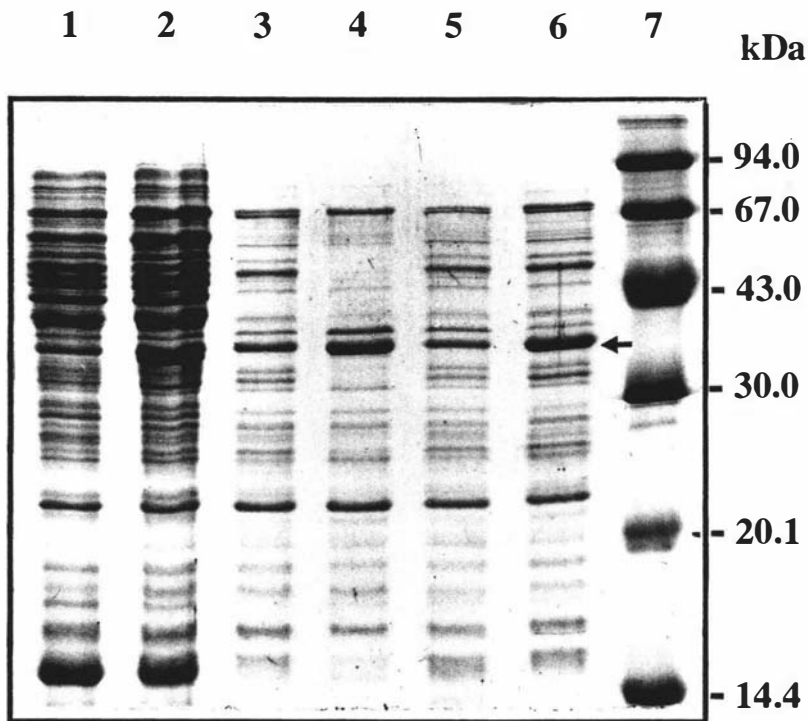


Figure 3.5 SDS-PAGE Analysis of the Effect of SDS Concentrations on the Yield of Extracted TR-ACO2 Fusion Protein from *E. coli* strain TB-1.

Lane 1, 2: lysed fractions using lysis buffer with 0.5 % (w/v) SDS from uninduced and induced cells.

Lane 3, 4: lysed fractions using lysis buffer with 0.1 % (w/v) SDS from uninduced and induced cells.

Lane 5, 6: lysed fractions using lysis buffer with 0.05 % (w/v) SDS from uninduced and induced cells.

Lane 7: BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

Cells were grown at 27°C and harvested 5.0 h after induction with 0.6 mM IPTG.

Arrow denotes the position of induced TR-ACO2 fusion protein.

3.2.1.3 Sephadex G-25 Gel Filtration Chromatography of Extracted Fusion Protein Preparation

Since the detergent Triton X-100 has been observed to cause an inactivation of activity of the enzyme from apple fruit (Dong *et al.*, 1992), it is important to remove the detergents added from the enzyme preparation as soon as feasible. This was obtained by passing the solubilised protein preparation through a Sephadex G-25 gel filtration column. However, Triton X-100 was not retained in the resin of the column. Therefore, only SDS was used in all subsequent experiments. In addition, removal of Triton X-100 from the protein preparation was achieved by passing through a DEAE-Sepharose column which adsorbed the protein (Dong *et al.*, 1992). This was not undertaken in the study.

3.2.2 Purification of Recombinant TR-ACO2 and TR-ACO3 Proteins

After optimisation of the production of TR-ACO2 and TR-ACO3 as His-tagged fusion proteins (Sections 3.2.1.1 and 3.2.1.2), the procedure was scaled up to 2 L cultures. The recombinant ACC oxidase proteins produced in *E. coli* were purified from the crude extracts by the Ni-NTA chelating agarose column affinity chromatography.

A total of 2.3 mg and 2.1 mg of TR-ACO2 and TR-ACO3 fusion proteins, respectively, extracted with 0.5 % (w/v) SDS lysis buffer, were obtained from a 2 L culture. After affinity chromatography, several impurities were removed as indicated by SDS-PAGE separation followed by Coomassie Brilliant Blue staining (Figure 3.6). In addition to the induced fusion proteins of *ca.* 38 kDa (expected size), another *ca.* 29 kDa contaminant protein was co-eluted from the column. The same result was also observed by Hunter (1998) and this has been identified as a nickel binding protein common to *E. coli* strains (Dr. M.T. McManus, *pers. comm.*).

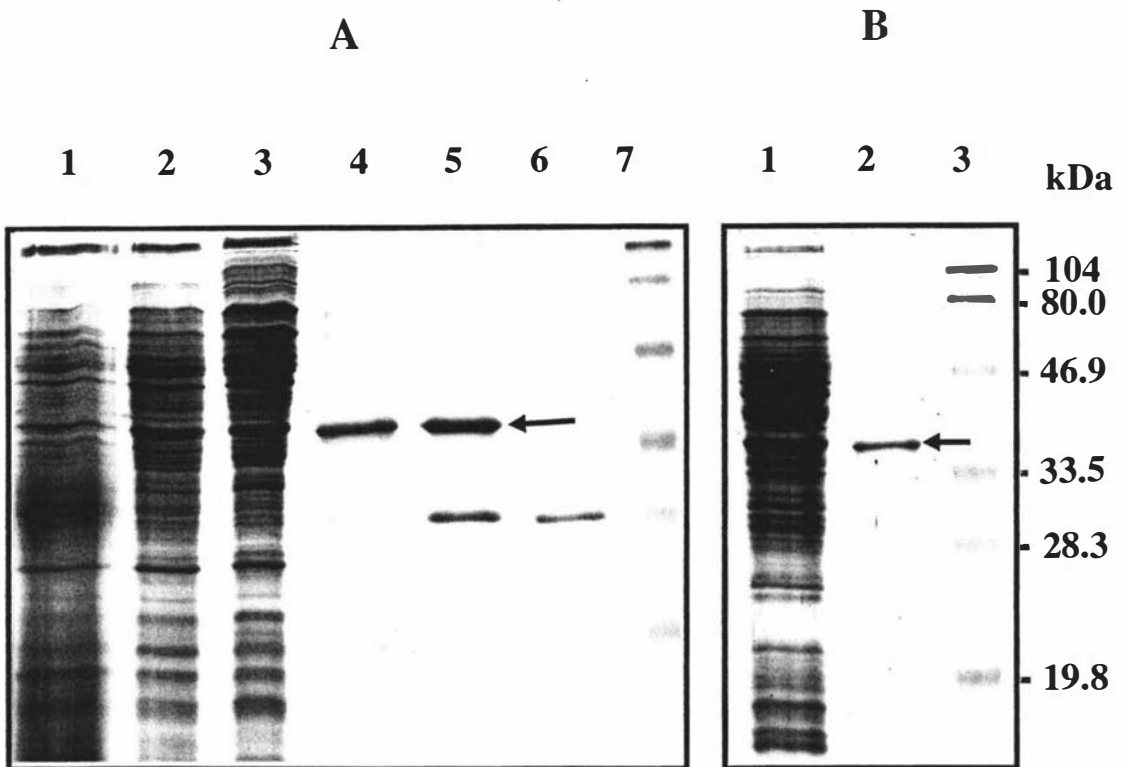


Figure 3.6 SDS-PAGE Analysis of Induced TR-ACO2 (A) and TR-ACO3 (B) Proteins in pPROEXTM-1 prior to and after Purification with the Ni-NTA Affinity Column.

A: Lanes 1, 2 and 3: lysed fractions of induced cells of *E. coli* TB-1 using lysis buffers with 1.5 % (w/v), 0.5 % (w/v) and 0.1 % (w/v) SDS.

Lanes 4, 5 and 6: pooled fractions after Ni-NTA chromatography of the lysed fractions using lysis buffers with 1.5 % (w/v), 0.5 % (w/v) and 0.1 % (w/v) SDS, respectively.

B: Lane 1: lysed fractions of induced cells of *E. coli* TB-1 using lysis buffer with 0.5 % (w/v) SDS.

Lane 2: pooled fractions after Ni-NTA chromatography of lysed fraction using lysis buffer with 0.5 % (w/v) SDS

Lanes 7 (A) and 3 (B): BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

3.2.3 Enzyme Activity of Purified Recombinant ACC Oxidases

The purified protein preparations after elution from the affinity column were exchanged into ACC oxidase activity assay buffer (Section 2.2.6.3.2) prior to being assayed for enzyme activity. Activity assays revealed that the purified TR-ACO2 and TR-ACO3 proteins produced 0.34 and 0.23 nmol ethylene/h/mg protein, respectively under the assay conditions used (Section 2.1.5). These activities were very low compared to, for example, a reported value of 2100 nmol ethylene/h/mg protein for a recombinant ACC oxidase from tomato (Zhang *et al.*, 1995). These results suggest that some critical amino acid residues may be missing from the truncated reading frames of TR-ACO2 and TR-ACO3. With the 3' sequence obtained by 3'-RACE (Hunter *et al.*, 1999), constructs could have been designed to include these regions. However, amino acid residues would be still missing from the N-terminus. Therefore, no further characterisation of these two reading frames of ACC oxidase expressed in *E. coli* was performed in this study. Instead, the extraction, purification and characterisation of ACC oxidase isoforms from leaf tissue of white clover at different developmental stages (mature green and senescent leaves) was undertaken.

3.3 Extraction and Purification of ACC Oxidase Isoforms from Leaves of White Clover

3.3.1 Extraction of ACC Oxidase from Mature Green and Senescent Leaves

The protocol (Section 2.2.3), used for extracting ACC oxidase from leaves of white clover plants (genotype 10F, cultivar Grasslands Challenge), was modified from the methods used by Kuai and Dilley (1992) for apple fruit and McGarvey and Christoffersen (1992) for avocado fruit. The same extraction procedure was used to extract the enzyme from both mature green and senescent leaf tissues. The recovery of enzyme activity *in vitro* in the supernatant ('crude extract') from the 26,800 x g centrifugation (Table 3.1) indicates that ACC oxidase is extracted as a soluble, active enzyme from both leaf tissues. A phenolic-binding compound, polyvinyl pyrrolidone (PVPP), or Triton X-100 was not absolutely required in the extraction

Table 3.1 Comparison of ACC Oxidase Activity in Mature Green and Senescent Leaves in Crude Extracts and after Ammonium Sulphate Precipitation and Sephadex G-25 Gel Filtration Chromatography.

Purification Step	Total Activity ¹		Total Protein		Specific Activity		Purification		Recovery ²	
	(nmol ethylene/h)		(mg)		(nmol ethylene/h/mg)		(fold)		(%)	
	MG ³	SE ³	MG	SE	MG	SE	MG	SE	MG	SE
Crude extract	295	239	1164.8	1148.0	0.25	0.207	1.0	1.0	100.0	100.0
(NH ₄) ₂ SO ₄ precipitation	228	203	576.0	599.9	0.44	0.388	1.8	1.9	77.3	84.9

¹ Enzyme activity was assayed at 1 mM ACC and pH 7.5 (Section 2.2.8).

² Recovery (yield) and purification fold are based on total activity in crude extract.

³ MG and SE represent mature green and senescent leaf tissue, respectively.

but the presence of ascorbic acid (30 mM) was found to be essential for the activity *in vitro* during the extraction (data not shown). Enzyme activity *in vitro* increased approximately in proportion to amount of added enzyme extract from both mature green and senescent leaf tissues (Figure 3.7; senescent leaf data not shown). Incubation of the enzyme extracts for 5 min at 100°C resulted in a complete inactivation of enzyme activity (data not shown), confirming that the release of ethylene from added ACC is enzymatically mediated. In addition, the storage of both mature green and senescent leaves at -80°C did not influence the recovery rate of ACC oxidase activity *in vitro* (data not shown).

The purification procedure used in this thesis included ammonium sulphate fractionation and subsequent Sephadex G-25 gel filtration chromatography and four successive FPLC steps involving hydrophobic interaction on Phenyl Superose and Phenyl Sepharose, anion exchange on Mono Q, chromatofocusing on Mono P and gel filtration on the Superose 12 column.

3.3.2 Ammonium Sulphate Precipitation and Sephadex G-25 Gel Filtration Chromatography of the Crude Extract from Mature Green and Senescent Leaves

The first purification step involved ammonium sulphate fractionation between 30 % and 90 % saturation, after which the protein pellet was resuspended in pre-cooled enzyme activity assay buffer (kept on ice). The protein suspension was then subjected to Sephadex G-25 gel filtration chromatography pre-equilibrated with the same buffer to remove putative low molecular mass (< 5 kDa) inhibitors. In addition to a reduction of volume of the protein preparation and increase in protein concentration, this salt fractionation step provided a 1.8 to 1.9-fold purification with 77.3 % to 84.9 % recovery from mature green and senescent leaf extracts (Table 3.1). Brown pigmented material in the senescent leaf protein extract was observed to be retained in the resin of the Sephadex G-25 column.

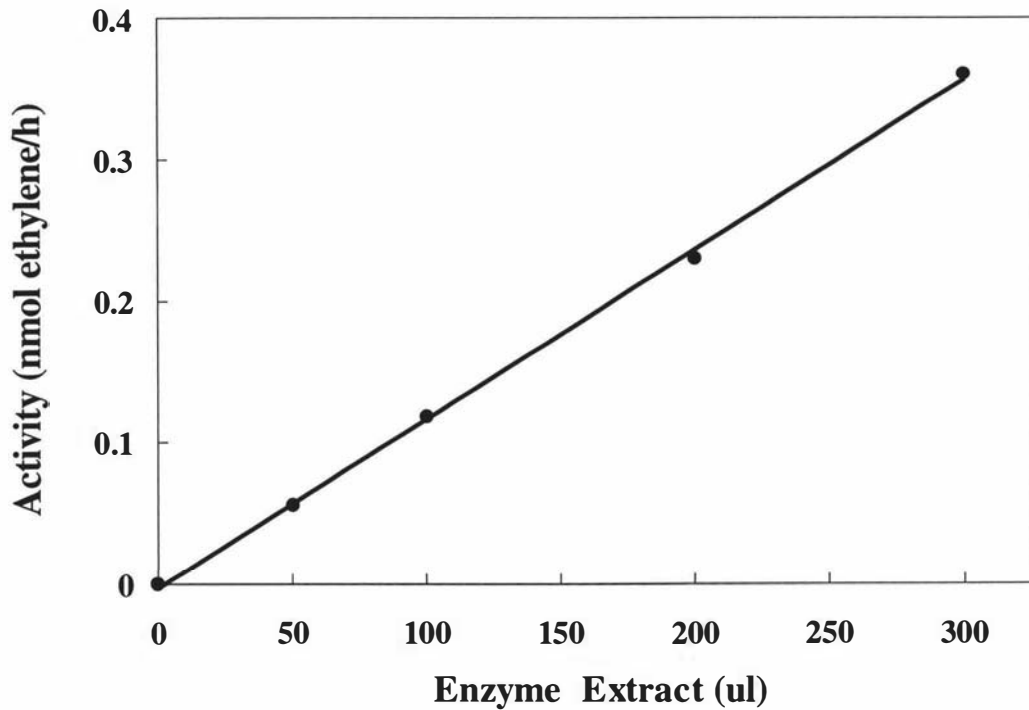


Figure 3.7 ACC Oxidase Activity *in vitro* as a Function of Added Enzyme Extract from Mature Green Leaves.

The enzyme extract was partially purified by ammonium sulphate precipitation (30 - 90 %) and Sephadex G-25 gel filtration chromatography (Section 3.3.2). Values are means (n = 4).

3.3.3 Stability and Stabilisation of ACC Oxidase Activity *in vitro*

3.3.3.1 Stability of ACC Oxidase Activity *in vitro*

ACC oxidase activity *in vitro* measured in several other plant species has been observed to be unstable during extraction, storage and purification. In order to determine the stability of the enzyme activity *in vitro*, enzyme extracts (with an initial specific activity of 0.538 nmol ethylene/h/mg protein) were assayed for activity after Sephadex G-25 gel filtration chromatography from mature green leaf tissue and incubated under a constant temperature of 20°C for 0 to 4 h (Figure 3.8) (20°C was chosen, since the FPLC system used for purification was in a 20°C constant temperature room rather than at 4°C in a cold room). The result showed that the enzyme extract lost 65 % of its initial activity after incubation at 20°C for only 2 h and no enzyme activity could be detected after 3 h (Figure 3.8), indicating that ACC oxidase from mature green leaf tissue was temperature labile once extracted.

3.3.3.2 Stabilisation of ACC Oxidase Activity *in vitro*

The stability of ACC oxidase activity *in vitro* from both mature green and senescent leaf tissues was examined under low temperature (4°C). At 4°C, 92.0 % of the initial enzyme activity in the mature green leaf extract was retained after 4 h incubation (Figure 3.9), and 84.2 % and 65.7 % in mature green leaf extract (Figure 3.9) and 71.4 % and 55.4 % in senescent leaf enzyme extract (Figure 3.10) of the initial activity was retained after 6 h and 10 h, respectively. The data indicate that the decline in enzyme activity *in vitro* at room temperature (20°C) could be due to the temperature lability of the enzyme or high protease activity at room temperature. Low temperature is necessary to retain ACC oxidase activity *in vitro* and so all manipulations were carried out on ice or at 4°C. The activity *in vitro* of the enzyme extract from senescent leaves seems to be more unstable, in common with observations by Hunter (1998).

To further determine the effect of proteases on enzyme activity *in vitro*, two protease inhibitors were added to senescent leaf enzyme extracts. PA (10 µM) or PMSF (1 mM) was added to the senescent leaf extracts and incubated at 4°C for 0, 6, 10, 14 h, respectively. The inclusion of 10 µM PA partially prevented the loss of enzyme activity

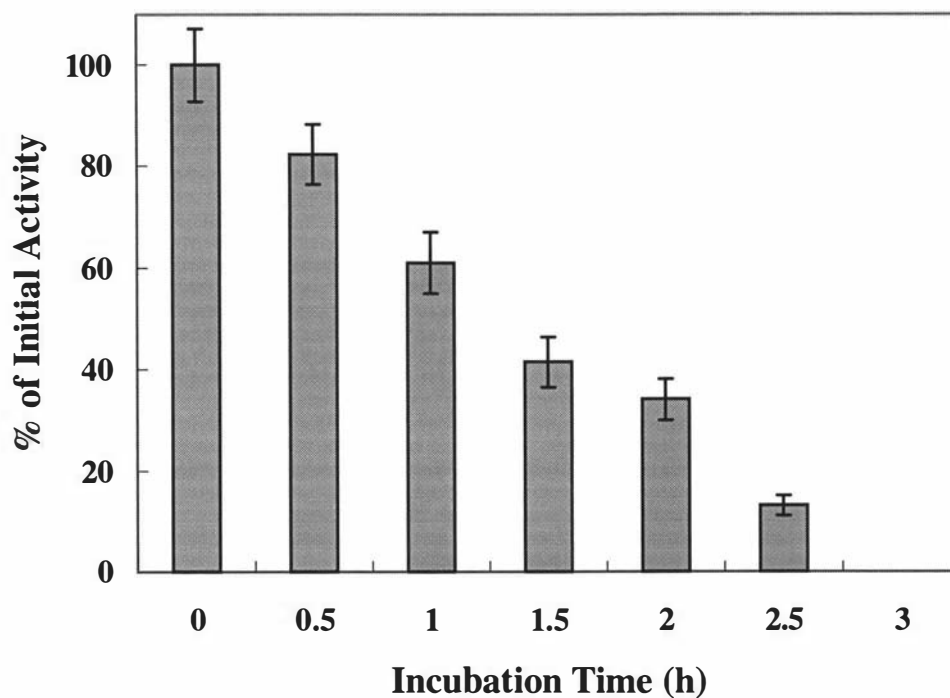


Figure 3. 8 ACC Oxidase Activity *in vitro* from Mature Green Leaf Enzyme Extracts Assayed after Different Incubation Times at 20°C.

The enzyme extract was partially purified by ammonium sulphate precipitation (30 - 90 %) and Sephadex G-25 gel filtration chromatography.

Values are means \pm SE (n = 6).

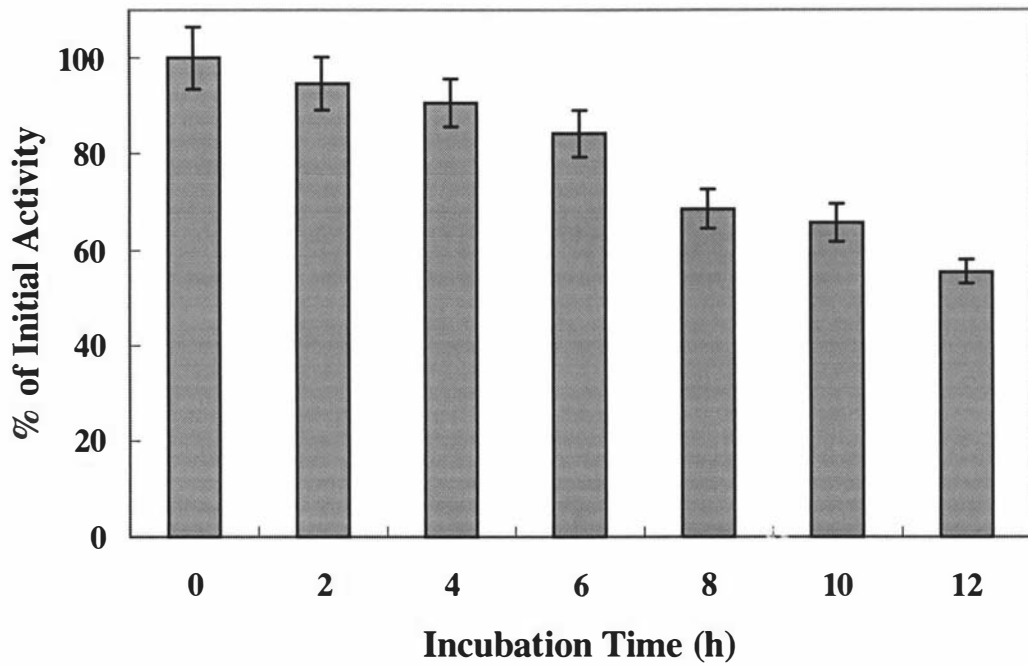


Figure 3.9 ACC Oxidase Activity *in vitro* of Enzyme Extracts from Mature Green Leaves Assayed after Different Incubation Time at 4°C.

The enzyme extract was partially purified by ammonium sulphate precipitation (30 - 90 %) and Sephadex G-25 gel filtration chromatography. Values are means \pm SE (n = 6).

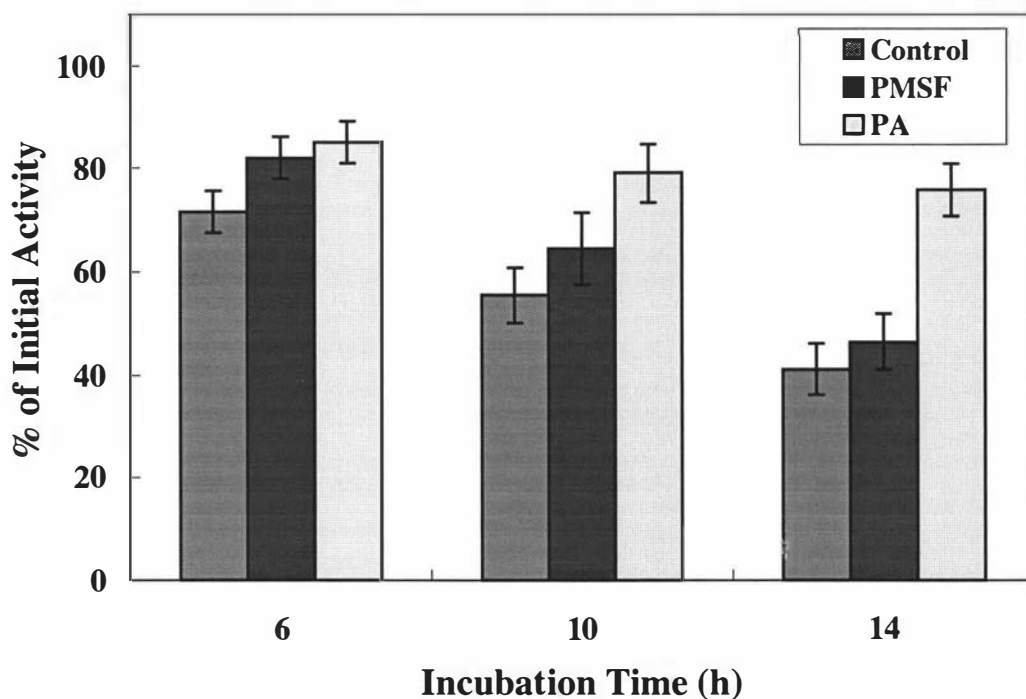


Figure 3.10 Stabilisation of ACC Oxidase Activity *in vitro* in Senescent Leaf Enzyme Extracts.

PA (10 μ M) or PMSF (1 mM) was added to enzyme extracts, partially purified by ammonium sulphate precipitation and Sephadex G-25 gel filtration chromatography, incubated at 4°C and assayed for ACC oxidase activity at the times indicated. Values are means \pm SE (n = 9).

observed at 4°C with 75.6 % of the initial enzyme activity retained for at least 14 h (Figure 3.10) while the untreated control enzyme extract lost about 59.0 % of its initial activity during the same incubation time. Therefore, 10 µM PA was routinely added to the extraction buffer and all buffers used for purification of ACC oxidase from mature green and senescent leaves. PMSF had little effect in preventing the loss of enzyme activity *in vitro* (Figure 3.10).

In addition, both mature green and senescent leaf enzyme extracts, partially purified by ammonium sulphate precipitation and subsequent Sephadex G-25 column chromatography, could be stored at -20°C for at least 30 days without significant loss of ACC oxidase activity *in vitro* (data not shown). Therefore, the enzyme preparations after extraction and each purification step were stored at -20°C as soon as possible to reduce the loss of activity *in vitro*.

3.3.4 Column Chromatography Purification of ACC Oxidase from Mature Green Leaves

3.3.4.1 Comparison of UV Absorbance of Different Components in FPLC Buffers

In the first separations using FPLC, a zero baseline was not obtained because of the high UV absorbance of components in the FPLC buffers used and so no protein elution profile at 280 nm was obtained. In order to determine which buffer components absorbed at 280 nm, different compounds were compared. The result showed that Tris-HCl buffers with different concentrations of sodium ascorbate (1, 3, 10 and 30 mM) gave very high UV absorbances (in excess of 1.97) whereas the buffer with 2 mM DTT or 10 µM PA had negligible absorbances (data not shown). However, sodium ascorbate was observed to be essential to retain enzyme activity *in vitro* during purification (data not shown). Therefore, after the elution of active enzyme from each column was determined, sodium ascorbate was removed from the buffers and the identical chromatographic steps repeated to obtain the protein elution profile.

3.3.4.2 Selection of Leaf Tissue for ACC Oxidase Purification

Initial purification experiments using protein extracts of pooled mature green leaves incorporating nodes 4 to 9 revealed two ACC oxidase protein bands of *ca.* 37 and 35 kDa. The 35 kDa protein was more weakly recognised by the TR-ACO2 antibody in active fractions eluted from both anion exchange Mono Q (fraction No.7, 3 µg protein loading per lane) and gel filtration Superose 12 (fraction No.21, 1 µg protein loading per lane) columns (Figure 3.11). It was proposed that the two proteins might be gene products of TR-ACO2 and TR-ACO3 since both genes are expressed in nodes 4 to 9 (Hunter *et al.*, 1999). To more specifically target an ACC oxidase isoform expressed in leaf tissue with only TR-ACO2 expression, younger mature (immature) green leaves were used. Likewise, to purify any isoforms expressed in senescent leaf tissue, nodes 12 to 16 were used. At these later developmental stages, TR-ACO3 is expressed exclusively (Hunter *et al.*, 1999).

From pooled leaves of nodes 1 to 3, only a very low abundance of ACC oxidase protein was detected by western analysis using the TR-ACO2 antibody in the active fractions eluted from both hydrophobic interaction Phenyl Sepharose and anion exchange Mono Q columns (data not shown). This was judged by more protein being loaded (6 µg and 5 µg protein for Phenyl Sepharose and Mono Q column fractions, respectively) and a longer developing time in the alkaline phosphatase substrate necessary to detect the protein bands when compared with the detection of protein extracts from nodes 4 to 9 (Figure 3.11). From pooled leaves of nodes 2 and 4, a higher ACC oxidase protein content was obtained as judged by the same protein loading (3 µg) as that used in the protein extract from mature green leaves (nodes 4 to 9, Figure 3.11). However, the signal was still not very strong since a longer detection time was needed (data not shown). Therefore, pooled leaves from nodes 2 to 6 were used to extract and purify the enzyme where a strongly recognised single band of *ca.* 37 kDa protein was observed from the active fractions eluted on the same columns in the immunoblot using the TR-ACO2 antibody. The purification of this ACC oxidase isoform is described.

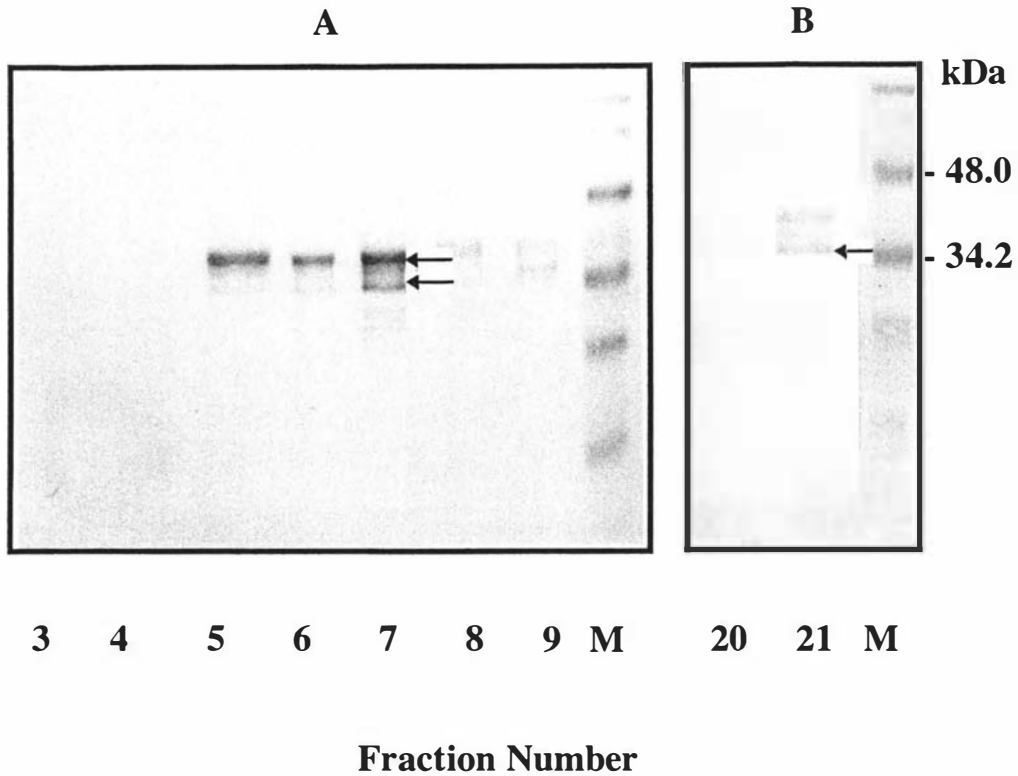


Figure 3.11 Western Analysis using the TR-ACO2 Antibody of Active Fractions Eluted from the Mono Q (A) and Superose 12 (B) Columns of Enzyme Extracts of Pooled Leaves from Nodes 4 to 9.

