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Studies on membrane-associated and soluble enoyl reductases from oil seed rape (Brassica napus)

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Studies on Membrane-Associated and Soluble Enoyl Reductases from Oil Seed Rape (*Brassica napus*).

By

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A thesis submitted for the degree of Doctor of Philosophy
in the University of Durham

Department of Biological Sciences

University of Durham

February, 1996.

To Mum and Dad

Studies on Membrane-Associated and Soluble Enoyl Reductases from Oil Seed Rape (*Brassica napus*).

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Three forms of enoyl reductase have been identified in *Brassica napus* seeds, one soluble, which is involved in the synthesis of fatty acids up to C18, and two membrane-associated forms, which elongate VLCFA's. NADH-utilising membrane-associated and soluble enoyl reductases have been differentiated by their pH profiles and the fact that they are immunologically distinct. Despite their differing properties, both enzymes reduce C20:2-CoA[$t2$, $c11$] with NADH and so adequate controls are essential to remove contaminating soluble enoyl-ACP reductase from microsomes when studying the membrane-associated enzyme. The importance of these controls and extensive washing procedures to remove such contamination have not previously been stressed in the literature. In addition to the NADH membrane-associated enoyl reductase, a NADPH-utilising form also exists and these two enzymes have been differentiated by their thermal stabilities.

Several methods were attempted to solubilise the membrane-associated enoyl reductases. Initial observations indicating their solubilisation by LDAO were later proved to be due to contaminating soluble enoyl-ACP reductase, again highlighting the importance of appropriate controls. Solubilisation of both forms was achieved using guanidinium thiocyanate, which is a inhibitor of soluble enoyl-ACP reductase. The solubilised enzymes were unusually stable at high temperature but were shown to be proteinaceous and to have molecular weights of greater than 30 kDa.

Attempts to obtain a cDNA encoding a membrane-associated enoyl reductase using a probe from a soluble enoyl-ACP reductase cDNA were unsuccessful. However, a cDNA encoding the soluble enzyme was isolated which showed high homology in the open reading frame to, but substantial differences in the 5' and 3' non-translated regions from, the previously isolated cDNA, and so represents a differently regulated enoyl-ACP reductase.

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No part of this thesis has been previously submitted for a degree in this or any other University. I declare that, unless otherwise indicated, this thesis is entirely the result of my own work.

Anne Fowler

February, 1996.

Acknowledgements.

Abbreviations.

Å	Angstrom
ACP	Acyl carrier protein
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Nucleotide base pair
BSA	Bovine serum albumin
C4:1-ACP[t2]	(trans)-2-Crotonyl Acyl Carrier Protein
C4:1-CoA[t2]	(trans)-2-Crotonyl Coenzyme A
C20:1-CoA[c11]	(cis)-11-Eicosaenoyl Coenzyme A
C20:2-CoA[t2, c11]	(trans)-2,(cis)-11-Eicosadienoyl Coenzyme A
cDNA	complementary DNA
CoA	Coenzyme A
cmc	Critical micelle concentration
dsDNA	double DNA
Diflufenican	N-(2,4-difluorophenyl)-2-[3-(trifluoromethyl)phenoxy]-3-pyridinecarboxamide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
FAS	Fatty acid synthase
g	Acceleration due to gravity
HEAR	High erucic acid rape
IPTG	Isopropyl β-D-thiogalactopyranoside
kDa	Kilodalton
K_M	Michaelis constant

LDAO	Lauryldimethylamine oxide
LEAR	Low erucic acid rape
MES	2-[N-Morpholino]ethanesulfonic acid
MOPS	3-[N-Morpholino]propanesulfonic acid
mRNA	messenger Ribonucleic acid
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
N. D.	Not determined
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
pfu	Plaque forming units
RNAse	Ribonuclease
r.p.m.	Revolutions per minute
SDS	Sodium dodecyl sulphate
ssDNA	single stranded DNA
TEMED	N,N,N'N'-tetramethylethylene diamine
Tris	Tris (hydroxymethyl) aminoethane
U	Unit of enzyme activity
U. V. light	Ultra-violet light
var	variety
VLCFA's	Very long chain fatty acids
v/v	Volume for volume
v/w	Volume for weight
w/v	Weight for volume
w/w	Weight for weight

1.1 The roles of fatty acids in plants.

Fatty acids play many different roles in plants. For example, they provide structure through membrane systems, are involved in biosynthesis as acyl-CoA's and acyl-ACP's, that act as intermediates in complex lipid biosynthesis, and are major storage components. The study of fatty acids has a long chemical history, and as a result, many of the fatty acids have trivial names; a summary of the systematic and trivial names of several of the common fatty acids is given in table 1.1.

Table 1.1 Systematic and trivial names of common fatty acids. (Adapted from Slabas *et al.*, 1992a.)

Structure	Systematic name	Trivial name
C2:0	*n*-ethanoic	acetic
C4:0	*n*-butanoic	butyric
C10:0	*n*-decanoic	capric
C12:0	*n*-dodecanoic	lauric
C16:0	*n*-hexadecanoic	palmitic
C18:0	*n*-octadecanoic	stearic
C18:1[*c*9]	*cis*-9-octadecanoic	oleic
C20:0	*n*-eicosanoic	arachidic
C22:0	*n*-docosanoic	behenic
C22:1[*c*13]	*cis*-13-docosenoic	erucic

The chemical nomenclature used for fatty acids is as follows: the number after the C denotes the length of the hydrocarbon chain with the largest number of carbon atoms, the number after the colon indicates the number of double bonds, and the type (*cis* [*c*] or *trans* [*t*]) and position of the double bond, defined from the carboxyl end of the hydrocarbon chain, is indicated in the square bracket.

The variability in fatty acid composition of storage oils is exceptionally high and due to their chemical properties, they have important commercial applications (Knauf, 1987). The major applications of some vegetable oils are summarised in table 1.2.

Table 1.2 Major applications of vegetable oils. (From Knauf, 1987.)

Fatty acid	Plant source	Application
Lauric acid (C12:0)	Palm, coconut	soaps, detergents
Palmitic acid (C16:0)	Palm	margarine
Oleic acid (C18:1[*c*9])	Olive, rapeseed	cooking oil
Erucic acid (C22:1[*c*11])	High erucic rapeseed	solvents and high temperature lubricants

Plant oils are of major economic importance and as a consequence, there is substantial interest in exploring the degree to which genetic methods can be used to modify the oil composition of seeds. In many cases, relatively large alterations can be made in the fatty acid composition of the storage triacylglycerol without exerting any obvious deleterious effects on the growth and development of the organism. For example, expression of cDNA encoding a laurate-ACP thioesterase from *Umbellularia californica* (california bay) in *Arabidopsis thaliana* seeds resulted in

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the accumulation of medium chain fatty acids at the expense of long chain (> C16) ones (Voelker *et al.*, 1992). Consequently, great potential exists for genetically engineering plant lipids, providing that there is sufficient information on the biochemical pathways involved, in addition to the temporal and tissue specific expression of the genes.

At present, there is interest in seed oils rich in erucic acid (C22:1[c13]) for the production of solvents and high temperature lubricants (Andrews and Ohlrogge, 1991). The original variety of *Brassica napus* (oil seed rape) contains high levels of erucic acid, which accounts for approximately 40 % of the total fatty acids accumulated in the seed (Hilditch and Williams, 1964). However, studies carried out on rats showed that consumption of large amounts of erucic acid caused cardiac lipidosis and necrosis (Roine *et al.*, 1960), and consequently, low erucic acid rape (LEAR) was developed, by classical breeding methods (Stefansson *et al.*, 1961). These observations caused several Western countries in the 1970's to limit the erucic acid content of oils destined for human consumption to less than 5 % (Sauer and Kramer, 1983), yet so far there have been no reports to suggest toxicity in human populations consuming rapeseed oil (Laryea *et al.*, 1992). Recently, the demand for erucic acid has increased because of its commercial value, and therefore, research is underway to increase the erucic acid content of *Brassica napus*, by genetic modification. Such modification of the triacylglycerol biosynthetic pathway in *Brassica napus* seeds, with the aim of an enhanced erucic acid production, requires a detailed understanding of the biosynthesis and storage of very long chain fatty acids (VLCFA's).

Genetic modification has already been used to alter the fatty acid composition of plants. For example, a decrease in the level of oleic acid, with an increase in stearic acid has been achieved in transgenic *Brassica napus* by modulation of stearoyl-ACP

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(Δ9) desaturase, using antisense technology (Knutzon *et al.*, 1992). As a consequence of this, up to 40 % of the fatty acid content was stearic acid, making the oil potentially useful as a substitute for cocoa butter. As previously stated, termination of fatty acid elongation can be altered by genetic engineering (Voelker *et al.*, 1992). Additionally, the chilling resistance of plants has been increased. Tobacco plants transformed with acyl-ACP: glycerol-3-phosphate acyltransferase from *Arabidopsis thaliana*, a plant resistant to chilling, had an increased level of unsaturation of phosphatidylglycerol and the plants were chilling tolerant (Murata *et al.*, 1992).

1.2 Fatty acid biosynthesis.

1.2.1 The reactions involved in fatty acid synthesis.

The component reactions involved in *de novo* fatty acid synthesis are shown in figure 1.1. The acyl-ACP product of the reaction is used as a substrate with malonyl-ACP in the next cycle of chain elongation resulting in the addition of two carbon units to the growing acyl-ACP chain per cycle, and this proceeds until the acyl chain is 18 carbons in length. The enzymes involved in fatty acid biosynthesis form the fatty acid synthase (FAS), of which there are two types, I and II.

Type I is a non-dissociable multi-functional protein found in animals and yeast. In animals, FAS consists of two identical multi-functional polypeptide subunits in an $\alpha 2$ dimer (Buckner and Kolattukudy, 1976). A schematic diagram of animal FAS is shown in figure 1.2.

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acetyl-CoA: ACP transacylase

acetyl-CoA + ACP-SH $\longrightarrow$ acetyl-ACP + CoA-SH

malonyl-CoA: ACP transacylase

malonyl-CoA + ACP-SH $\longrightarrow$ malonyl-ACP + CoA-SH

acetyl-ACP + malonyl-ACP

β-ketoacyl-ACP synthase

+ ACP—SH + CO_2

+ NAD(P)H + H^+ β-ketoacyl-ACP reductase

+ NAD(P)$^+$

β-hydroxyacyl-ACP dehydrase

+ H_2O

+ NAD(P)H + H^+ enoyl-ACP reductase

+ NAD(P)$^+$
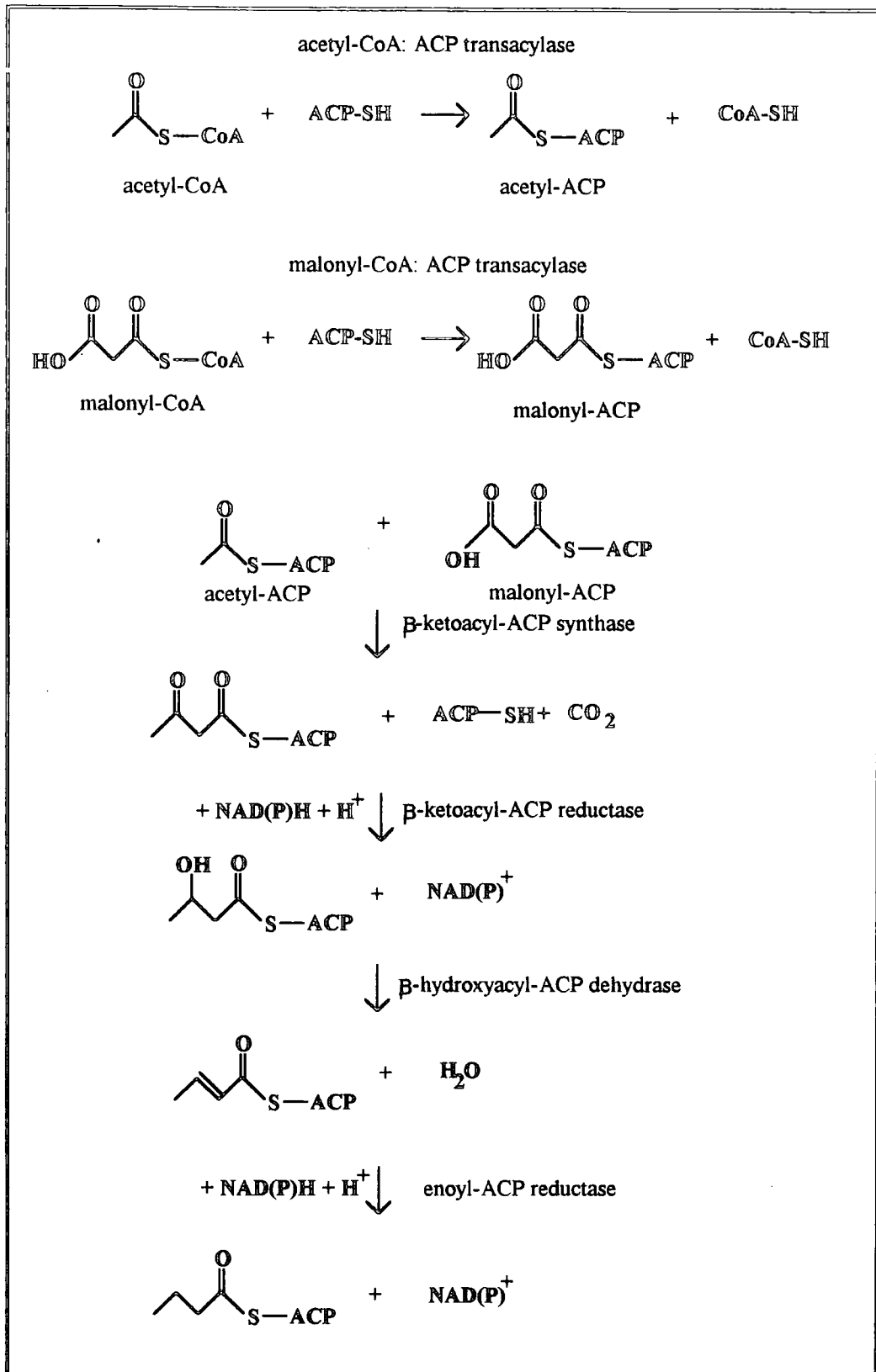
Figure 1.1 The component reactions in fatty acid biosynthesis.
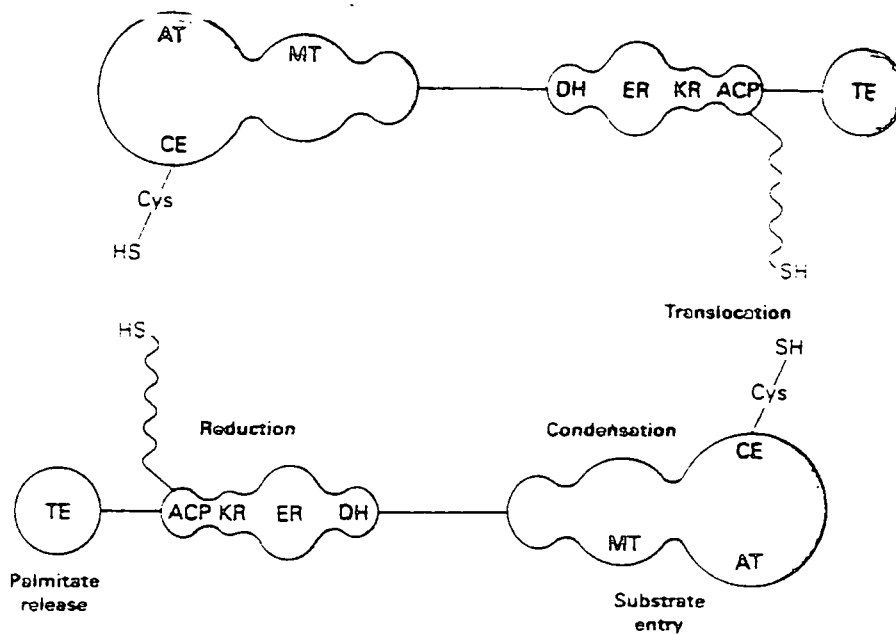
Figure 1.2 A schematic diagram of animal fatty acid synthase. (From Stryer, 1988.) Each chain consists of three domains. Domain 1 contains acetyl transferase (AT), malonyl transferase (MT) and β-ketoacyl-ACP synthase (condensing enzyme; CE); domain 2 contains acyl carrier protein (ACP), β-ketoacyl-ACP reductase (KR), β-hydroxyacyl-ACP dehydrase (DH) and enoyl reductase (ER); domain 3 contains thioesterase (TE).

Acetyl-CoA and malonyl-CoA enter FAS by attachment to the transferases. The acetyl group is then transferred to KAS (the condensing enzyme) and the malonyl unit is transferred to the ACP of the other chain in the dimer, hence domain 1 of each chain of the dimer interacts with domains 2 and 3 of the other chain. Therefore, each of the two functional units of the synthase consists of domains from both chains (Tsukamoto *et al.*, 1983).

In yeast, FAS consists of tri- and penta-functional subunits in an α6β6 hexamer. The α subunit comprises of KAS, β-ketoacyl-ACP reductase and ACP domains. The

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β subunit contains acetyl, malonyl and palmitoyl transferases, β-hydroxyacyl-ACP dehydrase and enoyl reductase (Schweizer *et al.*, 1986).

Type II FAS is typically present in plants and micro-organisms (Shimakata and Stumpf, 1982a and Simoni *et al.*, 1967, respectively). This FAS is composed of dissociable enzymes.

Since this research is based on membrane-associated and soluble enoyl reductases from *Brassica napus* (oil seed rape), a description of fatty acid biosynthesis will concentrate on the system found in plants.

1.2.2 Fatty acid biosynthesis in plants.

Fatty acid biosynthesis, i.e. the incorporation of [^{14}C] acetate into palmitate (C16:0) and oleate (C18:1 [*c*9]) in plants occurs in plastids; subcellular fractionation of disintegrated *Persea americana* (avocado) mesocarp on a sucrose density gradient produced a dense particulate fraction containing FAS activity, and electron microscopy showed that this fraction contained plastids (Weaire and Kekwick, 1975). Previous studies had lead to the conclusion that fatty acid synthesis occurred in the supernatant, but these experiments had been performed using a rigorous disintegration procedure that disrupted plastids (Weaire and Kekwick, 1975).