Proteins loaded for the fractions in A and B were 3 μ g and 1 μ g per lane, respectively. M = BIO-RAD prestained SDS-PAGE standards (the same standards were used in A and B and molecular masses are indicated in B). Arrows indicate the position of two ACC oxidase proteins of *ca.* 37 kDa and 35 kDa.

3.3.4.3 Hydrophobic Interaction Chromatography

3.3.4.3.1 Hydrophobic Interaction Chromatography on a Phenyl Superose Column

The first FPLC step, hydrophobic interaction chromatography, was observed to be the most efficient step in the purification of ACC oxidase from mature green leaf tissue in terms of increase in purification fold. A Phenyl Superose hydrophobic column was first equilibrated with Buffer A (50 mM Tris-HCl, pH 7.5, containing 30 mM sodium ascorbate, 2 mM DTT, 10 μ M PA and 2 M ammonium sulphate) and then the protein sample containing 2 M ammonium sulphate loaded. Bound proteins were eluted within a gradient of 100 % Buffer A : 0 % Buffer B (Buffer A with ammonium sulphate omitted) to 0 % Buffer A : 100 % Buffer B. On the hydrophobic interaction column, a large amount of protein with no apparent ACC oxidase activity was eluted within a decreasing linear gradient of ammonium sulphate (2.0 M to 0.0 M) while ACC oxidase activity eluted after fraction 28 with no ammonium sulphate in the buffer (Figure 3.12), suggesting a high degree of surface hydrophobicity of ACC oxidase protein molecule. Enzyme activity assays for all fractions eluted from the column revealed one major ACC oxidase activity peak, designated MGI, which consisted of fractions 30 to 34 and a second minor peak, designated MGII, which consisted of fraction 37 only (Figure 3.12). The same results were observed in many independent purification experiments.

To confirm that the enzyme activity peak eluted from the column was ACC oxidase, western blotting, using different polyclonal antibodies raised against TR-ACO1, TR-ACO2 and TR-ACO3 fusion proteins expressed in *E. coli*, was undertaken. In general, 1 μ g to 4 μ g of total protein from each fraction was used for western analysis using these antibodies. MGI was recognised by all three antibodies as a single band of *ca.* 37.0 kDa, with the TR-ACO2 antibody giving stronger recognition for ACC oxidase protein (determined by a shorter developing time in the alkaline phosphatase substrate) (Figure 3.13). This antibody therefore was used for western analysis in subsequent purification steps. In addition, a comparison experiment using 3.0 μ g and 1.5 μ g of protein from each ACC oxidase activity fraction from the Phenyl Superose column gave a similar result in terms of antibody recognition although the latter loading (1.5 μ g) gave

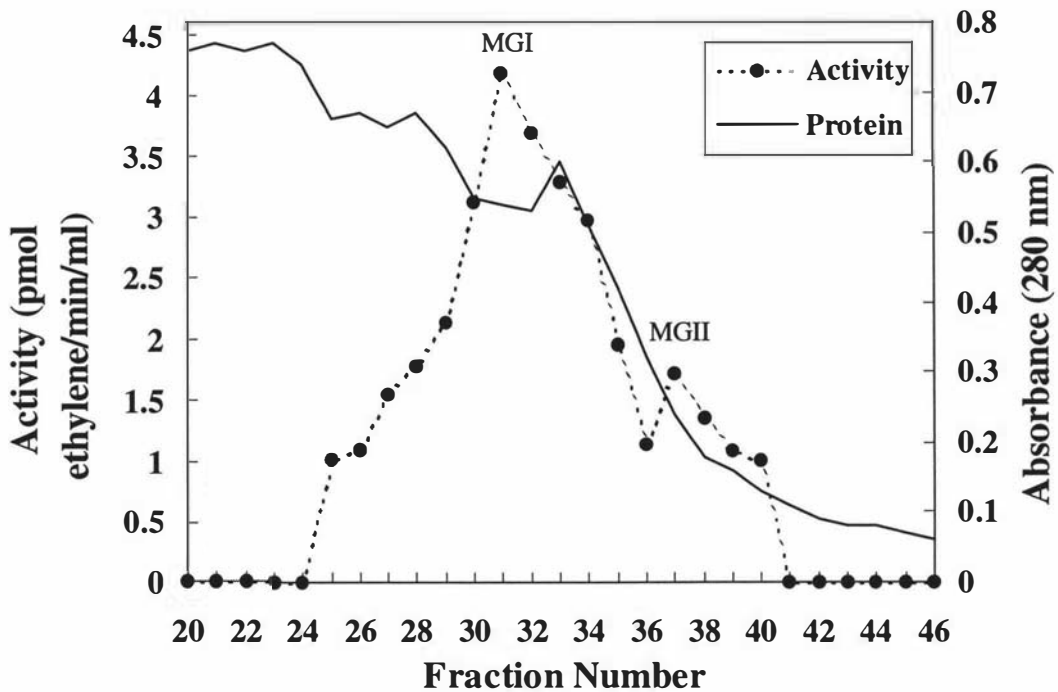


Figure 3.12 Protein Elution Profile and ACC Oxidase Activity from a Mature Green Leaf Protein Extract after Chromatography through a Phenyl Superose Hydrophobic Interaction Column.

The data from fractions 1 to 19 with no enzyme activity are not shown.

a weaker signal (data not shown). Therefore, 3 μg total protein from the first column fractions was used routinely for western analysis. The size of the detected protein is within the published range (34.8 kDa to 40 kDa) of apparent molecular masses determined by SDS-PAGE analysis for ACC oxidases reported from other plant species. Furthermore, the signal intensity of these fractions in immunoblotting is consistent with the ACC oxidase activity profile (Figures 3.12 and 3.13). The second minor peak, MGII (fraction 37), was not recognised by all antibodies at the same total protein loading as in MGI. This may be due to a much lower ACC oxidase protein content in this fraction and thus more protein and a longer developing time could be needed to detect the ACC oxidase. At this stage of the purification, the specific activity of MGI is 2.57 nmol ethylene/h/mg protein which represents a 10.2-fold purification (Table 3.2).

3.3.4.3.2 Hydrophobic Interaction Chromatography on a Phenyl Sepharose Column

To scale up the ACC oxidase protein for further purification, a Phenyl Sepharose hydrophobic interaction column was used. Its maximal protein loading is 150-fold higher than that of the Phenyl Superose column. Using this column, one major ACC oxidase activity peak, consisting of fractions 33 to 35, eluted with no ammonium sulphate in the buffer (Figure 3.14). This activity peak was recognised by the TR-ACO2 antibody as a *ca.* 37 kDa protein (Figure 3.14, top panel). From the elution property of the major active peak from the Phenyl Sepharose column, it is most likely that this is MGI. A second minor active peak, consisting of fraction 38 only, was also observed and this is most likely MGII. No western analysis was carried out on this peak.

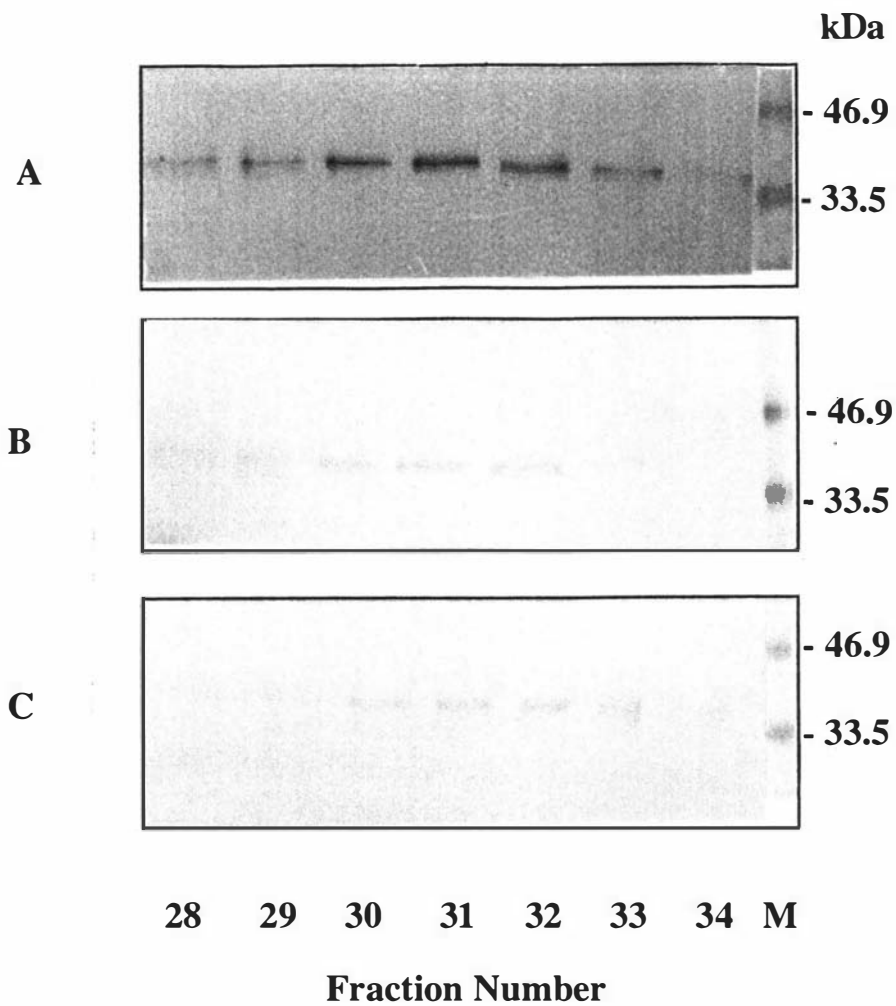


Figure 3.13 Immunodetection of ACC Oxidase Protein using the TR-ACO2 (A), TR-ACO3 (B) and TR-ACO1 (C) Antibodies in MGI Fractions from the Mature Green Leaf Protein Extract Eluted from the Phenyl Superose Column (Figure 3.12).

The protein amount loaded in SDS-PAGE for each fraction was 3 μg per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

Table 3.2 Summary of Purification of ACC Oxidase Isoform MGI from Mature Green Leaf Tissue of White Clover.

Purification Step	Total Activity ¹ (nmol ethylene/h)	Total Protein (mg)	Specific Activity (nmol ethylene/h/mg)	Purification (fold)	Recovery ² (%)
Crude extract	295	1164.8	0.25	1.0	100.0
(NH ₄) ₂ SO ₄ precipitation	228	576.0	0.44	1.8	77.3
Hydrophobic interaction (Phenyl Superose)	44.3	17.2	2.57	10.2	15.0
Anion exchange (Mono Q)	8.42	2.01	4.19	17.0	2.9
Chromatofocusing (Mono P)	0.361	0.04	9.03	35.7	0.12
Gel filtration (Superose 12)	0.227	0.009	25.2	99.6	0.077

¹ Enzyme activity was assayed at 1 mM ACC and pH 7.5 (Section 2.2.8).

² Recovery and purification fold are based on total activity in crude extract.

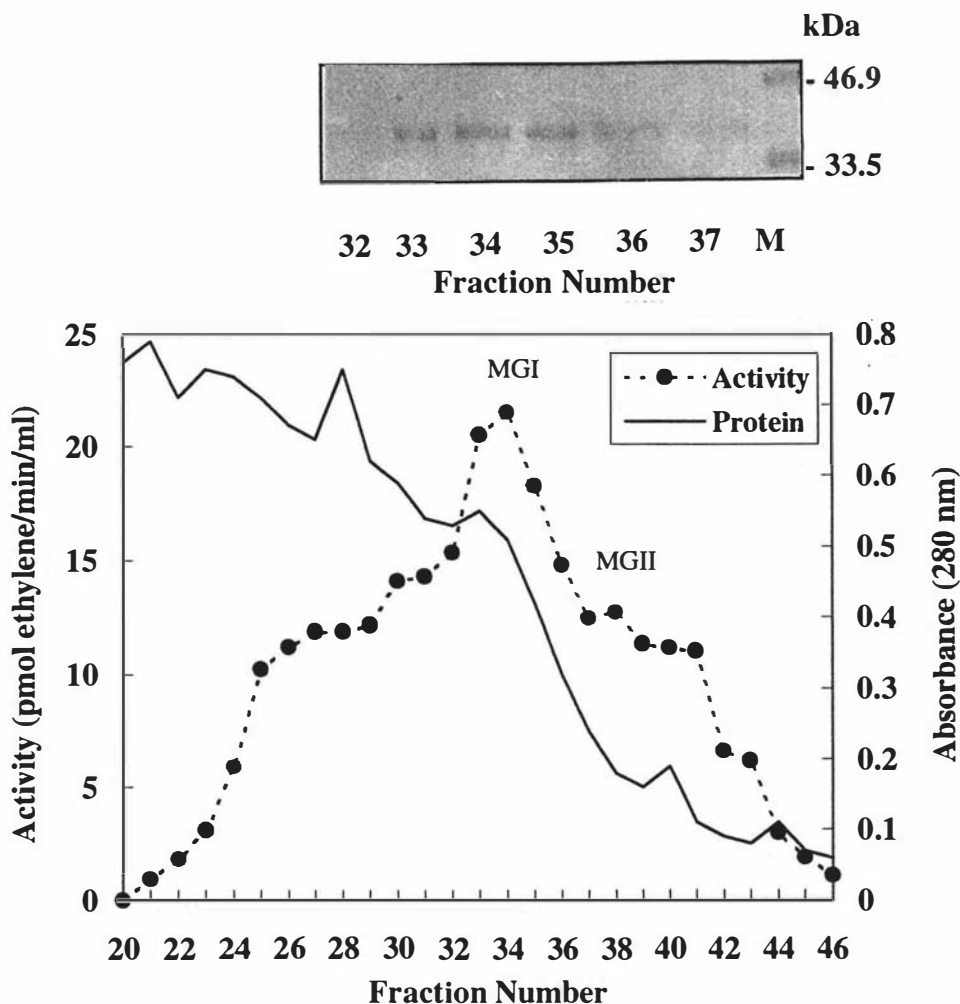


Figure 3.14 Protein Elution Profile and ACC Oxidase Activity from a Mature Green Leaf Protein Extract after Chromatography through a Phenyl Sepharose Hydrophobic Interaction Column.

The data from fractions 1 to 19 with no enzyme activity are not shown. The top panel is an immunodetection, using the TR-ACO2 antibody, of ACC oxidase protein in the active fractions from the Phenyl Sepharose column (Figure 3.14). The protein amount loaded was 2.0 μ g per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

3.3.5 Anion Exchange Chromatography on a Mono Q Column

Anion exchange chromatography on a Mono Q column was used as the next purification step to further purify MGI and MGII identified after hydrophobic column chromatography. To determine whether the ACC oxidase protein can bind to the groups of the Mono Q column resin at pH 7.5, a smaller Mono Q precolumn packed with similar material as the Mono Q column from Pharmacia Biotech was used for preliminary experiments. The result showed that ACC oxidase protein can bind to the resin and be eluted from it, with a linear sodium chloride gradient of 0 to 1.0 M observed to be most suitable for separation (data not shown).

A Mono Q anion exchange column was pre-equilibrated with Buffer A (50 mM Tris-HCl, pH 7.5, containing 30 mM sodium ascorbate, 2 mM DTT and 10 μ M PA), the most active MGI fractions from the Phenyl Superose (fractions 31 and 32) or Phenyl Sepharose (fraction 34) columns were concentrated and then applied separately onto the anion exchange column. MGI was eluted within an increasing salt gradient from 100 % Buffer A : 0 % Buffer B (50 mM Tris-HCl, pH 7.5, containing 30 mM sodium ascorbate, 2 mM DTT, 10 μ M PA and 1.0 M NaCl) to 0 % Buffer A : 100 % Buffer B. Activity assays for all fractions eluted from this column showed that the ACC oxidase activity was eluted as a single peak, designated isoform MGI, at a sodium chloride concentration of 265 mM to 371 mM (fractions 5 to 7, Figure 3.15), indicating that this isoform exists in its anionic form (negatively charged) at pH 7.5. Western analysis showed that this isoform was recognised as a single band of *ca.* 37 kDa by the TR-ACO2 antibody (Figure 3.15, top panel). The contaminant band of *ca.* 35 kDa protein which was observed in fraction 7 on the Mono Q column from the pooled mature green leaves of nodes 4 to 9 (Figure 3.11) is now not observed (Figure 3.15, top panel). The specific activity of MGI after the Mono Q step is 4.19 nmol ethylene/h/mg protein which represents a 17.0-fold purification (Table 3.2).

The MGII peak fraction was also loaded onto the Mono Q column. However, activity assays for all eluted fractions showed no apparent activity peak and no ACC oxidase protein was detected by western analysis using the TR-ACO2 antibody (data not shown). No further purification from this peak was undertaken.

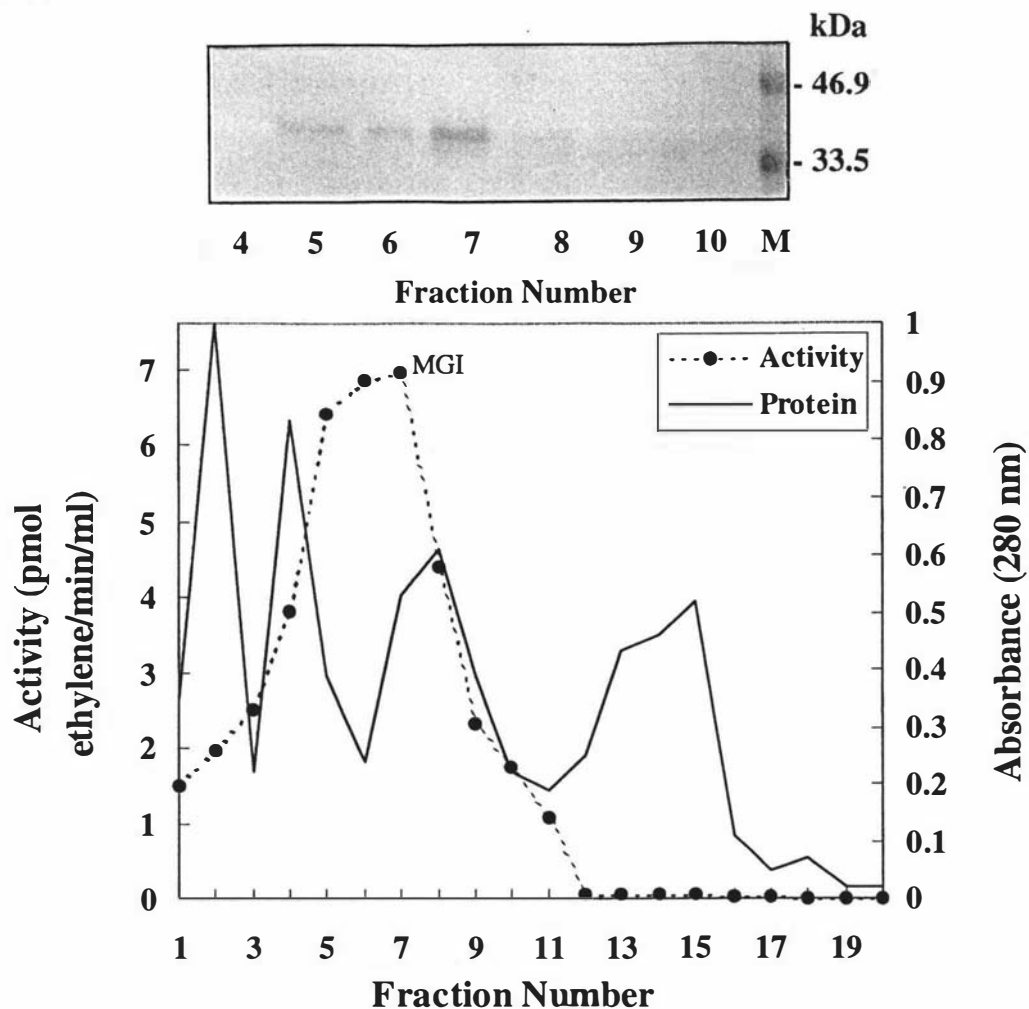


Figure 3.15 Protein Elution Profile and ACC Oxidase Activity of the MGI Preparation (fractions 30 to 34 from the Phenyl Superose column (Figure 3.12)) after Chromatography through a Mono Q Anion Exchange Column.

The data from fractions 21 to 32 with no enzyme activity are not shown. The top panel is an immunodetection of ACC oxidase protein, using the TR-ACO2 antibody, in the active fractions from the Mono Q column. The protein amount loaded was 2.0 μg per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

3.3.6 Chromatofocusing on a Mono P Column

The third FPLC step used in the purification is chromatofocusing on a Mono P column. Fractions with highest enzyme activity from the Mono Q column (fractions 6 and 7) were pooled, concentrated, desalted and exchanged into column equilibration buffer (Section 2.2.6.3.2), and then loaded onto a Mono P column which had been equilibrated with the same buffer (Section 2.2.6.3.1). Bound proteins were eluted with a Polybuffer 74 (pH 4.0), containing 15 mM sodium ascorbate, 2 mM DTT and 10 μ M PA, to provide a pH gradient of 7.5 to 4.0. ACC oxidase activity was eluted as a single peak at fraction 3 only (Figure 3.16) and this activity peak was also recognised as a single band of *ca.* 37 kDa by the TR-ACO2 antibody (Figure 3.16, top panel). The activity assay and pH measurement for each fraction eluted from this column showed that the apparent pI of the isoform MGI was at pH 7.36. The specific activity of MGI after this step is 9.03 nmol ethylene/h/mg protein which represents a 35.7-fold purification (Table 3.2).

3.3.7 Gel Filtration Chromatography on a Superose 12 Column

Gel filtration chromatography on a Superose 12 column was used as the final purification step. The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 30 mM sodium ascorbate, 2 mM DTT, 10 μ M PA and 150 mM NaCl. The inclusion of 150 mM NaCl in the equilibration and elution buffers was found to give good separation of proteins in preliminary experiments (data not shown). The same concentration of potassium chloride was also used in both equilibration and elution buffers for purification of the enzyme from banana (Moya-Leon and John, 1995) although no salt was included in the elution buffer in the same purification step for the enzyme from apple (Dupille *et al.*, 1993).

The most active fraction (fraction 3) from the Mono P column was concentrated and then loaded onto the pre-equilibrated gel filtration column. Proteins were eluted using the same buffer. A single activity peak was eluted at fraction 21 which corresponded to an elution volume of 13.8 ml (Figure 3.17). Western analysis showed that this peak cross-reacted with the TR-ACO2 antibody as a single band of *ca.* 37 kDa (Figure 3.17, top panel). The specific activity of the purified isoform MGI after this final step is 25.2

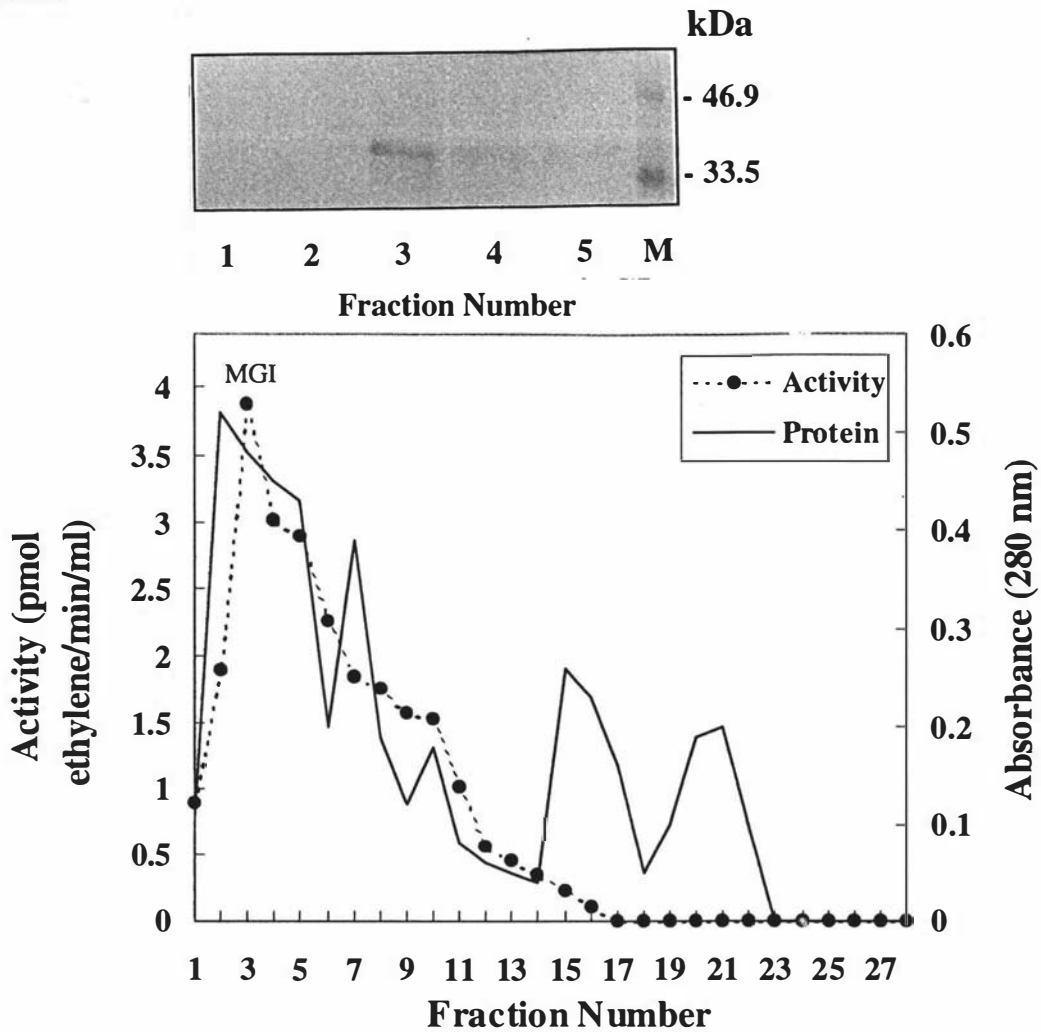


Figure 3.16 Protein Elution Profile and ACC Oxidase Activity of the MGI Preparation (fractions 5 to 7 from the Mono Q column (Figure 3.15)) after Chromatography through a Mono P Column.

The top panel is an immunodetection, using the TR-ACO2 antibody, of ACC oxidase protein in the active fraction from the Mono P column (Figure 3.16). The protein amount loaded was 1.5 μg per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

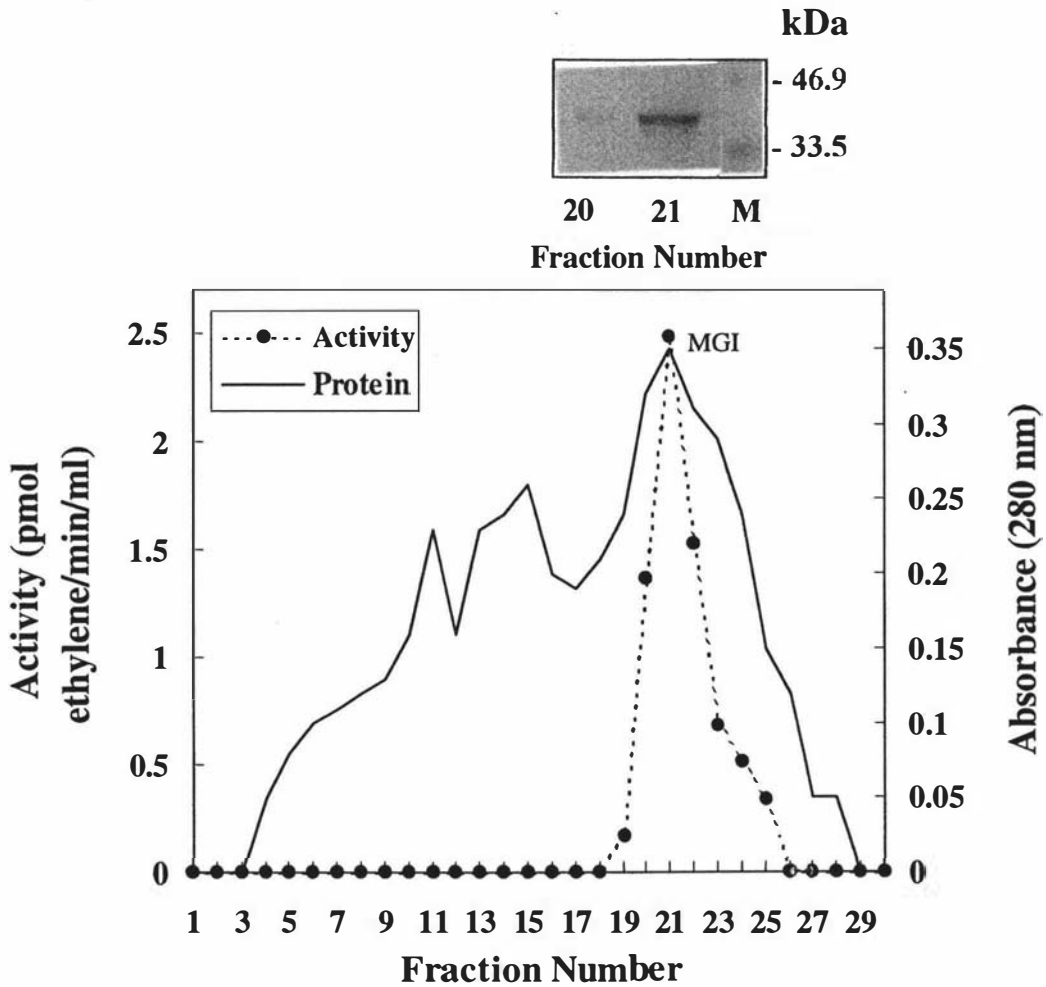


Figure 3.17 Protein Elution Profile and ACC Oxidase Activity of the MGI Preparation (fraction 3 from the Mono P column (Figure 3.16)) after Chromatography through a Superose 12 Gel Filtration Column.

The top panel is an immunodetection, using the TR-ACO₂ antibody, of ACC oxidase protein in the active fraction from the gel filtration column chromatography (Figure 3.17). The protein loaded was 1.0 μg per lane. M = BIO-RAD[®] prestained SDS-PAGE standards (molecular masses are indicated).

nmol ethylene/h/mg protein, assayed with 1 mM ACC and at pH 7.5, which represents a 99.6-fold purification with a total recovery of approximately 0.08 % (Table 3.2).

3.3.8 SDS-PAGE Analysis of Purified Isoform MGI

SDS-PAGE analysis (Figure 3.18) of the MGI protein preparation after the five purification steps showed that the isoform has been purified to homogeneity as judged by Coomassie Brilliant Blue staining and western analysis using the TR-ACO2 antibody. By reference to standards, an apparent molecular mass of *ca.* 37.0 kDa (Figure 3.18) was obtained.

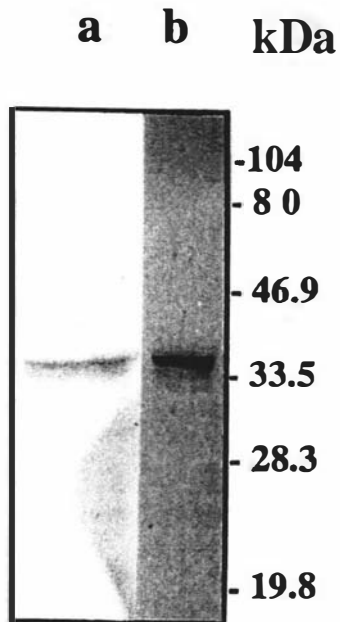


Figure 3.18 SDS-PAGE Analysis of the Purified Isoform MGI from Mature Green Leaf Tissue. (a) Coomassie Brilliant Blue Staining and (b) Western Analysis using the TR-ACO2 Antibody of the Separation Shown in (a).