The substrates required for the first round of fatty acid synthesis are acetyl-CoA and malonyl-CoA. Acetyl-CoA probably does not cross the chloroplast envelope and so it is presumed to be synthesised *in situ*, either from precursors derived from photosynthetic carbon fixation within the organelle, or from precursors imported from the cytosol. Incorporated acetate would be converted to acetyl-CoA by acetyl-CoA synthase, whereas pyruvate could be converted to acetyl-CoA by pyruvate dehydrogenase. Research into the true precursors for fatty acid biosynthesis

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is somewhat controversial since in pea leaf chloroplasts, citrate, pyruvate, acetate and acetylcarnitine all serve as sources of acetyl groups for fatty acid synthesis (Masterson *et al.*, 1990). Acetylcarnitine was the best substrate. It was incorporated into fatty acids at a rate that was at least 5 fold higher than those achieved with the other substrates. Acetate was incorporated at the second highest rate. Conversely, it was later reported that in chloroplasts isolated from spinach, pea or maize, acetylcarnitine was not a substrate for fatty acid synthesis (Roughan *et al.*, 1992). Additionally, in 1995, it was determined that in oat seedlings, acetate was used by chloroplasts for fatty acid biosynthesis, but in etioplasts, pyruvate was preferred (Lichtenthaler and Golz, 1995). Clearly, there is confusion over the mechanism used to import acetate groups. It is possible that there could be a variety of different sources of substrate for fatty acid synthesis dependent on the tissue and physiology of the plant. However, it is known that the second substrate, malonyl-CoA, is formed from acetyl-CoA.

1.2.2.1 Acetyl-CoA carboxylase.

Acetyl-CoA carboxylase (ACCase) catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA (figure 1.3).

$$1$$
$$\text{ATP} + \text{HCO}_3\text{-} + \text{BCCP} \Leftrightarrow \text{ADP} + \text{Pi} + \text{BCCP-CO}_2$$

$$2$$
$$\text{BCCP-CO}_2 + \text{acetyl-CoA} \Leftrightarrow \text{BCCP} + \text{malonyl-CoA}$$
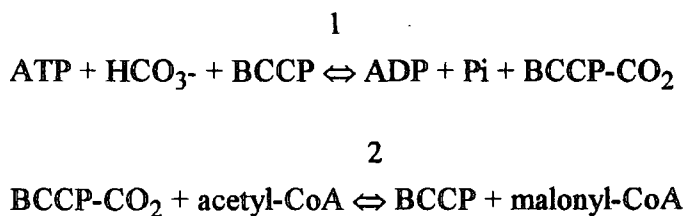
Figure 1.3 Acetyl-CoA carboxylase catalysed carboxylation of acetyl-CoA.

BCCP - biotin carboxyl carrier protein

1 - biotin carboxylase   2 - carboxyl transferase

This reaction is considered to be an important rate-limiting reaction of *de novo* fatty acid biosynthesis in chloroplasts (Post-Beittenmiller *et al.*, 1992). There is interest in studying plant ACCase because it is the primary target site of major classes of grass specific herbicides and the use of herbicides has been used to prove the existence of two ACCases in maize. The major isoform (230 kDa) is more sensitive to inhibition by aryloxphenoxypropionates and cyclohexanediones than the 220 kDa form (Herbert *et al.*, 1994).

The molecular organisation of ACCase differs with different enzyme sources. The mammalian form (type I) is a large multifunctional polypeptide of > 200 kDa (Takai *et al.*, 1988), consisting of three fused domains: biotin carboxylase, biotin carboxylase carrier protein and carboxyltransferase. By comparison, in *Escherichia coli* (*E. coli*), a type II ACCase exists, consisting of three separate proteins (Guchhait *et al.*, 1974).

Studies indicate that plants can contain both dissociable and non-dissociable forms of ACCase. The single polypeptide form of ACCase has been purified from wheat and has a molecular weight of 220 kDa (Elborough *et al.*, 1994). cDNA clones isolated from wheat (Elborough *et al.*, 1994) and maize (Ashton *et al.*, 1994a) represent a 220 kDa ACCase and the derived amino acid sequences have homology to ACCase from animal sources.

The second form of ACCase, which contains a 60 kDa biotinylated polypeptide, has been identified in carrot embryos (Nikolau *et al.*, 1995). Antibodies raised against this polypeptide result in the immunoprecipitation of half the ACCase activity present in extracts of carrot embryos, suggesting that the 60 kDa biotinylated polypeptide is a second form of ACCase. Using streptavidin, which detects biotin, it has been determined that pea chloroplasts contain a dissociable ACCase because a biotin protein of low molecular mass (approximately 35 kDa) was identified. In

comparison, pea leaves contain both forms of ACCase (Sasaki et al., 1995). It is not known why two types of ACCase exist, but they may synthesise malonyl-CoA for different pathways, since malonyl-CoA is required for fatty acid biosynthesis and synthesis of secondary compounds, such as flavonoids in plants (Sasaki et al., 1995).

1.2.2.2 Components of plant FAS.

Extensive research has been performed on the FAS components from plants and a summary of the findings is given below.

Acyl carrier protein (ACP) plays a vital role in fatty acid biosynthesis. The incorporation of [^{14}C] malonyl-CoA into fatty acids by homogenates of *Spinacia oleracea* (spinach) leaf is totally ACP dependent; the addition of an anti-ACP antibody results in 98 % inhibition of fatty acid biosynthesis (Ohlrogge et al., 1979). ACP contains a phosphopantetheine prosthetic group attached to a serine residue and the sulfhydryl group joins to the carboxyl carbon of fatty acids to form a thioester. This results in the activation of the acyl group so that it can be acted upon by the other FAS enzymes. Thus ACP is an essential component of fatty acid biosynthesis.

Immunogold labelling has been used to study the distribution of ACP in *Persea americana* (avocado) mesocarp and *Brassica napus* (oil seed rape) seeds; over 95 % of ACP was localised to plastids (Slabas and Smith, 1988). cDNA clones encoding ACP from *Brassica napus* embryos have been isolated and sequenced, and analysis of the sequence data revealed that ACP clearly has a transit peptide for entrance into the plastid (Safford et al., 1988).

ACP was the first protein of plant lipid metabolism to be purified to homogeneity and the purified protein from *Persea americana* (avocado) mesocarp and *Spinacia oleracea* (spinach) leaf is heat stable, contains an abundance of acidic amino acids and has a molecular weight of approximately 10 kDa (Simoni *et al.*, 1967).

Purification of ACP from *Brassica napus* (oil seed rape) seed required different procedures from the purification of the protein from leaf material, because the seed form is not freely soluble and requires detergent solubilisation; neither is the seed form heat or acid stable and biological activity is rapidly lost following chromatography. Despite this, purification to homogeneity was achieved using acyl-ACP synthase from *E. coli* to radiolabel partially purified ACP with [^{14}C] palmitic acid. This introduced a tag to follow ACP and converted the protein from a non-hydrophobic species to a hydrophobic one, and this was used as the basis for purification (Slabas *et al.*, 1987).

Two forms of ACP have been identified by Western blotting in *Spinacia oleracea* (spinach) leaves, yet only one form was detected in seed material (Ohlrogge and Kuo, 1985). It would have been predicted that seed would contain two forms of ACP, a core FAS component and a separate form involved in storage triacylglyceride biosynthesis (Slabas *et al.*, 1992b). There is also considerable evidence for different forms of ACP at the genetic level. For example, a cDNA encoding ACP, isolated from *Spinacia oleracea* leaves, does not cross-hybridise to *Spinacia oleracea* ACP in seeds or roots, suggesting that there are a number of forms of ACP in *Spinacia oleracea* (Scherer and Knauf, 1987). In addition, analysis of several *Brassica napus* embryo ACP clones revealed sequence heterogeneity, providing evidence for an ACP multigene family consisting of six genes encoding five different mature ACP polypeptides (Safford *et al.*, 1988). Northern blot analyses

using sequence specific probes have shown that at least one ACP cDNA in *Brassica napus* is seed specific and is not expressed in leaf tissue (Safford *et al.*, 1988).

Prior to the condensation reaction which adds an extra two carbons onto the growing acyl chain, malonyl-CoA is converted to malonyl-ACP by malonyl-CoA: ACP transacylase (MCAT) so that it can be used as a substrate by FAS. Two forms of MCAT exist in soybean leaves and both are soluble proteins with a molecular weight of approximately 43 kDa (Guerra and Ohlrogge, 1986). The isoforms were differentiated on the basis of their binding to ion-exchange or blue dye affinity chromatographic supports. Isoform I does not bind to these matrices, but isoform II does and requires salt for elution from them. In comparison to leaf material, only isoform I is present in seeds (Guerra and Ohlrogge, 1986). This tissue expression is the same as that for ACP which is present as two forms in *Spinacia oleracea* leaf material, but only one form in seed material.

The condensation between an activated acyl group and malonyl-ACP is catalysed by β-ketoacyl-ACP synthase (KAS), and therefore, the enzyme is often referred to as the condensing enzyme. Two forms of KAS were originally purified from *Spinacia oleracea* leaves; isoform I catalyses the condensation of C2 to C14 acyl-groups to malonyl-ACP, whereas KAS II can only utilise C14 and C16 acyl substrates (Shimakata and Stumpf, 1982b). Current evidence suggests that the elongation of fatty acids from C16 to C18 requires a specific KAS only; the other enzymes involved are the same as those involved in the elongation from C2 to C16. The two forms of KAS can be differentiated by the effect the antibiotic cerulenin has on them; 50 % inhibition of KAS I is achieved by the addition of 2 μM cerulenin, but for KAS II, 50 % inhibition is only obtained in the presence of 50 μM cerulenin (Shimakata and Stumpf, 1982b).

The role of KAS's was complicated in 1987, when KAS III was identified in *E. coli* as a cerulenin-resistant condensing enzyme that catalyses the initial steps in chain elongation (Jackowski and Rock, 1987). Furthermore, evidence suggested that KAS III utilised acetyl-CoA instead of acetyl-ACP and this finding promoted debate as to whether acetyl-ACP was the substrate for fatty acid biosynthesis. It was later shown that acetyl-ACP was the least effective primer for fatty acid synthesis in *Spinacia oleracea* extracts, when compared to acetyl-CoA, butyryl-ACP or hexanoyl-ACP (Jaworski, *et al.*, 1992). This lead to the hypothesis that the major primer of fatty acid biosynthesis in plants is acetyl-CoA, with acetyl-ACP playing only a minor role. This was substantiated when KAS III was purified from *Spinacia oleracea* and its properties investigated (Clough *et al.*, 1992). The enzyme was highly specific for acetyl-CoA and malonyl-ACP; acetyl-, butyryl- and hexanoyl-ACP's would not substitute for acetyl-CoA as substrates, suggesting that the physiological function of KAS III was to catalyse the initial condensation reaction in fatty acid synthesis. The presence of KAS III would eliminate the requirement for a separate acetyl-CoA: ACP transacylase (ACAT), which converts acetyl-CoA to acetyl-ACP, and raised doubts over the existence of ACAT (Clough *et al.*, 1992). However, previous results implied that ACAT was the rate-determining enzyme in fatty acid biosynthesis (Shimakata and Stumpf, 1983), suggesting a requirement for both enzymes.

The hypothesis that both KAS III and ACAT are required was substantiated by the separation of ACAT from KAS III in *Persea americana* (avocado) by ion exchange chromatography (Gulliver and Slabas, 1994). The sizes of the enzymes were compared; KAS III has a subunit molecular weight of 37 kDa and a native molecular mass of 69 kDa, suggesting that the enzyme exists as a dimer. In comparison, the native molecular mass of ACAT is 18.5 kDa. Clearly, both enzyme activities exist *in vivo*, yet, the roles are not fully understood since if the initial reaction of plant FAS is determined by KAS III, why does ACAT activity exist? The use of anti-sense

constructs in transgenic plants would enable the conditions under which the two systems are utilised to be investigated (Gulliver and Slabas, 1994).

The first reduction in fatty acid biosynthesis is catalysed by β-ketoacyl-ACP reductase. The enzyme has been purified from *Spinacia oleracea* (spinach) leaves and *Carthamus tinctorius* (safflower) seeds, and in both cases the reductant NADPH was much more effective than NADH (Shimakata and Stumpf, 1982c and Shimakata and Stumpf, 1982a, respectively). In *Carthamus tinctorius*, the activity detected with NADH was only 1 % of the activity detected with NADPH. It is not known if the activities with NADPH and NADH are caused by one enzyme that is non-specific for pyridine nucleotide, or whether two enzymes exist. In comparison, two separate forms of β-ketoacyl-ACP reductase have been partially purified from *Persea americana* (avocado) mesocarp, that utilise NADH and NADPH, respectively (Caughey and Kekwick, 1982). The two enzymes were affected differently by pH; the NADH-specific enzyme showed an unpronounced optimum at pH 7.0, whereas, the NADPH-specific form showed a sharp optimum at pH 6.5. The NADPH-specific β-ketoacyl-ACP reductase was later purified to homogeneity from the mesocarp of *Persea americana* and no activity was detected with the purified enzyme when NADH was used instead of NADPH (Sheldon *et al.*, 1990). In order to maintain enzymatic activity, purification of the enzyme was performed at room temperature using buffer solutions of as high an ionic strength as possible. Analysis of internal amino acid sequence data showed similarity with the putative gene products encoded by the *nodG* gene from the nitrogen-fixing bacterium, *Rhizobium meliloti*. The *nodE* and *nodF* gene products, which may be part of the same operon, show similarity to the condensing enzyme and ACP, respectively, therefore, it appears that these genes, which determine host specificity, encode enzymes catalysing a similar pathway to that of fatty acid synthesis (Sheldon *et al.*, 1990).

β-ketoacyl-ACP reductase was first purified from seed tissue in 1992. It was purified from *Brassica napus* (oil seed rape) seeds and like the enzyme from *Persea americana* mesocarp, activity was best maintained by high ionic strength and moderate rather than low temperature (Sheldon *et al.*, 1992). A cDNA clone encoding the NADPH-specific β-ketoacyl-ACP reductase was isolated from a *Brassica napus* developing seed library (Slabas *et al.*, 1992c) and was used to construct an over-expression vector that produces large amounts of the protein in *E. coli* BL21(DE3) (Thomas *et al.*, 1995). As a result of this, it is now possible to produce and purify milligram quantities of active β-ketoacyl-ACP reductase with ease, which will facilitate future detailed structural and mechanistic studies.

The keto group formed by β-ketoacyl-ACP reductase is converted to a *trans*-2 double bond, as a result of the removal of a water molecule by the action of β-hydroxyacyl dehydrase. This enzyme has been partially purified from *Carthamus tinctorius* (safflower) seeds (Shimakata and Stumpf, 1982a) and purified to homogeneity from *Spinacia oleracea* (spinach) leaves (Shimakata and Stumpf, 1982c). The *Spinacia oleracea* enzyme has a subunit molecular weight of 19 kDa and a native molecular mass of 85 kDa, suggesting that it exists as a tetramer. Unlike β-ketoacyl-ACP reductase and enoyl-ACP reductase which can reduce both acyl-CoA's and acyl-ACP's, β-ketoacyl-ACP reductase is inactive with acyl-CoA's (Shimakata and Stumpf, 1982c).

Reduction of the *trans*-2 double bond is catalysed by enoyl-ACP reductase which will be discussed in section 1.4.

After the synthesis of fatty acids, they can be subjected to desaturation. Desaturation affects the physical properties of lipids and several plants change the degree of unsaturation in response to cold acclimatisation so that membranes can retain their

degree of fluidity, allowing the biochemical activities that occur in membranes to continue (Murata, 1983). In plants, both soluble and membrane-bound desaturases exist; the latter present a much greater challenge in purification, due to their membrane requirement.

Stearoyl-ACP ($\Delta 9$) desaturase, a soluble enzyme, has been purified from several species including *Carthamus tinctorius* (safflower) seeds where it is a dimer with a molecular weight of 68 kDa and is highly specific for stearoyl-ACP (C18:0-ACP). Activity is dependent on both chain length and thiol content (McKeon and Stumpf, 1982). Anti-sense technology has been used to inhibit stearoyl-ACP ($\Delta 9$) desaturase resulting in an increase in stearic acid (Knutzon *et al.*, 1992). In coriander endosperm an unusual fatty acid, petroselinic acid (C18:1[$c6$]), is accumulated. Evidence suggests that the biosynthetic pathway involves $\Delta 4$ desaturation of palmitoyl-ACP (C16:0) by the action of a desaturase that is structurally related to stearoyl-ACP ($\Delta 9$) desaturase, followed by a 2 carbon elongation of the resulting $\Delta 4$-hexadecenoyl-ACP (Cahoon and Ohlrogge, 1994).

In comparison to soluble-ACP desaturases, alternative approaches have been used to characterise the membrane-bound desaturases rather than attempting their purification. For example, a series of lipid metabolism mutants in *Arabidopsis thaliana* were created so that genes could be isolated by gene walking (Somerville and Browse, 1991). In 1992, a gene was isolated from *Brassica napus* (oil seed rape) that complements an *Arabidopsis thaliana* mutant deficient in omega-3-unsaturated fatty acids and the derived amino acid sequence contains several strongly hydrophobic internal domains that could be transmembrane domains (Arondel *et al.*, 1992).

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Despite the existence of alternative characterisation methods, attempts have been made to purify membrane-bound desaturases. *Borago officinalis* (borage) seeds accumulate the polyunsaturated fatty acids, linoleate (C18:2[*c*9, *c*12]) and linolenate (C18:3[*c*6, *c*9, *c*15]) whose syntheses involve Δ12-desaturase, and Δ6 and Δ15-desaturases, respectively. Progress has recently been made in their characterisation following the solubilisation of Δ12-desaturase and Δ6-desaturase using 1 % w/v CHAPS (Galle *et al.*, 1995).

Following the synthesis of long-chain acyl-ACP's, they are either hydrolysed to free fatty acids, by acyl-ACP thioesterase and exported from the plastid, or they are incorporated into plastid lipids by acyl transferases.

Acyl-ACP thioesterase catalyses the hydrolysis of acyl-ACP's to free fatty acids and ACP. An acyl-ACP thioesterase has been purified to homogeneity from *Brassica napus* (oil seed rape) seed; the enzyme has a preference for C18:1-ACP[*c*9], but can also utilise C8:0, C10:0, C12:0, C14:0, C16:0 and C18:0-ACP's. In comparison, the enzyme has low activity towards acyl-CoA's, typically activity is less than 10 % of that with acyl-ACP's (Hellyer and Slabas, 1990). Such substrate specificity is consistent with a thioesterase which would fit into the termination step after the soluble stearoyl-ACP (Δ9) desaturase has acted.