The protein amount loaded for lanes a and b were 6 μg and 1 μg , respectively. The molecular masses of the standards used are indicated.

3.4 Column Chromatography Purification of ACC Oxidase from Senescent Leaf Tissue

3.4.1 Hydrophobic Interaction Chromatography on a Phenyl Superose Column

The first FPLC step, hydrophobic interaction chromatography, also produced the most efficient and reproducible separation of the proteins from the senescent leaf protein extract in terms of increase in purification fold. On a Phenyl Superose hydrophobic interaction column, a large amount of protein with no ACC oxidase activity eluted within a decreasing linear gradient of ammonium sulphate whereas the ACC oxidase activity peaks eluted in later fractions with no ammonium sulphate in the buffer (Figure 3.19). This result also suggests a very strong surface hydrophobicity of the ACC oxidase protein (s) in this extract. Enzyme activity assay on each fraction eluted from the column revealed two ACC oxidase activity peaks, designated SEI (consisting of fractions 27 to 30) and SEII (consisting of fractions 33 to 38) (Figure 3.19), indicating that SEI and SEII proteins differed in surface hydrophobicity. The second active peak, SEII, showed a higher ACC oxidase activity than SEI. Both peaks were recognised by both TR-ACO2 and TR-ACO3 antibodies (Figure 3.20, TR-ACO3 antibody data not shown). A major protein band of *ca.* 35 kDa was recognised by the TR-ACO2 antibody in SEI while no clear recognition by the TR-ACO3 antibody was observed. The TR-ACO2 antibody recognised a number of bands in the SEII preparation ranging in molecular weight from *ca.* 33 kDa to 37 kDa, particularly in fractions 33 to 34. The TR-ACO3 antibody more weakly recognised a similar number of protein bands. The two different enzyme activity peaks with similar activity ratio were observed at the same elution point on this column, and the different cross-reactivity with both antibodies was observed in several independent purification experiments. The specific activity of SEII after this step is 3.7 nmol ethylene/h/mg protein which represents a 17.9-fold purification (Table 3.3) while the specific activity of SEI is 1.69 nmol ethylene/h/mg protein which represents a 8.2-fold purification (Table 3.4).

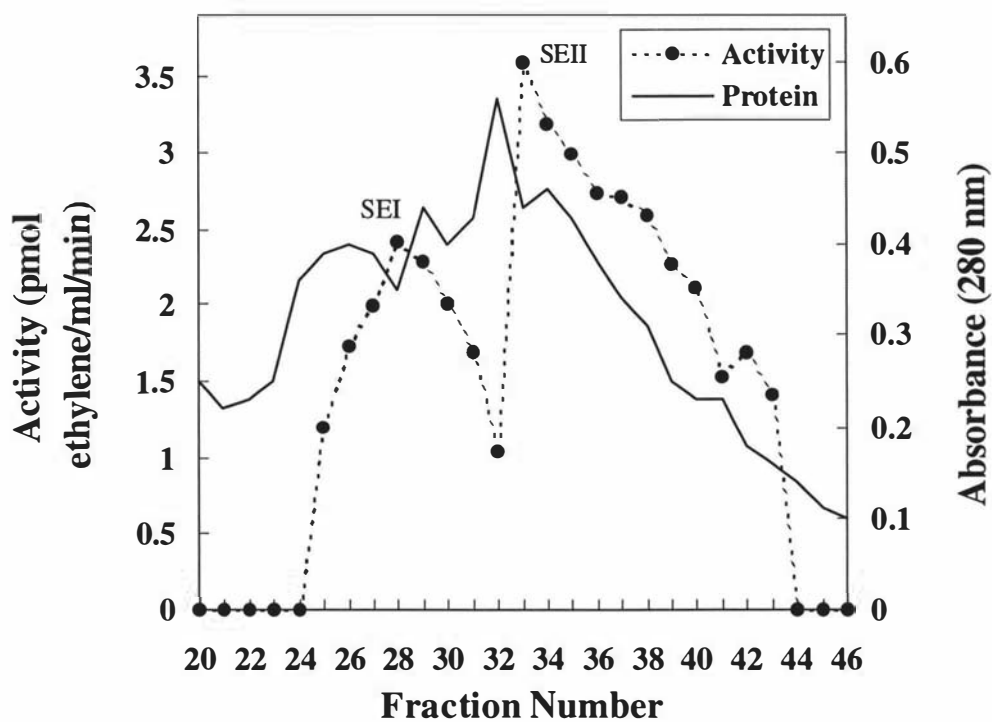


Figure 3.19 Protein Elution Profile and ACC Oxidase Activity from a Senescent Leaf Protein Extract after Chromatography through a Phenyl Superose Hydrophobic Interaction Column.

The data from fractions 1 to 19 with no enzyme activity are not shown.

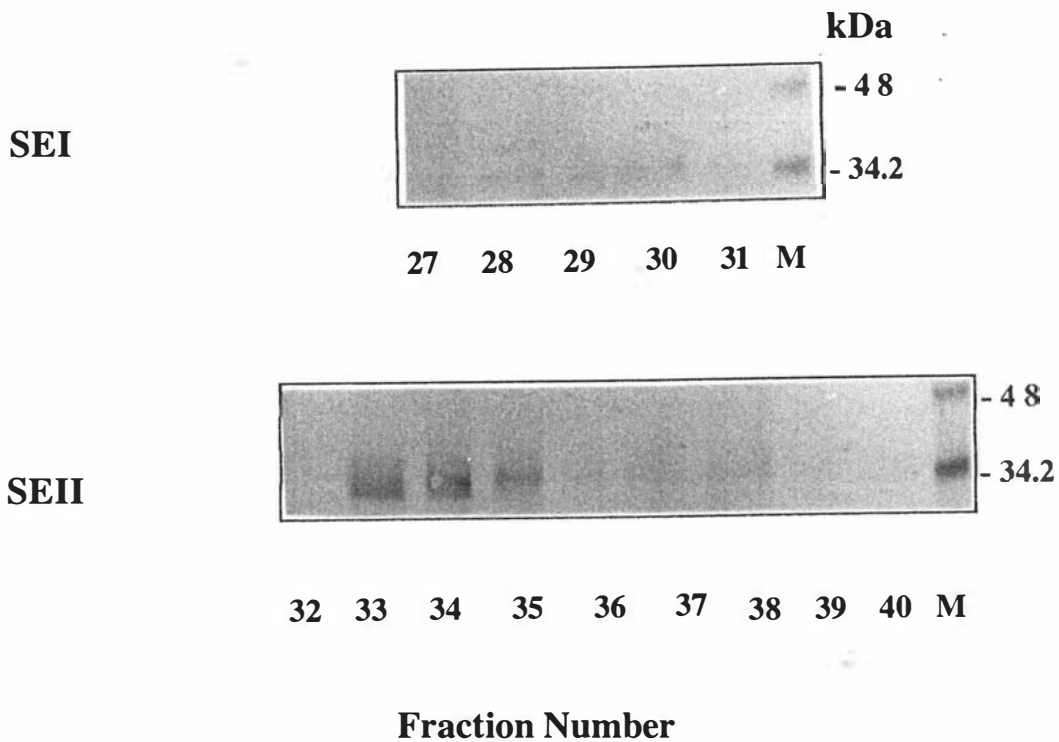


Figure 3.20 Immunodetection, using the TR-ACO2 Antibody, of ACC Oxidase Protein in the Two ACC Oxidase Activity Peaks, SEI and SEII, from the Senescent Leaf Protein Extract Eluted from the Phenyl Superose Hydrophobic Interaction Column (Figure 3. 19).

The protein amount loaded was 4 μ g per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

Table 3.3 Summary of Purification of ACC Oxidase Isoform SEII from Senescent Leaf Tissue of White Clover.

Purification Step	Total Activity ¹ (nmol ethylene/h)	Total Protein (mg)	Specific Activity (nmol ethylene/h/mg)	Purification (fold)	Recovery ² (%)
Crude extract	239	1148.0	0.207	1.0	100.0
(NH ₄) ₂ SO ₄ precipitation	203	599.9	0.338	1.9	84.9
Phenyl Superose	47.3	12.8	3.7	17.9	19.8
Mono Q	10.52	1.30	8.09	39.6	3.7
Mono P	0.562	0.031	18.12	87.5	0.104
Superose 12	0.119	0.004	29.75	143.7	0.050

¹ Activity assay was carried out at 1 mM ACC and pH 7.5 (Section 2.2.8).

² Recovery and purification fold are based on total activity in crude extract.

Table 3.4 Summary of Partial Purification of ACC Oxidase Isoform SEI from Senescent Leaf Tissue of White Clover.

Purification Step	Total Activity ¹ (nmol ethylene/h)	Total Protein (mg)	Specific Activity (nmol ethylene/h/mg)	Purification (fold)	Recovery ² (%)
Crude extract	239	1148.0	0.207	1.0	100.0
(NH ₄) ₂ SO ₄ precipitation	203	599.9	0.338	1.9	84.9
Phenyl Superose	6.1	3.60	1.69	8.2	12.2
Mono Q	0.7	0.18	3.89	18.8	0.04

¹ Enzyme activity was assayed at 1 mM ACC and pH 7.5 (Section 2.2.8).

² Recovery and purification fold are based on total activity in crude extract.

3.4.2 Anion Exchange Chromatography on a Mono Q Column

The most active fractions in SEI (fractions 28 to 29) and SEII (fractions 33 to 35) from the Phenyl Superose column separation were concentrated and loaded individually onto a Mono Q column. For SEI, a single activity peak consisting of fraction 2 only eluted at 106 mM NaCl from the same column (Figure 3.21) and was designated isoform SEI. The SEI isoform was recognised as a single band of *ca.* 35 kDa by the TR-ACO2 antibody (Figure 3.21, top panel). For SEII, only one activity peak consisting of fractions 7 to 9 (eluting at 371 mM to 480 mM NaCl) was recovered from the column (Figure 3.22) and was designated isoform SEII. The isoform was also recognised as a single band of *ca.* 35 kDa by the TR-ACO2 antibody with maximum recognition in fractions 7 and 8 (Figure 3.22, top panel, fraction 9 data not shown). These results indicate that both isoforms exist in their anionic form and that SEI is less negatively charged at pH 7.5 when compared with SEII and MGI. As well, the different elution points of these proteins on the Mono Q column indicate different pIs of these isoforms. The enzyme activity associated with SEI was much lower than SEII. Also, recognition by the TR-ACO2 antibody was lower because more SEI protein was needed (4 µg) when compared with 2 µg of SEII (Figure 3.21 and 3.22, top panel) and a longer developing time in the alkaline phosphatase substrate was needed to detect the SEI protein using the same antibody. The specific activities of SEII and SEI after this purification step is 8.09 and 3.89 nmol ethylene/h/mg protein, which represent 39.6- and 18.8-fold purifications, respectively (Tables 3.3 and 3.4). Because of the lower ACC oxidase protein concentration and enzyme activity of the isoform SEI, no further purification of this isoform was undertaken.

3.4.3 Chromatofocusing on a Mono P Column

The most active fractions (fractions 7 and 8) from the SEII separation using the Mono Q column were pooled, concentrated and exchanged into the Mono P column equilibration buffer (Section 2.2.6.3.2), and then loaded onto a Mono P column pre-equilibrated with the same buffer (Section 2.2.6.3.1). From this chromatofocusing step (with a pH gradient of 7.5 to 4.0), the ACC oxidase activity was eluted as a single peak in fraction 4 only (Figure 3.23) and this activity peak was also recognised as a single band of *ca.* 35 kDa by the TR-ACO2 antibody (Figure 3.23, top panel). The apparent pI of this

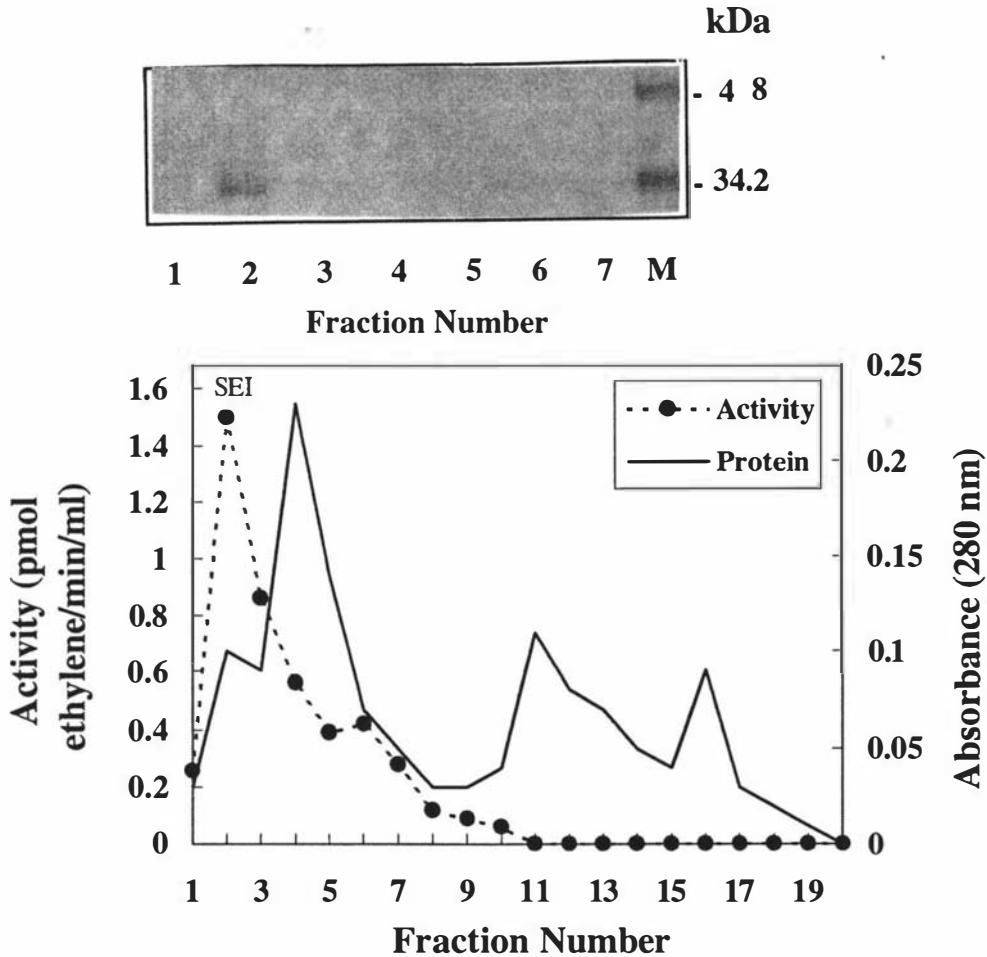


Figure 3.21 Protein Elution Profile and ACC Oxidase Activity of the SEI Preparation (fractions 28 to 29 from the Phenyl Superose column (Figure 3. 19)) after Chromatography through a Mono Q Anion Exchange Column.

The data from fractions 21 to 32 with no enzyme activity are not shown. The top panel is an immunodetection, using the TR-ACO2 antibody, of ACC oxidase protein in the active fraction after the Mono Q column (Figure 3.22). The protein amount loaded was 4.0 μg per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

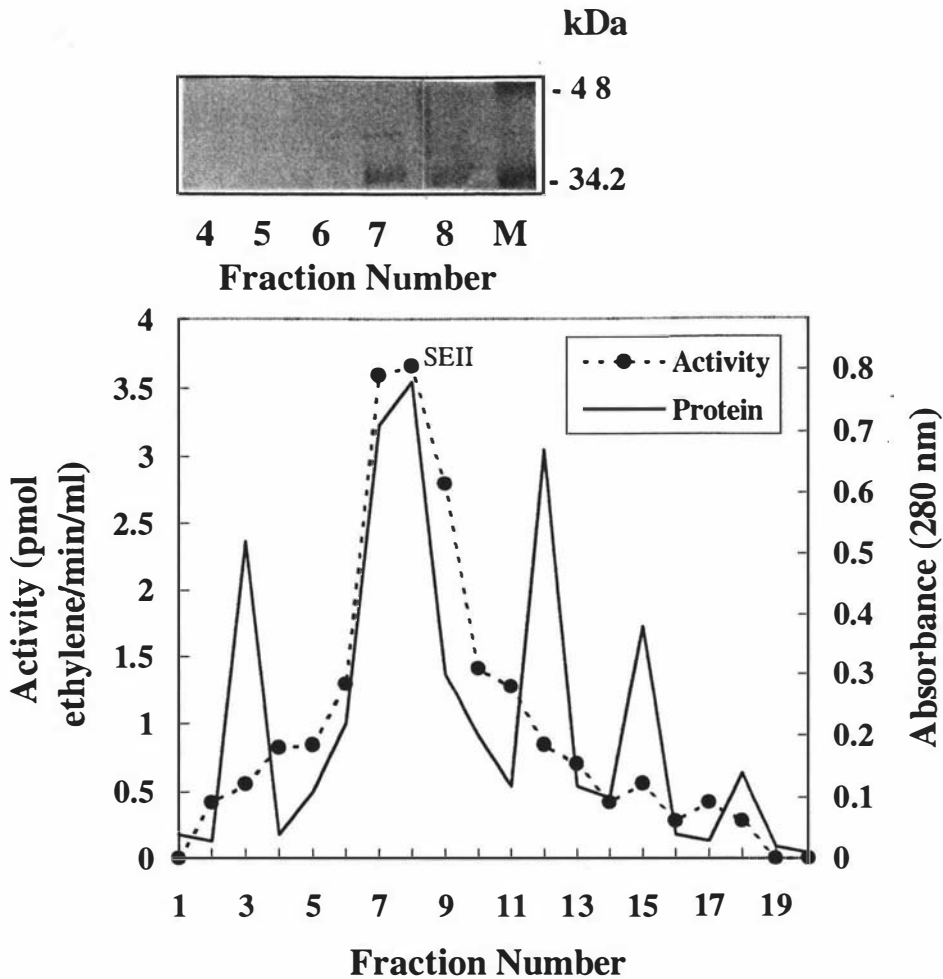


Figure 3. 22 Protein Elution Profile and ACC Oxidase Activity of the SEII Preparation (fractions 33 to 35 from the Phenyl Superose column (Figure 3.19)) after Chromatography through a Mono Q Anion Exchange Column.

The data from fractions 21 to 32 with no enzyme activity are not shown. The top panel is an immunodetection, using the TR-ACO2 antibody, of ACC oxidase protein in the active fractions after the Mono Q column (Figure 3.21). The protein amount loaded was 2.0 μ g per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

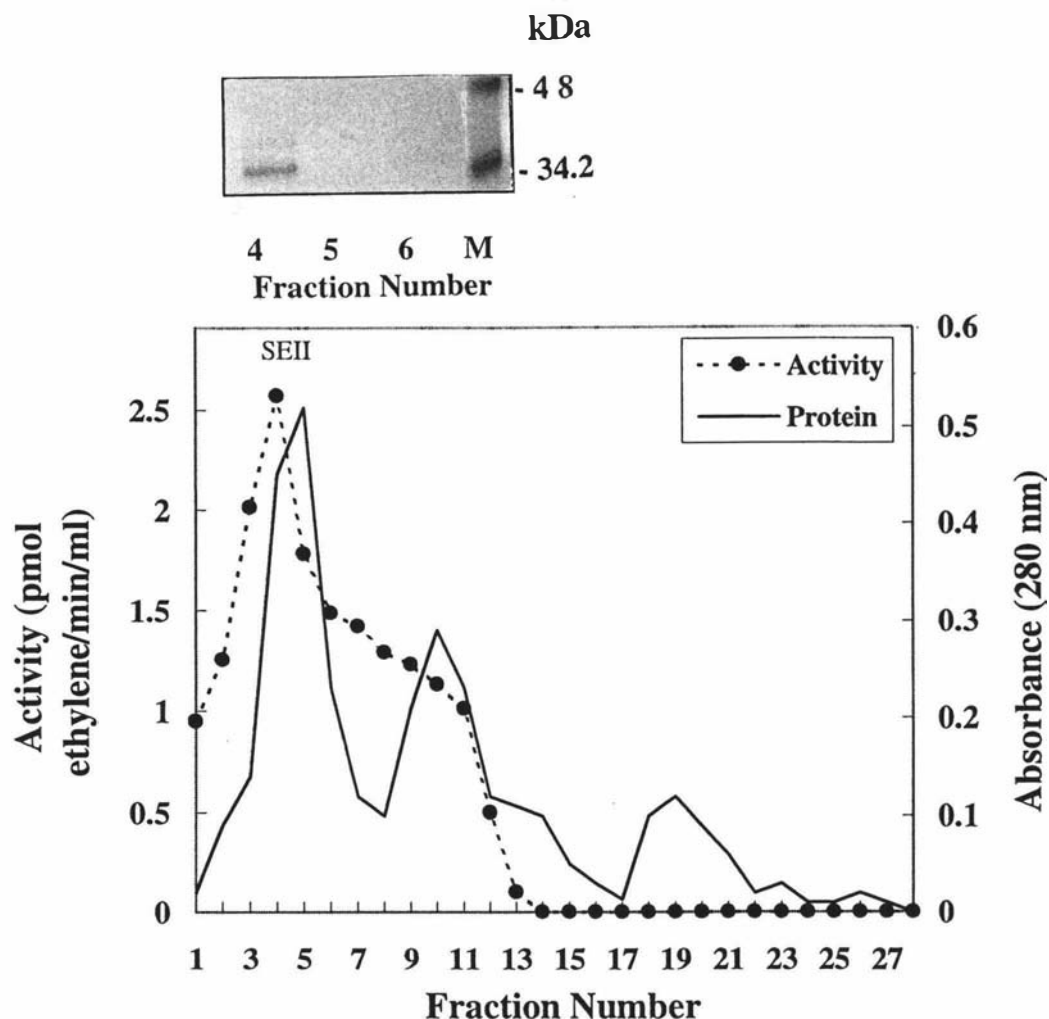


Figure 3.23 Protein Elution Profile and ACC Oxidase Activity of the SEII Preparation (fractions 7 to 8 from the Mono Q anion Exchange column (Figure 3.22)) after Chromatofocusing through a Mono P Column.

The top panel is an immunodetection, using the TR-ACO2 antibody, of ACC oxidase protein in the active fractions from the Mono P column (Figure 3.23). The protein amount loaded was 1.5 μg per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

isoform was determined to be at pH 7.0 by enzyme activity assay and pH measurement of each fraction. The specific activity of SEII after this step is 18.12 nmol ethylene/h/mg protein which represents an 87.5-fold purification (Table 3.3).

3.4.4 Gel Filtration Chromatography on a Superose 12 Column

As the last purification step, Superose 12 gel filtration chromatography was used and SEII was eluted as a single active peak at fraction 21 (Figure 3.24). This active fraction also cross-reacted with the TR-ACO2 antibody as a single band of *ca.* 35 kDa (Figure 3.24, top panel). The purified isoform had a specific enzyme activity of 29.8 nmol ethylene/h/mg protein when assayed with 1 mM ACC at pH 7.5 (Section 2.2.8) which represents a 144-fold purification and a 0.05 % recovery (Table 3.3).

3.4.5 SDS-PAGE Analysis of Purified Isoform SEII

SDS-PAGE analysis (Figure 3.25) of the purified isoform SEII revealed that the isoform has also been purified to homogeneity after the five purification steps as judged by Coomassie Brilliant Blue staining and western analysis using the TR-ACO2 antibody, with an apparent molecular mass of *ca.* 35 kDa.

3.4.6 Comparison of Purified Isoforms of ACC Oxidase from Leaf Tissue of White Clover

The three isoforms (MGI, SEI and SEII) identified from mature green and senescent leaf tissues in this thesis differ in their surface hydrophobicity and two (MGI and SEII) of the three isoforms purified to homogeneity differ in terms of net electric charge and pI (summaried in Table 3.5). After the final gel filtration step, approximately 9 μ g of purified MGI and 4 μ g of purified SEII were recovered with an overall purification of *ca.* 100-fold and 144-fold, respectively. The purified MGI and SEII had specific activities of 25.2 and 29.8 nmol ethylene/h/mg protein, respectively (Tables 3.2 and 3.3) under the assay conditions used (1 mM ACC and at pH 7.5, Section 2.2.8).

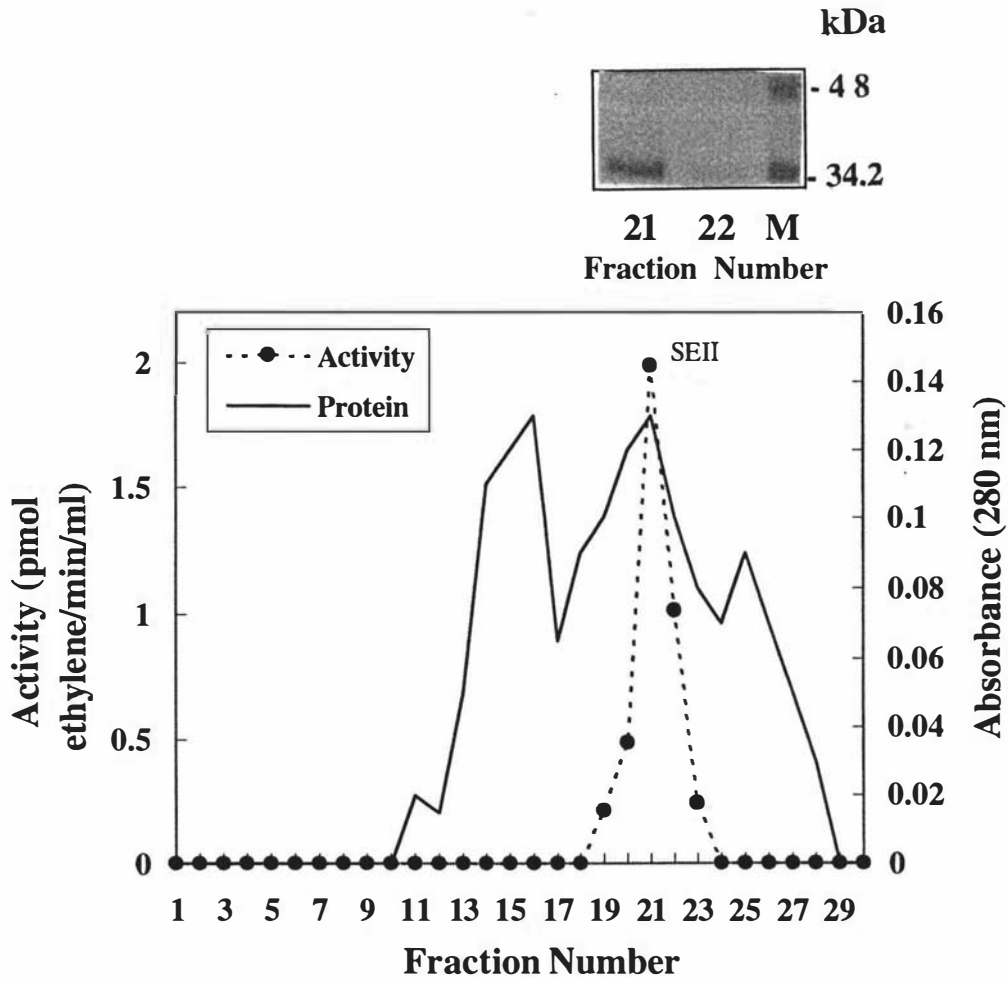


Figure 3.24 Protein Elution Profile and ACC Oxidase Activity of the SEII Preparation (fraction 4 from the Mono P chromatofocusing column (Figure 3.23)) after Separation through a Superose 12 Gel Filtration Chromatography.

The top panel is an immunodetection, using the TR-ACO2 antibody, of ACC oxidase protein in the active fraction from the gel filtration column (Figure 3.24). The protein amount loaded was 1.0 μg per lane. M = BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

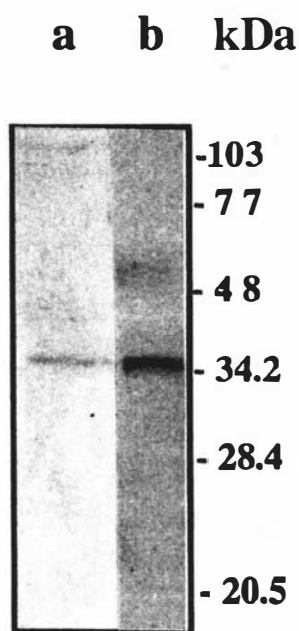


Figure 3.25 SDS-PAGE Analysis of the Purified Isoform SEII from a Senescent Leaf Extract. (a) Coomassie Brilliant Blue Staining and (b) Western Analysis using the TR-ACO2 Antibody of the Separation in (a).

The protein amount loaded for lanes a and b were 3 μg and 1 μg , respectively. The molecular masses of the standards used are indicated.

Table 3.5 Summary of Purification Properties of Three Isoforms of ACC Oxidase Identified from Mature Green and Senescent Leaves of White Clover.

Leaf Tissue	Isoform	Hydrophobic Interaction		Anion Exchange	Chromatofocusing
		% (NH ₄) ₂ SO ₄ (%)	Elution Active fractions (No.)	Elution [NaCl] (mM)	Elution pI (pH)
Mature green leaves	MGI	0.0	30 - 32	265 - 371	7.36
Senescent leaves	SEI	0.0	28	106	-
	SEII	0.0	33 - 35	371 - 480	7.0

3.5 Partial Purification of ACC Oxidase from Wounded Detached Mature Green Leaves by Hydrophobic Interaction Chromatography

The expression of the senescent leaf related gene, TR-ACO3, has been observed to be increased by wounding in mature green leaves. However, no increase in ACC oxidase enzyme activity *in vitro* was found in crude extracts from wounding mature green leaf tissue (Hunter, 1998). To determine if increased ACC oxidase activity *in vitro* can be observed in response to wounding, partial purification of ACC oxidase from extracts of wounded mature green leaves was undertaken. Using Phenyl Sepharose hydrophobic interaction column chromatography, one major activity peak (MGI, comprising fractions 33 to 35) and a second minor peak (MGII, comprising fraction 38) were observed in both unwounded and wounded (for 3 h) mature green leaf protein extracts (Figure 3.26; wounded 3h data not shown). However, two ACC oxidase activity peaks, designated MGI' (consisting of fractions 33 to 35) and MGII' (consisting of fraction 38), were

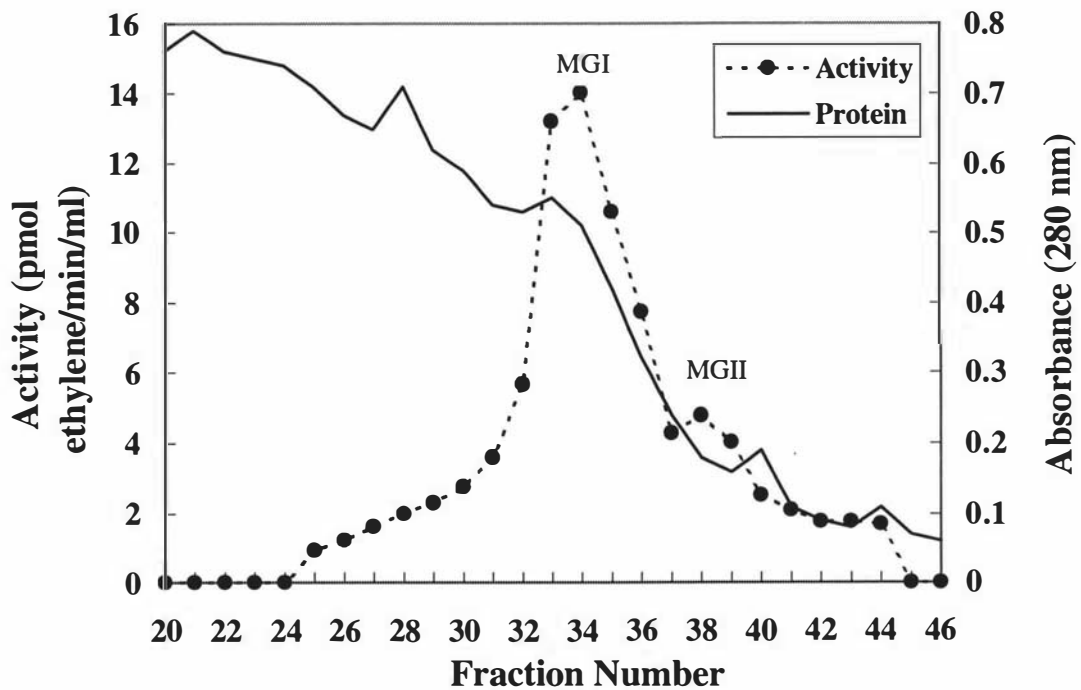


Figure 3. 26 Protein Elution Profile and ACC Oxidase Activity from a Non-Wounded Mature Green Leaf Protein Extract Subjected to Phenyl Sepharose Hydrophobic Interaction Chromatography.