Saturated fatty acids are required for wax biosynthesis in epidermal cells, but it seems unlikely that the non-specific oleoyl-ACP thioesterase activity with C16:0 and C18:0-ACP's would provide an adequate supply of saturated fatty acids. Recently, though, two thioesterase activities, C16:0-ACP and C18:0-ACP thioesterases have been identified in *Allium porrum* (leek) epidermis and the C18:0-ACP thioesterase,

which essentially resides in epidermal cells, has been purified to near homogeneity. In sharp contrast to oleoyl-ACP thioesterase, C18:0-ACP thioesterase had high substrate specificity with C18:0-ACP, suggesting its role may be the termination of fatty acid biosynthesis in the plastid to supply saturated fatty acids for wax biosynthesis (Liu and Post-Beittenmiller, 1995).

Umbellularia californica (california bay) accumulates the medium chain C10:0 and C12:0 fatty acids, which are synthesised due to the activity of C12:0-ACP thioesterase. This enzyme has been separated from C18:1-ACP thioesterase by affinity chromatography using immobilised ACP and separation of the two enzymes was used to confirm that the medium chain enzyme has negligible action on C18:1-ACP (Davies *et al.*, 1991). Sequencing of C12:0-ACP thioesterase enabled the cloning of a cDNA encoding the enzyme, and subsequent expression of the cDNA in *Arabidopsis thaliana* resulted in enzyme activity, causing the accumulation of medium chain fatty acids at the expense of long chain ones (Voelker *et al.*, 1992). *Cuphea* spp. also accumulates medium chain fatty acids; it contains decanoic acid (C10:0) rich triacylglycerols. Like *Umbellularia californica*, two acyl-ACP thioesterases, specific for medium chain acyl-ACP's and C18:1-ACP, respectively, have been separated using Mono Q chromatography and partially purified from developing seeds (Dörmann *et al.*, 1992).

1.2.2.3 Formation of complex lipids.

Triacylglycerides (TAG) are the major storage lipids in plants. The basic structure of TAG's consists of a glycerol backbone to which three fatty acids are esterified. The positions of the fatty acids on the glycerol backbone are referred to as *sn*-1, 2 and 3,

starting from the top carbon position. The formation of TAG's in plants involves acyltransferases and proceeds via the Kennedy pathway, which involves the two step acylation of glycerol-3-phosphate to form initially 1-acylglycerol-3-phosphate (lysophosphatidic acid; LPA) and then 1,2-diacylglycerol-3-phosphate (phosphatidic acid; PA). This is followed by a dephosphorylation to yield diacylglycerol, which is acylated at the sn-3 position to form triacylglycerol (Barron and Stumpf, 1962).

There is a correlation between the chilling sensitivity of plants and lipid composition. In chilling-resistant plants, such as pea and spinach, the acyl-ACP: glycerol-3-phosphate acyltransferase exhibits a very strong preference for the oleoyl (C18:1) group, whereas the acyltransferase from Cucurbita moschata (squash), a chilling-sensitive plant, is less selective and incorporates a high proportion of saturated acyl groups found at the sn-1 position (Frentzen et al., 1987). This soluble acyltransferase from Cucurbita moschata has been purified from greening cotyledons using ACP affinity chromatography and other conventional procedures, leading to the isolation of three isoforms. Acyltransferase (AT) 1 was separated from the other two forms by anion-exchange chromatography, whereas, hydroxyapatite chromatography was required to separate isoforms 2 and 3. All the isoforms are monomers with molecular masses of 30, 40 and 40 kDa, respectively (Nishida et al., 1987). An oleate (C18:1)-selective acyl-ACP: glycerol-3-phosphate acyltransferase has since been purified from pea chloroplasts and antibodies raised against this 40 kDa protein were used for cDNA cloning (Weber et al., 1991). However, comparison between a sensitive (pea) and resistant (squash) acyltransferase cDNA sequence (cloned by Ishizaki et al., 1988) did not provide a clue for recognising the structural differences resulting in different selectivities.

The substrate specificity of membrane-bound acyl-ACP: 1-acylglycerol-3-phosphate acyltransferase, which catalyses the acylation at the sn-2 position, has been studied in microsomes from palm, maize and *Brassica napus* (oil seed rape), and showed that the acyl group in the sn-1 position of 1-acylglycerol-3-phosphate is important in determining the acyl preference in the sn-2 position in 1,2-diacylglycerol-3-phosphate synthesis (Oo and Huang, 1989). The acyl transferases from *Brassica napus* and maize preferred 1-acylglycerol-3-phosphate containing C18:1 at sn-1 rather than C12:0. In comparison, the enzyme from palm also prefers C18:1, but not as strongly as in *Brassica napus* and maize. However, only the 1-acylglycerol-3-phosphate acyltransferase from palm can utilise C12:0. Therefore, due to the substrate specificities of acyl-ACP: 1-acylglycerol-3-phosphate acyltransferase, there is a limitation in the production of genetically engineered plants to produce oil with a high lauric acid content, which is required for the production of soaps and detergents.

There is interest in the production of oils from *Brassica napus* with a high erucic acid (C22:1) content, and consequently, the utilisation of erucoyl-CoA by acyltransferases from developing seeds of *Brassica napus* has been investigated. The fatty acid compositions at sn-1 and sn-3 depend on the acyl-CoA mixture available; however, at sn-2, selectivity is controlled by the acyl-ACP: 1-acylglycerol-3-phosphate acyltransferase and C22:1 can not be used (Bernerth and Frentzen, 1990). This means that the theoretical content of erucic acid in *Brassica napus* oil is 66 % and consequently, to increase the content of C22:1 in triacylgylcerols, it is not sufficient to increase the level of C22:1 within the cell, but the properties of acyl-ACP: 1-acylglycerol-3-phosphate acyltransferase must be modified as well.

A potential method of increasing the C22:1 content of *Brassica napus* seed oil is to transfer an acyl-ACP: 1-acylglycerol-3-phosphate acyltransferase from *Limnanthes*

douglasii (meadowfoam), whose seed microsomes synthesise dierucoyl phosphatidic acid (Hanke *et al.*, 1995). Since acyl-ACP: 1-acylglycerol-3-phosphate acyltransferases are membrane bound and have not been purified to homogeneity because of loss of activity on solubilisation, complementation cloning has been used to isolate cDNA's instead. Originally, a maize cDNA clone that complements an *E. coli* 1-acylglycerol-3-phosphate acyltransferase was isolated (Brown *et al.*, 1994) and subsequently this method has been used to isolate an erucoyl-CoA specific 1-acylglycerol-3-phosphate acyltransferase cDNA from *Limnanthes douglasii* (Hanke *et al.*, 1995). Consequently, this cloned cDNA is suitable for transforming *Brassica napus* with the aim of increasing the content of erucic acid in the fatty acid composition of *Brassica napus* oil and thus, improve its industrial applicability.

1.2.3 Fatty acid synthesis in *Escherichia coli.*

Like fatty acid biosynthesis in plants, the FAS components in *Escherichia coli* (*E. coli*) are dissociable. Several of the genes encoding FAS enzymes have been isolated from *E. coli* and it was found that they lie within a cluster of fatty acid biosynthetic genes. The order within the gene cluster is: KAS III, MCAT, β-ketoacyl-ACP reductase, ACP and KAS II (Rawlings and Cronan, 1992).

It has been well documented that from an evolutionary point of view the fatty acid biosynthetic pathways of plants and prokaryotes are considered to be closely related. Recently, it has been shown that one of the enzymes is so similar that the *E. coli* enzyme can be replaced by the same enzyme from plant. The diazaborine-sensitive enoyl-ACP reductase in *E. coli* has been replaced by *Brassica napus* enoyl-ACP reductase, which demonstrated for the first time *in vivo*, that a single component of

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the plant FAS can functionally replace its counterpart within the bacterial system (Kater *et al.*, 1994).

Even though FAS components of *E. coli* and plant are very similar, the systems involved in fatty acid biosynthesis are not identical. One difference is the presence of a specific dehydrase enzyme, β-hydroxydecanoyl-ACP dehydrase, in *E. coli*. This enzyme catalyses a key reaction at the point where unsaturated fatty acid biosynthesis diverges from saturated fatty acid biosynthesis. The enzyme catalyses the usual dehydration reaction, but also isomerises *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP, which is then elongated to C16:1[*c*9] and C18:1[*c*11] (Bloch, 1970).

1.2.4 Polyketide biosynthesis.

Polyketides make up a large family of structurally varied and complex secondary metabolites produced by bacteria, fungi and plants. Many are useful in human and veterinary medicine as effective antibiotics and chemotherapeutic agents; others are important as fungal aflatoxins or as plant pigments and flavour compounds (Sherman *et al.*, 1989).

The biosynthesis of polyketides appears to be analogous to that of long-chain fatty acids, although, it is potentially much more complex. Firstly, the cycle of reduction, dehydration, reduction that follows every 2-carbon addition in fatty acid biosynthesis is omitted, or curtailed, at some or all points in the polyketide chain. Secondly, many polyketide synthases (PKSs) make a choice of starter unit and of each successive extender unit from a range of possibilities, including acetate, propionate, butyrate and occasionally more complex residues (Sherman *et al.*, 1989).

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1.3 Elongation of fatty acids.

1.3.1 Fatty acid chain elongation in plants.

All plants need to synthesise saturated very long chain fatty acids (VLCFA's) for formation of surface coverings e.g. cutin, waxes (Harwood *et al*, 1990). In addition, some species accumulate very long chain monounsaturated fatty acids in triacylglycerols. High erucic acid rape (HEAR) synthesises VLCFA's including erucic acid (C22:1), which accounts for 44 % of the total fatty acid accumulated in the seed (Hilditch and Williams, 1964).

Research into the biosynthesis of VLCFA's, such as erucic acid, is of interest because of their commercial applications, yet even though many species synthesise VLCFA's, the processes involved have not been well characterised, and they are not as fully understood as the mechanisms involved in the biosynthesis of fatty acids up to C18. Synthesis of VLCFA's has been studied in many species including, *Arabidopsis thaliana* (Kunst *et al.*, 1992), *Limnanthes alba* (meadowfoam) (Lardans and Trémolières, 1991), HEAR (Creach *et al.*, 1992, Whitfield *et al.*, 1993), *Lunaria annua* (honesty) (Whitfield *et al.*, 1993, Fehling and Mukherjee, 1991) and *Allium porrum* (leek) (Bessoule *et al.*, 1989). *Allium porrum* epidermal cells accumulate very long chain saturated fatty acids for wax biosynthesis (Cassagne and Lessire, 1978); in comparison, the other species listed accumulate very long chain unsaturated fatty acids in seeds; the seed fatty acid composition of these plants is shown in table 1.3. For comparison, the composition of low erucic acid rape is included.

Table 1.3 A summary of seed fatty acid composition of plants

Plant	% composition of total fatty acids				Reference
	C16:0	C18:1[c9]	C20:1[c11]	C22:1[c13]	
Arabidopsis thaliana	10.2	15.4	16.7	3.5	Lemieux *et al.*, 1990
Limnanthes alba (meadowfoam)	<1	<1	69 (N. B. This is C20:1[c5])	11	Lardans and Trémolières, 1991
High erucic acid rape (HEAR)	3	14	8	44	Hilditch and Williams, 1964
Low erucic acid rape (LEAR)	3.5	64.5	<1	<1	Stumpf and Pollard, 1983
Lunaria annua (honesty)	1	26	2	48	Hilditch and Williams, 1964

The reactions involved in VLCFA biosynthesis are similar to those described for the synthesis of shorter chain fatty acids, i.e. condensation of acyl-CoA and malonyl-CoA to form a β-ketone, reduction of the β-ketone to a secondary alcohol, dehydration of the alcohol to form a *trans*-2 double bond and finally reduction of the double bond to form an acyl-CoA two carbons longer than the substrate. This pathway was proved by the identification of the intermediates from *Lunaria annua* (honesty; Fehling and Mukherjee, 1991). However, unlike the soluble FAS system

that synthesises fatty acids up to C18. VLCFA substrates, intermediates and products are mainly thioesterified to Co-ASH (Lessire *et al.*, 1989) and not ACP. An exception to this was found in *Simmondsia chinenis* (Jojoba), which was able to elongate, almost equally well, very long chain acyl-CoA's and acyl-ACP's *in vitro*, but this result was highly dependent on the age of seed (Pollard *et al.*, 1979). A further difference between this elongation system and soluble FAS, is that the elongation of VLCFA's is catalysed by a series of membrane-associated enzymes (Stumpf and Pollard, 1983), and because of the difficulties involved in solubilising membrane-associated enzymes, the elongase complex has not been well characterised.

1.3.1.1 Elongation systems.

At present, it is not known whether the two rounds of elongation required for the production of erucic acid (C22:1-CoA[c13]) from C18:1-CoA[c9] are catalysed by the same enzyme system or two separate ones (Slack and Browse, 1984). It has been proposed that in *Brassica juncea* two separate elongation systems exist, one to elongate C18:1[c9]→C20:1[c11] and the other to elongate C20:1[c11]→ C22:1[c13] (Agrawal and Stumpf, 1985). Evidence for this is based on sensitivity to inhibitors and different reductant requirements since the elongation of C20:1[c11]→ C22:1[c13] is more susceptible to inhibition by sodium metabisulphite and trichloroacetate than the elongation of C18:1[c9]→C20:1[c11]; and the elongation of C18:1[c9]→C20:1[c11] can utilise NADPH or NADH, but the elongation of C20:1[c11]→C22:1[c13] can only use NADPH (Agrawal and Stumpf, 1985). However, since little research has been performed on the elongating β-ketoacyl reductase and enoyl reductase, their pyridine nucleotide specificities still have to be determined.

The exact long chain acyl-CoA substrates used by the elongating system still have to be resolved. Evidence in *Brassica juncea* suggests that C18:0, C18:1[c9] and trace amounts of C16:0 can all be used by different elongases (Agrawal and Stumpf, 1985). In *Brassica napus*, since the major VLCFA accumulated is C22:1[c13], the most likely route for C22:1[c13] synthesis is from C18:1[c9], as Δ-11 and Δ-13 desaturases have not been identified.

1.3.1.2 Acyl-CoA elongases.

One of the best studied systems involved in VLCFA biosynthesis is that in *Allium porrum* (leek) epidermal cells where two elongases have been identified. Both have been solubilised using Triton X-100 and they were separated by gel filtration. The apparent molecular masses for the C18:0 and C20:0 elongases were 350 kDa and 600 kDa, respectively (Lessire *et al.*, 1985). The C18:0 elongase was chiefly located within the endoplasmic reticulum, whereas the C20:0 elongase was found in the Golgi apparatus (Moreau *et al.*, 1988). The solubilised C18:0-CoA elongase has been partially purified and three major bands (56, 61 and 65 kDa) have been seen on SDS-PAGE using these preparations (Bessoule *et al.*, 1989); however, a function has yet to be assigned to them.

VLCFA biosynthesis has also been well studied in *Limnanthes alba* (meadowfoam), in which the seed storage triacylglycerols are composed essentially of C20 and C22 fatty acids, containing an usual Δ5 double bond (Pollard and Stumpf, 1980). Two pathways have been proposed for the synthesis of these long chain fatty acids and they are summarised in figure 1.4.

$$C2:0 \rightarrow \rightarrow \quad C16:0 \quad \rightarrow C18:0 \quad \rightarrow \quad C18:1 \ [c9]$$

$$\downarrow \qquad\qquad\qquad\quad |$$

$$C18:0 \quad \rightarrow C18:1 \ [c5] \quad |$$

$$\downarrow \qquad\qquad\qquad\quad \downarrow$$

$$C20:0 \quad \rightarrow C20:1 \ [c5] \quad C20:1 \ [c11]$$

$$\downarrow \qquad\qquad\qquad\quad \downarrow$$

$$C22:0 \quad \rightarrow C22:1 \ [c5] \quad C22:1 \ [c13] \quad \rightarrow C22:2 \ [c5,13]$$
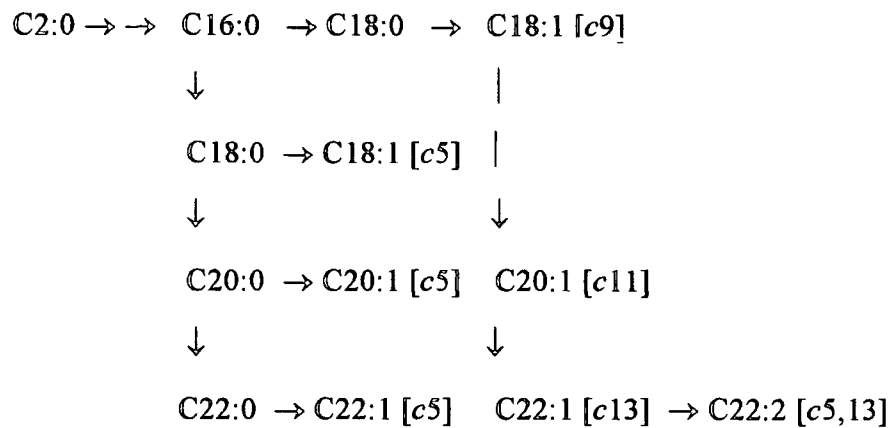
Figure 1.4 A summary of the proposed pathways for fatty acid chain elongation in *Limnanthes alba* seeds.

$\Delta 5$ desaturation occurs at the end of the biosynthetic pathway (Pollard and Stumpf, 1980). As shown in table 1.3, C20:1[c5] accounts for 69 % of the total fatty acid composition in *Limnanthes alba* mature seeds and C22:1[c13] accounts for 11 %, consequently, the major pathway is involved in the synthesis of C20:1[c5]. It has been reported that two elongation systems exist in *Limnanthes alba*, with differing substrate specificities; elongation of C18:0-CoA occurred in a 15 - 100 000 x g microsomal pellet, but elongation of C18:1-CoA[c9] occurred in a 2 - 15 000 x g pellet (Lardans and Trémolières, 1992). However, rates detected in the 2 - 15 000 x g pellet were much lower than those in the microsomal pellet, by approximately an order of magnitude. This is not unexpected considering the major elongation pathway is the production of C20:1[c5], which is presumably the product of C20:0 desaturation, as shown in figure 1.4. Therefore, considering the rates measured in the 2 - 15 000 x g pellet are much lower than in the microsomal pellet, it is difficult to conclude whether two elongation systems exist, or whether the difference in rate seen is due to substrate access to the system. In addition, during preparation of these pellets, EDTA was not present in the buffer, and it is known that EDTA is required

to prevent membranes and organelles clumping together due to the presence of divalent cations (Whitfield *et al.*, 1993). Hence, it is possible that during this experiment, some membranes and organelles aggregated, and as a result pelleted at 15 000 x g , whereas non-aggregated membranes pelleted at 100 000 x g.

In *Lunaria annua* (honesty), saturated and unsaturated acyl-CoA's are equally well elongated (Fehling and Mukherjee, 1991). C18:1-CoA[*c*9] elongation activity was detected in a 15 000 x g pellet and all components of the elongase have been solubilised using Triton X-100 (Fehling *et al.*, 1992). This 15 000 x g fraction elongates to C20:1[*c*11] and C22:1[*c*13], but after diethylaminoethyl (DEAE) chromatography, the active fraction only elongates to C20:1[*c*11], suggesting that either two elongases are present in *Lunaria annua*, or two different β-ketoacyl-CoA synthases exist (Fehling *et al.*, 1992). It has previously been determined that the soluble FAS contains multiple β-ketoacyl-ACP synthases, with different substrate specificities.

In *Brassica napus*, there is debate over the location of the C18:1-CoA[*c*9] elongase since activity has been detected in a 2 000 - 15 000 x g pellet (Creach *et al.*, 1992), oil bodies (Imai *et al.*, 1995) and a microsomal fraction (Whitfield *et al.*, 1993 and Fuhrmann *et al.*, 1994). Further studies suggest that microsomal membrane particles aggregate due to the presence of endogenous divalent cations if EDTA is not present, and as a result, pellet at a lower speed (Fuhrmann *et al.*, 1994). This may explain the presence of elongase activity in a 2 000 - 15 000 x g pellet, because EDTA was absent during the preparation of these membranes. However, since it has been shown that oil bodies containing elongase activity are not contaminated by endoplasmic reticulum (Imai *et al.*, 1995), this area requires further research. Elongase activity was solubilised from the 15 000 x g pellet using Triton X-100 (Creach and Lessire, 1993) and from oil bodies using zwittergent 3-10 (Imai *et al.*, 1995), though, it is thought that only β-ketoacyl-CoA synthase was solubilised by

zwittergent 3-10. In comparison, the solubilised C18:1-CoA[c9] elongase from the 15 000 x g pellet has been partially purified and contains three dominant bands (61, 62 and 67 kDa) when analysed by SDS-PAGE (Creach et al., 1995). This result is similar to the sizes obtained for the solubilised C18:0-CoA elongase from Allium porrum (56, 61 and 65 kDa; Bessoule et al., 1989). It has been proposed that two elongases exist in Brassica napus, elongating C18:1-CoA[c9] and C20:1-CoA[c11] respectively, and these can be separated by sucrose density gradient centrifugation (Whitfield et al., 1993). This was supported by the observation that C18:1-CoA[c9] elongation has an absolute requirement for both NADH and NADPH, whereas, the elongation from C20:1-CoA[c11] depends specifically on the presence of NADPH (Fuhrmann et al., 1994).

When studying VLCFA biosynthesis, it is important to consider the possibility of contamination due to soluble FAS enzymes that may utilise the same substrates, yet, so far, few groups have done this. A few studies have, however, been performed on the inhibition of the elongating KAS in comparison to soluble KAS's. KAS I (which elongates from C2 to C14) is inhibited 50 % by 2 μM cerulenin, whereas KAS II (which elongates from C14 and C16) is inhibited 50 % by 50 μM (Shimakata and Stumpf, 1982b). In comparison, 50 % inhibition of the elongating KAS from Lunaria annua was obtained with 120 μM cerulenin (Fehling et al., 1992), suggesting that it is different to KAS I and II. Further studies, though, showed that in Allium porrum, the elongating KAS was inhibited 46 % by 50 μM cerulenin (Schneider et al., 1993), and this result is very similar to that obtained for KAS II. Therefore, it is vitally important to determine whether the KAS activity detected in Allium porrum is due to an elongating KAS or contaminating KAS II.

As an alternative approach to studying VLCFA biosynthesis in plants, a series of mutants have been identified in Arabidopsis thaliana with deficiencies in the

elongation of C18:1 to C20:1 (James and Dooner, 1990) Fatty acid elongation1 (FAE1) was isolated by directed transposon tagging and is thought to be the elongating KAS (condensing enzyme) for the elongase since the predicted amino acid sequence shares homology with other condensing enzymes (chalcone synthase, stilbene synthases and β-ketoacyl-ACP III; James *et al.*, 1995).

1.3.2 Fatty acid chain elongation in animals.

In animals, as in higher plants, VLCFA biosynthesis occurs in microsomal fractions (Nugteren, 1965) and analysis of partial enzymatic activities has shown that the reactions involved in the biosynthesis are the same as those described for the soluble FAS system. Elongation systems have been studied in several tissues including the liver, brain, kidney and small intestine (Cinti *et al.*, 1992). However, because of the difficulty in solubilising and purifying membrane-associated enzymes, the structure of the enzymes remains unknown.

1.4 Enoyl reductase.

Enoyl reductase catalyses the reduction of *trans*-2 enoyl substrates to form a saturated acyl group for the next round of elongation. It is present in both the soluble and membrane-associated FAS systems.

1.4.1 Soluble enoyl-(acyl carrier protein) reductase in plants.

1.4.1.1 Types of enoyl-ACP reductase.

Different types of soluble enoyl-ACP reductase have been identified that differ by their substrate specificity; in castor bean seeds, enoyl-ACP reductase utilises NADPH with C4:1-ACP[*t*2] as substrate, but in *Chlorella vulgaris*, the enzyme requires NADH and can not utilise NADPH (Saito *et al.*, 1980). Two forms of

enoyl-ACP reductase have been separated by polyethylene glycol (PEG) precipitation from *Carthamus tinctorius* (safflower) seeds; enoyl-ACP reductase I was precipitated in the 5-15 % PEG fraction, utilised NADH only, and preferred C4:1-ACP[$t2$] to C10:1-ACP[$t2$] and C4:1-CoA[$t2$]. Conversely, isoform II was found in the 15 % PEG supernatant fraction, preferred NADPH and reduced C10:1-ACP[$t2$], but not C4:1-ACP[$t2$] or C4:1-CoA[$t2$] (Shimakata and Stumpf, 1982a).

In *Spinacia oleracea* (spinach) leaves, only one type of enoyl-ACP reductase can be detected, which has similar properties to the NADH-specific isoform I from *Carthamus tinctorius* seeds (Shimakata and Stumpf, 1982c). The fact that only one form of enoyl-ACP reductase is present in leaves is different to the situation with MCAT and ACP where two forms of the enzymes exist in leaves, yet in seed material, only one isoform is present.

In *Brassica napus* seeds, it is unclear how many types of enoyl-ACP reductase exist; a NADH-utilising enoyl-ACP reductase has been purified from developing seeds and NADPH-utilising activity was detected in the crude extract using C4:1-CoA[$t2$] as substrate, but this was lost during purification (Slabas *et al.*, 1986). NADPH-utilising soluble enoyl-ACP reductase has recently been detected in *Brassica napus* seeds using hexanoyl-N-acetylcysteamine as substrate, although this enzyme can not utilise acyl-CoA substrates (Dr. T. Fawcett, Department of Biological Sciences, University of Durham, personal communication).

1.4.1.2 NADH-utilising enoyl-ACP reductase.

NADH-utilising enoyl-ACP reductase has been purified from many species including *Spinacia oleracea* (spinach) leaves (Shimakata and Stumpf, 1982b), *Persea americana* (avocado) mesocarp (Caughey and Kekwick, 1982) and *Brassica napus*

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(oil seed rape) seeds (Slabas et al., 1986). In Brassica napus, enoyl-ACP reductase activity remains high even after fatty acid synthesis has ceased, which is similar to the situation with ACP activity, whereas ACCase activity is rapidly lost following attainment of the maximum level of stored fatty acid. This suggests that either enoyl-ACP reductase is a more stable enzyme than ACCase, or the mRNA is functional for longer (Slabas et al., 1986).

Initially, it was thought that Brassica napus enoyl-ACP reductase existed as a doublet, with components of 34.8 and 33.6 kDa (Slabas et al., 1986), but it was later shown, using an antibody raised against Brassica napus seed enoyl-ACP reductase, that the lower molecular weight enzyme is not present in fresh seed material and is a proteolysis product (Slabas et al., 1990). A subunit molecular weight for enoyl-ACP reductase of 32.89 kDa was determined from the amino acid sequence derived from the plant cDNA (Kater et al., 1991) and since the enzyme has a native molecular weight of 140 kDa (Slabas et al., 1986), it can be deduced that soluble Brassica napus enoyl-ACP reductase is an $\alpha 4$ homotetramer; this was later confirmed from the crystal structure (Rafferty et al., 1994).

The enzyme has an absolute specificity for NADH, the apparent K_M being 7.6 μM. The apparent K_M's for C4:1-CoA[$t2$] and C4:1-ACP[$t2$] are 178 μM and less than 1 μM, respectively (Slabas et al., 1986). Enzyme activity is inhibited by two cysteine-modifying inhibitors, 100 μM p-chloromercuribenzoate and 10 mM N-ethylmaleimide, suggesting that cysteine residues play an important role in enzymic activity (Slabas et al., 1986), and by phenylglyoxal, an arginine modifying reagent. Protection studies using coenzyme A indicate that phenylglyoxal affects acyl-CoA binding (Cottingham et al., 1989).

A cDNA for soluble enoyl-ACP reductase has been cloned from a *Brassica napus* var. rafal seed library (Kater *et al.*, 1991) and sequence analysis showed that enoyl-ACP reductase mRNA encodes a 73 amino acid leader sequence which is removed during the translocation of the protein through the plastid membrane. To prove that the cDNA isolated did encode enoyl-ACP reductase, it was expressed in *E. coli*, and by using this expression system, large amounts of recombinant enoyl-ACP reductase can easily be purified. However, during production of the construct required to express *Brassica napus* seed soluble enoyl-ACP reductase cDNA in *E. coli*, the nucleotide sequence had to be changed, for convenient subcloning, resulting in the conversion of the first amino acid of the mature protein from serine to alanine (Kater *et al.*, 1991).

Southern blot analysis, using this cDNA as a probe, showed that four genes encoding enoyl-ACP reductase exist in *Brassica napus* and two pairs are inherited from each of its ancestors, *Brassica oleracea* and *Brassica campestris* (Kater *et al.*, 1991).

Enoyl-ACP reductase was also cloned from *Arabidopsis thaliana* leaf (Fawcett *et al.*, 1992) and comparison with the *Brassica napus* seed cDNA sequence showed that there was a high degree of identity between the two coding regions; 90 % at the nucleotide level and 95 % at the amino acid level. A high degree of similarity was also apparent in the plastid-targetting transit peptide; the two transit peptides shared 66 % homology at the nucleotide level and 80 % homology at the amino acid level.

Using pure recombinant enzyme, NADH-dependent enoyl-ACP reductase from *Brassica napus* has been crystallised (Rafferty *et al.*, 1994) and the crystal structure has been determined at 1.9 Å resolution, showing that the enzyme consists of a homotetramer. Each subunit forms a single domain comprising of a 7-stranded parallel β sheet flanked by 7 α helices; a schematic representation of the enzyme structure is shown in figure 1.5. The subunits have topology highly reminiscent of a dinucleotide binding fold and there is a high degree of three dimensional similarity between enoyl-ACP reductase and 3α, 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Rafferty *et al.*, 1995).

1.4.1.5 Isoforms of enoyl-ACP reductase.

Amino acid sequence data suggest that isoforms of NADH-specific enoyl-ACP reductase exist, due to sequence heterogeneity. At position 3 of the mature protein sequence it is unknown whether the residue is serine or phenylalanine (Cottingham *et al.*, 1988) and additionally, the tryptic fragments T1-28 and T1-f29 have the same sequence except for an isoleucine to valine change, suggesting the presence of isoforms (Slabas *et al.*, 1991). The possible existence of enoyl-ACP reductase isoforms was further studied using two-dimensional electrophoresis and the results obtained showed that enoyl-ACP reductase from both *Brassica napus* seed and leaf material has four isoforms (Fawcett *et al.*, 1994). The ratio of isoforms is the same in seed and leaf material, even though the isoforms in leaf are present at a much lower level. Consequently, the greater demand for enoyl-ACP reductase activity in seed material is met by an increase in the expression of all four isoforms rather than by expression of a seed-specific gene (Fawcett *et al.*, 1994).
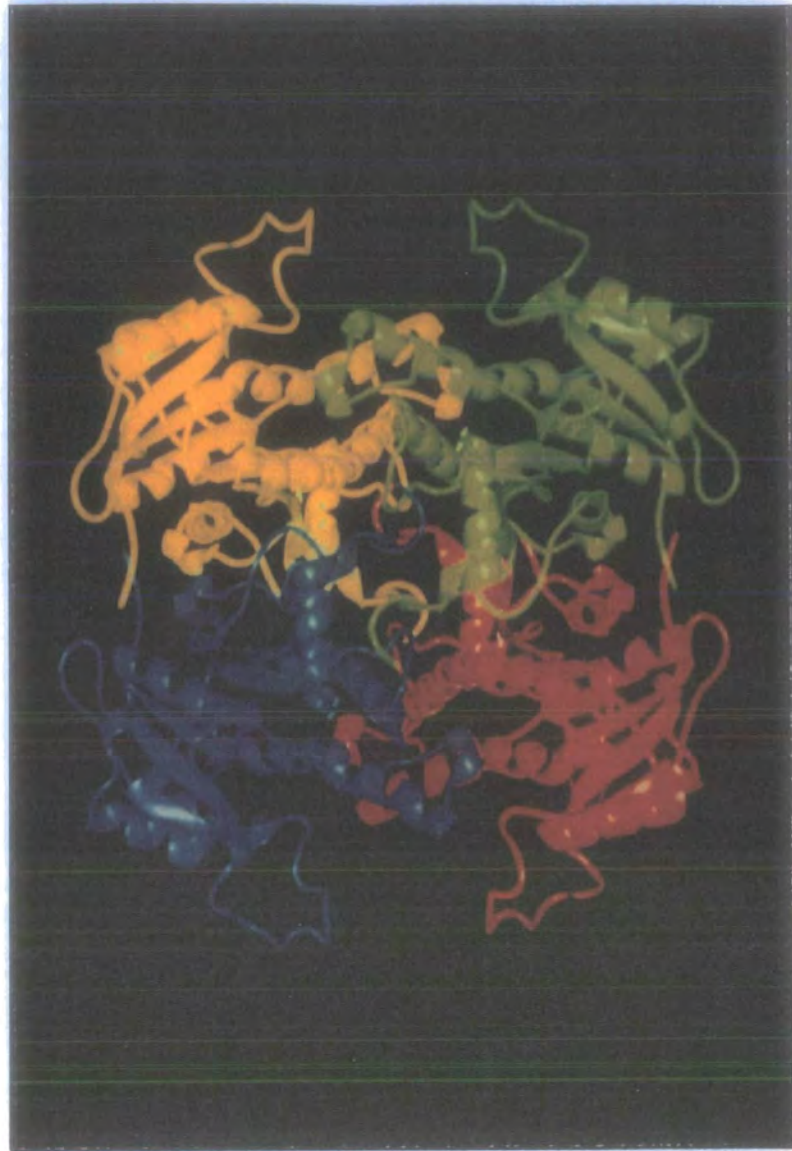
Figure 1.5 A schematic representation of the polypeptide backbone within the enoyl-ACP reductase tetramer. (From Rafferty *et al.*, 1995.)

The domain structures are defined with arrows for β strands and coils for α helices.

Two forms of enoyl reductase have been identified in *E. coli*; one form utilises NADH, while the other uses NADPH (Weeks and Wakil, 1968). The NADPH-utilising enzyme will reduce both C4:1-ACP[*t*2] and C10:1-ACP[*t*2], but will not reduce acyl-CoA's. In addition, it is unstable at pH values greater than pH 7.5. The NADH-utilising form reduces both acyl-ACP and acyl-CoA derivatives and is more active with C10:1-ACP[*t*2] than C4:1-ACP[*t*2], and in contrast with the NADPH enzyme, the NADH form is relatively stable at higher pH values. The reaction catalysed by *E. coli* is essentially irreversible and the stoichiometry of the reaction is consistent with 1 mole of reduced pyridine nucleotide being oxidised for every mole of acyl-ACP reduced (Weeks and Wakil, 1968).

Diazaborine, a synthetic antibiotic, inhibits cellular growth of *E. coli* by inhibiting the enzyme EnvM. This protein was purified from an overproducing *E. coli* strain and was shown to be a NADH-dependent enoyl-ACP reductase. As a consequence of this, the gene has been renamed *fabI*. It is thought that diazaborine inhibits this enzyme by mimicking part of the substrate molecule, since it binds in the presence of pyridine nucleotide (Bergler *et al.*, 1994). FabI is the only enoyl-ACP reductase required for fatty acid synthesis, since it is involved in the elongation of both long-chain saturated (C14:0-ACP) and unsaturated (C14:1-ACP) fatty acids. By use of a *fabI* mutant, data have ruled out the existence of the NADPH dependent enoyl-ACP reductase participating in the elongation of either long chain saturated or unsaturated fatty acids (Heath and Rock, 1995).

A novel NADPH-dependent enoyl reductase has been purified from *Streptomyces collinus* (Reynolds *et al.*, 1992) and this work reports the first isolation of an enoyl-CoA reductase involved in secondary metabolism. The enzyme catalyses the conversion of 1-cyclohexenylcarbonyl-CoA to cyclohexylcarbonyl-CoA and is thought to participate in ansatrienin A (an antibiotic) biosynthesis. The enzyme has similarities with other enoyl reductases in that it is inhibited by thiol reagents such as *N*-ethylmaleimide and *p*-chloromercuribenzoate (Reynolds *et al.*, 1992). A second enoyl-CoA reductase was later purified from *Streptomyces collinus*, that reduces C4:1-CoA[*t*2] to C4:0-CoA (Reynolds, 1993); only C4:1-CoA[*t*2] is reduced and the enzyme requires NADPH. This enzyme, a homodimer with subunits of 46 kDa, is thought to be involved in a novel fatty acid metabolism.

An NADH-specific enoyl-ACP reductase from *Mycobacterium tuberculosis* has been isolated (Dessen *et al.*, 1995); the enzyme is inhibited by isoniazid. However, if alanine is substituted for serine 94, the enzyme is resistant to the drug. Both wild type and resistant forms of the enzyme have been crystallised and analysis revealed that resistance is directly related to a change in the hydrogen bonding network that stabilises NADH binding (Dessen *et al.*, 1995).

1.4.3 Membrane-associated enoyl reductase.

Membrane-associated enoyl reductase is involved in the elongation of fatty acids by reducing a *trans* 2 double bond in the acyl chain, as shown in figure 1.6.