Data from fractions 1 to 19 with no enzyme activity are not shown.

eluted from protein extracts of wounded (for 6 h) detached mature green leaves (Figures 3.27 and 3.28). In terms of specific activity, MGII' was more active than MGI' (Figure 3.28). The first activity peak from the 0, 3 and 6 h wounded detached mature green leaf protein extracts was eluted at the same point, suggesting that it is the same protein. The second enzyme activity peak, observed only in the 6 h wounded treatment, may be an ACC oxidase that is induced by wounding. No further purification of these two activity peaks was carried out in this study.

3.6 Immunoaffinity Chromatography of ACC Oxidase Protein from Mature Green Leaves

The aim of this section was to obtain sufficient purified ACC oxidase protein for amino acid sequencing by exploiting the TR-ACO2 antibody's high specificity for ACC oxidase protein as determined by western analysis. Immunoaffinity chromatography has not been used in the purification of this enzyme from any source. In this study, an immunoaffinity column was prepared using CNBr-activated Sepharose 4B. The TR-ACO2 IgG was coupled directly to CNBr-activated Sepharose 4B and was used to purify ACC oxidase protein from enzyme preparations recovered from Sephadex G-25 gel filtration chromatography.

3.6.1 Affinity Purification of Native ACC Oxidase Protein

An enzyme extract after Sephadex G-25 gel filtration was dialysed against 1 x PBSalt buffer (pH 7.4) and then slowly applied to the column which had been pre-equilibrated in the same buffer (pH 7.4) at room temperature. Bound protein was eluted with a low pH (pH 2.5) buffer followed by a high pH (pH 11.0) buffer. SDS-PAGE analysis of the eluted protein preparation from the affinity column showed that no ACC oxidase protein of the expected size was eluted from the column as judged from Coomassie Brilliant Blue staining (data not shown). This indicates that the TR-ACO2 antibody might not bind the ACC oxidase protein in its native conformation or the protein has bound to the column too tightly to be eluted by the pH change. Therefore, other conditions may be necessary to elute the bound protein.

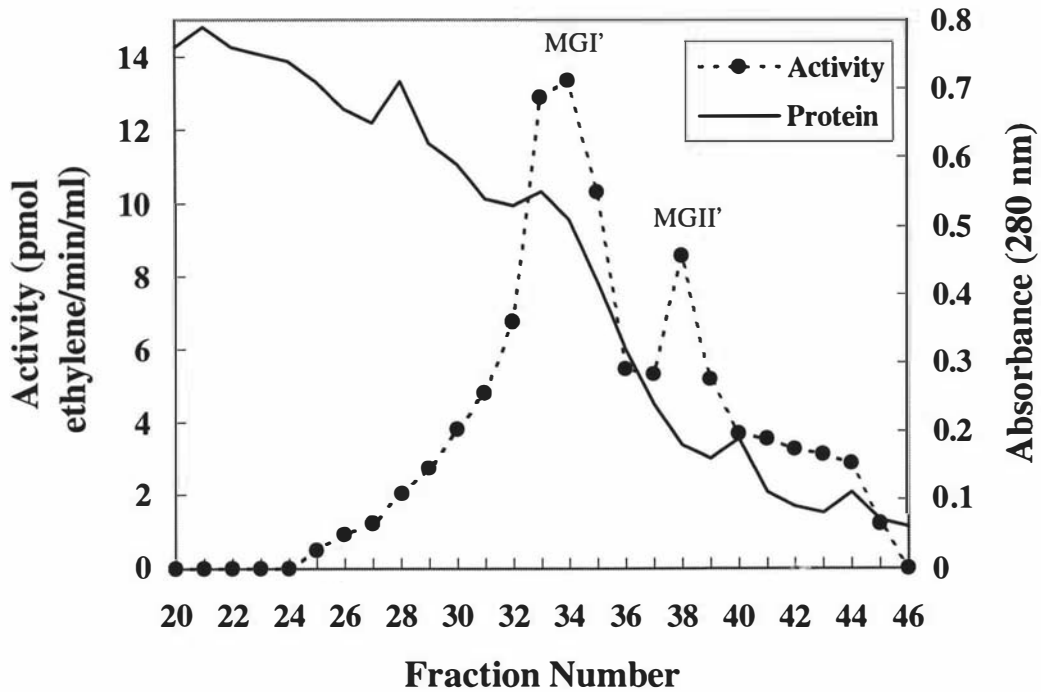


Figure 3.27 Protein Elution Profile and ACC Oxidase Activity from Extracts of Wounded (for 6 h) Mature Green Leaf Tissue Subjected to Phenyl Sepharose Hydrophobic Interaction Chromatography.

Data from fractions 1 to 19 with no enzyme activity are not shown.

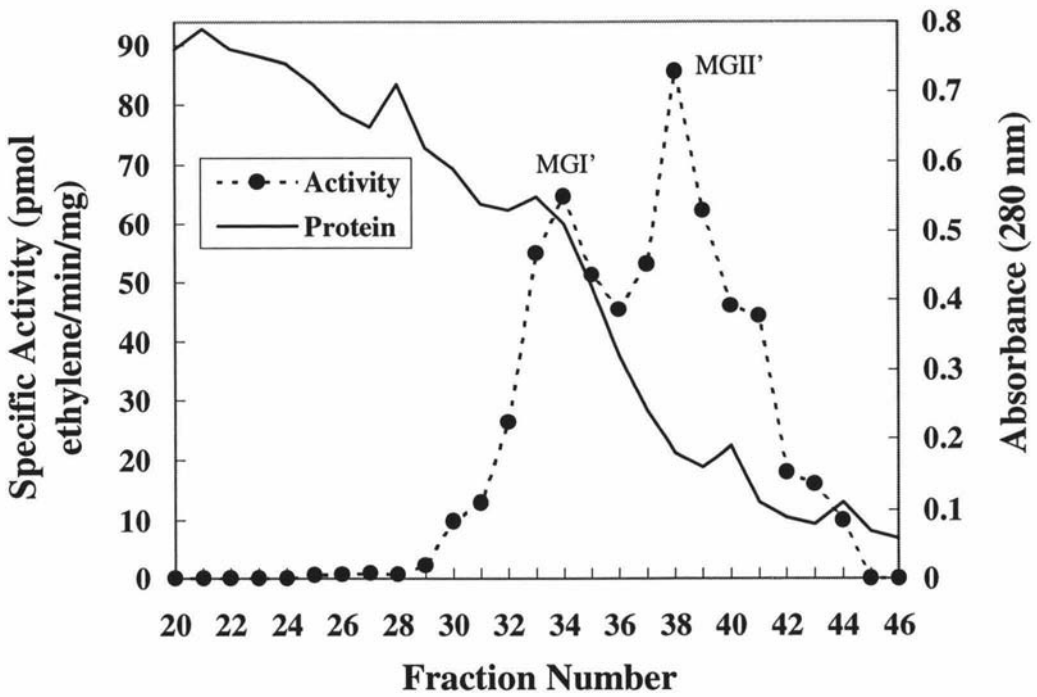


Figure 3.28 Protein Elution Profile and ACC Oxidase Specific Activity from Extracts of Wounded (for 6 h) Mature Green Leaf Tissue Subjected to Phenyl Sepharose Hydrophobic Interaction Chromatography.

Data from fractions 1 to 19 with no enzyme activity are not shown.

3.6.2 Affinity Purification of Denatured ACC Oxidase Protein

To determine if denatured protein will bind to the column, 1.0 % (w/v) of SDS was added to the protein extract and then the denatured protein preparation subjected to the affinity column chromatography as described (Section 3.6.1). SDS-PAGE analysis (Figure 3.29A) showed that several proteins were eluted from the column. One of these proteins was ACC oxidase as judged from the molecular mass and recognition by the TR-ACO2 antibody using western analysis (Figure 3.29B). However, the amount of ACC oxidase protein eluted was very low and so amino acid sequencing was not pursued further.

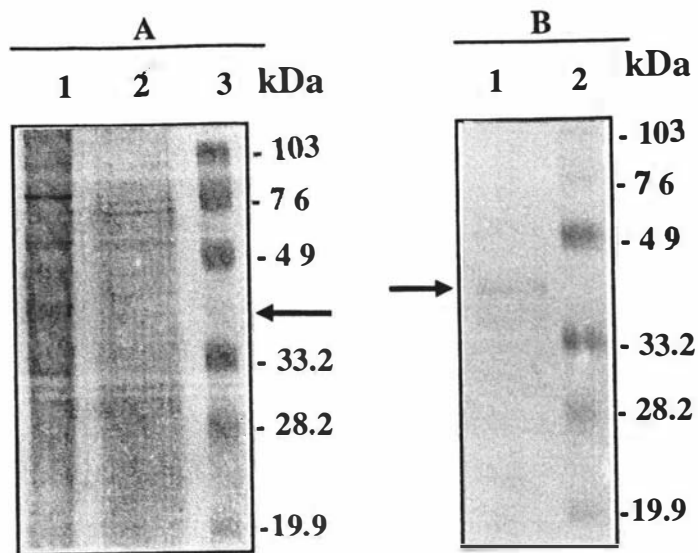


Figure 3.29 (A) SDS-PAGE Analysis of the Eluate from a TR-ACO2 Antibody Immunoaffinity Column after Chromatography and Elution of Bound Protein from a Mature Green Leaf Extract Denatured with 1.0 % (w/v) SDS.

Lane 1: enzyme extract (20 μg) from mature green leaves before application to the column.

Lane 2: protein preparation (5 μg) eluted from the immunoaffinity column.

Lane 3: BIO-RAD prestained SDS-PAGE standards (molecular masses are indicated).

(B) Western Analysis of (A) using the TR-ACO2 Antibody.

Lane 1: protein preparation (5 μg) eluted from the immunoaffinity.

Lane 2: BIO-RAD prestained SDS-PAGE standards (molecular weights are indicated).

Arrows indicate the ACC oxidase protein.

3.7 Characterisation of Two Isoforms of ACC Oxidase from Leaves of White Clover

Three isoforms (MGI, SEI and SEII) of ACC oxidase have been identified and two of the three (MGI and SEII) purified to homogeneity from white clover mature green and senescent leaf tissue, respectively. In this thesis, the two purified isoforms are compared in terms of several important physicochemical and kinetic properties: apparent and native molecular mass, pI, pH optimum, apparent K_m for the substrate ACC and optimal requirements of enzyme activity for co-substrate and cofactors.

3.7.1 Physicochemical Properties of Two Purified Isoforms

3.7.1.1 Molecular Mass

3.7.1.1.1 Apparent Molecular Mass Determined by SDS-PAGE

Using SDS-PAGE followed by western analysis, one or two lanes of prestained protein markers (low range) were loaded alongside the ACC oxidase samples. By measuring the distance of migration of the prestained protein markers, a standard curve was drawn as shown in Figures 3.30 and 3.31. The $\text{Log}(M_r)$ values for MGI and SEII were determined to be 4.57 and 4.54, respectively from the graphs (Figures 3.30 and 3.31), which were converted to molecular masses for MGI and SEII of 37.0 kDa and 35.0 kDa, respectively.

3.7.1.1.2 Native Molecular Mass Determined by Gel Filtration Chromatography

The native molecular mass of each isoform was determined by gel filtration column chromatography. The gel filtration column was calibrated using various protein molecular mass standards according to the manufacturer's instructions. The void volume (V_o) was determined by Dextran Blue 2000 elution to be 7.8 ml. The elution volumes (V_e) of the markers were periodically checked and remained unchanged throughout the course of the experiment. A curve between a ratio of elution volume (V_e) to void volume (V_o) (as V_e/V_o) and $\text{Log}(M_r)$ of the standard proteins used was constructed as shown in Figure 3.32. The two purified isoforms were chromatographed through the column in an identical manner to the standard proteins. The same elution volume of these two isoforms (V_e , 13.8 ml) was determined for the same active fraction

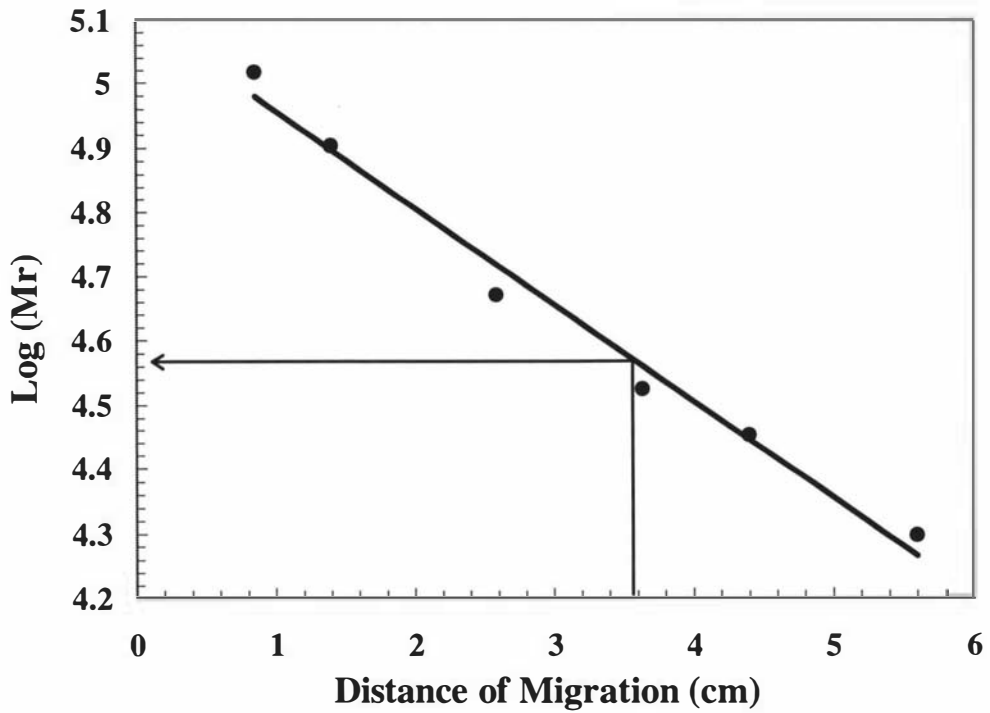


Figure 3.30 Determination of the Molecular Mass of Isoform MGI using SDS-PAGE.

The mobility of MGI was compared to the mobilities of BIO-RAD SDS-PAGE prestained standards. The distance migrated is plotted against Log (M_r) of each standard and a line of best fit was drawn between the plotted points using Excel 5.0. Log (M_r) for MGI is 4.57 (denoted by the arrow).

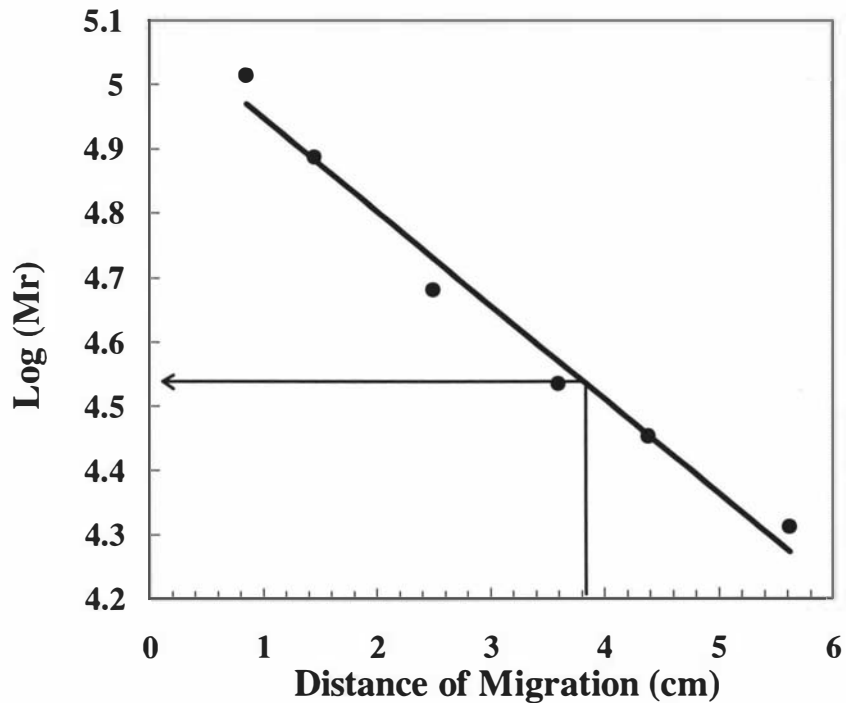


Figure 3.31 Determination of the Molecular Mass of Isoform SEII using SDS-PAGE.

The mobility of SEII was compared to the mobilities of BIO-RAD SDS-PAGE prestained standards. The distance migrated is plotted against Log (M_r) of each standard and a line of best fit was drawn between the plotted points using Excel 5.0. Log (M_r) for SEII is 4.54 (denoted by the arrow).

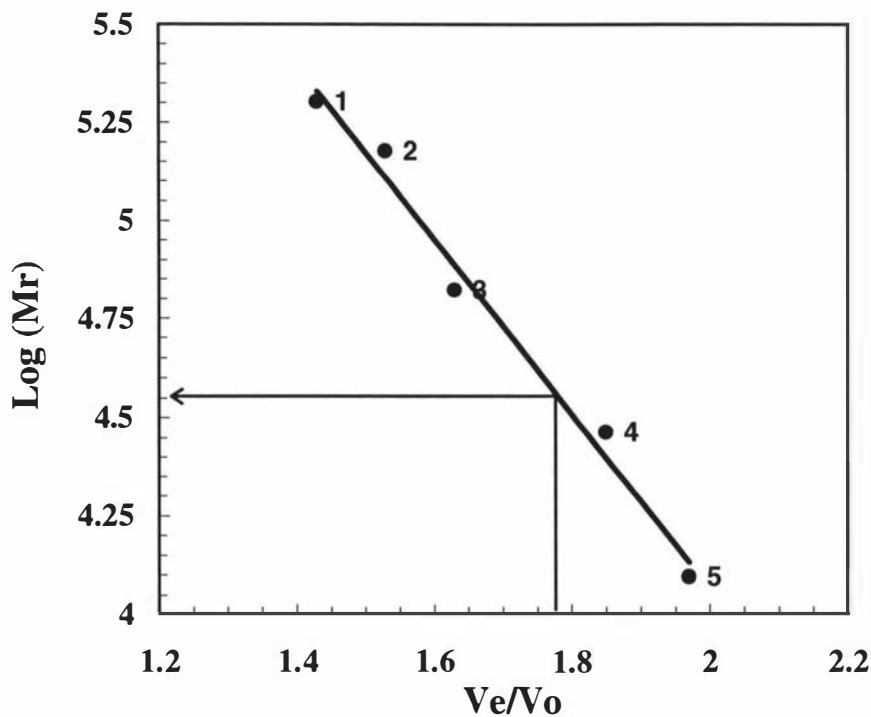


Figure 3.32 Determination of the Molecular Masses of MGI and SEII using Gel Filtration Column Chromatography.

A calibration curve of $\text{Log}(M_r)$ against V_e/V_o was plotted from a series of molecular mass standards. The V_e/V_o and corresponding molecular mass for both isoforms are marked with arrows. The molecular mass markers are: 1, β -amylase (200 kDa); 2, alcohol dehydrogenase (150 kDa); 3, albumin (66 kDa); 4, carbonic anhydrase (29 kDa); 5, cytochrome (12.4 kDa).

(No.21, Figures 3.17 and 3.24). From this plot, the native molecular mass of the two active isoforms was estimated to be 37.5 kDa which is similar to the apparent molecular masses (37 kDa and 35 kDa for MGI and SEII, respectively) determined by SDS-PAGE analysis (Figures 3.30 and 3.31). These data indicate that both isoforms from mature green and senescent leaf tissue are active as monomers.

3.7.1.2 pH Optimum

Because of the instability of ACC oxidase activity *in vitro*, partially purified preparations of the two isoforms after the third purification step (Mono Q anion exchange) were used to analyse pH optimum and kinetic properties in terms of apparent K_m for ACC and optimal requirements for co-substrate and cofactors. The effect of pH on the enzyme activities *in vitro* of these two isoforms as partially purified preparations was investigated by activity assay *in vitro* performed over a pH range of 4.0 to 10.0. Isoform MGI was active over a pH range of 5.50 to 8.75 with maximum activity at pH 7.5 using Tris-HCl buffer. In contrast, SEII was active at pH 6.0 to 9.0 with an optimal pH at 8.5 using Tris-HCl buffer (Figure 3.33). To further confirm the basic pH optimum for SEII, 100 mM phosphate buffer (pH from 7.0 to 10.0) was used and the same optimal pH observed (Figure 3.34) although the enzyme activity was lower when compared with the activity determined using Tris-HCl buffer. With the determination of pH optima, all subsequent kinetic studies were carried out at pH 7.5 for MGI and 8.5 for SEII using Tris-HCl buffer.

3.7.2 Kinetic Properties of Partially Purified Isoforms MGI and SEII

3.7.2.1 Apparent K_m for ACC

The dependence of ACC oxidase activity of both isoforms on the concentration of ACC, assayed at the optimal pHs (pH 7.5 for MGI and pH 8.5 for SEII) and using the standard concentrations of bicarbonate (30 mM), sodium ascorbate (30 mM) and ferrous iron (20 μ M), exhibited Michaelis-Menten kinetics (Figures 3.35 and 3.36). Enzyme activities of isoforms MGI and SEII were saturated at ACC concentrations of 2 mM and 4 mM, respectively (Figures 3.35 and 3.36). The apparent K_m and V_{max} values for ACC were determined from Eadie-Hofstee plots (Figures 3.37 and 3.38). The apparent K_m of

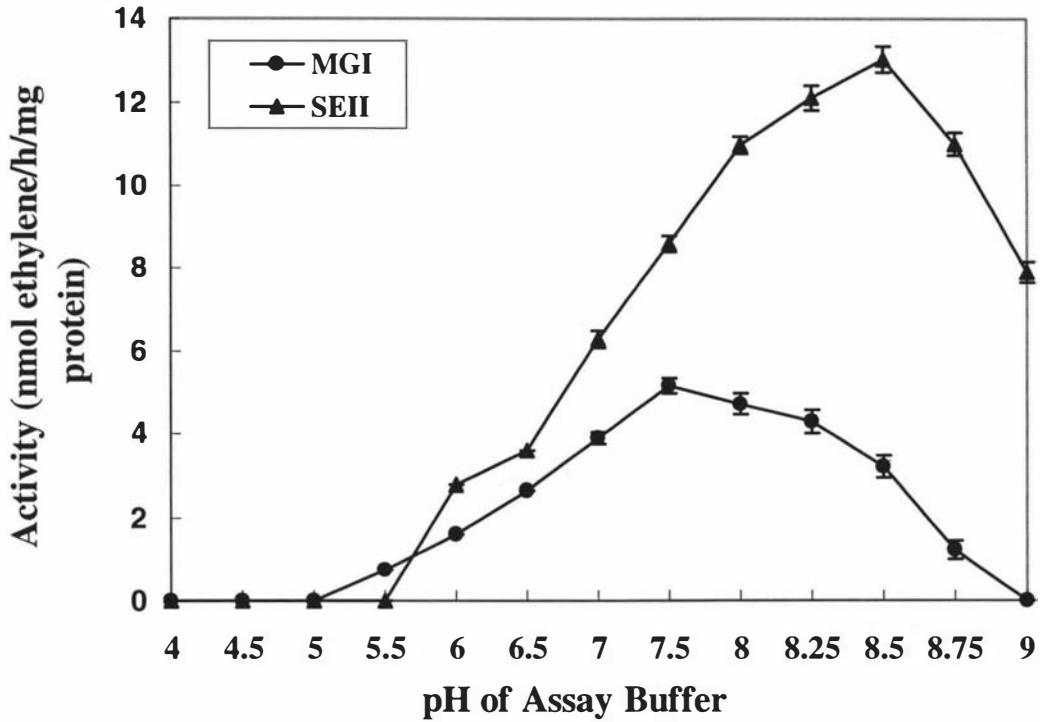


Figure 3.33 ACC Oxidase Activity of Isoforms MGI and SEII over a pH Range from 4.0 to 9.0.

Buffers used were 50 mM sodium acetate-HCl (pH 4.0 to 5.0), MOPS-NaOH (pH 6.0 to 6.5) and Tris-HCl (pH 7.0 to 9.0). Values are means \pm SE (n = 4).

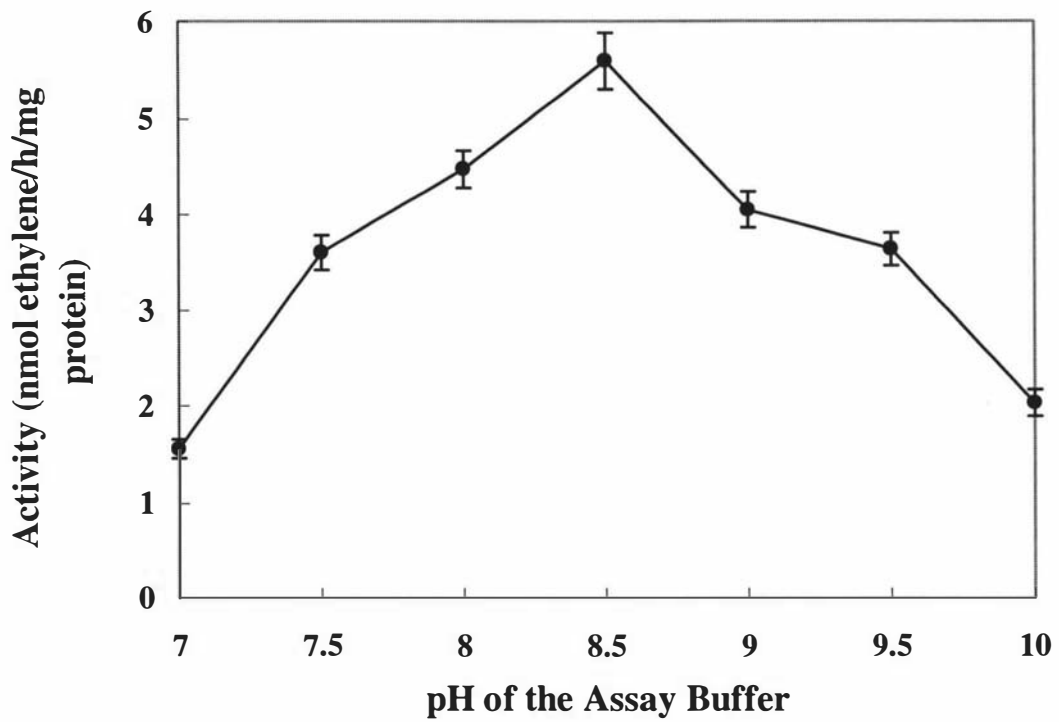


Figure 3.34 ACC Oxidase Activity of Isoform SEII over a pH Range from 7.0 to 10.0 using a 100 mM Phosphate Buffer.

Values are means \pm SE (n = 4).

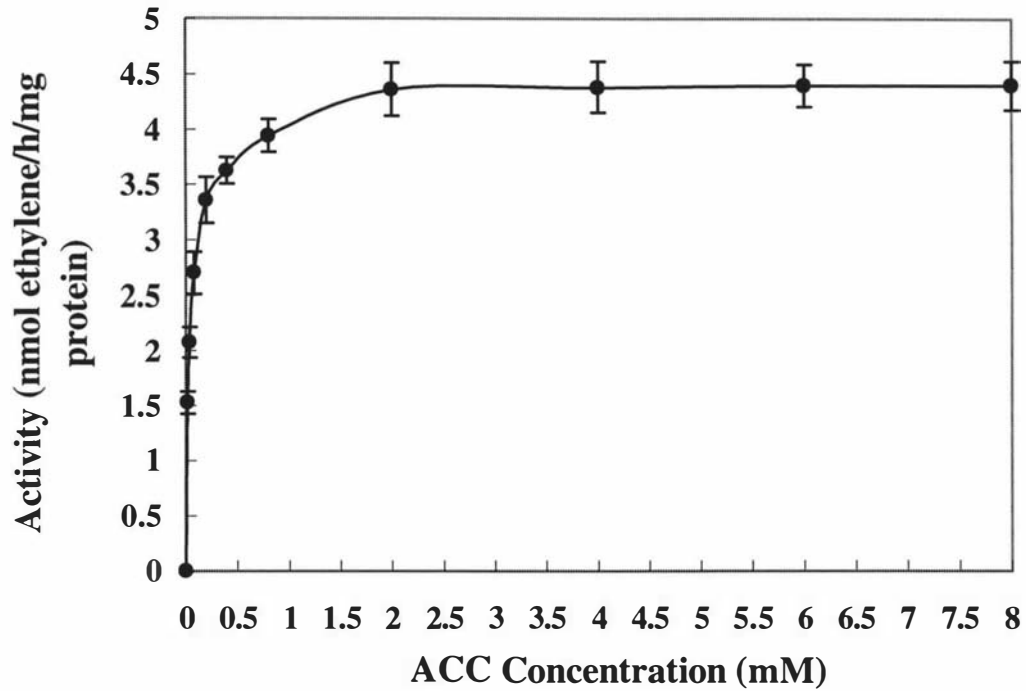


Figure 3.35 Dependence of ACC Oxidase Activity *in vitro* of Isoform MGI on ACC Concentration at pH 7.5 in the Presence of 30 mM Sodium Bicarbonate.

Values are means \pm SE (n = 6).

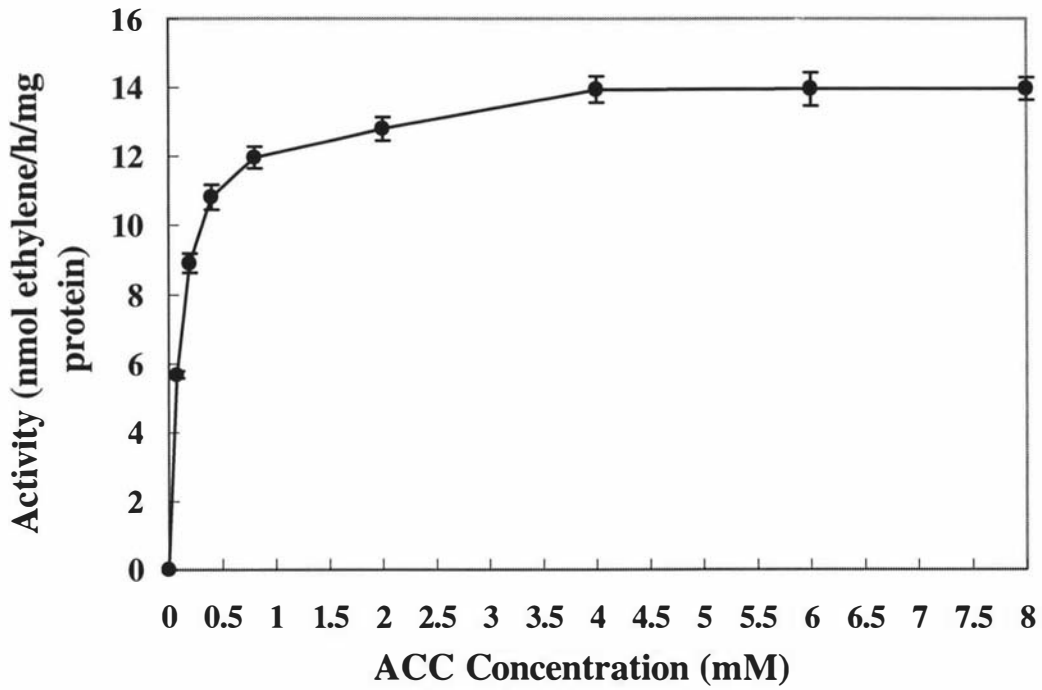


Figure 3. 36 Dependence of ACC Oxidase Activity *in vitro* of Isoform SEII on ACC Concentration at pH 8.5 in the Presence of 30 mM Sodium Bicarbonate.

Values are means \pm SE (n = 6).

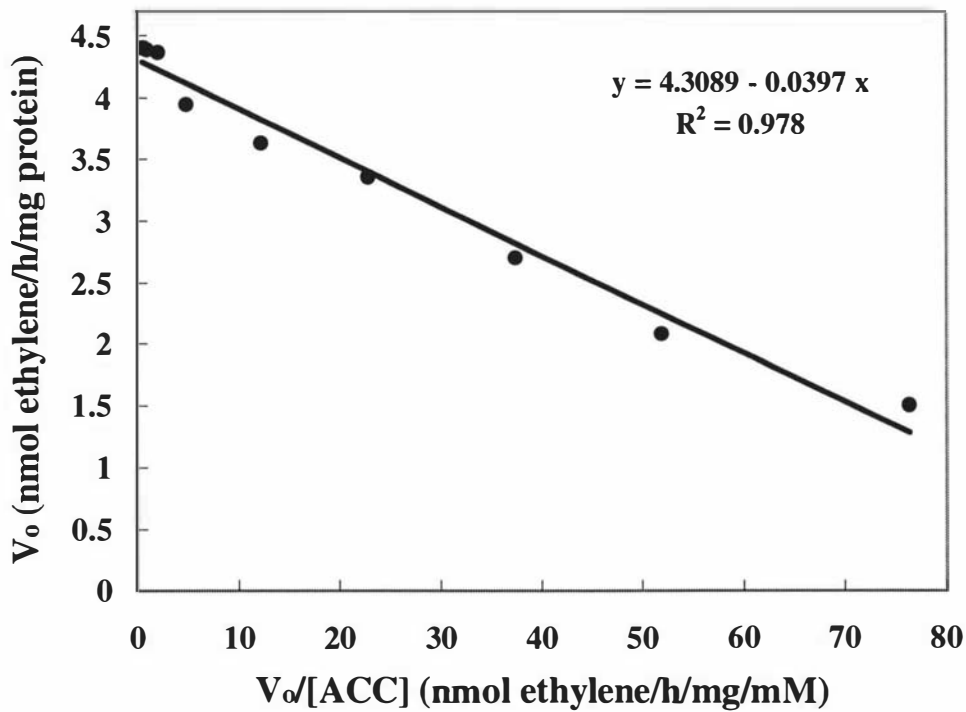


Figure 3.37 Eadie-Hofstee Plot for ACC Oxidase Activity of Isoform MGI with ACC as Substrate (data derived from Figure 3.35).

Ninety-five percent confidence interval of the slope of the Eadie-Hofstee regression did not overlap. The kinetic parameters derived from this curve are shown in Table 3.6.

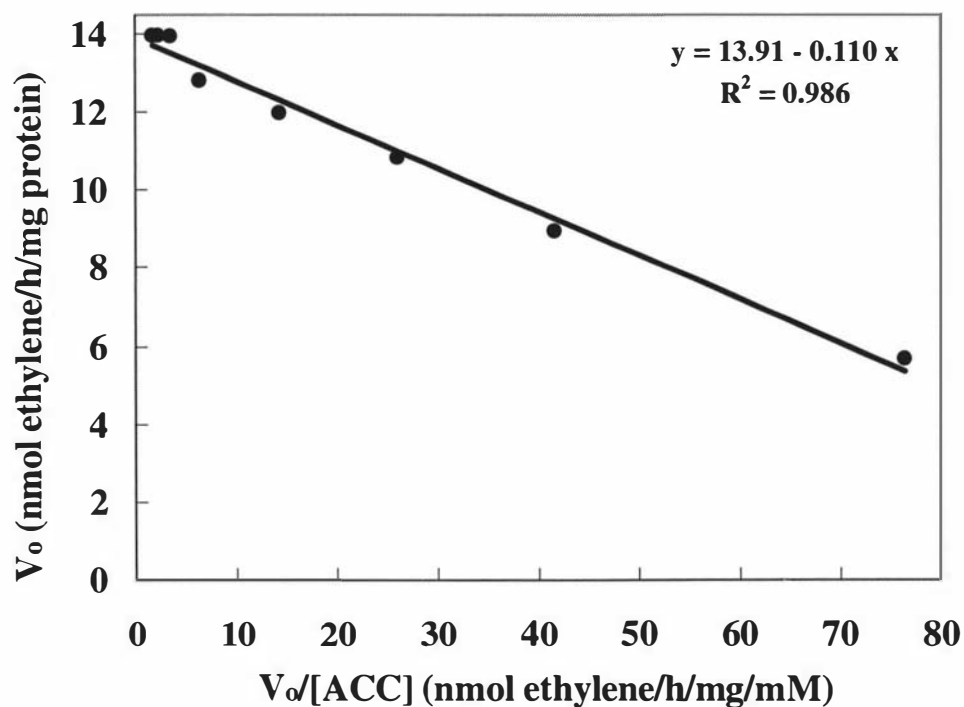


Figure 3.38 Eadie-Hofstee Plot for ACC Oxidase Activity of Isoform SEII with ACC as Substrate (data derived from Figure 3.36).

Ninety-five percent confidence intervals of the slope of the Eadie-Hofstee regression did not overlap. The kinetic parameters derived from this curve was shown in Table 3.6.

SEII for ACC (110.0 μM) in the presence of bicarbonate was approximately 2.8-fold higher than MGI (39.7 μM), and SEII had a higher V_{max} for ACC (13.91 nmol ethylene/h/mg protein) when compared with MGI (4.31 nmol ethylene/h/mg protein) (Table 3.6). The results indicate that isoform MGI in mature green leaves has a higher affinity for its substrate ACC than SEII in the senescent leaves. SEII requires a higher ACC concentration to achieve a higher reaction velocity and thus produce higher amount of ethylene when compared with MGI. Due to the very low enzyme activity when bicarbonate was removed from the assay buffer, it was not possible to estimate the K_m value for its substrate ACC in the absence of bicarbonate (data not shown).

Table 3.6 Summary of Kinetic Parameters Determined for Isoforms MGI and SEII from White Clover Leaves.

Apparent K_m and V_{max} values for ACC were determined from Eadie-Hofstee plots in the presence of sodium bicarbonate.

Isoform (Leaf Tissue)	Optimal pH	V_{max} for ACC (nmol ethylene/h/mg protein)	Apparent K_m for ACC (μM)
MGI (Mature green leaves)	7.5	$4.31^b \pm 0.21$	$39.7^b \pm 7.1$
SEII (Senescent leaves)	8.5	$13.91^a \pm 1.1$	$110.0^a \pm 12.0$

a, b, significant difference at $P = 0.05$. Values are means \pm SE ($n = 6$).

3.7.2.2 Optimal Requirements of ACC Oxidase Activity *in vitro* of Two Isoforms for Co-substrate and Cofactors

Both isoforms show an absolute requirement for the co-substrate ascorbate *in vitro*. In the absence of ascorbate, no enzyme activity was observed (Figure 3.39) and increasing ascorbate concentrations stimulated the enzyme activity of both isoforms. The optimal concentrations of ascorbate for MGI and SEII were determined to be 8 mM and 4 mM, respectively, with higher concentrations becoming inhibitory to enzyme activity *in vitro* (Figure 3.39).

ACC oxidase activity *in vitro* of both isoforms was stimulated by the addition of sodium bicarbonate to the reaction medium (Figure 3.40) and increasing sodium bicarbonate concentrations stimulated enzyme activity *in vitro*. The maximum activities for MGI and SEII was found to be at 24 mM NaHCO₃ and a higher concentration of bicarbonate resulted in inhibition of the enzyme activity. Furthermore, when bicarbonate was omitted from the reaction mixture, the enzymatic activity was dramatically reduced (Figure 3.40).

Both MGI and SEII were observed to require ferrous iron for enzyme activity *in vitro*, with maximum activity obtained at 16 μM and 24 μM FeSO₄, respectively (Figure 3.41). Higher concentrations of ferrous iron were also inhibitory to enzyme activity.

Differences between these two isoforms of ACC oxidase in terms of physiochemical and kinetic properties are summarized in Table 3.7.

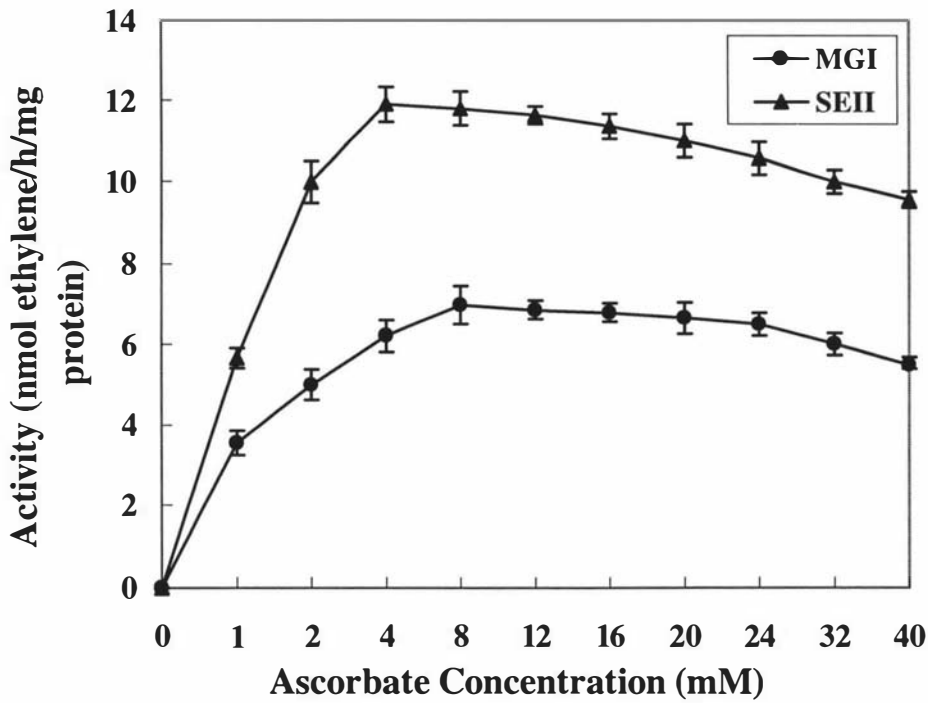


Figure 3.39 Dependence of ACC Oxidase Activity of Isoforms MGI and SEII on Ascorbate Concentration.

Values are means \pm SE (n = 4).

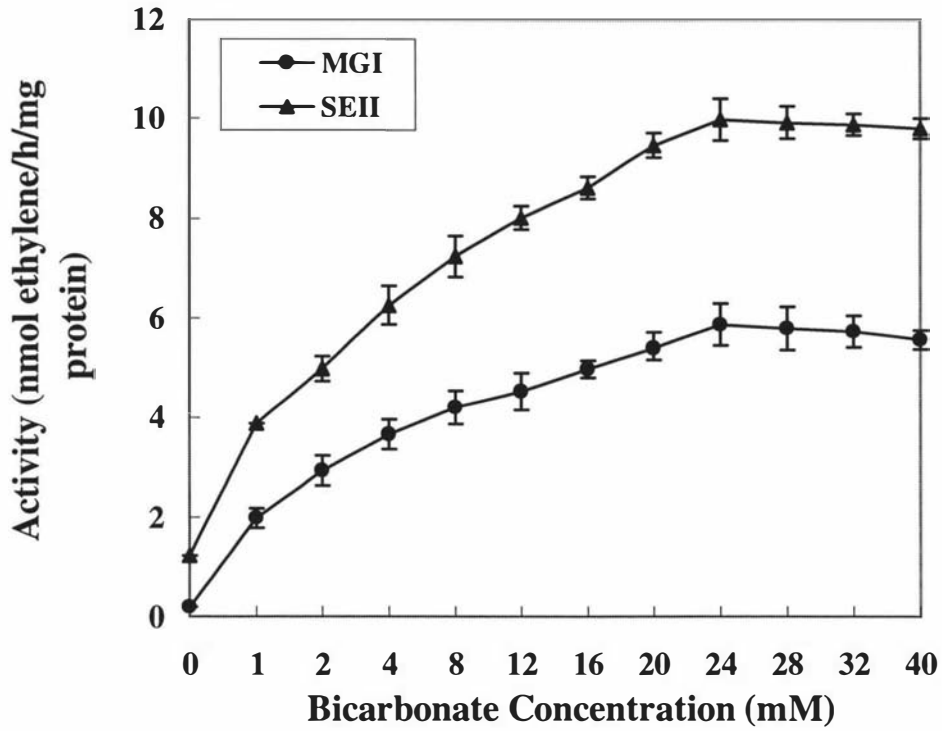


Figure 3.40 Dependence of ACC Oxidase Activity of Isoforms MGI and SEII on Bicarbonate Concentration.

Values are means \pm SE (n = 4).

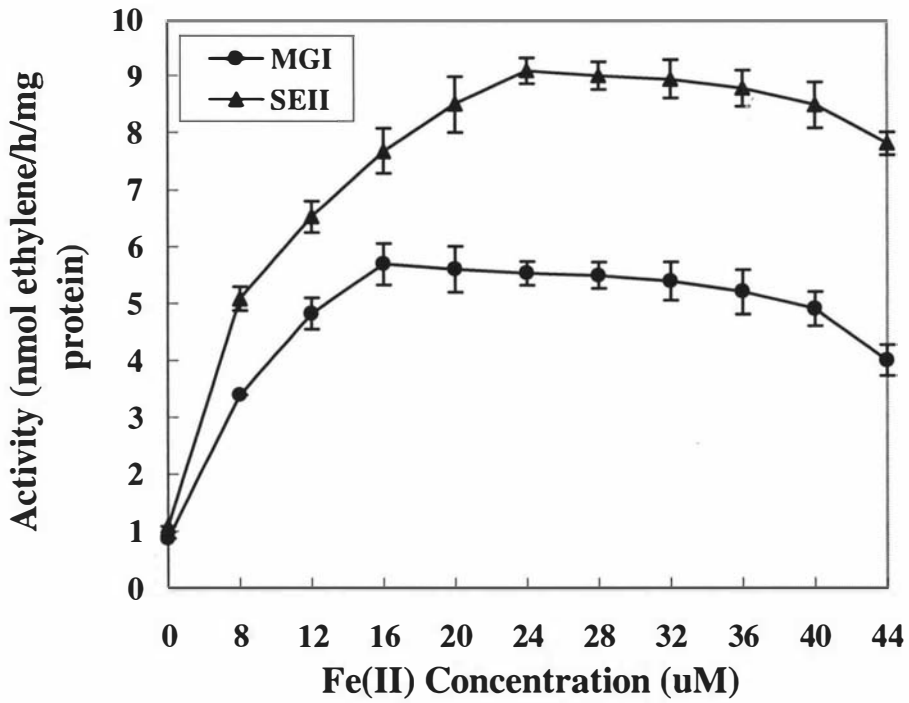


Figure 3.41 Dependence of ACC Oxidase Activity of Isoforms MGI and SEII on Fe^{2+} Concentration.

Values are means \pm SE (n = 4).

Table 3.7 Summary of Biochemical Properties of ACC Oxidase Isoforms MGI and SEII from Mature Green and Senescent Leaves of White Clover.

Biochemical Properties	Isoforms of ACC Oxidase	
	MGI	SEII
A. Physicochemical characteristics		
Molecular mass (kDa)		
Determined by gel filtration	37.5	37.5
Determined by SDS-PAGE	37.0	35.0
pI		
Determined by chromatofocusing	7.36	7.0
pH optimum	7.5	8.5
B. Kinetic properties		
K_m for ACC (μM) ¹	39.70 ± 7.1	110.0 ± 12
V_{\max} (nmol ethylene/h/mg) ²	4.31 ± 0.21	13.91 ± 1.1
Optimum for co-substrate and cofactors		
Ascorbate (mM)	8.0	4.0
Bicarbonate (mM)	24.0	24.0
Fe ²⁺ (μM)	16.0	24.0

¹ Values are means \pm SE (n = 6).

² Values are means \pm SE (n = 6).

3.7.2.3 Relative Abundance of Partially Purified Isoforms of ACC Oxidase Identified from Mature Green and Senescent Leaves

With the elucidation of optimal pHs and saturating ACC concentrations for each isoform an attempt was made to determine the relative abundance of each isoform in each particular tissue. Assaying the activities *in vitro* of the two partially purified isoforms (after the Phenyl Superose hydrophobic interaction step) at optimal pHs and saturating concentrations of ACC (2 mM for MGI and 4 mM for SEII) revealed an approximately 2.8-fold higher enzyme specific activity for SEII in senescent leaf tissue when compared with MGI in mature green leaf tissue (Table 3.8).

Table 3.8 Comparison of Relative Abundance of Isoforms MGI and SEII from White Clover Leaves.

Partially purified enzyme preparations, recovered from the hydrophobic interaction column, were assayed for activity at saturating ACC concentrations and optimal pHs for both isoforms.

Leaf Tissue	Isoform Specific Activity (nmol ethylene/h/mg protein)	
	MGI (pH 7.5)	SEII (pH 8.5)
Mature green leaves	2.89 ^b ± 0.16	-
Senescent leaves	-	7.95 ^a ± 0.87

a,b, significant difference at P = 0.05. Values are means ± SE (n = 6).

4. Chapter Four: Discussion

In the first section of this thesis, two truncated reading frames from white clover ACC oxidase genes, TR-ACO2 and TR-ACO3, have been expressed in *E. coli*, and the enzyme activity of the purified proteins assayed. The enzyme activity obtained was much lower when compared with ACC oxidase genes from other plant species expressed in *E. coli*. Further characterisation therefore was not pursued. In white clover, mature green and senescent leaf tissues have recently been shown to express two ACC oxidase genes differentially (Hunter *et al.*, 1999). The second section of this thesis involved the identification and purification to homogeneity of two distinct isoforms of ACC oxidase from mature green and senescent leaf tissues and the detailed biochemical characterisation of each.

4.1 Expression and Purification of ACC Oxidases in *E. coli*

There has been little systematic study on the effect of different growth conditions on the synthesis of foreign proteins in *E. coli* (Old and Primrose, 1995). It has been shown that lowering the incubation temperature increased the yield of correctly folded soluble protein (Schein and Noteborn, 1988; Zhang *et al.*, 1995). In this study, the incubation temperature after IPTG induction was lowered from 37°C to 27°C, but the solubility of the ACC oxidase proteins was still very low as judged from the very low protein yield in the supernatant of lysed *E. coli* cells (Figure 3.4). It is most likely that the ACC oxidase proteins were incorporated into inclusion bodies although experiments to test this directly were not undertaken. Many eukaryotic proteins, when expressed in bacteria, accumulate as insoluble inclusion bodies inside the cell (Marston, 1986). This has been observed to occur with the expression in *E. coli* of ACC synthase genes expressed in fruits of tomato and zucchini (Zhou *et al.*, 1998). The formation of inclusion bodies in *E. coli* can be a problem as the induced protein within the inclusion bodies is inactive. For ACC synthase, Zhou *et al.* (1998) found that the induction conditions did not influence the solubility of the protein. However, soluble expression increased significantly when the enzyme was cloned into vector pET11d compared with another vector, pET30a. In addition, Wyatt (1997) observed that a fusion protein of glutathione S-transferase-aldehyde dehydrogenase (GST-hAIDH) induced with 1.0 mM IPTG was more soluble when compared with induction with 0.04 mM and 0.1 mM IPTG. In this

thesis, a range of IPTG concentrations and different induction times with IPTG were tested to maximise production of the fusion proteins. However, an investigation on the effect of different expression vectors and IPTG concentrations on the solubility of the TR-ACO2 and TR-ACO3 fusion proteins when induced in *E. coli* should also be performed.

In this study, in addition to the induced ACC oxidase protein band of *ca.* 38 kDa, another protein band of *ca.* 29 kDa was co-eluted from the affinity column (Figure 3.6). This protein has been identified as a nickel binding protein common to strains of *E. coli* (Dr. M.T. McManus, *pers. comm.*). This suggests that this protein might possess surface His residues since any protein with these residues could also bind to the column. The more His residues available, the higher affinity for the column. Therefore, it is necessary to optimise the washing buffer to exclude all other proteins (including the nickel binding protein) except for the six-His tagged fusion protein.

The purified TR-ACO2 and TR-ACO3 fusion proteins had specific activities of 0.34 and 0.23 nmol ethylene/h/mg protein, respectively under the assay conditions used (Section 2.1.5). These activities were very low when compared to the reported values of 2100 nmol ethylene/h/mg protein for the recombinant purified enzyme from tomato fruit (Zhang *et al.*, 1995), 1200 nmol ethylene/h/mg protein for the purified recombinant ACC oxidase from tomato fruit (Smith *et al.*, 1994) and 514 nmol ethylene/h/mg protein for the purified recombinant enzyme from kiwifruit (Xu *et al.*, 1998). Three explanations for this low enzyme activity in this study can be considered:

- Absence of some important amino acid residues for ACC oxidase activity *in vitro*

Although both ACC oxidase sequences contain some critical amino acids which have been considered to be important for maximal enzyme activity *in vitro* (Hunter, 1998), there are some amino acid residues which are absent in both gene sequences. For example, one residue, Pro⁵, was found to be conserved among 12 plant ACC oxidases (Lin *et al.*, 1997), and this residue was also conserved among all ferrous iron and ascorbate-requiring superfamily of enzymes (Tang *et al.*, 1993). Another residue, Arg³⁰⁰, which was suggested as a putative site for CO₂-dependent activation of ACC

oxidase from kiwifruit (Lay *et al.*, 1996), was conserved in all three active isoforms of ACC oxidase from tomato expressed in yeast (Bidonde *et al.*, 1998). This residue was also available in one active ACC oxidase from papaya fruit expressed in *E. coli* (Lin *et al.*, 1997). This residue may be involved in CO₂ activation of ACC oxidase by a carbamylation process. As well, Arg²⁹⁹ has been found to be important for the activity of an enzyme from apple fruit and CO₂ activation (Kadyrzhanova *et al.*, 1998). However, none of these residues are available in both TR-ACO2 and TR-ACO3 sequences. In addition, two residues (Lys²⁹² and Lys²⁹⁶) of the eight highly conserved Lys residues among 38 known or putative ACC oxidases (Kadyrzhanova *et al.*, 1998) are absent in both ACC oxidase sequences. Recently, Arg²⁹⁹ and Glu³⁰¹ have been identified as being very critical for enzyme activity as they are proposed to be important for ACC oxidase protein folding (Professor David Dilley, Michigan State University, USA, *pers. comm.*). Both residues are not present in the truncated reading frame used for expression. However, 3'-RACE has been used to extend the TR-ACO2 and TR-ACO3 cDNAs into the 3'-UTR (Hunter *et al.*, 1999), and comparison of the deduced amino acid sequence with a consensus constructed by Kadyrzhanova *et al.* (1997) reveals that these critical amino acid residues are present.

- Incubation time post-induction with IPTG

In this study, although the culture temperature was lowered from 37°C to 27°C, the growth time post-induction with IPTG was as long as 5 h in order to obtain more induced protein. *E. coli* cells transformed with an ACC oxidase from tomato fruit, grown at 27°C with shaking at 250 rev./min and harvested 2.5 h after induction with 0.4 mM IPTG, yielded an active enzyme (Zhang *et al.*, 1995). However, *E. coli* cells harvested 5.0 h post induction with both a lower rate of shaking (120 rev./min) and lower temperature (25°C), also yielded an active enzyme (Lin *et al.*, 1997). It has been observed that the activity of a recombinant enzyme from apple fruit expressed in *E. coli* declined as the incubation time post IPTG induction increased (Professor David Dilley, Michigan State University, USA, *pers. comm.*). Therefore, the effect of the incubation time after the addition of IPTG on the enzyme activity of both TR-ACO2 and TR-ACO3 fusion proteins needs to be examined in the future.

- Activity assay for the fusion proteins from *E. coli*

The enzyme activity assay protocol (Section 2.1.5) used in this study is similar to that used for the enzyme from leaf tissues (Section 2.2.8) although both catalase and BSA were added in the reaction mixture. It is quite possible that the enzyme activity assay for the fusion ACC oxidase proteins may require further optimisation. However, while conditions of enzyme assay need to be optimised further to improve activity, the very low enzyme activity obtained is most likely due to missing critical amino acid residues. When full length clones of white clover ACC oxidase are obtained, such optimisation experiments can be pursued further.

4.2 Extraction and Purification of ACC Oxidase Isoforms from Mature Green and Senescent Leaves

ACC oxidase has been extracted and purified to homogeneity or partially purified from fruits of apple (Dong *et al.*, 1992; Kuai and Dilley, 1992; Dupille *et al.*, 1993; Pirrung *et al.*, 1993), banana (Moya-Leon and John, 1995), cherimoya (Escribano *et al.*, 1996), papaya (Dunkley and Golden, 1998) and pear (Kato and Hyodo, 1999) which are climateric fruits with a surge in ethylene production at the onset of ripening and also partially purified from senescing carnation flowers (Kosugi *et al.*, 1997) (Table 1.1). However, as yet there have been no studies on the purification of this enzyme from vegetative tissue. This study therefore is the first in which different isoforms of ACC oxidase expressed differentially have been identified and purified to homogeneity and characterised in detail from a specific plant organ at different stages of development.

In this thesis, recovery of ACC oxidase activity *in vitro* in the supernatant of the 26,800 x g centrifugation indicates that ACC oxidase from white clover leaf tissue is soluble as was observed in fruits of melon (Ververidis and John, 1991), avocado (McGarvey and Christoffersen, 1992), banana (Moya-Leon and John, 1995), cherimoya (Escribano *et al.*, 1996), papaya (Dunkley and Golden, 1998) and pear (Kato and Hyodo, 1999) and senescing carnation flowers (Nijenhuis-de Vries *et al.*, 1994; Kosugi *et al.*, 1997). However, ACC oxidases from apple fruits have been observed to be membrane-

associated and PVPP was necessary to solubilise the enzyme (Kuai and Dilley, 1992; Dupille *et al.*, 1993; Smith and John, 1993). The addition of PVPP was not required to extract active ACC oxidase from leaf tissue of white clover.

One of the major problems encountered during purification of ACC oxidase from fruits is believed to be its rapid loss of enzyme activity *in vitro*, making purification difficult for the enzyme from fruits of apple (Dupille *et al.*, 1993) and cherimoya (Escribano *et al.*, 1995) and senescing carnation flower tissue (Kosugi *et al.*, 1997). In this thesis, the activity *in vitro* of the enzyme from both mature green and senescent leaf tissues was found to be labile once extracted (Figures 3.8, 3.9 and 3.10). At 20°C, approximately 65 % of initial activity was lost after 2 h and activity lost completely after 3 h while at 4°C, 55 % to 71 % of activity was lost after 10 h. In comparison, an enzyme from pear fruit lost 30 % of the initial activity if stored at 2°C for 24 h, 60 % after 3 days and 85 % after 7 days storage (Vioque and Castellano, 1994). Smith *et al.* (1992) and Escribano *et al.* (1996) also observed the instability of activity *in vitro* from melon and cherimoya fruits. When the crude homogenate from papaya fruit was stored at -15°C with a buffer containing 1.5 M ammonium sulphate, activity was detectable for 8 days only (Dunkley and Golden, 1998). Also, the enzyme from senescing carnation flowers lost approximately 50 % of the initial activity after storage at 4°C for 8 h and the activity was lost completely after 4 days at 14°C (Kosugi *et al.*, 1997). Storage of crude enzyme extract from chick pea seed at -20°C also lowered the initial activity by 27 % and 90 % after 10 days and 45 days, respectively (Munoz De Rueda *et al.*, 1995).

Loss of activity during purification has been reported for other members of the 2-oxoacid-dependent dioxygenase family (Britsch, 1993). This instability could be due to the aerobic auto-oxidation of ascorbate catalysed by Fe²⁺ and / or the denaturation of the enzyme by Fe³⁺ (Dupille *et al.*, 1993). In this study, the inclusion of sodium ascorbate in the extraction and column chromatography buffers was found to be important to prevent the loss of the enzyme activity during purification (data not shown). The requirement of sodium ascorbate during extraction and purification was also reported by Moya-Leon and John (1995) in banana fruit. It was included in buffers

used in the purification of the enzyme from fruits of apple (Dong *et al.*, 1992; Dupille *et al.*, 1993) and papaya (Dunkley and Golden, 1998) and senescing carnation flowers (Kosugi *et al.*, 1997). It was also included in the extraction of the enzyme from fruits of apple (Fernandez-Maculet and Yang, 1992; Kuai and Dilley, 1992) and mandarin (Dupille and Zacarias, 1996) and from white clover leaves (Butcher, 1997; Hunter, 1998; Hunter *et al.*, 1999). Ascorbate has been proposed to act as an antioxidant (Mathews and van Holde, 1996) to help exclude oxygen or reduce enzyme-bound Fe^{2+} . Alternatively, it could be acting as a co-substrate (Dong *et al.*, 1992; Moya-Leon and John, 1995) to help stabilise the enzyme as found in the enzyme flavanone 3-hydroxylase (Prescott, 1993) where the inclusion of its co-substrate, 2-oxoglutarate (Britsch and Grisebach, 1986), stabilised the enzyme during purification. However, ascorbate was not required to retain activity during the extraction and partial purification of ACC oxidase from cherimoya fruit (Escribano *et al.*, 1996).