$+\ \text{NAD(P)H}\ +\ \text{H}^+$

C20:2-CoA[*t*2, *c*11]

C20:1-CoA[*c*11]
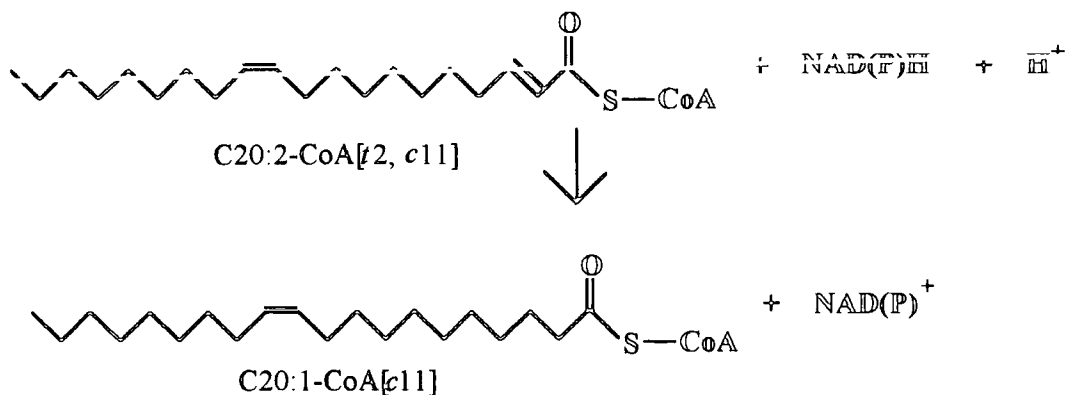
$+\ \text{NAD(P)}^+$

Figure 1.6 Membrane-associated enoyl reductase catalysed reduction.

To date, the enzyme in plants has not been well characterised, however, its activity has been detected in *Allium porrum* (leek) microsomes, where the enzyme prefers NADPH to NADH; the preferred acyl-CoA substrates are C20:1-CoA[*t*2], C18:1-CoA[*t*2] and C16:1-CoA[*t*2] (Spinner *et al.*, 1995). However, it is unknown whether the utilisation of both pyridine nucleotides is due to the presence of one or two enzymes.

Membrane-associated enoyl reductase has also been studied in animals. Research performed on rat liver microsomes discovered that two forms of membrane-associated enoyl reductase existed; one isoform was NADPH-specific and utilised short chain acyl-CoA's. The second form reduced long chain acyl-CoA's and utilised both pyridine nucleotides. Since the ratio of activities with NADPH and NADH was 100:40, the preferred reductant was NADPH (Nagi *et al.*, 1983). It was thought that the activity with NADPH was due to two enzymes since C4:1-CoA[*t*2] did not inhibit C16:1-CoA[*t*2] reduction (Prasad *et al.*, 1983). However, further research suggested that two long chain acyl-CoA specific enoyl reductase activities were present (Prasad *et al.*, 1985). The two forms, separated by ion exchange chromatography, differed in cofactor requirements; one form was NADPH-specific, but the other utilised either pyridine nucleotide. The NADPH form was purified to

one band on SDS PAGE (51 kDa) and catalysed the reduction of *trans-2-enoyl* CoA's from C4 to C16, yet it is unknown whether this enzyme contained two independent active sites for long and short chains, or whether two enzymes with the same molecular weight are present (Prasad *et al.*, 1985). Therefore, the number of enoyl reductase isoforms present in liver microsomes requires further clarification and a role needs to be allocated for the isoforms.

1.5 Membrane-associated proteins.

Since one of the aims of this research was to characterise membrane-associated enoyl reductase, a knowledge of membrane proteins was required. Two types of membrane proteins exist in cells; peripheral membrane proteins, e.g. cytochrome c and succinic dehydrogenase, which are associated with the membrane surface through electrostatic interactions, and integral membrane proteins. e.g. rhodopsin and Ca^{2+}-ATPase. Several methods exist for the solubilisation of peripheral membrane proteins, including use of chelating agents, salts, variations in pH and chaotropic ions. Chelating agents, such as EDTA, cause destabilisation of membranes by complexing Mg^{2+} and Ca^{2+}, and salts, such as KCl or NaCl, decrease electrostatic interactions between proteins and charged lipids (van Renswoude and Kempf, 1984). Acidic (pH 3 - 5) and basic (pH 8 - 12) buffers, also interrupt electrostatic interactions (Thomas and McNamee, 1990). Chaotropic ions, e.g. I^{-}, Br^{-}, SCN^{-} disorder the structure of water, causing the disruption of hydrophobic bonds near the surface of the membrane structures, resulting in the transfer of hydrophobic groups from an apolar environment to an aqueous phase (van Renswoude and Kempf, 1984).

Methods used to solubilise integral membrane proteins include detergent solubilisation. Ionic detergents are generally very effective at solubilising integral

membrane proteins and because they usually have high critical micelle concentrations (cmc's), can be removed by dialysis (Thomas and McNamee, 1990). There are three groups of ionic detergents; cationic e.g. cetyltrimethylammonium bromide (Neugebauer, 1994), zwitterionic e.g. CHAPS and Zwittergent (Thomas and McNamee, 1990) and anionic e.g. SDS, cholate and deoxycholate (Neugebauer, 1994). Care has to be taken when using some detergents, though, e.g. SDS is very denaturing and is normally only used when recovery of biological activity is not important; cholate and deoxycholate cannot be present during ion-exchange chromatography due to their negative charges (Thomas and McNamee, 1990); neither can cholate be used with enzymes that require divalent cations for biological function because the carboxylic acid polar head group of cholate forms insoluble complexes with divalent metals (Hjelmeland and Chrambach, 1984). Nonionic detergents are less effective at dissociating protein complexes, but many proteins are more stable in nonionic detergents than ionic ones, however, they have low cmc's and as a result are difficult to remove (Thomas and McNamee, 1990). Examples of nonionic detergents include octyl glucoside and Triton X-100 (Thomas and McNamee, 1990). Again, care has to be taken when using these detergents since Triton X-100, for example, cannot be used if absorbance at 280 nm is an important parameter to monitor the enzyme being solubilised as Triton X-100 also absorbs at 280 nm (Hjelmeland and Chrambach, 1984).

When solubilising membrane proteins with detergents, there are a number of important factors that have to be considered, including the type and amount of detergent used, the protein to detergent ratio and whether salts need to be added (Hjelmeland and Chrambach, 1984).

Other methods that can be used to solubilise membrane proteins include use of phospholipase A and lipase to digest membrane lipids, and extraction of proteins into

organic solvents (Penefsky and Tzagoloff, 1971). Most membrane proteins are denatured in organic solvents, though, and only a small percentage are soluble in an organic phase (Thomas and McNamee, 1990). Yet, extraction into acetone does solubilise some membrane proteins e.g. diglyceride acyltransferase (Akao and Kusaka, 1976).

Proof of solubilisation of membrane proteins is usually the retention of biological function in a soluble phase after centrifugation at 105 000 x g for 1 hour, however, care has to be taken as this will depend on the density of the medium. Further studies could involve the separation of solubilised proteins from membranes by gel filtration since the void volume will contain aqueous insoluble membranes, whereas the included volume of a gel filtration column will contain solubilised proteins (Hjelmeland and Chrambach, 1984).

1.6 The aims of this research.

Since various fatty acids of storage lipids are industrially important, there is enormous potential of genetically modified transgenic plants. However, to obtain industrially important plant oils by genetic engineering, a fundamental knowledge of the general biochemical factors governing fatty acid chain elongation, desaturation and esterification to glycerol backbones is required. At present, there is much interest in the biosynthesis of erucic acid, $C22:1[c13]$, because it is an excellent lubricating oil at high temperature. In HEAR (high erucic acid rape) erucic acid accounts for 44 % of the total fatty acid composition; to increase this, detailed knowledge of the elongation of fatty acids to erucic acid is required, since at present, the processes limiting elongation have not been identified.

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The aim of this research was to study and characterise membrane-associated enoyl reductase, part of the system involved in the elongation of VLCFA's in *Brassica napus* seeds. To be certain that activity measured in microsomes is not due to contaminating soluble enoyl-ACP reductase, a component of soluble FAS which synthesises fatty acids up to C18, membrane-associated enoyl reductase needs to be compared to the soluble enzyme. Soluble enoyl-ACP reductase has previously been well characterised and Southern blot analysis has shown that in *Brassica napus*, four genes encoding enoyl-ACP reductase exist, however, only one cDNA has so far been isolated (Kater *et al.*, 1991). The isolation of the other cDNA clones is necessary to determine the expression of the individual genes. It might also be possible to use a probe designed from soluble enoyl-ACP reductase to isolate a clone for membrane-associated enoyl reductase; this clone would be expected to contain a membrane-binding domain.

As an alternative approach to studying membrane-associated enoyl reductase direct enzymatic detection is possible. This would require a rapid, reproducible optical assay to be designed and such as assay could be based on the one used to measure soluble enoyl-ACP reductase. This would require the synthesis of a suitable very long chain acyl-CoA substrate, since the major product of the soluble FAS is C18:1-CoA[c9], and so C20:2-CoA[t2, c11] would be the desired substrate for the first enoyl reductase reaction in elongation. This acyl-CoA is not available commercially, and therefore, its synthesis is critical for direct assays of the elongase enoyl reductase.

It is not known if soluble enoyl-ACP reductase can reduce very long chain fatty acyl-groups, such as C20:2-CoA[t2, c11] and this will need to be determined, since the utilisation of this substrate by soluble enoyl-ACP reductase could lead to misinterpretation of membrane-associated enoyl reductase assays, if soluble enoyl-ACP reductase contamination of the membrane-associated enzyme extract occurred.

The lack of availability of a suitable substrate has not allowed this to be investigated to date.

Development and optimisation of an assay for membrane-associated enoyl reductase is necessary to study the pyridine nucleotide specificity of the enzyme, the effect pH has on activity and it will allow the K_M of the substrates to be calculated, in order to characterise the enzyme. To be certain that soluble enoyl-ACP reductase and membrane-associated enoyl reductase are distinct enzymes, detailed knowledge of both enzymes is required to compare the properties of the two. No such comparison of these enoyl reductases has previously been reported in the literature.

Before attempts are made to purify membrane-associated enoyl reductase, the enzyme will need to be solubilised, but as it is not known how the enzyme is associated with membranes, a range of methods are needed for solubilisation attempts.

As an alternative approach to studying membrane-associated enoyl reductase, the enzyme could be detected immunologically, if antibodies raised against soluble enoyl-ACP reductase cross-reacted with the membrane-associated form. Cross-reactivity would indicate that the two enzymes share regions of structural homology and immobilisation of the antibody to agarose beads would allow cross-reacting membrane-associated enoyl reductase to be purified by affinity chromatography.

As similar studies are performed on the other components of the elongase, detailed knowledge on the elongation system will be gained, which is necessary before plants can be genetically modified to increase the amount of industrially important VLCFA's synthesised.

2.1 Materials.

Bacto-tryptone, bacto-yeast and agar were obtained from DIFCO laboratories, PO box 14B, Central Avenue, West Molesey, Surrey, KT8 2SE.

Ampicillin and tetracycline were obtained from NBL Gene Sciences Ltd., South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL.

C20:1-CoA[c11], C4:1-CoA[t2], acyl-CoA oxidase, horseradish peroxidase, Proteinase K, NADH and NADPH were obtained from Sigma Chemical Co., Fancy Road, Poole, Dorset, BH17 7NH.

IPTG and DTT were obtained from Melford Laboratories Ltd., Chelsworth, Ipswich, Suffolk, IP7 7LE.

Acrylamide, bis acrylamide, SDS and TEMED were from Bio-Rad Laboratories LTD., Bio-Rad, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD.

Guanidinium thiocyanate was obtained from BDH, Merck Ltd., Merck House, Poole, BH15 1TD.

Restriction endonucleases were from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG.

[α-^{32}P] dCTP, ^{125}I-labelled donkey anti-rabbit IgG antibody and ^{35}S-methionine (1000 Ci/mmol) were purchased from Amersham International Plc., Little Chalfont, Amersham, Buckinghamshire, HP7 9NA.

Fuji X-Ray film was obtained from Fuji Photo Film [UK] Ltd., Fuji Film House, 125 Finchley Road, Swiss Cottage, London, NW3 6JH. Developer and fixer were obtained from H.A. West [X-Ray] Ltd., 41 Watson Crescent, Edinburgh, EH11 1ES.

Ultrafree®-MC filters were purchased from Millipore (UK) Limited, The Boulevard, Blackmoor Lane, Watford, Hertfordshire, WD1 8YW.

Smart chromatography media (Mono Q and Superose 12) were obtained from Pharmacia, Milton Keynes, Buckinghamshire, and used on a Pharmacia Smart chromatography system.

All other chemicals used were of molecular biology standard or the highest purity available and were obtained from either Sigma or BDH.

2.1.1 Plasmids and recombinant protein.

Recombinant pBluescript containing the cDNA for *Brassica napus* soluble enoyl-ACP reductase (λEAR7) isolated from a seed library was a gift from Dr. M. M. Kater, Vrije Universiteit, Amsterdam.

Biologically active soluble enoyl-ACP reductase was over-expressed in *E. coli* strain BL21, transformed with pEAR2, a plasmid containing a *Brassica napus* enoyl-ACP reductase cDNA (Kater *et al.*, 1991). Purified recombinant soluble enoyl-ACP reductase was a gift from Mr. J. W. Simon and Miss S. Bithell, Department of Biological Sciences, University of Durham.

2.2 Seed material.

Two varieties of *Brassica napus* (oil seed rape) seeds, Miranda, a high erucic acid rape variety, and Falcon, a low erucic acid rape variety, were collected from plants grown both in the field and in greenhouses. Seeds were removed from freshly harvested pods and frozen in liquid nitrogen, before being stored at -80°C.

2.3 Bacterial growth media and conditions.

The bacterial growth media Luria broth (LB) and 2 x YT were used throughout this research.

LB (Luria broth) consists of 10 g Bacto-tryptone, 5 g Bacto-yeast, 10 g sodium chloride per litre, (pH 7.5)

2 x YT consists of 16 g Bacto-tryptone, 10 g Bacto-yeast, 5 g sodium chloride per litre, (pH 7.0)

Liquid cultures were incubated on an orbital shaker at 200 r.p.m. at 37°C. Five ml cultures were grown in 15 ml culture tubes and 50 ml cultures were grown in 250 ml conical flasks.

Liquid media was solidified where appropriate by the addition of 1.5 % w/v agar or 0.7 % w/v agarose.

Antibiotics were added as required to LB media after autoclaving to final concentrations of 50 μg/ml for ampicillin and 10 μg/ml for tetracycline. Ampicillin was stored as a 50 mg/ml stock in Milli Q water at -20°C, after being filter sterilised through a 0.22 μm filter. Tetracycline was stored as a 12.5 mg/ml stock in 50 % v/v ethanol at -20°C, in a foil covered bottle. Both antibiotics were destroyed by autoclaving, therefore, were added to media after it had been autoclaved and cooled.

2.4 Genetic procedures.

The genetic procedures were based on Sambrook *et al.*, 1989, unless otherwise stated.

2.4.1 Preparation of plasmid DNA.

Plasmid DNA was prepared by the alkaline lysis method. Large scale and plasmid minipreps were performed, using 50 ml and 5 ml cultures, respectively.

An overnight culture in LB media containing 50 μg/ml ampicillin was centrifuged to harvest the cells (5 000 x g for 15 minutes for a 50 ml culture, 10 000 x g for 2 minutes for a 5 ml culture). The pellet was resuspended in 5 ml or 100 μl respectively, of ice-cold solution 1 (25 mM Tris/HCl, 50 mM glucose, 10 mM EDTA [pH 8.0]) and incubated at room temperature for 5 minutes. Ten ml or 200 μl respectively, of freshly prepared solution 2, (0.2 M sodium hydroxide, 1 % w/v SDS) was added to lyse cells and release DNA. The contents of the tube were mixed by gentle inversion, so that genomic DNA was not sheared, before being placed on ice for 5 minutes. Subsequently, 7.5 ml or 150 μl respectively, of ice-cold solution 3 was added and the tube vortexed briefly. (Solution 3 consisted of 11.5 ml glacial acetic acid and 28.5 ml of distilled water added to 60 ml 5 M potassium acetate [pH 4.8] with no pH adjustment, the final solution being 3 M with respect to potassium and 5 M with respect to acetate). This caused the precipitation of chromosomal DNA, but not plasmid DNA. After a further 5 minutes on ice, the tube was centrifuged at 5 000 x g for 20 minutes, or 10 000 x g for 10 minutes, respectively. The supernatant was removed to a fresh tube and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) to remove protein. The tube was vortexed and then centrifuged at 10 000 x g for 5 minutes. Two volumes of 96 % v/v ethanol (room temperature) were added to the aqueous phase to precipitate the plasmid DNA. After 5 minutes at room temperature, the DNA was recovered by centrifugation at 10 000 x g for 10 minutes, washed with 200 μl 70 % v/v ethanol (-20°C) and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA [pH 7.5]) containing 50 μg/ml DNAse-free pancreatic RNAse.

DNA was electrophoresed through a 0.7 % w/v agarose gel to check quality and quantity (section 2.4.3), by comparison to known standards.

Restriction digests, using EcoRI and Hinf I, were carried out according to the manufacturer's recommendations. Plasmid DNA was digested in a volume of 50 μl with 20 U of the required restriction enzyme and 0.1 volume of the appropriate 10 x concentrated enzyme buffer. The reaction was incubated at 37°C for 3 hours. An aliquot of digested DNA was analysed by gel electrophoresis following the addition of 1 μl of 10 x loading buffer.

2.4.3 Separation of DNA fragments by agarose gel electrophoresis.

Gel electrophoresis was performed in Scotlab electrophoresis tanks. Gels were made with the appropriate concentration of agarose (dependent on the size of DNA to be separated, with 0.7 % w/v routinely being used) in TAE buffer (40 mM Tris/acetate, 1 mM EDTA [pH 8.0]). The agarose mixture was dissolved by heating in a microwave for approximately 2 minutes. Ethidium bromide (0.5 μg/ml) was added and the solution cooled before being poured into a gel mould (15 x 11 cm for maxigels, 6 x 5 cm for minigels) and allowed to set.

DNA samples were prepared by the addition of 0.1 volume of 10 x loading buffer (0.2 % w/v bromophenol blue, 50 % w/v sucrose, 100 mM EDTA [pH 8.0]). Gels were run in TAE buffer containing 0.5 μg/ml ethidium bromide at 100 V until the bromophenol blue dye reached the end of the gel. Permanent records of gels were made by photographing the fluorescence of DNA-ethidium bromide complexes under U. V. light. Lambda DNA digested with the restriction enzyme Hind III and φx 174 'phage DNA digested with Hae III were used to estimate the size of unknown fragments.

DNA was digested and electrophoresed through a 2 % w/v agarose gel as described previously. DNA was visualised with long wave U.V. light and the appropriate band cut out with a sterile scalpel blade and sliced into small pieces. Electroelution was performed using a Schleicher and Schuell Biotrap, following the manufacturer's instructions (Anderman and Company Ltd., Laboratory Supplies Division, 145 London Road, Kingston-Upon-Thames, Surrey, KT2 6NH).

The apparatus was assembled as shown in figure 2.1. Gloves were worn during the assembly of the apparatus and membranes were mounted using tweezers. The BT1 membrane retained all charged macromolecules with a molecular weight larger than 5000 Da, whereas buffer ions passed freely. The BT2 membrane functioned as a prefilter for the trap. Pieces of gel containing the DNA to be electroeluted were placed in the chambers between the BT2 membranes. TAE buffer (200 µl) was added to the trapchamber and enough TAE was added to the remainder of the apparatus to cover the gel slices. The Biotrap was placed in a horizontal electrophoresis tank containing enough TAE to half cover the Biotrap. Electrophoresis was performed at 100 V for 1 hour, then to remove DNA from the BT1 membrane, the voltage was reversed for 1 minute. The DNA solution was recovered from the trapchamber with a Pasteur pipette, care being taken not to perforate the BT2 membrane.

DNA was precipitated in 0.15 M sodium acetate (pH 4.8), 50 % v/v isopropanol (-20°C) and oyster glycogen (20 µg/ml) at -20°C for 1-2 hours. After centrifugation for 15 minutes at 10 000 x g, the pellet obtained was washed with 70 % v/v (-20°C) ethanol and resuspended in TE buffer. An aliquot was electrophoresed through a 0.7 % w/v agarose gel to check recovery.

A and G - BT1 membranes

B to F - BT2 membranes

1 - location for gel containing DNA to be eluted

2 - trapchamber

Figure 2.1 A schematic diagram of a Scleicher and Schuell Biotrap, used for the electroelution of DNA fragments.

DNA restriction fragments for use in screening libraries or Southern blot analysis were labelled with [α-^{32}P] dCTP using a random nonanucleotide primer system (Amersham International Plc.), following the manufacturer's recommendations. The reaction mixture contained 300 ng DNA to be labelled (in a total volume of 23 μl), which was denatured by boiling for 5 minutes in the presence of 5 μl primer solution (containing random nonamer primers). To this, 10 μl of labelling buffer, 100 μCi [α-^{32}P] dCTP and 2 μl of the Klenow fragment of DNA polymerase I were added and the reaction mixture incubated at room temperature for 90 minutes. The reaction was stopped by the addition of stop mix which contains 20 mM EDTA [pH 8.0], 2 mg/ml blue dextran, 0.02 mg/ml xylene cyanol. EDTA inhibits DNA polymerase I by chelating Mg^{2+}, whereas blue dextran and xylene cyanol are dyes used to locate DNA on a Spin column. Unincorporated nucleotides were removed using a Bio-Rad Bio-Spin Chromatography column containing Bio-Gel P-6 polyacrylamide gel, which has an exclusion limit of approximately 5 base pairs. The column was centrifuged at 1 100 x g for 2 minutes, to remove storage buffer. The reaction mixture, containing radiolabelled DNA fragment, was carefully applied to the centre of the top of the column, then the column was placed in a collection tube and centrifuged for 4 minutes at 1 100 x g. The purified sample was recovered from the collection tube and the specific activity (dpm/μg DNA) was calculated after counting 1 μl of the probe solution in a scintillation counter.

2.4.6 Screening a *Brassica napus* leaf cDNA library.

In order to clone cDNA's for enoyl-ACP reductase, a λZAP II library made from *Brassica napus* leaf mRNA was screened with a radiolabelled probe from a

previously isolated enoyl-ACP reductase cDNA. Prior to screening the library, it was titred using the method described in section 2.4.6.1. 2×10^5 pfu were screened and once plaques had formed on the bacterial lawn, the bacteriophage DNA was transferred to a nitrocellulose membrane (section 2.4.6.3) for hybridisation with the labelled probe (section 2.4.7). Positive plaques were removed from the plate and used in a second round of screening. This procedure was continued until all plaques were positive for the probe.

2.4.6.1 Titration of a recombinant cDNA library.

Tenfold serial dilutions of bacteriophage stocks in SM (0.1 M sodium chloride, 8 mM magnesium sulphate, 50 mM Tris/HCl [pH 7.5], 0.01 % w/v gelatin) were prepared and 100 μl of each dilution was added to 100 μl of plating bacteria (prepared as described below) and incubated for 20 minutes at 37°C to allow the bacteriophage particles to adsorb to the bacteria. The mixture was then added to 2.5 ml of molten top agarose (LB media containing 0.7 % w/v agarose) at approximately 50°C, gently mixed and immediately poured on top of a pre-warmed plate (9 cm diameter) of LB containing 10 μg/ml tetracycline and 1.5 % w/v agar. Top agarose was used rather than top agar, as agar tends to lift off with the nitrocellulose filter during plaque lifts (Ausubel et al., 1992). Once the top agarose had set, the plates were inverted and incubated at 37°C overnight. From the number of plaques that appeared, the amount of plaque forming units (pfu)/ml was calculated.

2.4.6.2 Preparation of plating bacteria.

XL1-Blue E. coli was grown in a 50 ml LB culture containing 0.2 % w/v maltose and 10 μg/ml tetracycline at 37°C for 4 hours. Maltose was included in the culture to

induce the *lamB* gene which encodes for outer membrane receptors; bacteriophage λ adsorbs to these receptors that normally function to transport maltose into the cell. Tetracycline was present because it causes expression of the F' pili, which permits M13 superinfection; this is required for the excision of the Bluescript plasmid from bacteriophage λ. Cells were harvested by centrifugation at 4 000 x g for 10 minutes, resuspended in 5 ml of 10 mM magnesium sulphate, to facilitate the adsorption of 'phage λ and were stored at 4°C for up to 1 week.

2.4.6.3 Immobilisation of bacteriophage λ plaques on nitrocellulose filters.

In order to screen bacteriophage plaques by hybridisation with a ^{32}P-labelled DNA probe, bacteriophage DNA was transferred to a Hybond C nitrocellulose (Amersham International Plc.) filter by capillary action. Prior to transfer, the plate was chilled at 4°C for 1 hour to allow the top agarose to harden. A nitrocellulose filter was placed on the plate for 1 minute and allowed to wet from beneath. The filter and plate were marked in three asymmetric positions, in order to orientate the filter to the plate, by stabbing a needle through the filter into the LB agar. Using sterile tweezers, the filter, with DNA uppermost was transferred to 3MM paper saturated with denaturing solution (1.5 M sodium chloride, 0.5 M sodium hydroxide) for 5 minutes to denature DNA. This was followed by 5 minutes incubation on 3MM paper soaked in neutralising solution (1.5 M sodium chloride, 0.5 M Tris/HCl [pH 7.4]) and finally, on 3MM paper soaked in 2 x SSC buffer (0.3 M sodium chloride, 30 mM sodium citrate [pH 7.0]) for 5 minutes. The filter was then air dried on 3MM for 10 minutes and baked at 80°C in a vacuum oven for 20 minutes to fix the DNA to the filter.

2.4.7 Hybridisation of radiolabelled probes to nucleic acids.

After baking, membranes were pre-hybridised in a solution of 6 x SSC, 1 x Denhardts solution, 0.5 % w/v SDS, 0.05 % w/v sodium pyrophosphate and 0.05 mg/ml herring sperm DNA at 65°C for 2 hours. (20 x SSC consisted of 3 M sodium chloride, 300 mM sodium citrate [pH 7.0]; 50 x Denhardts solution consisted of 0.1 % w/v ficoll, 0.1 % w/v polyvinylpyrrolidine, 0.1 % w/v BSA and herring sperm DNA was prepared as in Sambrook *et al.* 1989.)

After pre-hybridisation, the solution was removed and replaced by 10 ml of a hybridisation solution containing 6 x SSC, 1 x Denhardts solution, 0.5 % w/v SDS, 0.05 % w/v sodium pyrophosphate, 1 mM EDTA (pH 8.0) and boiled radiolabelled probe (approximately 1×10^6 dpm and 4 ng DNA were added per ml of hybridisation solution). Prior to addition, the probe was boiled to denature the DNA and then rapidly cooled so that it would hybridise to ssDNA on the membranes. Hybridisation was performed at 65°C overnight after which the hybridisation solution was removed and the membrane washed to remove non-specific binding.

The washing procedure employed was: two washes in 2 x SSC containing 0.1% w/v SDS for 2 minutes at room temperature to remove excess probe, followed by two washes in 1 x SSC containing 0.1 % w/v SDS for 30 minutes at 65°C. The membrane was blotted to remove excess washing solution, wrapped in Saran Wrap and exposed to Fuji X-ray film for varying lengths of time. Film sheets were pre-flashed once to increase sensitivity (Laskey and Mills, 1975) and film cassettes with intensifying screens were used. Exposure was carried out at -80°C to reduce the effects of background radiation. Exposed films were developed with Ilford Phenisol developer for 2 minutes and fixed with Kodak Unifix fixer for a further 2 minutes.

2.4.8 Excision of the Bluescript plasmid from bacteriophage λ.

Throughout this procedure, it is important to grow bacteria in tetracycline to maintain expression of the F' pili. An overnight XL1-Blue culture (200 μl) was used to inoculate 5 ml LB containing 10 μg/ml tetracycline and 0.2 % w/v maltose, and the culture was incubated at 37°C for 2 hours until the cells entered the log phase.

An aliquot of this culture (200 μl) was added to 200 μl recombinant bacteriophage (1 x 10^5 - 1 x 10^6 pfu/ml) and 1 μl of helper 'phage R408 (from Stratagene). After incubation at 37°C for 15 minutes, 3 ml of 2 x YT media was added, and the culture grown at 37°C for 3 hours. The culture was then heated at 70°C for 20 minutes to kill the bacteria but not destroy the M13 'phage, and centrifuged at 4 000 x g for 5 minutes. The supernatant, which contained single stranded phagemids, was recovered.

Phagemids (20 μl) were added to 200 μl of XL1-Blue (from the 5 ml culture) and after incubation at 37°C for 10 minutes, the culture was plated out on LB plates containing 10 μg/ml tetracycline and 50 μg/ml ampicillin, and incubated at 42°C overnight. This higher temperature kills the helper 'phage as it is temperature sensitive. Tetracycline and ampicillin were included in the media because XL1-Blue carries a tetracycline resistance gene and pBluescript carries an ampicillin resistance gene. Colonies that grew were used to set up cultures so that DNA plasmid preparations could be undertaken (section 2.4.1).

2.4.9 Transfer of DNA to nitrocellulose for Southern analysis.

DNA was transferred from agarose gels to Hybond C nitrocellulose (Amersham International Plc.) for hybridisation studies, by capillary action, essentially as

described by Southern (1975). The gel containing DNA to be blotted was photographed next to a ruler, then the DNA was denatured by soaking the gel in 1.5 M sodium chloride, 0.5 M sodium hydroxide for 1 hour. The gel was washed twice in neutralising solution (1.5 M sodium chloride, 0.5 M Tris/HCl [pH 7.4]) for 15 minutes and finally rinsed in 2 x SSC buffer (0.3 M sodium chloride, 30 mM sodium citrate [pH 7.0]). The blot was constructed as follows:- the gel with wells face down was placed on filter paper positioned on a glass plate so that its ends were dipped in a reservoir of 20 x SSC (3 M sodium chloride, 300 mM sodium citrate [pH 7.0]). A nitrocellulose membrane, cut to the same size of the gel, was soaked in 2 x SSC and placed on top of the gel and air bubbles removed. The nitrocellulose was covered with two pieces of filter paper and the whole blot covered with Saran wrap. The Saran wrap directly over the gel was cut and folded back. This was to ensure that the only buffer route was via the gel. Two layers of disposable nappies were put on top of the blot, and covered with a glass plate with a weight of approximately 500 g on top; the blot was left at room temperature overnight.

After blotting, the positions of the gel wells were marked on the nitrocellulose, and then the nitrocellulose was baked at 80°C for 30 minutes in a vacuum oven to fix the DNA to the nitrocellulose. Hybridisation was undertaken as described in section 2.4.7.

2.4.10 Polyethylene glycol precipitation of plasmid DNA.

To prepare DNA for sequencing, it was precipitated by polyethylene glycol (PEG), using the following procedure. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the plasmid DNA solution (prepared as described in section 2.4.1) and the mixture vortexed, then centrifuged in a microfuge for 5 minutes. The aqueous phase was removed and to it two volumes of ethanol and 1/10

volume of 3 M sodium acetate (pH 4.8) were added and the DNA precipitated after incubation at room temperature for 5 minutes. Following centrifugation for 5 minutes, the pellet was washed in 70 % v/v ethanol and the air-dried pellet was resuspended in 6 µl Milli Q water. Eight µl of 2 M sodium chloride was added and after mixing, 20 µl of 13 % w/v PEG 8000 was added. After shaking, the mixture was incubated on ice water for 2 hours. DNA was recovered by centrifugation at 10 000 x g for 5 minutes, and washed three times with 70 % v/v ethanol. The final DNA pellet was resuspended in 10 µl TE. An aliquot of the DNA was electrophoresed through a 0.7 % w/v agarose gel to estimate the concentration of DNA by comparison to known standards (section 2.4.3).

2.4.11 DNA sequencing.

PEG precipitated DNA was sequenced using an Applied Biosystems (ABI) 373 DNA sequencer, which uses the Sanger method of dideoxy-mediated chain termination. Automated sequencing was performed by Ms. J. Bartley, Department of Biological Sciences, University of Durham. For each sequencing run, 1.5 µg DNA in 6 µl water or TE was required. To initially characterise cDNA clones, they were sequenced at the 5' and 3' ends, using -21M13 (forward) and M13 (reverse) primers; the position of these primer sites in the Bluescript vector is shown in figure 2.2. The primers used to fully sequence one of the clones are described in the main text.

Oligonucleotide primers for DNA sequencing were synthesised on an Applied Biosystems ([ABI], Warrington) 381A DNA Synthesiser by Mr. J. Gilroy, Department of Biological Sciences, University of Durham, using the standard protocol.
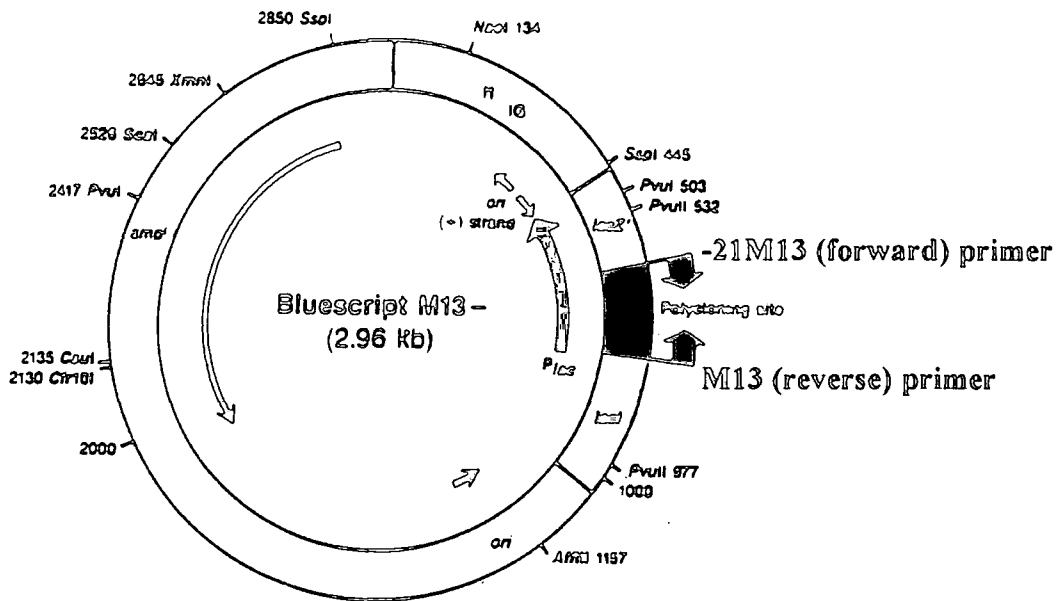
-21M13 (forward) primer

       3' TGA CCG GCA GCA AAA TGT 5'

M13 (reverse) primer

       5' CAG GAA ACA GCT ATG ACC ATG 3'

Figure 2.2 pBluescript and -21M13 (forward) and M13 (reverse) primer sites.

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2.4.12 Calcium chloride transformation of bacteria.

To prepare competent cells, 500 μl of a 5 ml overnight *E. coli* BL21 culture was used to inoculate a 50 ml culture. The culture was incubated at 37°C until the concentration of cells was 5×10^7 per ml, which corresponded to an absorbance at 550 nm of 0.2, and usually took 2-4 hours. The culture was then incubated on ice for 10 minutes and the cells harvested by centrifugation at 4 000 x g for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in half the original volume of sterile, ice-cold 100 mM $CaCl_2$ (pH 8.0). After a further incubation on ice for 15 minutes, the cells were centrifuged again at 4 000 x g for 5 minutes at 4°C. The pellet obtained was resuspended in 1/15th of the original volume of ice-cold 100 mM $CaCl_2$ (pH 8.0). Competent cells were stored on ice for 1-2 hours before being transferred for long term storage as 200 μl aliquots in 20 % v/v glycerol at -80°C.

Before transformation, a 200 μl aliquot of competent cells was made to 500 μl with 100 mM $CaCl_2$ (pH 8.0) and 100 μl used per transformation. The DNA to be transformed (pEAR2), approximately 20 ng, was added to the competent cells and the mixture incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 2 minutes and placed back on ice for a further 2 minutes. Pre-warmed LB (1 ml) was added and the cells incubated at 37°C for 1 hour to allow antibiotic resistance to be expressed. The cells were then plated on LB agar plates containing 50 μg/ml ampicillin. Colonies were allowed to grow overnight, before they were picked off and stored at -80°C in 100 μl LB containing 15 % v/v glycerol. An aliquot was used to inoculate a 5 ml culture. The method was based on Mandel and Higa, (1970).