In this study, the addition of PA, an effective inactivator of metallo-proteases (Salvesen and Nagase, 1989; Seynon, 1989), in the enzyme extract was observed to partially prevent the loss of initial activity for at least 14 h (Figure 3.10). It has also been reported that ACC oxidase activity *in vitro* from apple fruit was completely stabilised by the inclusion of 10 μM PA for at least 18 h (Dupille *et al.*, 1993), making it possible to undertake purification of this enzyme, probably by preventing the enzyme from aerobic oxidative damage in the presence of iron. PA was then included in the chromatography buffers for the purification of the enzyme from apple fruit (Dupille *et al.*, 1993) and also the recombinant enzyme from tomato fruit (Zhang *et al.*, 1995). However, the inclusion of PA did not have any significant effect on the enzyme from banana fruit (Moya-Leon and John, 1995). The addition of an inhibitor of serine proteases, PMSF, failed to prevent the loss of enzyme activity in this thesis, in common with the enzyme from apple fruit (Dupille *et al.*, 1993). In addition, the inactivation of the enzyme from senescing carnation flowers was not prevented by adding a mixture of protease inhibitors (0.5 mM PMSF, 0.7 $\mu\text{g/ml}$ pepstatin and 0.5 $\mu\text{g/ml}$ leupeptin) to the incubation medium, suggesting that this decline in enzyme activity *in vitro* is not protease-dependent (Nijenhuis-de Vries *et al.*, 1994).

During the extraction and purification of the enzyme from cherimoya fruit, the addition of catalase (1 mg/ml) stimulated enzyme activity and was increasingly effective during the course of purification (Escribano *et al.*, 1996). This stimulation by catalase was also observed in the purification of the recombinant enzyme from tomato fruit in the form of a fusion protein with glutathione S-transferase (GST) (Smith *et al.*, 1994), in the non fusion recombinant ACC oxidase from tomato fruit (Zhang *et al.*, 1995) and other members of the 2-oxoacid-dependent dioxygenase family (Blanchard *et al.*, 1982). However, it did not stimulate the activity of the enzyme extracted from melon fruit (Smith *et al.*, 1992) possibly because of the existence of endogenous catalase (Smith *et al.*, 1994). Catalase is believed to be able to protect oxidative damage mediated *via* hydrogen peroxide on ACC oxidase (Barlow *et al.*, 1997). Therefore, in the future, the inclusion of catalase during the purification of the enzyme from white clover can be tested to determine if an increase in enzyme activity is observed. In this thesis, sufficient enzyme activity was observed without the addition of catalase.

Protein precipitation by salting out (increasing the ionic strength) with ammonium sulphate makes use of the surface hydrophobic property of the protein molecule. In this study, this step not only significantly reduced the total volume of protein extract from leaf tissues but also resulted in *ca.* 2-fold purification (Table 3.1). The concentration used (30 % to 90 % saturation) was the same as that of Dupille *et al.* (1993; who observed a similar purification fold for the enzyme from apple fruit) but is higher than the concentrations used by other researchers. For example, McGarvey and Christoffersen (1992) observed that 30 % to 50 % ammonium sulphate fractionation was necessary and sufficient to separate the enzyme from endogenous ACC. Similarly, Dunkley and Golden (1998) used 50 % ammonium sulphate to precipitate protein from crude extracts of papaya fruit and a 26-fold purification was achieved. Also, the use of 50 % to 65 % saturated ammonium sulphate gave a 9.3-fold purification for the enzyme from senescing carnation flowers (Kosugi *et al.*, 1997). Therefore, to optimise the purification of this step that offers further fractionation, a different series of concentrations needs to be tried and the proteins fractionated in both the pellet and the supernatant tested for protein content and enzyme activity. In addition, another precipitation method, PEG fractionation, can also be tried. This method utilises the

polar nature of proteins rather than the hydrophobic property, and has been used to purify ACC oxidase from apple fruit with *ca.* 3.5-fold purification (Kuai and Dilley, 1992). Several polar amino acid residues (Asp, Glu, Arg, Lys, Tyr and His) were found to occupy the outer surface of the ACC oxidase protein molecule from papaya fruit (Dunckley and Golden, 1998). A novel technique using a combination of PEG and ammonium sulphate fractionation was used to concentrate ACC oxidase from banana fruit crude extract with a nearly 3-fold purification obtained (Moya-Leon and John, 1995).

In this study, the enzymes from both mature green and senescent leaf tissue were eluted with buffers containing no ammonium sulphate on both Phenyl Superose and Phenyl Sepharose hydrophobic interaction columns. The enzymes from fruits of both apple (Dupille *et al.*, 1993) and banana (Moya-Leon and John, 1995) were also eluted with no ammonium sulphate in the elution buffer on a Phenyl Sepharose CL4B hydrophobic column. Furthermore, significant purification of both isoforms was accomplished by hydrophobic interaction chromatography with a 10.2- and 17.9-fold purification obtained for MGI and SEII, respectively (Tables 3.2 and 3.3). A 17.6-fold purification of the enzyme from apple fruit was observed using the Phenyl Sepharose CL4B column (Dupille *et al.*, 1993). The purification of the enzyme from apple fruit was primarily accomplished by a hydrophobic interaction column (15.5-fold), and the purity of the enzyme preparation after this purification step was considered to be sufficient for inhibition studies (Pirrung *et al.*, 1993). These results suggest a significant surface hydrophobicity of ACC oxidase protein. One molecular explanation for the hydrophobic characteristic is that the protein possesses an amphipathic helix resulting from the hydrophobic amino acids present every four to five positions within the highly helical region of the enzyme (Pirrung *et al.*, 1993). However, the possible biological role for this property remains unclear. The different elution points of the three ACC oxidase isoforms on this column indicate their different hydrophobicity. This difference may be due to different number and/or content of hydrophobic amino acid residues (e.g. Ile, Leu and Phe) on the surface of protein molecules, and suggests that they are distinct ACC oxidase proteins.

Northern analysis revealed the induction of an ACC oxidase transcript (TR-ACO3) in senescent leaf tissue although no concomitant increase in ACC oxidase activity *in vitro* was observed in enzyme extracts from senescent leaf tissue after ammonium sulphate precipitation and Sephadex G-25 chromatography (Hunter *et al.*, 1999). In the current study, higher ACC oxidase activity, assayed at optimal pH and ACC concentration, is observed in the senescent leaf enzyme preparation partially purified by hydrophobic interaction chromatography when compared with mature green leaf enzyme preparation (Table 3.8). This is the first demonstration, to the author's knowledge, of ACC oxidase activity *in vitro* from any senescent leaf tissues of plants although leaf tissues from many plant species have been shown to be capable of converting applied ACC to ethylene (Osborne, 1991). This result suggests the occurrence of proteinaceous inhibitors which have a molecular weight above 5 kDa since they cannot be separated from ACC oxidase by Sephadex G-25 gel filtration. A proteinaceous inhibitor has been shown to inhibit the conversion of applied ACC to ethylene (Sakai and Maniwa, 1994) although it has not been shown to inhibit ACC oxidase activity *in vitro* directly. The unmasking of ACC oxidase activity in senescent leaf extracts of white clover may provide an useful experimental system with which to examine the presence of proteinaceous inhibitors.

The unmasking of activity was also demonstrated in response to wounding. Hunter (1998) has shown that transcript of the leaf senescence-associated gene, TR-ACO3, was induced by wounding after 6 h but no increase in enzyme activity was detected. In this thesis, in respect to wounding, two ACC oxidase activity peaks were observed in protein extracts of 6 h wounded mature green leaves after hydrophobic interaction chromatography (Figures 3.27 and 3.28). One of the two peaks, MGII', was apparently induced by wounding since this activity peak was small in both the non-wounded control and 3 h wounded mature green leaves (Figure 3.26, wounded 3 h data not shown).

Using anion exchange column chromatography, all three isoforms from white clover leaf tissues were found to exist as the anionic form. This was also observed by Dunkley and Golden (1998) for the enzyme from papaya fruit. Isoform SEI was eluted at 106 mM NaCl in the elution buffer, being broadly similar to the enzymes from fruits of apple (125 mM; Dupille *et al.*, 1993) and banana (*ca.* 140 mM; Moya-Leon and John, 1995). Both

MGI and SEII were eluted with a 2 to 3-fold higher sodium chloride concentration (265 mM to 371 mM for MGI; 371 mM to 480 mM for SEII). These results indicate that both MGI and SEII are more negatively charged at pH 7.5 when compared with the enzymes examined from fruit tissues.

In this thesis, two distinct isoforms of ACC oxidase, SEI and SEII, have been identified from senescent leaf tissues of white clover. Not only did they have different elution points on both hydrophobic interaction (Figure 3.19) and anion exchange (Figures 3.21 and 3.22) columns (that is, different surface hydrophobicity and net charge), they also displayed different immunological properties in terms of cross-reactivity with the TR-ACO2 antibody (Figure 3.20).

To confirm the existence of two isoforms of ACC oxidase in senescent leaves, two further experiments need to be undertaken. The first is to do reinjection experiments. If reinjection of either SEI or SEII onto a hydrophobic interaction column results in the same chromatographic profile as originally obtained, it can be concluded that one isoform is not a breakdown product of the other. The second is to do a control experiment. Protein is extracted under denaturing conditions (e.g. with the addition of SDS), thereby eliminating the potential action of proteases during extraction and purification. If the two activity peaks are eluted at the original point on the same column and western analysis of the two active peaks reveals the same size (*ca.* 35 kDa) of the two proteins, it could also be concluded that one isoform is not a breakdown product of the other. Enhanced proteolysis occurs during leaf senescence in plants (Huffaker, 1990), and cDNAs encoding tomato cysteine proteases expressed during leaf senescence have been isolated (Drake *et al.*, 1996). However, the potential for proteolytic digestion of the enzyme has been minimised in this study through the inclusion of an effective protease inhibitor, PA, ascorbic acid, glycerol and the use of low temperature during enzyme extraction and purification. No decrease in the molecular mass of the ACC oxidase proteins from both mature green and senescent leaves was observed during each purification step. Although there is no direct evidence for the existence of proteolytic activity during leaf maturation and senescence in white clover, it cannot be ruled out that one of the two isoforms from senescent leaves, SEI, might be the degradation product of isoform MGI *in vivo* during leaf senescence.

The identification of two distinct leaf senescence-associated isoforms is consistent with the molecular investigation on the senescence-associated ACC oxidase gene TR-ACO3 which suggests that there may be another closely related ACC oxidase gene (Hunter, 1998). Southern analysis, using the 3'-UTR of TR-ACO3 (the more divergent region of the gene; Fluhr and Mattoo, 1996) as probe, showed that this region hybridised to a subset of sequences recognised by the reading frame of TR-ACO3. Since none of the restriction enzymes used in the Southern analysis had cleavage sites within the reading frame or 3'-UTR of TR-ACO3, the result suggests that there is another gene which is closely related to TR-ACO3 but with a divergent 3'-UTR (Hunter, 1998). Therefore, these two distinct genes might encode for the two isoforms of ACC oxidase (SEI and SEII) identified in this study although the expression of any further ACC oxidase gene would need to be determined - that is, is it also senescence-associated?

The purification of the isoforms from both mature green and senescent leaves (Tables 3.2, 3.3 and 3.4) in this study suggests that ACC oxidase is not an abundant enzyme in both mature green and senescent leaf tissues of white clover. Early in 1984, Yang and Hoffman also reported that ACC oxidase was an enzyme of low abundance. However, both Dong *et al.* (1992) and Pirrung *et al.* (1993) observed that the enzyme from apple fruit was abundant; "it is present at a significant level even in the crude extract" (Pirrung *et al.*, 1993). This is probably because apple is a climateric fruit and the fruits used were pretreated by storage at low temperature (4°C) and ethylene production monitored to ascertain that the fruit was at the climateric stage (and hence should contain maximal ACC oxidase activity) before being extracted for active enzyme (apple fruit; Dong *et al.*, 1992).

In this thesis, two (MGI and SEII) of the three isoforms (MEI, SEI and SEII) identified have been purified to homogeneity. The purified isoforms MGI and SEII displayed specific activities of 25.2 and 29.8 nmol ethylene/h/mg protein when assayed with 1 mM ACC and pH 7.5 (Section 2.2.8) with *ca.* 100- and 144-fold purification, respectively (Tables 3.2 and 3.3) after the final gel filtration chromatography step. These assay conditions are not optimal for SEII and 1 mM ACC is not a saturating

concentration for MGI. The enzyme activities and purification fold for the two purified isoforms would be expected to be higher when assayed at saturating concentrations of ACC (2 and 4 mM for MGI and SEII, respectively, Figures 3.35 and 3.36) and optimal pH (7.5 and 8.5 for MGI and SEII, respectively, Table 3.7). When assayed at the saturating ACC concentration and optimal pH conditions, the partial purified enzyme preparations (after hydrophobic interaction step) had specific enzyme activities of 2.89 (for MGI) and 7.95 (for SEII) nmol ethylene/h/mg protein (Table 3.8), being near 1.13- and 2.20-fold higher than those (2.57 and 3.70 nmol ethylene/h/mg protein for MGI and SEII, respectively, Tables 3.2 and 3.3) assayed at the same purification stage but using the non-optimal conditions.

However, even at suboptimal assay conditions the specific activity is much lower when compared with those from fruit tissues, such as 1200 nmol ethylene/h/mg protein for the purified enzyme from apple fruit (Dong *et al.*, 1992). This different activity may be due to a different ethylene production rate from leaf tissue and fruit tissue. The rate of ethylene production observed in senescing leaves (nodes 11 to 16) of white clover ranged from 0.8 to 2.0 nl/g FW/h and *ca.* 0.25 nl/g FW/h in mature green leaves (nodes 4 to 9) (Hunter, 1998). However, ethylene production in whole banana fruit is negligible at preclimateric stage but steadily increases until reaching a value of *ca.* 9 nl/g FW/h (Lopez-Gomez *et al.*, 1997) and 115 nl/g FW/h in ripening avocado fruit (Hoffman and Yang, 1980). Therefore, the enzyme activity of the leaf senescence-related isoform of ACC oxidase may not be expected to increase to the same relative level as those in other fruit tissues.

In this study, the purification fold for MGI and SEII is higher when compared to the enzyme from apple fruit (21.3-fold; Pirrung *et al.*, 1993) and similar to the purified enzymes from fruits of papaya (81-fold; Dunkley and Golden, 1998), banana (145-fold; Moya-Leon and John, 1995) and apple (180-fold; Dong *et al.*, 1992; 170-fold; Dupille *et al.*, 1993). However, the yields of the isoforms (*ca.* 0.08 % for MGI; 0.05 % for SEII) are lower compared with the enzymes purified to homogeneity from fruits of banana (1.2 %; Moya-Leon and John, 1995), apple (2.6 %; Dupille *et al.*, 1993; 3.2 %; Pirrung *et al.*, 1993) and papaya (6.4 %; Dunkley and Golden, 1998). This may be

because either the enzyme is more stable in fruit tissue or may reflect the lower abundance of ACC oxidase in leaf tissue.

4.3 Physicochemical Characteristics of Two Isoforms of ACC Oxidase from Mature Green and Senescent Leaves

In terms of physicochemical properties, both isoforms (MGI and SEII) purified in this thesis resemble purified or partially purified ACC oxidases reported from other plant species in molecular mass and pH optimum for MGI but differ in a higher pI value and pH optimum reported for SEII. When compared together, the two isoforms had the same native molecular weight but displayed differences in apparent molecular mass, pI value and pH optimum.

In this study, the SDS-denatured and native isoforms of ACC oxidase had similar molecular masses (Table 3.7), indicating that both isoforms are active as monomers as is observed for the enzymes from fruits of apple (Dong *et al.*, 1992; Dupille *et al.*, 1993), banana (Moya-Leon and John, 1995), papaya (Dunkley and Golden, 1998) and pear (Kato and Hyodo, 1999). Using SDS-PAGE analysis, apparent molecular weights of 37.0 kDa for MGI and 35.0 kDa for SEII were determined. Both values are within the published apparent molecular masses (35 kDa to 40 kDa) although an unusual low apparent molecular mass (27.5 kDa) of a purified ACC oxidase has been reported from papaya fruit (Dunkley and Golden, 1998). The native molecular masses of both isoforms were determined by elution of enzyme activity on gel filtration chromatography, in which 150 mM sodium chloride was included in the gel filtration buffer to minimise the interaction between the proteins and the matrix. Using gel filtration chromatography, a molecular mass of 37.5 kDa for both isoforms was determined. The estimated native molecular weights for both isoforms are also within the published range determined by gel filtration chromatography (35 kDa to 41 kDa) from senescing carnation flowers (35 kDa; Kosugi *et al.*, 1997), fruits of apple (39 kDa; Dong *et al.*, 1992; 39 kDa to 39.5 kDa; Dupille *et al.*, 1993), banana (40 kDa; Moya-Leon and John, 1995) and melon (41 kDa; Smith and John, 1992) and for the highly purified enzyme from apple fruit (35.332 ± 5 kDa by EMS; Pirrung *et al.*, 1993). However, the native molecular mass of a partially purified enzyme from cherimoya fruit

was determined to be 62 kDa to 66 kDa (Escribano *et al.*, 1996), and the enzyme was suggested to be active as a dimer rather than a monomer.

The TR-ACO2 antibody has been shown to recognise a protein of 36.4 kDa which is expressed in developing and mature green leaves with maximum expression in mature green leaf tissue (Hunter *et al.*, 1999; McManus *et al.*, 1999). In this study, however, this antibody recognised MGI as a protein of 37.0 kDa. This discrepancy may be because the 36.4 kDa value was determined from a crude mixture of proteins which together may influence the mobility of MGI protein during SDS-PAGE.

Both partially purified isoforms of ACC oxidase are found to be neutral or near neutral proteins in this study with apparent pIs of 7.36 for isoform MGI and 7.0 for SEII as determined by chromatofocusing (Table 3.7). The pI values are higher than those from fruits of both banana (4.35; Escribano *et al.*, 1996) and cherimoya (4.90; Moya-Leon and John, 1995) determined using the same method. Using denaturing IEF (pH gradient from 4.5 to 7.5) followed by immunoblotting, Bidonde *et al.* (1998) observed that three recombinant isoforms of tomato ACC oxidase expressed in yeast had pIs of 6.1 for LE-ACO1 and LE-ACO3 and 6.8 for LE-ACO2. In this study, IEF using Ampholine PAG plates was also performed to determine the pI values of both isoforms. The proteins were separated by IEF as determined by a co-separation of protein markers after Coomassie Brilliant Blue staining (data not shown). However, no IEF separated proteins were transferred to PVDF membrane by capillary action as no recognition was observed by western analysis using the TR-ACO2 antibody. The higher pIs of the two isoforms from white clover when compared with fruits of banana and cherimoya suggest that they may have a relatively more basic amino acid residues (e.g. His, Lys and Arg) and less acidic residues (e.g. Asp and Glu). The higher pI value of MGI when compared with SEII indicates that MGI may have relatively more basic or less acidic amino acid residues than SEII.

The partially purified isoform MGI had a pH optimum of 7.5 in Tris-HCl buffer (Figure 3.33). This optimum is the same as that of the crude enzyme extract from mature green

leaf tissue (Hunter, 1998). This near neutral pH optimum has also been observed for the partially purified enzymes from fruits of apple (pH 7.2 in Tris-Mes and 7.6 in phosphate buffer; Kuai and Dilley, 1992; pH 7.4 in Tris-HCl; Dupille *et al.*, 1993), melon (pH 7.5 in both MOPS-NaOH and Tris-HCl; Smith *et al.*, 1992), cherimoya (pH 7.4 in Tricine-HCl; Escribano *et al.*, 1996) and papaya (pH 7.0 in Tris-HCl; Dunkley and Golden, 1998) and also from senescing carnation flowers (pH 7.0 to 7.5 in MOPS-NaOH; Kosugi *et al.*, 1997). The partially purified isoform SEII, however, was found to have a higher pH optimum (pH 8.5) determined in both 50 mM Tris-HCl (Figure 3.33) and 100 mM phosphate buffer (Figure 3.34) although the latter gave lower enzyme activity. A pH optimum of 7.5 to 8.0 in Tris-HCl buffer was also observed for the crude enzyme from avocado fruit (McGarvey and Christoffersen, 1992). No shift in pH optimum for SEII in two buffer systems is in agreement with that in melon (Smith *et al.*, 1992) although a shift of 0.4 was observed when two buffer systems were used in apple fruit (Kuai and Dilley, 1992). Hunter (1998) observed an optimum of pH 7.5 for the enzyme in senescent leaf crude extracts. However, FPLC was not used to unmask the leaf senescence-related isoform (s), and so residual mature green leaf enzyme was most likely being measured since this activity decreased during leaf senescence (Hunter *et al.*, 1999). A pH optimum of 8.5 has not been reported for the enzyme from any other plant species. The following alternative explanations for an ACC oxidase with an alkaline pH optimum can be presented:

Firstly, plant cells undergoing senescence may become more alkaline, particularly as a consequence of an increase in ammonia content as part of enhanced proteolysis and aminotransferase activity (Smart, 1994). Secondly, during leaf senescence, chloroplast breakdown occurs and some basic compounds, such as basic amino acids, may be released into the cytoplasm and therefore the pH in cytoplasm may increase.

Thirdly, the two isoforms could have different subcellular locations with different pH environments. The presence of an alkaline optimum of SEII indicates the possibility that the isoform may reside in an alkaline compartment, such as the chloroplast stroma where the pH reaches 8.0 in the light (Avron, 1981). Isoform MGI may be cytosolic, in common with ACC oxidases from other plants. In *Canavalia lineata* leaf tissue,

cytoplasmic glutamine synthetase (GS) had an optimal pH of 7.5 while chloroplastic GS has a pH optimum at around 8 (Choi and Kwon, 1998). Similarly, in another green tissue of *Datura stramonium*, one isoform (CM1) of chorismate mutase (CM, EC 5. 4. 99. 5) is localised in the chloroplast while CM2 is cytosolic (Leuschner *et al.*, 1995). CM1 showed an optimal pH at pH 8.0 whereas CM2 showed activity at pH 5 to 9. Ethylene may play a role in degrading chlorophyll, so it is possible that SEII may be localised in the chloroplast stroma. An examination of the subcellular location of the isoform in senescent leaf tissue using immunocytological methods should provide significant clues as to its cellular localisation. In addition, the lack of enzyme activity of both isoforms below pH 5.0 may rule out the possibility that both isoforms are localised within the vacuole as was observed in an ACC oxidase from avocado fruit (McGarvey and Christoffersen, 1992).

In this study, 30°C was used in all activity assays, in common with Hunter (1998). Any difference in temperature optima between the two isoforms was not determined. Distinct temperature optima for ACC oxidases have been observed in crude enzyme extracts from pear fruit (28°C and 38°C; Vioque and Castellano, 1994), chick-pea seeds (28°C and 35°C; Munoz De Rueda *et al.*, 1995) and citrus peel (35°C and 45°C; Dupille and Zacarias, 1996). ACC oxidases extracted from roots and leaves of sunflower seedlings were observed to display temperature optima of 30°C and 35°C, respectively. These results indicate that MGI and SEII may also have different optimal temperatures.

4.4 Kinetic Properties of Two Isoforms of ACC Oxidase

In this study, both partially purified isoforms have been found to resemble those partially purified enzymes from other plant species in terms of low apparent K_m values for the substrate ACC and absolute requirement for co-substrate and cofactors. Repeated determination of the kinetic parameters of the two isoforms gave similar results. A low K_m value for ACC is believed to be diagnostic of authentic ACC oxidase activity *in vitro*; the K_m values of artifactual activity are in the millimolar range (McKeon and Yang, 1984; Ververidis and John, 1991). Therefore, the low K_m of both isoforms for ACC and the complete inactivation of enzyme activity by boiling confirms the authenticity of the enzyme activity of these isoforms from mature green and senescent leaf tissues.

The apparent K_m value of isoform MGI for ACC (39.7 μM) determined in this study is similar to those reported for the partially purified enzymes from fruits of papaya (37 μM ; Dunkley and Golden, 1998) and pear (42.4 μM ; Kato and Hyodo, 1999), higher than apple fruit (6.4 μM ; Kuai and Dilley, 1992; 12.0 μM ; Pirrung *et al.*, 1993; 20.0 μM ; Dupille *et al.*, 1993) and lower than fruits of banana (56 μM ; Moya-Leon and John, 1995), melon (60 μM ; Smith *et al.*, 1992) and cherimoya (82 μM ; Escribano *et al.*, 1996) and senescing carnation flowers (111 μM to 125 μM ; Kosugi *et al.*, 1997). In contrast, the K_m of the isoform SEII for ACC (110 μM) is higher than all K_m values published for several fruits ranging from 6.4 (apple fruit; Kuai and Dilley, 1992) to 82 μM (cherimoya fruit; Escribano *et al.*, 1996) and similar to that of senescing carnation flowers (111 μM to 125 μM ; Kosugi *et al.*, 1997). The higher K_m of SEII for ACC compared with the 'ripening-related' isoform (s) in fruits may be due to the distinct difference in kinetic characteristics between the isoform in the senescent leaf tissue of white clover and those in fruit tissues used by previous workers. It may be significant that the higher K_m value for the leaf senescence-associated isoform from white clover is similar to the value determined for the enzyme from another senescing nonfruit tissue, carnation floral tissue.

When compared together, the two isoforms display differences in K_m values for ACC and optimal requirements for ascorbate and Fe^{2+} (Tables 3.7). The enzyme activities *in vitro* of the isoforms MGI and SEII were saturated by *ca.* 2 mM and 4 mM ACC, respectively. The apparent K_m value of MGI for ACC was higher than SEII determined at their optimal pHs and ACC concentrations using Eadie-Hofstee plots with a stringent test of linearity. SEII had a nearly 3-fold higher V_{max} for ACC than MGI (Table 3.7). These results indicate that MGI displays a higher affinity for the substrate ACC than SEII, and SEII requires a higher concentration of ACC to achieve a higher enzyme activity. The results are consistent with the higher endogenous ACC concentration and ethylene production observed in the senescent leaf tissues when compared with those in developing and mature green leaves in white clover (Butcher, 1997; Hunter *et al.*, 1999; McManus *et al.*, 1999). An increase in endogenous ACC concentration was also observed in ripening fruits of avocado, banana and tomato (Hoffman and Yang, 1980)

and senescing carnation flowers (Hanley *et al.*, 1989). ACC oxidase activities *in vitro* in the crude enzyme extracts from roots and leaves of corn and sunflower seedlings had different K_m values for ACC (Finlayson *et al.*, 1997). This means that the putative organ-specific isoenzymes had different affinities for ACC. In a similar approach to comparing single isoforms, three isoforms of ACC oxidase from tomato overexpressed in yeast have also been shown to display significant differences in apparent K_m values for ACC and specific activity (Bidonde *et al.*, 1998). However, different isoforms of ACC oxidase have not been identified and purified from corn, sunflower and tomato plants in which they are optimally expressed, and so any definitive comparison in terms of the regulation of isoforms *in planta* cannot yet be made.

Both isoforms of white clover ACC oxidase have also been found to exhibit absolute requirements for co-substrate and cofactors for maximal activity *in vitro*. The optimal requirements were similar to those observed for the partially purified enzymes from fruits of apple (Kuai and Dilley, 1992; Dong *et al.*, 1992; Dupille *et al.*, 1993; Pirrung *et al.*, 1993), banana (Moya-Leon and John, 1995), cherimoya (Escribano *et al.*, 1997), papaya (Dunkley and Golden, 1998) and pear (Kato and Hyodo, 1999). The two isoforms display a 2-fold difference in the optimal requirement for the co-substrate ascorbate (8 mM and 4 mM for MGI and SEII, respectively) and a 1.5-fold difference for the cofactor Fe^{2+} (16 μ M and 24 μ M for MGI and SEII, respectively) (Table 3.7). The root and leaf ACC oxidases of both corn and sunflower also displayed different optimal requirements for ascorbate. The K_m values for ascorbate for the corn and sunflower leaf enzyme were 4.42 mM and 8.29 mM, respectively whereas the root enzyme of corn and sunflower gave the values of 2.67 mM and 1.83 mM, respectively (Finlayson *et al.*, 1997). The measured ascorbate levels in the different organs were consistent with the K_m values. Therefore, it can be hypothesised that the different optimal ascorbate and Fe^{2+} requirements of the two isoforms from mature green and senescent leaf tissues may also reflect the differences in ascorbate and Fe^{2+} levels between these tissues. Further determination of both ascorbate and ferrous iron concentrations in mature green and senescent leaves should, in part, explain the different optimal requirements observed.

The two isoforms displayed the same optimal requirement (24 mM) for another cofactor bicarbonate which is comparable to the partially purified enzymes from fruits of banana (15 mM; Moya-Leon and John, 1995) and cherimoya (10 mM; Escribano *et al.*, 1996). In white clover, Hunter (1998) also observed that activities of the enzyme extracts from both mature green and senescent leaves displayed similar optima for bicarbonate. No optimal concentration, however, was observed up to 50 mM in the partially purified enzyme from senescing carnation flowers (Kosugi *et al.*, 1997). Also, all three recombinant isoforms of tomato ACC oxidase expressed in yeast had the same optimal requirement for bicarbonate (10 mM) (Bidonde *et al.*, 1998). The same optimal requirement of both isoforms for bicarbonate indicates that the putative amino acid residues and their numbers for CO₂-dependent activation in these two proteins may be the same. Two residues (Lys¹⁷² and Arg³⁰⁰) which have been suggested as putative sites for CO₂-dependent activation of ACC oxidase (Lay *et al.*, 1996) could be fully conserved in these two proteins. Arg¹⁷⁵, recently proposed as the binding site for HCO₃⁻ (Professor David Dilley, Michigan State University, USA, *pers. comm.*), should also occur in both MGI and SEII.

Finally, the relative abundance data in this thesis (Table 3.8) supports a positive correlation between ACC oxidase activity *in vitro* and ethylene production. The MGI and SEII isoforms were measured at their respective optimal pH and saturating ACC concentration, and the activity expressed as per unit protein in each tissue. Highest ethylene production is associated with the senescent leaf tissue (Hunter *et al.*, 1999), and it is isoform SEII which demonstrates higher activity in senescent leaf tissue when compared with MGI in mature green leaf tissue.

4.5 Isoforms MGI and SEII Might Be Encoded by TR-ACO2 and TR-ACO3

TR-ACO2 and TR-ACO3 show 84 % and 87 % homology in their nucleotide and predicted amino acid sequences, respectively although only 61 % similarity in the 3'-UTRs (Hunter *et al.*, 1999). However, the two purified isoforms of ACC oxidase differ

not only in hydrophobicity and net charge but also in molecular mass, pI, pH optimum, K_m for ACC and optimal requirements for sodium ascorbate and Fe^{2+} . Although the tomato LE-ACO1 and LE-ACO3 cDNA had up to 95 % homology in their predicted amino acid sequences, the respective isoforms translated in yeast did display different pI, K_m values for ACC and specific enzyme activity (Bidonde *et al.*, 1998). Therefore, small difference in predicted amino acid sequence may result in significantly distinct enzyme kinetic properties.

In this thesis, the identification and purification to homogeneity of a single isoform of ACC oxidase (MGI) expressed in mature green leaf tissue, correlates well with the expression of a single ACC oxidase gene in this tissue, TR-ACO2 (Hunter *et al.*, 1999). This isoform displays a similar pH optimum as the enzymes from other plant species. The lower K_m of MGI for ACC is consistent with lower ACC content in this tissue which has been demonstrated by Hunter *et al.* (1999). Therefore, it may be that isoform MGI is encoded for by TR-ACO2.