2.5 Biochemical Methods.

2.5.1 Enzyme Assays.

2.5.1.1 Detection of the biological activity of catalase.

The biological activity of catalase was assayed by monitoring the production of O_2

from H_2O_2 at 25°C. Catalase was added to a reaction chamber in an oxygen

electrode containing Milli Q water and a blank reading was taken. As small aliquots

of H_2O_2 (8 μmoles) were added, using a microsyringe, evolution of O_2 was

monitored, confirming the activity of catalase. A unit of catalase will decompose

1 μmole of H_2O_2 per minute at pH 7.0 at 25°C.

2.5.1.2 Soluble enoyl-ACP reductase assay.

Soluble enoyl-ACP reductase was assayed by following the substrate specific

decrease in absorbance at 340 nm due to the oxidation of NADH, based on the

method of Slabas et al., (1986). The standard reaction mixture contained 10 mM

sodium phosphate (pH 6.2), 140 μM NADH, 80 μM acyl-CoA (either C4:1-CoA[t2]

or C20:2-CoA[t2, c11]) and an appropriate amount of enzyme in a total volume of

125 μl. Endogenous NADH oxidation was measured in the absence of acyl-CoA and

the reaction was then initiated by its addition. Any alteration to this basic reaction

mixture is explained in the main text. All assays were carried out at 25°C.

An enzyme unit is defined as the amount of enzyme that oxidises 1 μmol NADH per

minute at pH 6.2 at 25°C. The absorption coefficient of NADH at 340 nm was taken

to be 6.22×10^3 M^{-1} cm^{-1}.

All optically based assays were monitored in either a Pharmacia Ultrospec®III
U.V./visible spectrophotometer or a Varian DMS 90 U.V./visible
spectrophotometer.

2.5.1.3 Membrane-associated NADH and NADPH enoyl reductase assays.

The assay for membrane-associated enoyl reductase was a modification of the
soluble enoyl-ACP reductase assay.

The standard reaction mixture contained 80 mM MOPS/NaOH (pH 6.0), 140 µM
NADH, 40 µM BSA, 0.8 mM magnesium chloride, 1.6 mM DTT, 80 µM
C20:2-CoA[t2, c11] and an appropriate amount of enzyme (routinely 10 µl) in a
total volume of 125 µl. C20:2-CoA[t2, c11] was used to initiate the reaction after
any endogenous pyridine nucleotide oxidation had been measured. When NADPH
was used as reductant, its concentration was 140 µM and the buffer used was 80
mM MES/NaOH (pH 5.0). Any alteration to this basic reaction mixture is explained
in the main text.

2.5.2 Preparation of dialysis tubing.

Before use, dialysis tubing was boiled in 20 % w/v sodium bicarbonate and 1 mM
EDTA for 10 minutes. After rinsing in distilled water, the tubing was boiled in
1 mM EDTA for 10 minutes. After cooling, it was stored in 25 % ethanol and
1 mM EDTA at 4°C, to prevent microbial contamination. This treatment is required
to remove chemical contaminants from the manufacturing process. Prior to use,
tubing was washing in Milli Q water to remove ethanol.

2.5.3 Protein concentration determination.

Protein concentration was estimated using the Bio-Rad protein assay. This is a dye-binding assay based on the differential colour change of Coomassie Brilliant Blue G-250 when bound to various concentrations of protein. The absorbance maximum for an acidic solution of the dye shifts, when bound to protein, from 465 to 595 nm. The assay was performed following the manufacturer's recommendations, which are based on the method of Bradford, (1976).

Protein solutions (1-20 μg protein) were made up to 1 ml by the addition of distilled water, then 250 μl of dye reagent were added and the absorbance at 595 nm was read after 5 minutes. Ovalbumin was used as the standard protein, since in comparison to other proteins bovine serum albumin would have resulted in an over-estimation.

2.5.4 Preparation of crude *Brassica napus* seed extract.

Crude *Brassica napus* seed extract, for SDS PAGE, was prepared from 1 g of seeds that were ground in liquid nitrogen, before being resuspended in 5 ml of 1 x SDS sample buffer (2 % w/v SDS, 0.06 M DTT, 0.01 % w/v bromophenol blue, 62.5 mM Tris-HCl [pH 6.8] and 10 % v/v glycerol). The mixture was boiled for 2 minutes and spun for 5 minutes in a microfuge, before an aliquot of the supernatant was loaded on a gel.

2.5.5 Concentration of proteins by chloroform methanol precipitation.

Protein samples for electrophoresis, were concentrated using the quantitative recovery method of Wessel and Flügge, (1984). Methanol (400 μl) was added to

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100 µl protein sample, and after vortexing and centrifuging for 10 seconds at 10 000 x g, 100 µl of chloroform was added and the sample was vortexed and centrifuged as before. After the addition of 300 µl Milli Q water, the sample was centrifuged at 10 000 x g for 5 minutes. The upper phase was discarded, care being taken not to remove the interface, and 300 µl methanol was added to the lower phase. After vortexing and centrifugation for 3 minutes, the supernatant was discarded and the pellet left to air dry for approximately 20 minutes. This results in both total concentration and complete desalting.

2.5.6 Separation of proteins by SDS polyacrylamide gel electrophoresis.

Polyacrylamide gels, 7 x 10 cm, were cast and run using the Bio-Rad Protean II gel electrophoresis kit, based on the method of Laemmli, (1970). The thickness of the gels was 0.75 mm. The gels were made with two phases, routinely a 10 % w/v polyacrylamide resolving gel and a 5 % w/v polyacrylamide stacking gel.

The resolving gel was made using 37.5:1 acrylamide/bis-acrylamide in 0.1 % w/v SDS, 0.375 M Tris-HCl (pH 8.8), 0.014 % w/v ammonium persulphate and 0.2 % v/v TEMED. The solution was immediately poured into the gel former to approximately 2 cm from the top. Water saturated *n*-butanol was layered on the top of the gel to ensure a level surface and the gel was allowed to set at room temperature. Once the resolving gel had set, the *n*-butanol was washed off with distilled water. The stacking gel consisted of acrylamide in 0.1 % w/v SDS, 0.125 M Tris-HCl (pH 6.8), 0.014 % w/v ammonium persulphate and 0.4 % v/v TEMED. This was poured on top of the formed resolving gel and the comb was inserted to create wells. The stacker was allowed to set at room temperature.

Protein samples were prepared for electrophoresis by the addition of 1/5 volume of 5 x SDS sample buffer (10 % w/v SDS, 0.3 M DTT, 0.05 % w/v bromophenol blue, 312.5 mM Tris-HCl [pH 6.8] and 50 % v/v glycerol). The samples were boiled for 2 minutes and spun for a few seconds in a microfuge before being applied to the gel.

Electrophoresis was carried out in 0.1 % w/v SDS, 192 mM glycine and 25 mM Tris-HCl buffer [pH 8.3]. The proteins were stacked at 100 V for 10 minutes and resolved at 200 V for approximately 30 minutes until the blue dye front reached the bottom of the gel.

Proteins were detected by Coomassie blue protein staining. Gels were stained for 5 minutes in approximately 50 ml of 25 % v/v propan-2-ol, 10 % v/v glacial acetic acid and 0.025 % w/v Coomassie Brilliant Blue, which had been heated to approximately 60°C by microwaving on 80 % power for 1 minute. Gels were destained using approximately 50 ml of 60°C destain buffer (10 % v/v acetic acid and 1 % v/v glycerol). Destaining was repeated until sufficient background staining was removed. The gels were washed in distilled water before drying.

2.5.7 Separation of proteins by native gel electrophoresis.

This was essentially the same as SDS polyacrylamide gel electrophoresis, except no SDS was present in the gel or running buffer. A 7.5 % w/v polyacrylamide resolving gel with a 5 % w/v polyacrylamide stacking gel was routinely used. Protein samples were prepared by the addition of 1/5 the volume of 5 x native gel sample buffer (250 mM Tris-HCl [pH 6.8], 50 % v/v glycerol, 0.3 M DTT, 0.5 % w/v bromophenol blue) and incubated at room temperature for 5 minutes. As a

molecular weight standard bovine serum albumin, as a monomer and dimer (66 kDa and 132 kDa, respectively) was used.

2.5.8 Two-Dimensional electrophoresis of proteins.

Two-dimensional electrophoresis was performed using a Pharmacia Multiphor II electrophoresis system, following the manufacturer's recommendations, based on the method of O'Farrell, (1975). Protein samples were prepared by concentrating crude protein extracts by precipitating with methanol and chloroform, as described in section 2.5.5. Dried pellets were resuspended in 50 μl sample buffer (8 M urea, 268 mM 2-mercaptoethanol, 2 % v/v Pharmalyte 3-10 (these are ampholines which are used to establish the pH gradient needed for iso-electric focusing [IEF]) and 0.5 % v/v Triton X-100) and incubated for 1 hour at room temperature. The first dimension, iso-electric focusing, was performed using precast Immobiline Drystrips, pH 3.0-10.5. These were rehydrated for 6 hours in 8 M urea, 0.5 % v/v Triton X-100, 10 mM DTT and 2 mM acetic acid. A few crystals of Orange G were added to stain the strips faintly yellow, which helped with strip alignment. Protein samples were loaded on to the Drystrips under oil and focused overnight (300 V for 3 hours [900 Vh], 300 V for 5 hours [5750 Vh], 2000 V for 8 hours [16000 Vh]) at 15°C. (Phase 2 is a ramping phase going from 300 V to 2000 V.)

After iso-electric focusing, the Drystrips could be stored at -20°C for later use. Before the second dimension, the Drystrips were equilibrated in solution A (50 mM Tris/HCl [pH 6.8], 6 M urea, 30 % v/v glycerol and 1 % w/v SDS) containing 16 mM DTT for 10 minutes at room temperature, then solution A containing 243 mM iodoacetamide and a few grains of bromophenol blue, again for 10 minutes at room temperature. DTT is used as it helps to solubilise proteins, but it may cause streaks, however, this can be eliminated by the addition of iodoacetamide. The

second dimension gels were precast 8-18 % gradient SDS. The gels were run at 20 mA for 20 minutes, to electrophorese proteins out of the IEF gels, then 50 mA for 10 minutes, to stack the proteins. The gel strips were then removed and the gel-buffer strip moved over the loading area. Finally, the gel was run at 50 mA for 70 minutes, until the blue dye front reached the end of the gel. Proteins were electrophoretically transferred to Hybond C nitrocellulose membrane using a Pharmacia semi-dry blotter, for detection by immuno-screening, as described below.

2.5.9 Electrophoretic transfer of proteins to nitrocellulose membrane.

Proteins separated by electrophoresis were blotted onto Hybond C nitrocellulose membrane using a Pharmacia semi-dry blotter, based on the method of Towbin *et al.*, (1979).

Nitrocellulose membrane and nine pieces of 3MM filter paper (Whatman), both cut to the size of the gel, were soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 0.0375 % w/v SDS and 20 % v/v methanol; the final pH was 8.5). Methanol increases the capacity and affinity of nitrocellulose for proteins, but also causes a general reduction in gel pore size, thus restricting the transfer of some molecules. However, the addition of SDS results in improved elution efficiency of high molecular weight proteins (Bers and Garfin, 1985). For transfer of proteins separated by native gel electrophoresis, no SDS was present in the transfer buffer. The blot was assembled as follows: 6 layers of 3MM filter paper were placed on the anode, then nitrocellulose and the gel were placed on top, followed by 3 layers of 3MM filter paper next to the cathode. Air bubbles were removed by smoothing and the blot was run at 0.8 mA per cm^2 for 60 minutes.

To check the blotting efficiency and to locate the position of any markers, the membrane was stained in Ponceau S (0.1 % w/v Ponceau S in 1 % v/v glacial acetic acid) for 1 minute, destained in three 1 % v/v glacial acetic acid washes for 1 minute each and then washed once in Milli Q water. The blot was then used for immuno-screening.

2.5.10 Direct transfer of proteins to nitrocellulose.

Proteins were directly transferred to nitrocellulose under vacuum using the Bio-Rad Bio-Dot SF apparatus, following the manufacturer's recommendations. Three sheets of filter paper wetted in Tris-buffered saline (TBS) [137 mM sodium chloride, 3 mM potassium chloride, 25 mM Tris/HCl (pH 7.4)] were placed onto the membrane support. Pre-wetted nitrocellulose (soaked in TBS) was placed on top of the filter papers, care being taken not to trap any air bubbles. The sample template was placed on top of the membrane and screwed down tightly. TBS (100 µl) was added to each sample well to rehydrate the membrane, then the buffer was removed by a gentle vacuum. Protein was added to the sample wells in a volume of 200 µl, and allowed to filter through the membrane by gentle vacuum. The wells were then washed with 200 µl TBS, again under gentle vacuum. The membrane was removed and the efficiency of blotting determined by Ponceau S staining.

2.5.11 Immuno-screening of proteins.

2.5.11.1 Detection by ^{125}I-labelled antibody.

Following transfer of proteins to nitrocellulose, they were detected using immuno-screening. Non specific antibody binding sites on nitrocellulose were blocked by incubation in approximately 100 ml of blocking solution (1 % w/v haemoglobin in

phosphate-buffered saline containing 0.1 % v/v Tween-20 and 0.02 % w/v sodium azide) at 4°C overnight. Phosphate-buffered saline (PBS) consisted of 137 mM sodium chloride, 3 mM potassium chloride, 8 mM di-sodium hydrogen orthophosphate 2-hydrate, 2 mM potassium dihydrogen orthophosphate (pH 7.4). Primary antibody was added in 10 ml fresh blocking solution, usually at a 1:1000 dilution and incubated at room temperature for 2 hours in a roller bottle. Non-bound antibody was removed by extensive washing: three quick washes with 50 ml of PBS containing 0.1 % v/v Tween-20, followed by three 10 minute washes. Tween-20 was added because it prevents non-specific hydrophobic reactions (Bers and Garfin, 1985). After incubation, the antibody solution was stored for future use. If the primary antibody used was raised in mouse, sheep or pig, the blot was incubated in rabbit anti-mouse IgG antibody (Pierce, 44 Upper Northgate Street, Chester), rabbit anti-sheep IgG antibody (Miles-Yeda Ltd., Israel) or rabbit anti-pig IgG antibody (Miles-Yeda Ltd., Israel) respectively, at the usual dilution of 1:1000 in blocking solution at room temperature for 1 hour, then washed as above. This was so that ^{125}I-labelled donkey anti-rabbit IgG antibody could be used for detection; this extra step was not required if the primary antibody was raised in rabbit. Five μCi ^{125}I-labelled antibody were used in 10 ml of blocking solution and incubated at room temperature for 1 hour, before washing as above. The radiolabelled blot was wrapped in Saran Wrap and exposed to Fuji X-ray film at -80°C overnight, using the method described in section 2.4.7.

2.5.11.2 Enhanced chemiluminescence detection.

Enhanced chemiluminescence (ECL) Western blotting is a light emitting non-radioactive method for the detection of immobilised specific antigens conjugated with horseradish peroxidase labelled antibodies. Horseradish peroxidase (HRP) catalyses the oxidation of luminol in the presence of hydrogen peroxide. Once

oxidised, the luminol is in an excited state which decays to ground state via a light emitting pathway as shown in figure 2.3.
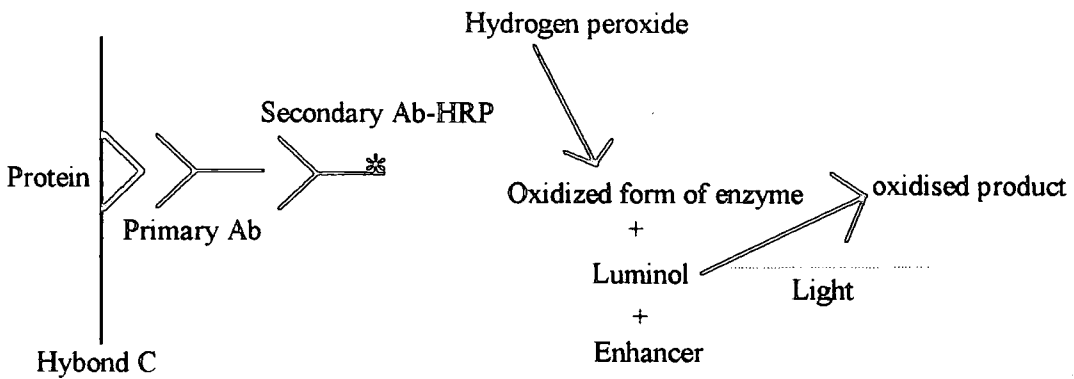
Figure 2.3 A schematic diagram of the principles of ECL Western blotting.

The method used was essentially the same as for ^{125}I-labelled antibody detection, except the blocking solution contained 5 % w/v skimmed milk powder in PBS containing 0.01 % v/v Tween-20 and 0.02 % w/v sodium azide. Haemoglobin was not used in the blocking solution because it contains endogenous peroxidase activity and would interfere with the detection mechanism (Bers and Garfin, 1985). Primary antibody was added in 10 ml of fresh blocking solution at a 1:1000 dilution and incubated at room temperature for 2 hours in a roller bottle. Non-bound antibody was removed by extensive washing: three quick washes with 50 ml of PBS containing 0.1 % v/v Tween-20, followed by three 10 minute washes. If the primary antibody was raised in sheep, the blot was incubated in rabbit anti-sheep IgG antibody at a 1:1000 dilution in blocking solution at room temperature for 1 hour, then washed as described above. Horseradish peroxidase-labelled anti-rabbit IgG or anti-mouse IgG antibody was added at a 1:4000 dilution in blocking buffer containing no sodium azide and incubated at room temperature for 1 hour. Care

was taken to exclude sodium azide since it inhibits horseradish peroxidase activity. After washing, detection was performed using a kit from Amersham International Plc., following the manufacturer's instructions. Excess buffer was removed from the washed nitrocellulose, which was then incubated in a equal mixture of solutions 1 and 2 for 1 minute. Excess detection reagent was removed by blotting the nitrocellulose on tissue before it was wrapped in Saran wrap and exposed to X-ray film for 15 seconds to 5 minutes (dependent on the strength of the signal). Exposed films were developed as described in section 2.4.7.

2.5.12 Preparation of crude animal FAS from a lactating rat mammary gland.

Animal FAS was prepared from the mammary gland of lactating rat using the method of Smith and Abraham, 1975. The procedure was maintained at 4°C throughout. Tissue (2.5 g) was washed with 5 ml of 0.25 M sucrose, then homogenised in 6 ml of 0.25 M sucrose using a polytron with a 2 cm diameter probe (Kinematica, Switzerland) for 2 minutes at 20 second intervals. The temperature, which never exceeded 10°C, was allowed to drop to 4°C between intervals. The extract was centrifuged at 200 000 x g for 30 minutes in a Beckman Optima™ TLX ultracentrifuge with a 8 x 3.2 ml fixed angle rotor. After centrifugation, the supernatant was filtered through four layers of muslin, to remove floating fat, and stored at -80°C.