In this study, two ACC oxidase isoforms (SEI and SEII) were identified from senescent leaf tissue. SEII was observed to have higher enzyme activity and protein content and was purified to homogeneity from a senescent leaf extract. Activity assays *in vitro* using optimal pH and saturating ACC concentration showed that SEII, the predominant isoform in the senescent leaf tissue, had a 2.2-fold higher relative abundance when compared with MGI (Table 3.8). SEII was also found to have a higher K_m for ACC, and therefore require a higher ACC concentration to achieve higher enzyme activity and produce a higher level of ethylene. These findings coincide with the endogenous ACC concentration and ethylene evolution data over the stolon of white clover (Butcher, 1997; Hunter *et al.*, 1999; Sang Dong Yoo, *pers. comm.*); both endogenous ACC concentration and ethylene production in senescent leaves have been found to be several fold higher than those in developing and mature green leaves (Hunter *et al.*, 1999). Since the expression of TR-ACO3 mRNA correlated well with the increase of ethylene evolution and ACC accumulation during leaf senescence, it may be that SEII is encoded by TR-ACO3. However, until the putative fourth ACC oxidase gene is cloned, and its expression pattern determined during leaf ontogeny, as well as SEI characterised further, the postulation that SEII is encoded by TR-

ACO3 remains as speculation.

In wounding experiments performed by Hunter (1998), the highest transcript level of the TR-ACO3 was observed at 6 h after wounding in mature green leaves. In this study, an increase in enzyme activity associated with MGII' was observed from wounded (6h) mature green leaves (Figures 3.27 and 3.28). Therefore, the induced activity peak could be the wound response-related isoform of ACC oxidase encoded by the TR-ACO3. However, SEII was not applied to the Phenyl Sepharose hydrophobic interaction column and its point of elution from this column is as yet determined. If the MGII' preparation is eluted at the same point as SEII on both hydrophobic interaction and anion exchange columns, this would provide further evidence that this isoform is encoded by TR-ACO3. These experiments were not performed as part of this thesis.

In this study, both purified MGI and SEII have been subjected to tryptic digestion to obtain some peptides. However, no data were obtained from these digestions followed by reverse-phase high performance liquid chromatography (HPLC). This may be due to a low concentration of proteins in the initial trypsin-digested samples (Ms Catriona Knight, Auckland University, Auckland, New Zealand, *pers. comm.*). So far, partial amino acid sequences of the purified ACC oxidase proteins were obtained only from apple fruit (Dong *et al.*, 1992; Dupille *et al.*, 1993). In the future, the purification protocol developed in this thesis needs to be scaled up considerably to obtain a higher concentration of each isoform for amino acid sequencing. Due to the low ACC oxidase protein concentration in leaf tissues of white clover, a large quantity of leaf sample must be used in order to obtain enough purified enzyme for amino acid sequencing. When the amino acid sequences of tryptic peptides are obtained, the difference in the amino acid sequence between the two isoforms could explain the different kinetic and physicochemical properties observed in this thesis. A comparison of the amino acid sequences of tryptic peptides from each isoform with the deduced amino acid sequences from TR-ACO2 and TR-ACO3 could also determine unequivocally whether a particular isoform is encoded for by one of the ACC oxidase genes.

4.6 Conclusions

Two coding regions of white clover ACC oxidase genes, TR-ACO2 and TR-ACO3, have been expressed in *E. coli* as fusion proteins. The recombinant TR-ACO2 and TR-ACO3 proteins were purified by 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 (0.34 and 0.23 nmol ethylene/h/mg protein, respectively) were much lower when compared with other plant ACC oxidase genes expressed in *E. coli*. The low enzyme activity may be due to missing amino acid residues which are important for ACC oxidase activity *in vitro*.

In this thesis, enzyme activity *in vitro* of ACC oxidase, extracted from both mature green and senescent leaves of white clover, has been shown to be unstable at 20°C with low temperature, ascorbate and PA required to help stabilise the enzyme activity during extraction and purification.

Using a six-step purification procedure, three distinct isoforms of ACC oxidase (designated MGI, SEI and SEII) have been successfully identified, and two of the three (MGI and SEII) were purified to homogeneity from mature green and senescent leaf tissue of white clover. The purification was achieved at the expense of protein yield by collecting the most active fractions at each stage starting with hydrophobic interaction chromatography. Both purified proteins are enzymatically active with specific enzyme activities of 25.2 (for MGI) and 29.8 (for SEII) nmol ethylene/h/mg protein assayed at pH 7.5 and 1 mM ACC with approximately 100- (for MGI) and 144-fold (for SEII) purification. Both purified MGI and SEII proteins corresponded to a single band on SDS-PAGE analysis followed by Coomassie Brilliant Blue staining. The apparent molecular masses were determined to be 37.0 kDa (for MGI) and 35.0 kDa (for SEII) by SDS-PAGE analysis. A native molecular mass of *ca.* 37.5 kDa was calculated for both isoforms based on size exclusion chromatography, indicating that both isoforms are active as monomers. During the four FPLC purification steps, all three isoforms were recognised by an antibody raised against the protein product of mature green leaf ACC oxidase, TR-ACO2, but with different cross-reactivity indicating differences in the native conformation of each isoform.

Both isoforms (MGI and SEII) purified to homogeneity from white clover leaves resemble ACC oxidases purified or partially purified from several fruits and senescing carnation flowers in terms of molecular mass, apparent K_m value for ACC and absolute requirement for co-substrate and cofactors for maximal enzyme activity *in vitro*. However, among their important biochemical properties, the two isoforms display different pI, pH optimum, apparent K_m value for ACC, optimal requirements for co-substrate ascorbate and cofactor Fe^{2+} and relative abundance in each tissue. These results indicate that these isoforms are differentially regulated by pH, ACC and sodium ascorbate concentrations, and are activated by different levels of the cofactor (Fe^{2+}) during leaf ontogeny in white clover. In addition, the relative abundance data supports a positive correlation between ACC oxidase activity *in vitro* and ethylene production during leaf maturation and senescence in white clover. Highest ethylene production is observed from senescent leaf tissue, and SEII, the ACC oxidase isoform which accumulates in leaf tissue at this developmental stage, demonstrates highest enzyme activity.

Different isoforms of ACC oxidase with different physiochemical and kinetic properties will make it possible for white clover leaves to effectively utilise its substrate ACC, co-substrate and cofactor at different stages of development and in response to different stimuli. The significant differences in their biochemical characteristics reflect the differential expression of distinct ACC oxidase genes, which have recently been demonstrated during leaf ontogeny in white clover (Hunter *et al.*, 1999). The distinct properties of the isoforms may also reflect the different physiological status of leaf tissue at different developmental stages in white clover. Further, the work in this thesis extends our earlier findings of differential expression of ACC oxidase genes during leaf development. It can now be added that ACC oxidase isoforms are subject to regulation at the physiological level which represents an extra tier of control of ethylene biosynthesis in higher plants.

4.7 Suggestions for Future Work

The work in this thesis involved mainly the purification and biochemical characterisation of the two distinct isoforms of ACC oxidase from mature green and senescent leaves of

white clover. It also provides some interesting avenues for further investigation on the isoforms of the enzyme in white clover.

4.7.1 Investigation on Rhythmicity of ACC Oxidase Activity and Protein in Leaf Tissue

In this study, the active fractions (on hydrophobic interaction column), recovered from the protein extract of mature green leaves harvested in the late afternoon (4 to 5 pm), were observed to give a lower ACC oxidase activity and protein concentration when compared with those taken in late morning (10:30 am to noon) (data not shown). The observation suggests the existence of rhythmic property of the ACC oxidase activity and protein accumulation in white clover leaves. ACC oxidase activity *in vitro* from a mature green leaf crude extract was also observed to be the highest at 11:30 am and the lowest at 9:00 pm although this experiment has been performed only once (Hunter, 1998). Also, TR-ACO2 mRNA accumulation was observed to be highest at 2:30 pm and lowest at 7:30 pm. In *Stellaria longipes*, Kathiresan *et al.* (1996) found that circadian ethylene evolution was correlated with both ACC oxidase mRNA and enzyme activity *in vitro*. ACC synthase mRNA abundance were not observed to be contributing to the rhythm of ethylene production (Emery *et al.*, 1997; Kathiresan *et al.*, 1998). More recently, Finlayson *et al.* (1999) have found both circadian ACC oxidase mRNA (sbACO2) accumulation and enzyme activity *in vitro* from sorghum. Therefore, it is necessary to investigate the rhythmicity of ACC oxidase activity and protein accumulation to determine optimal sampling time for mature green and senescent leaves of white clover.

4.7.2 Investigation on How to Improve ACC Oxidase Stability during Extraction and Purification

To improve enzyme stability during extraction and purification, the following two experiments can be performed in the future.

- Increasing glycerol concentration in both extraction and FPLC buffers

In this study, 10 % (v/v) glycerol was included in the extraction buffer and only 5 % (v/v) was used in all FPLC buffers to reduce the back pressure of the columns. However, 30 % (v/v) glycerol was included in the extraction and activity assay buffer for the enzyme from melon fruit. This higher concentration of glycerol was shown to improve enzyme stability (Smith and John, 1993). The same concentration of glycerol was used in all purification buffers for the enzyme from apple fruit (Pirrung *et al.*, 1993). As well, ACC oxidase activity of the partially purified enzyme from apple fruit was retained when stored at -20°C in 30 % (v/v) glycerol (Kuai and Dilley, 1992; Poneleit and Dilley, 1993).

- Addition of ACC in the extraction and FPLC buffers

The inclusion of ACC during extraction resulted in a slight increase in ACC oxidase activity *in vitro* of the enzyme from senescing carnation flowers (Nijenhuis-de Vries *et al.*, 1994), and 0.5 mM ACC was routinely added to the extraction buffer. The same effect has been ascribed to the stabilising effect of ACC on the enzyme from melon fruit (Smith and John, 1992).

4.7.3 Further Characterisation of These Two Isoforms

While this work uncovered key biochemical properties of the two isoforms identified, some important questions still need to be addressed. These include what is the structural basis for the differences in the distinct biochemical characteristics of the two isoforms of ACC oxidase, especially pH optimum, K_m for ACC and optimal requirements for ascorbate and Fe^{2+} ; how are they expressed and regulated under different internal and external factors, and what are their physiological functions during leaf maturation and senescence in white clover.

5. Bibliography

- Adams, D.O. & Yang, S.F.** 1979. Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Sciences, USA* **76**: 170-174.
- Aharoni, N., Anderson, J., Lieberman, M.** 1979. Production and action of ethylene in senescing leaf discs. *Plant Physiology* **64**: 805-809.
- Aharoni, N. & Lieberman, M.** 1979. Ethylene as a regulator of senescence in tobacco leaf discs. *Plant Physiology* **64**: 801-804.
- Alejar, A.A., De Visser, R., Spencer, M.S.** 1988. Ethylene production by attached leaves or intact shoots of tobacco cultivars differing in their speed of yellowing during curing. *Plant Physiology* **64**: 801-804.
- Atkinson, R.G., Bolitho, K.M., Wright, M.A., Iturriagaitia-Bueno, T., Reid, S.J., Ross, G.S.** 1998. Apple ACC oxidase and polygalacturonase: ripening-specific gene expression and promoter analysis in transgenic tomato. *Plant Molecular Biology* **38**: 449-460.
- Avni, A., Baily, B.A., Mattoo, A.K., Anderson, J.D.** 1994. Induction of ethylene biosynthesis in *Nicotiana tabacum* by a trichoderm viride xylanase is correlated to the accumulation of ACC synthase and ACC oxidase transcripts. *Plant Physiology* **106**: 1049-1055.
- Avron, M.** 1981. Photosynthetic electron transport and photophosphorylation. In: *The Biochemistry of Plants: A Comprehensive Treatise, Vol 8, Photosynthesis*. Hatch, M.D. & Boardman, N.K. (Eds), Academic Press, San Francisco, pp 164-191.
- Balague, C., Watson, C.F., Turner, A.J., Rouge, P., Picton, S., Pech, J.C., Grierson, D.** 1993. Isolation of a ripening and wound-induced cDNA from *Cucumis melo* L. encoding a protein with homology to the ethylene-forming enzyme. *European Journal of Biochemistry* **212**: 27-34.
- Banks, N.H.** 1985. The oxygen affinity of 1-aminocyclopropane-1-carboxylic acid oxidation in slices of banana fruit tissue. In: *Ethylene in Plant Development*. Roberts, J.A. & Tucker, G.A. (Eds), Butterworths, London, pp 29-36.
- Barlow, J.N., Zhang, Z.H., John, P., Baldwin, J.E., Schofield, C.J.** 1997. Inactivation of 1-aminocyclopropane-1-carboxylate oxidase involves oxidative modifications. *Biochemistry* **36**: 3563-3569.

- Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J., Grierson, D.** 1996. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *The Plant Journal* **9**: 525-535.
- Beynon, R.J.** 1989. Prevention of uncontrolled proteolysis. In: *Protein Purification Methods: A Practical Approach*. Harris, E.L.V. & Angal, S. (Eds), IRL Press, Oxford, UK, pp 40-49.
- Bidonde, S., Ferrer, M.A., Zegzouti, H., Ramassamy, S., Latche, A., Pech, J.C., Hamilton, A.J., Grierson, D., Bouzayen, M.** 1998. Expression and characterization of three tomato 1-aminocyclopropane-carboxylate oxidase cDNAs in yeast. *European Journal of Biochemistry* **253**: 20-26.
- Blanchard, J.S., England, S., Kondo, A.** 1982. γ -butyrobetaine hydroxylase: a unique protective effect of catalase. *Archives of Biochemistry and Biophysics* **219**: 327-334.
- Bleecker, A.B., Kenyon, W.H., Somerville, S.C., Kende, H.** 1986. Use of monoclonal antibodies in the purification and characterization of 1-aminocyclopropane-1-carboxylate synthase, an enzyme in ethylene biosynthesis. *Proceedings of the National Academy of Sciences, USA* **83**: 7755-7759.
- Blume, B. & Grierson, D.** 1997. Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana glauca* regulated by developmental and environmental stimuli. *The Plant Journal* **12**: 731-746.
- Bonghici, C., Ruperti, B., Rasori, A., Tonutti, P., Ramina, A.** 1998. Organization and structure of 1-aminocyclopropane-1-carboxylate oxidase gene family from peach. In: *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kanellis, A.K., Chang, C., Grierson, D., Klee, H., Pech, J.C. (Eds), European Union TMR-Euroconference Programme, pp 67.
- Bouquin, T., Lasserre, E., Pradier, J., Pech, J.C., Balague, C.** 1997. Wound and ethylene induction of the ACC oxidase melon gene CM-ACO1 occurs via two direct and independent transduction pathways. *Plant Molecular Biology* **35**: 1029-1035.
- Bouzayen, M., Cooper, W., Barry, C., Zegzouti, H., Hamilton, A.J., Grierson, D.** 1993. EFE multigene family in tomato plants: expression and characterization. In: *Cellular and Molecular Aspects of the Plant Hormone Ethylene*. Pech, J.C., Latche, A., Balague, C. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 76-81.
- Braford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

- Britsch, L.** 1993. Purification of flavanone-3-hydroxylase from *Putunia hybrida*: antibody preparation and characterization of a chemogenetically defined mutant. *Archives of Biochemistry and Biophysics* **276**: 348-354.
- Britsch, L. & Grisebach, H.** 1986. Purification and characterization of flavanone 3-hydroxylase from *Petunia hybrida*. *European Journal of Biochemistry* **156**: 569-577
- Bufler, G.** 1986. Ethylene-promoted conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in peel of apple at various stages of fruit development. *Plant Physiology* **80**: 539-543.
- Burg, S.P.** 1973. Ethylene in plant growth. *Proceedings of the National Academy of Sciences, USA* **70**: 591-597.
- Butcher, S.M., Fountain, D.W., McManus, M.T.** 1996. Leaf senescence and clonal growth of white clover. *Agronomy Society of New Zealand Special Publication No.11/ Grassland Research and Practice Series* **6**: 171-173.
- Butcher, S.M.** 1997. Ethylene biosynthesis during leaf maturation and senescence in white clover. Ph.D. thesis, Massey University, Palmerston North, New Zealand.
- Callahan, A.M., Morgens, P.H., Wright, P., Nichol, K.E.** 1992. Comparison of Pch313 (pTOM 13 homolog) RNA accumulation during fruit softening and wounding of two phenotypically different cultivars. *Plant Physiology* **100**: 482-488.
- Cameron, A.C., Fenton, C.A.L., Yu, Y., Adams, D.O., Yang, S.F.** 1979. Increased production of ethylene from plant tissues treated with 1-aminocyclopropane-1-carboxylic acid. *HortScience* **14**: 178-180.
- Casas, J.L., Garcia-Canovas, F., Tudela, J., Acosta, M.** 1993. A kinetic study of simultaneous suicide inactivation and irreversible inhibition of an enzyme. application to 1-aminocyclopropane-1-carboxylate (ACC) synthase inactivation by its substrate S-adenosylmethionine. *Journal of Enzyme Inhibition* **7**: 1-14.
- Castiglione, S., Pirola, B., Sala, F., Ventura, M., Pancaldi, M., Sansavini, S.** 1996. Allelic forms of ripening-related ACC oxidase and ACC synthase genes in *Malus* clones and cultivars. Genbank Accession number X98627.
- Cazzonelli, C.I., Cavallaro, A.S., Botella, J.R.** 1998. Cloning and characterization of ripening induced ethylene biosynthetic genes from non-climacteric pipeapple (*Ananas comosus*) fruits. *Australia Journal of Plant Physiology* **25**: 513-518.
- Chalutz, E., Mottoo, A.K., Sollomos, T., Anderson, J.D.** 1984. Enhancement by ethylene of cellulysin-induced ethylene production by tobacco leaf discs. *Plant Physiology* **74**: 99-103.

- Charng, Y.Y., Dong, J.G., Yang, S.F.** 1996. Structure-function studies on the 1-aminocyclopropane-1-carboxylate acid (ACC) oxidase carbon dioxide binding site. In : NATO Advanced Research Workshop, *Biology and Biotechnology of the Plant Hormone Ethylene*, June 9-13, Chania, Crete, Greece, Poster Abstract No 9.
- Choi, Y.A. & Kwon, Y.M.** 1998. Comparative characterization of glutamine synthetase isoforms from *Canavalia lineata* in biochemical and immunological aspects. *Plant Science* **134**: 171-180.
- Christofferson, R.E., McGarvey, D.J., Savarese, P.** 1993. Biochemical and molecular characterization of ethylene-forming enzyme from avocado. In: *Cellular and Molecular Aspects of the Plant Hormone Ethylene*. Pech, J.C., Latche, A., Balague, C. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 65-69.
- Clark, D.G., Richards, C., Hilioti, Z., Lindiversen, S., Brown, K.** 1997. Effect of pollination on accumulation of ACC synthase and ACC oxidase transcripts, ethylene production and flower petal abscission in geranium (*Pelargonium X hortorum* L H Bailey). *Plant Molecular Biology* **34**: 855-865.
- Cooper, W., Bouzayen, M., Hamilton, A., Barry, C., Rossall, S., Grierson, D.** 1998. Use of transgenic plants to study the role of ethylene and polygalacturonase during infection of tomato fruit by *Colletotrichum gloeosporioides*. *Plant Pathology* **47**: 308-316.
- Destefano-Beltran, L.J.C., Van Caeneghem, W., Gielen, J., Richard, L., Van Montagu, M., Van Der Straeten, D.** 1995. Characterization of three members of the ACC synthase gene family in *Solanum tuberosum* L. *Molecular & General Genetics* **246**: 496-508.
- Dilley, D.R., Kuai, J., Poneleit, L., Zhu, Y., Pekker, Y., Wilson, I.D., Burmeister, D.M., Gran, C., Bowers, A.** 1993. Purification and characterization of ACC oxidase and its expression during ripening in apple fruit. In: *Cellular and Molecular Aspects of the Plant Hormone Ethylene*. Pech, J.C., Latche, A., Balague, C. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 46-52.
- Dilley, D.R., Kuai, J., Wilson, I.D., Pekker, Y., Zhu, Y., Burmeister, D.M., Beaudry, R.M.** 1995. Molecular biological investigations of ACC oxidase and its expression attending apple fruit ripening. *Acta Horticulture* **379**: 25-39.
- Dominguez, M. & Vendrell, M.** 1993. Wound ethylene biosynthesis in preclimateric banana slices. *Acta Horticulture* **343**: 270-274.
- Dominguez, M. & Vendrell, M.** 1993. Ethylene biosynthesis in banana fruit: Evolution of EFE activity and ACC level in peel and pulp during ripening. *Journal of Horticultural Sciences* **68**: 63-70.

- Dong, J.G., Fernandez-Maculet, J.C., Yang, S.F.** 1992. Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from ripe apple fruit. *Proceedings of the National Academy of Sciences, USA* **89**: 9789-9793.
- Dong, J.G., Olsen, D., Silverstone, A., Yang, S.F.** 1992. Sequence of a cDNA coding for an 1-aminocyclopropane-1-carboxylate oxidase homolog from apple fruit. *Plant Physiology* **98**: 1530-1531.
- Drake, R., John, I., Farreu, A., Cooper, W., Schuch, W.M., Grierson, D.** 1996. Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. *Plant Molecular Biology* **30**: 755-767.
- Drory, A., Mayak, S., Woodson, W.R.** 1993. Expression of ethylene biosynthetic pathway mRNAs is spatially regulated within carnation flower petals. *Journal of Plant Physiology* **141**: 663-667.
- Dunkley, H.M. & Golden, K.D.** 1998. ACC oxidase from *Carica papaya*: Isolation and characterization. *Physiologia Plantarum* **103**: 225-232.
- Dupille, E., Rombaldi, C., Lelievre J.M., Cleyet-Marel J.C., Latche, A.** 1993. Purification, properties and partial amino acid sequence of 1-aminocyclopropane-1-carboxylate oxidase from apple fruits. *Planta* **190**: 65-70.
- Dupille, E. & Zacarias, L.** 1996. Extraction and biochemical characterization of wound-induced ACC oxidase from citrus peel. *Plant Science* **114**: 53-60.
- Emery, R.J.N., Kathiresan, A., Reid, D.M., Chinnappa, C.C.** 1997. Ecotypic differences in rhythmicity of ethylene production in *Stellaria longipes*: the possible roles of ACC, MACC, and ACC oxidase. *Canada Journal of Botany* **75**: 1027-1033.
- English, P.J., Lycett, G.W., Roberts, J.A., Jackson, M.B.** 1995. Increased 1-aminocyclopropane-1-carboxylic acid oxidase activity in shoots of flooded tomato plants raises ethylene production to physiologically active level. *Plant Physiology* **109**: 1435-1440.
- Esaka, M., Fukui, H., Suzuki, K., Kubota, K.** 1989. Secretion of ascorbate oxidase by suspension-cultured pumpkin cells. *Phytochemistry* **28**: 117-119.
- Escribano, M.I., Merodio, C., John, P.** 1996. Characterization of 1-aminocyclopropane-1-carboxylate oxidase partially purified from cherimoya fruit. *Journal of Agricultural Food Chemistry* **44**: 730-735.
- Espartero, J., Pintor-Toro, J.A., Pardo, J.M.** 1994. Differential accumulation of S-adenosylmethionine synthase transcript in response to salt stress. *Plant Molecular Biology* **25**: 217-227.

- Fernandez-Maculet, J.C. & Yang, S.F.** 1992. Extraction and partial characterization of the ethylene-forming enzyme from apple fruit. *Plant Physiology* **99**: 751-754.
- Fernandez-Maculet, J.C., Dong, J.G., Yang, S.F.** 1993. Activation of 1-aminocyclopropane-1-carboxylate oxidase by carbon dioxide. *Biochemical and Biophysical Research Communications* **193**: 1168-1173.
- Finlayson, S.A. & Reid, D.M.** 1994. Influence of CO₂ on ACC oxidase activity from roots of sunflower (*Helianthus annuus*) seedlings. *Phytochemistry* **35**: 847-851.
- Finlayson, S.A., Reid, D.M., Morgan, P.W.** 1997. Root and leaf specific ACC oxidase activity in corn and sunflower seedlings. *Phytochemistry* **45**: 869-877
- Finlayson, S.A., Lee, I.J., Mullet, J.E., Morgan, P.W.** 1999. The mechanism of rhythmic ethylene production in sorghum. The role of phytochrome B and simulated shading. *Plant Physiology* **119**: 1083-1089.
- Fluhr, R. & Mattoo, A.K.** 1996. Ethylene - biosynthesis and perception. *Critical Review in Plant Science* **15**: 479-523.
- Gomez-Gomez, L. & Carrasco, P.** 1998. Hormonal regulation of S-adenosylmethionine synthase transcripts in pea ovaries. *Plant Molecular Biology* **30**: 821-832.
- Go'mez-Jime'nez, M.C., Matilla, A.J., Garrido, D.** 1998. Isolation and characterization of a cDNA encoding an ACC oxidase from *Cicer arietinum* and its expression during embryogenesis and seed germination. *Australian Journal of Plant Physiology* **25**: 765-777.
- Grierson, D., Barry, C., Wilson, I., Hackett, R., Hamilton, A., Alpuche-Solis, A., Lin, Z., Santalla, M.** 1998. Control of ethylene synthesis and action. In: *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kanellis, A.K., Chang, C., Grierson, D., Klee, H., Pech, J.C. (Eds), European Union Tmr-Euroconference Programme, pp 30.
- Guillen, P., Domenech, A., Larrigaudiere, C., Vendrell, M.** 1998. Ethylene-induced rise of abscisic acid levels and ACC oxidase activity in immature melons. *The Journal of Horticultural Science and Biotechnology* **73**: 313-316.
- Guis, M., Bouquin, T., Zegzouti, H., Ayub, R., Ben Amor, M., Lasserre, E., Botondi, R., Raynal, J., Latche, A., Bouzayen, M., Balague, C., Pech, J.C.** 1997. Differential expression of ACC oxidase genes in melon and physiological characterization of fruit expressing an antisense ACC oxidase gene. In: *Biology and Biotechnology of the Plant Hormone Ethylene*. Kanellis, A.K., Chang, C., Klee, H., Kende, H., Grierson, D. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 327-337.
- Guy, M. & Kende, H.** 1984. Conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene by isolated vacuoles of *Pisum sativum* L. *Planta* **160**: 281-287.

- Hamilton, A.J., Bouzayen, M., Grierson, D.** 1991. Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. *Proceedings of the National Academy of Sciences, USA* **88**: 7434-7437.
- Hamilton, A.J., Lycett, G.W., Grierson, D.** 1990. Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature* **346**: 284-287.
- Hanley, K.M., Meir, S., Bramlage, W.J.** 1989. Activity of ageing carnation flower parts and the effects of 1-(malonylamino) cyclopropane-1-carboxylic acid-induced ethylene. *Plant Physiology* **91**: 26-1130.
- Harris, E.L.V.** 1989. Concentration of the extract. **In:** *Protein Purification Methods: A Practical Approach*. Harris, E.L.V. & Angal, S. (Eds), IRL Press, Oxford, England, pp 154-157.
- Hill, C.R., Thompson, L.G., Kenney, A.C.** 1989. Immunopurification. **In:** *Protein Purification Methods: A Practical Approach*. Harris, E.L.V. & Angal, S. (Eds), IRL Press, Oxford, England, pp 282-290.
- Hoffman, N.E. & Yang, S.F.** 1980. Changes of 1-cyclopropane-1-carboxylic acid content in ripening fruits in relation to their ethylene production rates. *Journal of the American Society for Horticultural Science* **105**: 492-495.
- Hoffman, N.E., Yang, S.F., Ichihara, A., Sakamura, S.** 1982. Stereospecific conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in plant tissues. Conversion of stereoisomers of 1-amino-2-ethylcyclopropane-carboxylic acid to 1-butene. *Plant Physiology* **70**: 195-199.
- Holdsworth, M.J., Bird, C.R., Ray, J., Schuch, W., Grierson, D.** 1987. Structure and expression of an ethylene-related mRNA from tomato. *Nucleic Acid Research* **15**: 731-739.
- Hong, S.B., Kuo, J.M., Mullins, L.S., Raushel, F.M.** 1995. CO₂ is required for the assembly of the binuclear metal center of phosphotriesterase. *Journal of American Chemistry Society* **117**: 7580-7581.
- Huang, P.L. & Do, Y.Y.** 1998. Cloning and characterization of genes encoding 1-aminocyclopropane-1-carboxylate oxidase specific for fruit ripening in banana. **In:** *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kanellis, A.K., Chang, C., Grierson, D., Klee, H., Pech, J.C. (Eds), European Union Tmr-Euroconference Programme, pp 67.
- Huffaker, R.C.** 1990. Proteolytic activity during senescence of plants. Tansley Review No.25. *New Phytologist* **116**: 199-231.

- Hunter, D.A.** 1998. Characterisation of ACC oxidase during leaf maturation and senescence in white clover. Ph.D. thesis, Massey University, Palmerston North, New Zealand.
- Hunter, D.A., Yoo, S.D., Butcher, S.M., McManus, M.T.** 1999. Expression of 1-aminocyclopropane-1-carboxylate oxidase during leaf ontogeny in white clover. *Plant Physiology* **120**: 131-141.
- Hyodo, H., Hashimoto, C., Morozumi, S., Hu, W., Tanaka, K.** 1993. Characterization and induction of the activity of 1-aminocyclopropane-1-carboxylate oxidase in the wounded mesocarp tissue of *Cucurbita maxima*. *Plant and Cell Physiology* **34**: 667-671.
- Imaseki, H.** 1999. Control of ethylene synthesis and metabolism. In: *Biochemistry and Molecular Biology of Plant Hormone Ethylene*. Hooykaas, P.J.J., Hall, M.A., Libbenga, K.R. (Eds), Elsevier Science B.V., pp 209-245.
- Itturriagoitia-Bueno, T., Gibson, E.J., Schofield, C.J., John, P.** 1996. Inhibition of 1-aminocyclopropane-1-carboxylate oxidase by 2-oxoacids. *Phytochemistry* **43**: 343-349.
- Jin, E., Lee, J.H., Park, J.A., Kim, W.T.** 1999. Temporal and spatial regulation of the expression of 1-aminocyclopropane-1-carboxylate oxidase by ethylene in mung bean (*Vigna radiata*). *Physiologia Plantarum* **105**: 132-140.
- John, P., Iturriagoitia-Bueno, T., Lay, V., Thomas, P.G., Hedderson, T.A.J., Prescott, A.G., Gibson, E.J., Schofield, C.J.** 1997. 1-Aminocyclopropane-1-carboxylate oxidase: Molecular structure and catalytic function. In: *Biology and Biotechnology of the Plant Hormone Ethylene*. Kanellis, A.K., Chang, C., Kende, H., Grierson, D. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 15-22.
- John, P.** 1997. Ethylene biosynthesis: The role of 1-aminocyclopropane-1-carboxylate (ACC) oxidase, and its possible evolutionary origin. *Physiologia Plantarum* **100**: 583-592.
- John, P., Drake, R., Farrell, A., Cooper, W., Lee, P., Horton, P., Grierson, D.** 1995. Delayed leaf senescence in ethylene-deficient ACC oxidase antisense tomato plants: molecular and physiological analysis. *The Plant Journal* **7**: 483-490.
- Johnstone, A. & Thorpe R.** 1982. Purification of immunoglobulins, constituent chains and fragments. In: *Immunochemistry in Practice*. Blackwell Scientific Publications, Oxford, England, pp 44-46.
- Jones, M.L. & Woodson, W.R.** 1999. Differential expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in carnation. *Plant Physiology* **119**: 755-764.

- Kadyrzhanova, D.K., McCully, S.A., Jaworski, P., Ververidis, K.E., Vlachonasios, K.G., Murakami, K.G., Dilley, D.R.** 1997. Structure-function analysis of ACC oxidase by site-directed mutagenesis. In: *Biology and Biotechnology of the Plant Hormone Ethylene*. Kanellis, A.K., Chang, C., Kende, H., Grierson, D. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 5-13.
- Kadyrzhanova, D., McCully, T.J., Warner, T., Vlachonasios, K., Dilley, D.R.** 1998. Analysis of ACC oxidase by site-directed mutagenesis of conserved amino acid residues. In: *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kanellis, A.K., Chang, C., Kende, H., Grierson, D. (Eds), European Union Tmr-Euroconference Programme, pp 31.
- Kathiresan, A., Nagarathna, K.C., Moloney, M.M., Reid, D.M., Chinnappa, C.C.** 1998. Differential regulation of 1-aminocyclopropane-1-carboxylate synthase gene family and its role in phenotypic plasticity in *Stellaria longipes*. *Plant Molecular Biology* **36**: 265-274.
- Kathiresan, A., Reid, D.M., Chinnappa, C.C.** 1996. Light and temperature entrained circadian regulation of activity and mRNA accumulation of 1-aminocyclopropane-1-carboxylic acid oxidase in *Stellaria longipes*. *Planta* **199**: 329-335.
- Kato, M. & Hyodo, H.** 1999. Purification and characterization of ACC oxidase and increase in its activity during ripening of pear fruit. *Journal of the Japanese Society for Horticultural Science* **68**: 551-557.
- Kende, H.** 1993. Ethylene biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**: 283-307.
- Kende, H. & Zeevaart, J.A.D.** 1997. The five "classical" plant hormones. *The Plant Cell* **9**: 1197-1210.
- Kim, W.T. & Yang, S.F.** 1994. Structure and expression of cDNA encoding 1-aminocyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. *Planta* **194**: 223-229.
- Kim, W.T., Campbell, A., Kang, B.G., Yang, S.F.** 1997. Induction of 1-aminocyclopropane-1-carboxylate oxidase mRNA by ethylene in mung bean hypocotyls. *Journal of Plant Physiology* **150**: 77-84.
- Kim, Y.S., Choi, D., Lee, M.M., Lee, S.H., Kim, W.T.** 1998. Biotic and abiotic stress-related expression of 1-aminocyclopropane-1-carboxylate oxidase gene family in *Nicotiana glutinosa* L. *Plant and Cell Physiology* **39**: 565-573.
- Kosugi, Y., Oyamada, N., Satoh, S., Yoshioka, T., Onodera, E., Yamada, Y.** 1997. Inhibition by 1-aminocyclobutane-1-carboxylate of the activity of 1-aminocyclopropane-1-

carboxylate oxidase obtained from senescing petals of carnation (*Dianthus caryophyllus* L.) flowers. *Plant and Cell Physiology* **38**: 312-318.

Kruzmane, D. & Ievinsh, G. 1999. Characterization of 1-aminocyclobutane-1-carboxylic acid oxidase from barley (*Hordeum vulgare* L.) seedlings and pine (*Pinus sylvestris* L.). *Plant Science* **142**: 13-19.

Kuai, J. & Dilley, D.R. 1992. Extraction, partial purification and characterization of 1-aminocyclopropane-1-carboxylic acid oxidase from apple fruit. *Postharvest Biology and Technology* **1**: 203-211.

Kwak, S.H. & Lee, S.H. 1997. The requirements for Ca²⁺, protein phosphorylation and dephosphorylation for ethylene signal transduction in *Pisum sativum* L. *Plant and Cell Physiology* **38**: 1142-1149.

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

Lasserre, E., Bouquin, T., Hernandez, J.A., Bull, J., Pech, J.C., Balague, C. 1996. Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Molecular and General Genetics* **251**: 81-90.

Latche, A., Dupille, E., Rombaldi, C., Ceyet-marel, J.C., Lelievre, J.M., Pech, J.C. 1993. Purification, characterization and subcellular localization of ACC oxidase from fruits. In: *Cellular and Molecular Aspects of the Plant Hormone Ethylene*. Pech, J.C., Latche, A., Balague, C. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 39-45.

Lawrence, C.L., Sobey, W.J., Field, R.A., Baldwin, J.E., Schofield, C.J. 1996. Purification and initial characterization of proline 4-hydroxylase from *Streptomyces griseoviridis* P8648: a 2-oxoacid, ferrous-dependent dioxygenase involved in etamycin biosynthesis. *Biochemistry Journal* **313**: 185-191.

Lay, V.J., Prescott, A.G., Thomas, P.G., John, P. 1996. Heterologous expression and site-directed mutagenesis of the 1-aminocyclopropane-1-carboxylate oxidase from kiwifruit. *European Journal of Biochemistry* **242**: 228-234.

Leuschner, C., Walton, N., Herzog, G., Schultz, G. 1995. Chorismate mutase isoenzymes in transformed roots of *Datura stramonium*. *Plant Physiology and Biochemistry* **33**: 367-371.

Liang, X., Abel, S., Keller, J.A., Shen, N.F., Theologis, A. 1992. The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **89**: 11046-11050.

- Lin, C.T., Lin, M.T., Shaw, J.F.** 1997. Cloning and characterization of a cDNA for 1-aminocyclopropane-1-carboxylate oxidase from papaya fruit. *Journal of Agricultural Food Chemistry* **45**: 526-530.
- Lincoln, J.E., Campbell, A.D., Oetiker, J., Rottmann, W.H., Oeller, P.W., Shen, N.F., Theologis, A.** 1993. LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene of tomato (*Lycopersicon esculentum*). *The Journal of Biological Chemistry* **268**: 19422-19430.
- Lineweaver, H. & Burk, D.** 1934. The determination of enzyme dissociation constants. *Journal of the American Chemical Society* **56**: 658-666.
- Liu, Y., Hoffman, N.E., Yang, S.F.** 1985. Promotion by ethylene of the capability to convert 1-aminocyclopropane-1-carboxylic acid to ethylene in preclimateric tomato and cantaloupe fruits. *Plant Physiology* **77**: 407-411.
- Liu, J.H., Lee Tamon, S.H., Reid, D.M.** 1997. Differential and wound-inducible expression of 1-aminocyclopropane-1-carboxylate oxidase genes in sunflower seedlings. *Plant Molecular Biology* **34**: 923-933.
- Longnecker, N. & Welch, R.M.** 1990. Accumulation of apoplastic iron in plant roots. *Plant Physiology* **92**: 17-22.
- Lopez-Gomez, R., Campbell, A., Dong, J.G., Yang, S.F., Gomez-Lim, M.A.** 1997. Ethylene biosynthesis in banana fruit: isolation of a genomic clone to ACC oxidase and expression studies. *Plant Science* **123**: 123-131.
- Lorimer, G.H. & Miziorko, H.M.** 1980. Carbamate formation of the amino group of a lysyl residue as a basis for the activation of ribulose biphosphate carboxylase by CO₂ and Mg²⁺. *Biochemistry* **19**: 5321-5328
- Lorimer, G.H.** 1983. Carbon dioxide and carbamate formation: the makings of a biochemical control system. *Trends Biochemical Science* **8**: 65-68
- Lurssen, K., Naumann, K., Schroder, R.** 1979. 1-aminocyclopropane-1-carboxylic acid - an intermediate of the ethylene biosynthesis in higher plants. *Z.Pflanzen Physiology* **92**: 2850294.
- Luwe, M.W.F., Takahama, U., Heber, U.** 1993. Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves. *Plant Physiology* **101**: 969-976.
- MacDiarmid, C.W.B. & Gardner, R.C.** 1993. A cDNA sequence from kiwifruit homologous to 1-aminocyclopropane-1-carboxylic acid oxidase. *Plant Physiology* **101**: 691-692.

- Marston, F.A.O.** 1986. The purification of eukaryotic polypeptides synthetases in *Escherichia coli*. *Biochemical Journal* **240**: 1-12.
- Massiah, A.J. & Hartley, M.R.** 1995. Wheat ribosome-inactivating proteins: seed and leaf forms with different specificities and cofactor requirements. *Planta* **197**: 633-640.
- Mathews, C.K. & van Holde, K.E.** 1996. Biological oxidations, electron transport and oxidative phosphorylation. In: *Biochemistry* (second edition), The Benjamin/Cummings Publishing Company, Menlo Park, CA 94025, USA, pp 551.
- Mathooko, F.M., Inaba, A., Nakamura, R.** 1998. Characterization of carbon dioxide stress-induced ethylene biosynthesis in cucumber (*Cucumis sativus* L) fruit. *Plant and Cell Physiology* **39**: 285-293.
- Maunder, M.J., Holdsworth, M.J., Slater, A., Knapp, J.E., Bird, C.R., Schuch, W., Grierson, D.** 1987. Ethylene stimulates the accumulation of ripening-related messenger RNAs in tomatoes. *Plant, Cell and Environment* **10**: 177-184.
- Mbeguie-A-Mbeguie, D., Chahine, H., Gomez, R.M., Gouble, B., Reich, M., Audergon, J.M., Souty, M., Albagnac, G., Fils-lycaon, B.** 1999. Molecular cloning and expression of a cDNA encoding 1-aminocyclopropane-1-carboxylate (ACC) oxidase from apricot fruit (*Prunus armeniaca*). *Physiologia Plantarum* **105**: 294-303
- McGarvey, D. & Christoffersen, R.D.** 1992. Characterization and kinetic parameters of ethylene-forming enzyme from avocado fruit. *Journal of Biological Chemistry* **267**: 5964-5967.
- McKeon, T.A., Fernandez-Maculet, J.C., Yang, S.F.** 1995. Biosynthesis and metabolism of ethylene. In: *Plant Hormones*. Davies, P.J. (Ed), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp1 18-139.
- McKeon, T.A. & Yang, S.F.** 1984. A comparison of the conversion of 1-amino-2-ethylcyclopropane-1-carboxylic acid stereoisomers to 1-butene by pea epicotyls and by a cell free system. *Planta* **160**: 84-87.
- McManus, M.T., Hunter, D.A., Yoo, S.D., Gong, D.** 1999. ACC oxidase expression and leaf ontogeny in white clover. In: *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kanellis, A.K., Charng, C., Kende, H., Grierson, D. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands (in press).
- McManus, T. M. & Osborne, D.J.** 1990. Evidence for the preferential expression of particular polypeptides in leaf abscission zones of the bean (*Phaseolus vulgaris* L.). *Journal of Plant Physiology* **136**: 391-397.

- McRae, D.G., Coker, J.A., Legge, R.L., Thompson, J.E. 1983. Biocarbonate/CO₂ facilitated conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in model systems and intact tissues. *Plant Physiology* **73**: 784-790.
- Mehta, A.M., Jordon, R.L., Anderson, J.D., Mattoo, A.K. 1988. Identification of a unique isoform of 1-aminocyclopropane-1-carboxylate synthase by monoclonal antibody. *Proceedings of the National Academy of Sciences, USA* **85**: 8810-8814.
- Mita, S., Kawamura, S., Yamawaki, K., Nakamura, K., Hyodo, H. 1998. Differential expression of genes involved in the biosynthesis and perception of ethylene during ripening of passion fruit (*Passiflora edulis* Sims). *Plant and Cell Physiology* **39**: 1209-1217.
- Mizutani, F., Dong, J.G., Yang, S.F. 1995. Effect of pH on CO₂-activated 1-aminocyclopropane-1-carboxylate oxidase activity from apple fruit. *Phytochemistry* **39**: 751-755.
- Morgan, P.W. & Durham, J.I. 1980. Ethylene production and leaflet abscission in *Melia azedarach*. *Plant Physiology* **66**: 88-92.
- Morgan, P.W., He, C., Drew, M.C. 1992. Intact leaves exhibit a climacteric-like rise in ethylene production before abscission. *Plant Physiology* **100**: 1587-1590.
- Moya-Leon, M.A. & John, P. 1995. Purification and biochemical characterization of 1-aminocyclopropane-1-carboxylate oxidase from banana fruit. *Phytochemistry* **39**: 15-20.
- Munoz De Rueda, P., Gallardo, M., Matilla, A.J., Sanchez-Calle, I.M. 1995. Preliminary characterization of 1-aminocyclopropane-1-carboxylate oxidase properties from embryonic axes of chick-pea (*Cicer arietinum* L.) seeds. *Journal of Experimental Botany* **46**: 695-700.
- Nadeau, J.A., Zhang, X.S., Nair, H., O'Neill, S.D. 1993. Temporal and spatial regulation of 1-aminocyclopropane-1-carboxylate oxidase in the pollination-induced senescence of orchid flowers. *Plant Physiology* **103**: 31-39.
- Nakajima, N. & Imaseki, H. 1986. Purification and properties of 1-aminocyclopropane-1-carboxylate synthase of mesocarp of *Cucurbita maxima* Duch. fruits. *Plant and Cell Physiology* **27**: 969-980.
- Nakatsuka A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y., Inaba, A. 1998. Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, 1-aminocyclopropane-1-carboxylate oxidase and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiology* **118**: 1295-1305.

- Nakatsuka A., Shiomi, S., Kubo, Y., Inaba, A.** 1997. Expression and internal feedback regulation of ACC synthase and ACC oxidase genes in ripening tomato fruit. *Plant and Cell Physiology* **38**: 1103-1110.
- Neal, M.W. & Florini, J.R.** 1973. A rapid method for desalting small volumes of solution. *Analytical Biochemistry* **55**: 328-330.
- Nijenhuis-De Vries, M.A., Woltering, E.J., De Vrije, T.** 1994. Partial characterization of carnation petal 1-aminocyclopropane-1-carboxylate oxidase. *Plant Physiology* **144**: 549-554.
- Oetiker, J.H., Olson, D.C., Shiu, O.Y., Yang, S.F.** 1997. Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). *Plant Molecular Biology* **34**: 275-286.
- Old, R.W. & Primrose, S.B.** 1995. Expression in *E.coli* of cloned DNA molecules. **In** : *Principles of Gene Manipulation*. Blackwell Scientific Publications, Oxford, England (fifth edition), pp 165.
- O'Neill, S.D., Nadeau, J.A., Zhang, X.S., Bui, A.Q., Halevy, A.H.** 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. *Plant Cell* **5**: 419-432.
- Osborne, D.J.** 1991. Ethylene in leaf ontogeny and abscission. **In**: *The Plant Hormone Ethylene*. Mattoo, A.K. & Suttle, J.C. (Eds). CRC Press Inc., Boca Raton, USA, pp 193-214.
- Park, K.Y., Drory, A., Woodeson, W.R.** 1992. Molecular cloning of an 1-aminocyclopropane-1-carboxylate synthase from senescing carnation flower petals. *Plant Molecular Biology* **18**: 377-386.
- Peck, S.C. & Kende, H.** 1995. Sequential induction of the ethylene biosynthetic enzymes by indole-3-acetic acid in etiolated peas. *Plant Molecular Biology* **28**: 293-301.
- Picton, S., Barton, S.L., Bouzayen, M., Hamilton, A.J., Grierson, D.** 1993. Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. *The Plant Journal* **3**: 469-481.
- Pirrung, M.C., Cao, J., Chen, C.** 1995. Ethylene biosynthesis 12. Analog approach to the active site topography of the ethylene-forming enzyme. Novel hydroxamate inhibitors. *Journal of Organic Chemistry* **60**: 5790-5794.
- Pogson, B.J., Downs, C.G., Davies, K.M.** 1995. Differential expression of two 1-aminocyclopropane-1-carboxylic acid oxidase genes in broccoli after harvest. *Plant Physiology* **108**: 651-657.

- Poneleit, L.S. & Dilley, D.R.** 1993. Carbon dioxide activation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase in ethylene biosynthesis. *Postharvest Biology and Technology* **3**: 191-199.
- Preneta, A.Z.** 1989. Separation on the basis of size: gel permeation chromatography. **In:** *Protein Purification Methods: A Practical Approach*. Harris, E. L. & Angal, S. (Eds), IRL Press, Oxford, England, pp 293-306.
- Prescott, A.G.** 1993. A dilemma of dioxygenases (or where biochemistry and molecular biology fail to meet). *Journal of Experimental Botany* **44**: 849-861.
- Prescott, A.G. & John, P.** 1996. Dioxygenases: Molecular structure and role in plant metabolism. *Annual Review in Plant Physiology and Plant Molecular Biology* **47**: 245-271.
- Ramassamy, S., Olmos, E., Bouzayen, M., Pech, J.C., Latche, A.** 1998. 1-aminocyclopropane-1-carboxylate oxidase of apple fruit is periplasmic. *Journal of Experimental Botany* **49**: 1909-1915.
- Ramassamy, S., Stella, L., Fournel, A., Pech, J.C., Latche, A.** 1998. Structural modification of ACC oxidase during catalytic inactivation. **In:** *Biology and Biotechnology of the Plant Hormone Ethylene II*. Kanellis, A.K., Charng, C., Kende, H., Grierson, D. (Eds), European Union Tmr-Euroconference Programme, pp 68 .
- Reinhardt, D., Kende, H., Boller, T.** 1994. Subcellular localisation of 1-aminocyclopropane-1-carboxylate oxidase in tomato cells. *Planta* **195**: 142-146.
- Roach, P.L., Clifton, I.J., Fulop, V., Barton, G.J., Haijdu, J., Andersson, I., Schofield, C.J., Baldwin, J.E.** 1995. Crystal structure of isopenicillin-N-synthase is the first from a new structural family of enzymes. *Nature* **375**: 700-704.
- Roe, S.** 1989. Separation based on structure. **In:** *Protein Purification Methods: A Practical Approach*. Harris, E.L. & Angal, S. (Eds), IRL Press, Oxford, England, pp 175-244.
- Roe, S.** 1989. Separation based on structure. **In:** *Protein Purification Methods: A Practical Approach*. Harris, E.L. & Angal, S. (Eds), IRL Press, Oxford, England, pp 293-306.
- Rombaldi, C., Lelievre, J.M., Latche, A., Petitprez, M., Bouzayen, M., Pech, J.C.** 1994. Immunocytolocalization of 1-aminocyclopropane-1-carboxylic acid oxidase in tomato and apple fruit. *Planta* **192**: 453-460.
- Rosenfeld, J., Capdevielle, J., Guillemot, J.C., Ferrara, P.** 1992. In-gel digestion of proteins for internal sequence analysis after one or two-dimensional gel electrophoresis. *Analytical Biochemistry* **203**: 173-179.

- Rottmann, W.H., Peter, G.F., Oeller, P.W., Keller, J.A., Shen, N.F., Nagy, B.P., Taylor, L.P., Campbell, A.D., Theologis, A.** 1991. 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *Journal of Molecular Biology* **222**: 937-961.
- Sakai, S. & Maniwa, Y.** 1994. Inhibition of ethylene synthesis in mung bean hypocotyl segments by the proteinaceous inhibitors of ethylene synthesis. *Plant Physiology and Biochemistry* **32**: 385-389.
- Salvesen, G. & Nagase, H.** 1989. Inhibition of proteolytic enzymes. In: *Proteolytic Enzymes: A Practical Approach*. Beynon, R.J. & Bond, J.S. (Eds), IRL Press, Oxford, England, pp 91.
- Sato, T., Oeller, P.W., Theologis, A.** 1991. The 1-aminocyclopropane-1-carboxylate synthase of *cucurbita*. Purification, properties, expression in *Escherichia coli*, and primary structure determination by DNA sequence analysis. *Journal of Biological Chemistry* **266**: 3752-3759.
- Schein, C.H. & Noteborn, M.H.M.** 1988. Formation of soluble recombinant proteins in *E. coli* is favored by lower growth temperature. *Biotechnology* **6**: 291-294.
- Schierle, J., Rohwer, F., Bopp, M.** 1989. Distribution of ethylene synthesis along the etiolated pea shoot and its regulation by ethylene. *Journal of Plant Physiology* **134**: 331-337.
- Schroder, G., Eichel, J., Breinig, S., Schroder, J.** 1997. Three differentially expressed S-adenosyl methionine synthetases from *Catharanthus roseus*: molecular and functional characterisation. *Plant Molecular Biology* **33**: 211-222.
- Shaw, J.F., Chou, Y.S., Chang, R.C., Yang, S.Y.** 1996. Characterization of the ferrous iron binding sites of apple 1-aminocyclopropane-1-carboxylate oxidase by site-directed mutagenesis. *Biochemical and Biophysical Research Communications* **225**: 697-700.
- Shiomi, S., Yamamoto, M., Ono, T., Kakiuchi, K., Nakamoto, J., Nakatsuka, A., Kubo, Y., Nakamura, R., Inaba, A., Imaseki, H.** 1998. cDNA cloning of ACC synthase and ACC oxidase genes in cucumber fruit and their expression by wounding and auxin. *Journal of Japanese Society of Horticultural Science* **67**: 685-692.
- Shiomi, S., Yamamoto, M., Nakamura, R., Inaba, A.** 1999. Expression of ACC synthase and ACC oxidase genes in melon harvested at different stages of maturity. *Journal of Japanese Society of Horticultural Science* **68**: 10-17.
- Shiu, O.Y., Oetiker, J.H., Yip, W.K., Yang, S.F.** 1998. The promoter of LE-ACS7, an early flooding-induced 1-aminocyclopropane-1-carboxylate synthase gene of the tomato, is

- tagged by a SOL3 transposon. *Proceedings of the National Academy of Sciences, USA* **95**: 10334-10339.
- Sisler, E.C., Serek, M., Dupille, E.** 1995. Comparison of cyclopropene, 1-methylcyclopropene and 3,3-dimethylcyclopropene as ethylene antagonists in plants. *Plant Growth Regulation* **17**: 1-16.
- Slater, A., Maunder, M.J., Edwards, K., Schuch, W., Grierson, D.** 1985. Isolation and characterisation of cDNA clones for tomato polygalacturonase and other ripening-related proteins. *Plant Molecular Biology* **5**: 137-147.
- Smart, C.** 1994. Gene expression during leaf senescence. *New Phytologist* **126**: 419-448.
- Smart, C.M., Hosken, S.E., Thomas, H., Grieaves, J.A., Blair, B.G., Schuch, W.** 1995. The timing of maize leaf senescence and characterisation of senescence-related cDNAs. *Physiologia Plantarum* **93**: 673-682.
- Smith, J.J. & John, P.** 1993. Activation of 1-aminocyclopropane-1-carboxylate oxidase by bicarbonate/carbon dioxide. *Phytochemistry* **32**: 1381-1386.
- Smith, C.J.S., Slater, A., Grierson, D.** 1986. Rapid appearance of a mRNA correlated with ethylene synthesis encoding a protein of molecular weight 35000. *Planta* **168**: 94-100.
- Smith, J.J., Ververidis, P., John, P.** 1992. Characterization of the ethylene-forming enzyme partially purified from melon. *Phytochemistry* **31**: 1485-1494.
- Smith, J.J., Zhang, Z.H., Schofield, C.J., John, P., Baldwin, J.E.** 1994. Inactivation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase. *Journal of Experimental Botany* **45**: 521-527.
- Spanu, P., Reinhardt, D., Boller, T.** 1991. Analysis and cloning of the ethylene-forming enzyme from tomato by functional expression of its mRNA in *Xenopus laevis* oocytes. *EMBO Journal* **10**: 2007-2013.
- Spanu, P., Boller, T., Kende, H.** 1993. Differential accumulation of transcripts of 1-aminocyclopropane-1-carboxylate synthase genes in tomato plants infected with phytophthora infestans and in elicitor-treated tomato cell suspension. *Journal of Plant Physiology* **141**: 557-562.
- Tabor, C.W. & Tabor, H.** 1984. Methionineadenosyltransferase (S-adenosylmethionine synthetase) and S-adenosylmethionine decarboxylase. *Advances in Enzymology* **56**: 251-282.
- Takahama, U. & Oniki, T.** 1992. Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. *Plant and Cell Physiology* **33**: 379-387.

- Tang, X., Wang, H., Brandt, A.S., Woodson, W.R.** 1993. Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *Petunia hybrida*. *Plant Molecular Biology* **23**: 1151-1164.
- Tang, X., Gomes, A.M.T.R., Bhatia, A., Woodson, W.R.** 1994. Pistil-specific and ethylene-regulated expression of 1-aminocyclopropane-1-carboxylate oxidase genes in petunia flowers. *The Plant Cell* **6**: 1227-1239.
- Tayeh, M.A., Howe, D.L., Salleh, H.M., Sheflyan, G.Y., Son, J.K., Woodard, R.W.** 1999. Kinetic and mutagenic evidence for the role of histidine residues in the *Lycopersicon esculentum* 1-aminocyclopropane-1-carboxylate oxidase. *Journal of Protein Chemistry* **18**: 55-68.
- ten Have, A. & Woltering, E.J.** 1997. Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. *Plant Molecular Biology* **34**: 89-97.
- Thomas, R.G.** 1987. Vegetative growth and development. In: *White Clover*. Baker, M.J. & William, W.M. (Eds), CAB International, Oxon, England, pp 31-62.
- Towbin, H., Staehelin, T., Gordon, J.** 1979. Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76**: 4350-4354.
- Tsai, D.S., Arteca, R.N., Bachman, J.M., Phillips, A.T.** 1988. Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from etiolated mungbean hypocotyls. *Archives of Biochemistry and Biophysics* **264**: 632-640.
- Tsai, D.S., Arteca, R.N., Arteca, J.M., Phillips, A.T.** 1991. Characterization of 1-aminocyclopropane-1-carboxylate synthase from etiolated mungbean hypocotyls. *Journal of Plant Physiology* **137**: 301-306.
- Van Der Straeten, D., Van Wiemeersch, L., Van Damme, J., Goodman, H., Van Montagu, M.** 1989. Purification and amino acid sequence analysis of characterization of 1-aminocyclopropane-1-carboxylate synthase from tomato pericarp. In: *Biochemical and Physiological Aspects of Ethylene Production in Lower and Higher Plants*. Clijsters, H., De Proft, M., Marcelle, R., Van poucke, M. (Eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 93-100.
- Verlinden, S. & Woodson, W.R.** 1998. The physiological and molecular responses of carnation flowers to higher temperature. *Postharvest Biology and Technology* **14**: 185-192.
- Ververidis, P. & John, P.** 1991. Complete recovery *in vitro* of ethylene-forming enzyme activity. *Phytochemistry* **30**: 725-727.

- Vioque, B. & Castellano, J.M.** 1994. Extraction and biochemical characterization of 1-aminocyclopropane-1-carboxylate oxidase from pear. *Physiologia Plantarum* **90**: 334-338.
- Vioque, B. & Castellano, J.M.** 1998. *In vivo* and *in vitro* 1-aminocyclopropane-1-carboxylic acid oxidase activity in pear fruit: role of ascorbate and inactivation during catalysis. *Journal of Agricultural and Food Chemistry* **46**: 1706-1711.
- Vogel, J.P., Woeste, K.E., Theologis, A., Kieber, J.J.** 1998. Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of *Arabidopsis* confer cytokinin-insensitivity and ethylene overproduction respectively. *Proceedings of the National Academy of Sciences, USA* **8**: 4766-4771.
- Vries, M.A.N., Woltering, E.J., Vrije, T.** 1994. Partial characterization of carnation petal 1-aminocyclopropane-1-carboxylate oxidase. *Journal of Plant Physiology* **144**: 549-554.
- Walker, J.R.L.** 1997. Software review, *NZ BioScience* **2**: 8-9
- Whittaker, D.J., Smith, G.S., Gardner, R.C.** 1997. Expression of ethylene biosynthetic genes in *Actinidia chinensis* fruit. *Plant Molecular Biology* **34**: 45-55.
- Wilkinson, J.Q., Lanahan, M.B., Conner, J.W., Klee, H.J.** 1995. Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. *Plant Molecular Biology* **27**: 1097-1108.
- Wilson, I.D., Zhu, Y., Burmeister, D.M., Dilley, D.R.** 1993. Apple ripening related cDNA clone pAP4 confers ethylene-forming ability in transformed *Saccharomyces cerevisiae*. *Plant Physiology* **102**: 783-788.
- Woodson, W.R., Park, K.Y., Drory, A., Larsen, P.B., Wang, H.** 1992. Expression of ethylene biosynthetic pathway transcripts in senescing carnation flowers. *Plant Physiology* **99**: 526-532.
- Wyatt, J.M.** 1997. Expression, purification and mutagenesis of recombinant class 1 aldehyde dehydrogenase. MSc thesis, Massey University, Palmerston North, New Zealand.
- Xu, Z.C., Hyodo, H., Ikoma, Y., Yano, M., Ogawa, K.** 1998. Biochemical characterization and expression of recombinant ACC oxidase in *Escherichia coli* and endogenous ACC oxidase from kiwifruit. *Postharvest Biology and Technology* **14**: 41-50.
- Yang, S.F. & Hoffman, N.E.** 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual Review in Plant Physiology* **35**: 155-189.
- Yang, S.F. & Dong, J.G.** 1993. Recent progress in research of ethylene biosynthesis. *Botany of Bulletin Academy Sinica* **34**: 89-101.

- Yang, S.F. & Oetiker, J.H.** 1998. Molecular biology of ethylene biosynthesis and its application in horticulture. *Journal of Japanese Society of Horticultural Science* **67**: 1209-1214.
- Yip, W.K., Dong, J.G., Yang, S.F.** 1991. Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from apple fruits. *Plant Physiology* **95**: 251-257.
- Yip, W.K., Jiao, X.Z., Yang, S.F.** 1988. Dependence of *in vivo* ethylene production rate on 1-aminocyclopropane-1-carboxylic acid content and oxygen concentration. *Plant Physiology* **88**: 553-558.
- Yoon, I.S., Park, D.H., Mori, H., Imaseki, H., Kang, B.G.** 1999. Characterization of an auxin-inducible 1-aminocyclopropane-1-carboxylate synthase gene, VR-ACS6, of mungbean (*Vigna radiata* (L.) Wilczek) and hormonal interactions on the promoter activity in transgenic tobacco. *Plant and Cell Physiology* **40**: 431-438.
- Yu, S.J., Kim, S., Lee, J.S., Lee, D.H.** 1998. Differential accumulation of transcripts for ACC synthase and ACC oxidase homologs in etiolated mung bean hypocotyls in response to various stimuli. *Molecular Cells* **8**: 350-358.
- Zhang, Z., Barlow, J.N., Baldwin, J.E., Schofield, C.J.** 1997. Metal-catalyzed oxidation and mutagenesis studies on the iron (II) binding site of 1-aminocyclopropane-1-carboxylate oxidase. *Biochemistry* **36**: 15999-16007.
- Zhang, Z.H., Schofield, C.J., Baldwin, J.E., Thomas, P., John, P.** 1995. Expression, purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from tomato in *Escherichia coli*. *Biochemical Journal* **307**: 77-85.
- Zhou, H.Q., Huxtable, S., Xin, H., Li, N.** 1998. Enhanced high-level expression of soluble 1-aminocyclopropane-1-carboxylate synthase and rapid purification by expanded-bed adsorption. *Protein Expression and Purification* **14**: 178-184.
- Zor, T. & Selinger, Z.** 1996. Linerization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Analytical Biochemistry* **236**: 302-308.