2.5.13 Immobilisation of affinity-purified rabbit anti-native soluble enoyl-ACP reductase antibody to protein A agarose.

Rabbit anti-native soluble enoyl-ACP reductase antibody was immobilised to protein A using a Bio-Rad Affi-Gel® Protein A MAPS® II (monoclonal antibody purification system) kit; Affi-Gel Protein A consists of purified protein A coupled to

agarose beads. All procedures were performed at room temperature, based on the manufacturer's instructions. Affi-gel protein A agarose (1 ml) was placed in a 50 ml Falcon tube and the matrix was equilibrated with 5 ml of binding buffer (pH 9.0), by inverting the tube for a few minutes. The tube was centrifuged at 1 000 x g for 1 minute, so that the supernatant could be removed and its pH could be confirmed to be pH 9.0. The antibody sample (4 mg) was diluted 1:1 with binding buffer, added to the matrix and the mixture gently agitated for 1 hour. After 1 hour, the tube was centrifuged at 1 000 x g for 1 minute and the supernatant removed. The matrix was washed in 5 ml of binding buffer by gently agitating the tube for approximately 5 minutes. Again, the tube was centrifuged at 1 000 x g for 1 minute and the supernatant removed. This washing procedure was repeated twice. The antibody was then coupled to the protein A matrix, using a method based on Schneider *et al.*, 1982. The matrix was washed with 5 ml of 0.2 M triethanolamine (pH 8.2) and the supernatant removed after centrifugation, as above, before the protein A agarose was resuspended in 20 ml of 20 mM dimethyl pimelimidate dihydrochloride, freshly made up in 0.2 M triethanolamine (pH 8.2). The mixture was incubated with gentle agitation for 45 minutes prior to the supernatant being removed after centrifugation. The matrix was then resuspended in 1 ml of 20 mM ethanolamine (pH 8.2) and gently agitated for 5 minutes. The supernatant was removed and finally the matrix was washed with 5 ml of borate buffer (pH 8.2) containing 0.02 % w/v sodium azide. This washing procedure was repeated once, before the matrix was packed in to a column, which was stored at 4°C.

2.5.14 Labelling soluble enoyl-ACP reductase with ^{35}S-methionine.

2.5.14.1 Growth in LB media.

A 5 ml culture of BL21 containing pEAR2 was grown overnight at 37°C in LB containing 100 µg/ml ampicillin and this was used to inoculate a 50 ml LB culture

containing 100 μg/ml ampicillin. The cells were grown at 37°C for approximately 3 hours until the absorbance of the culture at 600 nm was 1.0, then 1 mM IPTG and 100 μCi ^{35}S-methionine were added to two 5 ml cultures, which were incubated at 37°C for 3 hours. This method of induction was based on Kater *et al.*, (1991).

Cells were harvested by centrifugation at 4 000 x g for 6 minutes and the pellet obtained was washed in 1 ml PBS (137 mM sodium chloride, 3 mM potassium chloride, 8 mM di-sodium hydrogen orthophosphate 2-hydrate, 2 mM potassium dihydrogen orthophosphate [pH 7.4]). The final pellet was resuspended in 100 μl TGE (25 mM Tris/HCl, 50 mM glucose, 10 mM EDTA [pH 8.0]) and a few grains of lysozyme, and incubated at room temperature for 10 minutes. To this, 100 μl of lysis buffer (150 mM sodium chloride, 1 % v/v NP-40, 50 mM Tris/HCl [pH 8.0]) was added and the cells incubated on ice for 30 minutes with occasional mixing. The cells were then centrifuged at 4 000 x g for 6 minutes and the supernatant removed (Harlow and Lane, 1988).

2.5.14.2 Growth in minimal media.

A 5 ml culture of *E. coli* BL21 (pEAR2) was grown overnight at 30°C in 2 x YT media containing 0.4 % w/v glucose and 200 μg/ml ampicillin and 1.25 ml of it was used to inoculate a 50 ml culture of 2 x YT media containing 0.4 % w/v glucose and 200 μg/ml ampicillin. The cells were grown at 30°C until the absorbance of the culture at 600 nm was 0.4 - 0.5. A 10 ml sample was centrifuged at 4 000 x g for 10 minutes to pellet the cells, which were washed in 10 ml of M9 media (1 x salts, 0.2 % w/v glucose, 1 mM magnesium sulphate). 5 x M9 medium salts stock solution contained 93 mM ammonium chloride, 43 mM sodium chloride, 211 mM di-sodium hydrogen orthophosphate 2-hydrate, 110 mM potassium dihydrogen orthophosphate. The washed pellet was resuspended in 10 ml of M9 media

supplemented with 0.005 % w/v amino acid mix, containing all amino acids except cysteine and methionine, and 100 μg/ml ampicillin, and grown at 30°C in a 50 ml flask. After 2 hours, 1 mM IPTG was added and the cells grown for a further 20 minutes before 200 μg/ml rifampicin was added and the cells incubated at 42°C for 10 minutes. Rifampicin inhibits *E. coli* RNA polymerase, and therefore, prevents expression of host proteins, but does not affect the T7 RNA polymerase. The culture was grown at 42°C because rifampicin is more effective at this temperature (Ausubel *et al.*, 1992.) The culture was then incubated at 30°C for 20 minutes, before 20 μCi of ^{35}S-methionine was added, and the cells grown for a further 15 minutes. The cells were then pelleted and washed in 10 ml wash buffer (50 mM Tris/HCl [pH 7.5], 150 mM sodium chloride). The re-pelleted cells were frozen in liquid nitrogen and stored at -80°C. This method was based on Ausubel *et al.*, 1992.

Cells were lysed using a method based on Johnson and Hecht, (1994). The frozen pelleted cells were incubated in ethanol and dry ice for 3 minutes, before being thawed to 4°C in a 40°C waterbath. The cells were then incubated in ice-water for 5 minutes and this procedure was repeated twice, before the pellet was resuspended in 400 μl cold 10 mM sodium phosphate buffer (pH 6.2) and incubated on ice for 1 hour with occasional agitation. The cells were centrifuged at 4 000 x g for 10 minutes and the supernatant removed.

3.1 Introduction.

Soluble enoyl-ACP reductase is a key enzyme involved in fatty acid biosynthesis. During the synthesis of fatty acids, the enzyme reduces a *trans* 2 double bond to leave a saturated acyl chain which is attached to acyl carrier protein. Enoyl-ACP reductase has been purified from several sources including *Brassica napus* (oil seed rape) seeds (Slabas *et al.*, 1986) and *Spinacia oleracea* (spinach) leaves (Shimakata and Stumpf, 1982c).

In 1991, Kater *et al.*, isolated a cDNA clone encoding enoyl-ACP reductase from a *Brassica napus* seed library. Initially, a *Brassica napus* var. jet neuf λgt 11 cDNA expression library was screened with an antibody raised against enoyl-ACP reductase purified from *Brassica napus* seeds. However, since the largest clone isolated was not full length, it was used as a probe to screen a second λgt 11 cDNA library, which had been prepared from mRNA isolated during an earlier stage in seed development. The largest clone, λEAR7, was 1358 bp long and sequence analysis showed it contained a leader peptide for translocation of the protein into the plastid. Subsequent expression of the clone in *E. coli* proved it encoded NADH-utilising enoyl-ACP reductase.

Southern blot analysis, using this cDNA as a probe, showed that *Brassica napus* has four genes encoding enoyl-ACP reductase - two pairs inherited from each of its ancestors, *Brassica oleracea* and *Brassica campestris* (Kater *et al.*, 1991); yet only one cDNA for enoyl-ACP reductase has so far been isolated. Isolation of other

enoyl-ACP reductase cDNA clones is necessary to determine whether all four genes are expressed.

It would also be of considerable interest to determine whether a probe designed from soluble enoyl-ACP reductase could hybridise to a clone which contains a membrane binding domain, and therefore, may encode the membrane-associated enoyl reductase, which is involved in the elongation of VLCFA's. Due to the problems associated with solubilising and purifying membrane-bound proteins, isolation of membrane-associated enoyl reductase cDNA would facilitate the characterisation of this enzyme in a more rapid manner.

3.2 Preparation of a radiolabelled probe to screen a *Brassica napus* leaf cDNA library.

The methods used throughout this chapter are described in sections 2.4.1 - 2.4.11.

Plasmid DNA containing cloned *Brassica napus* enoyl-ACP reductase cDNA, a gift from Dr. M. M. Kater, Vrije Universiteit, Amsterdam, was digested with EcoR I and Hinf I restriction enzymes to produce an 881 bp fragment that encodes part of the mature enoyl-ACP reductase protein (figure 3.1).
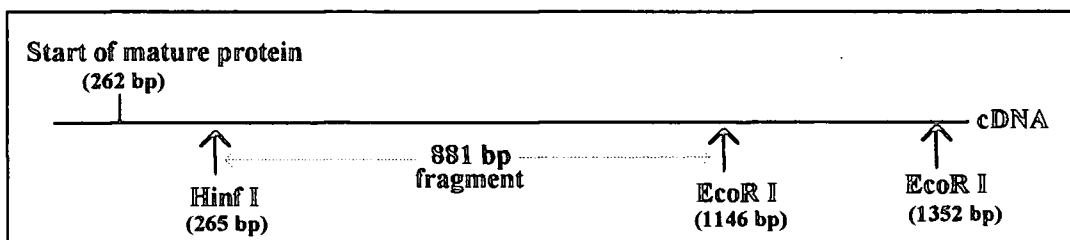
Figure 3.1 Positions of EcoR I and Hinf I restriction sites in *Brassica napus* seed enoyl-ACP reductase cDNA (λEAR7).

This fragment was chosen because the region encoding the mature protein would be more conserved between isoforms than non-translated regions.

The restriction digest products were analysed by gel electrophoresis and the required 881 bp fragment, visualised with long wavelength U.V. light, was cut out of the gel with a sterile scalpel blade. Figure 3.2 shows the gel before and after the 881 bp fragment was cut out. DNA was removed from the gel slice by electroelution, and quantification of the recovered fragment was determined by comparison to molecular weight markers after electrophoresis (figure 3.3); approximately 25 % of the isolated DNA fragment was recovered.

The isolated 881 bp probe was labelled with [α-^{32}P] dCTP using a random nonanucleotide primer system (Amersham International Plc.) and the reaction was stopped by the addition of stop mix which contained 20 mM EDTA [pH 8.0], 2 mg/ml blue dextran, 0.02 mg/ml xylene cyanol. Unincorporated nucleotides were removed using a Bio-Rad Bio-Spin Chromatography column containing Bio-Gel P-6 polyacrylamide. Incorporation of [α-^{32}P] into the DNA was estimated by counting an aliquot of the probe solution in a scintillation counter, and it was calculated that the labelled DNA had a specific activity of 8.2×10^8 dpm/μg DNA.

3.3 Screening a *Brassica napus* leaf cDNA library.

The library screened in this experiment was an amplified λZAP II cDNA library prepared from mRNA isolated from *Brassica napus* var. jet neuf leaves, which was available within the research group. A leaf library was used to try and isolate clones that were different from the one that had previously been isolated from a seed library. This library had previously been successfully used to isolate a β-ketoacyl-ACP reductase cDNA clone, when 2×10^5 pfu were screened. Therefore, assuming
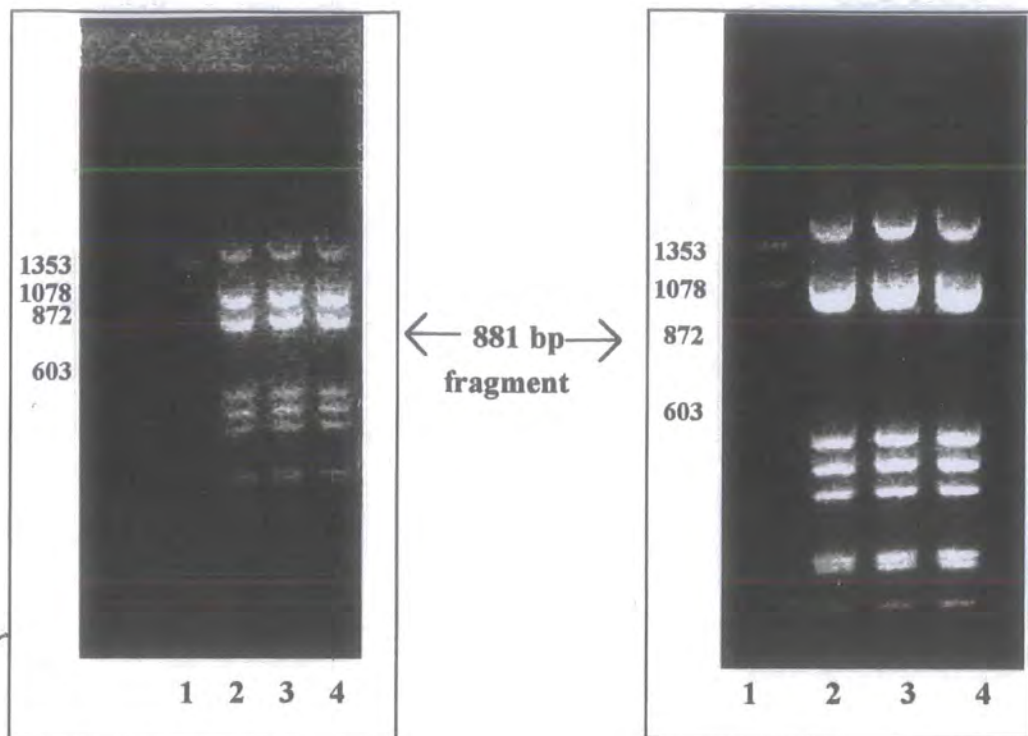
Figure 3.2 Band extraction of an 881 bp EcoR I - Hinf I restriction fragment from λEAR7.

The above photographs show the extraction of an 881 bp EcoR I - Hinf I restriction fragment from a 2 % w/v agarose gel.

Lane 1) Hae III digested 'phage φx 174 DNA molecular weight markers (1353, 1078, 872, 603 bp)

Lanes 2 - 4) pBluescript with λEAR7 insert, digested with EcoR I and Hinf I
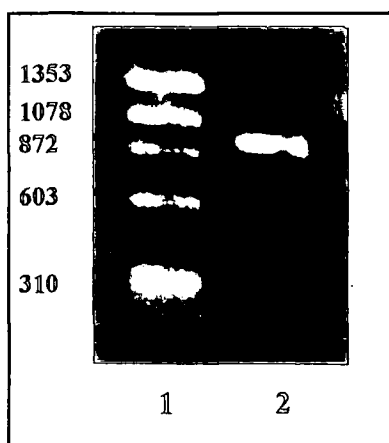
Figure 3.3 The recovered 881 bp EcoR I - Hinf I restriction fragment.

The above photograph shows a 0.7 % w/v agarose gel of the recovered restriction fragment.

Lane 1) Hae III digested 'phage ϕx 174 DNA molecular weight markers (1353, 1078, 872, 603, 310 bp)

Lane 2) recovered 881 bp restriction fragment

similar expression of other FAS genes, 2×10^5 pfu were screened with the 881 bp radiolabelled probe to obtain enoyl-ACP reductase cDNA clones.

Hybridisation was performed as described previously and the washing procedure employed was: two washes in 2 x SSC containing 0.1 % w/v SDS for 2 minutes at room temperature, to remove excess probe, followed by two washes in 1 x SSC containing 0.1 % w/v SDS for 30 minutes at 65°C. The membrane was exposed to X-ray film overnight and developed to identify to which plaques the radiolabelled probe hybridised.

Positive plaques were removed from the agar plate by stabbing the plaque area with a sterile pasteur pipette and the plug was placed in 1 ml of SM containing 10 μl chloroform to kill any bacteria. After vortexing briefly, the plug was incubated at room temperature for 2 hours to allow the bacteriophage to diffuse out of the agar. These supernatants could be stored for 6 - 12 months at 4°C.

Four rounds of screening were necessary to obtain pure positive plaques, after which six independent positive clones were isolated.

3.4 DNA preparation and characterisation.

An advantage of λZAP II is that it contains bacteriophage-f1-derived sequences for *in vivo* conversion of DNA from the bacteriophage λ vector to the Bluescript plasmid. If F' cells are infected with a helper bacteriophage, ssDNA is synthesised between the bacteriophage f1 sequences through the polycloning site and any cDNA sequences inserted into it. The ssDNA synthesised, circularises and becomes packaged into bacteriophage M13 and is secreted from the cell. If F' bacteria are infected with these particles, the incoming ssDNA is converted into dsDNA by

bacterial machinery and replicates as a conventional plasmid (Sambrook *et al.*, 1989).

Therefore, this method of plasmid rescue was used to obtain pBluescript containing the isolated clones, rather than isolating λ DNA for subcloning. Two colonies were selected from each of the six plasmid excised transformants and termed pERL 1 to pERL 12, respectively.

The sizes of the inserts were determined by Southern blot analysis of EcoR I restriction digests. EcoR I was used to excise the inserts because the library had been produced by insertion of cDNA into λZAP II at the EcoR I restriction site in the multiple cloning site. The digested fragments were separated by agarose gel electrophoresis using a 0.7 % w/v agarose gel, transferred to Hybond C nitrocellulose by capillary action and hybridised to the 881 bp ^{32}P-labelled probe isolated from *Brassica napus* seed enoyl-ACP reductase cDNA. The results, in figure 3.4, show that, as expected, the probe hybridises to all the inserts. Two sizes of inserts were obtained and by comparison to molecular weight markers, it was calculated that the sizes were 1450 and 2300 bp, respectively. The higher molecular weight bands are probably due to undigested clones. As no low molecular weight bands are seen, the isolated cDNA's not do contain internal EcoR I restriction sites.

Northern blot analysis has shown that mRNA encoding enoyl reductase in leaf material has the same molecular weight as the mRNA from seeds (Kater *et al.*, 1991). Consequently, since the size of pERL 8 was similar to the clone isolated from a seed library (1358 bp), the two clones were compared by sequence analysis at the 5' and 3' ends. 1.5 µg of PEG-precipitated pERL 8 DNA was sequenced on an ABI 373 DNA sequencer using -21M13 (forward) and M13 (reverse) primers, which prime either side of the polycloning site.
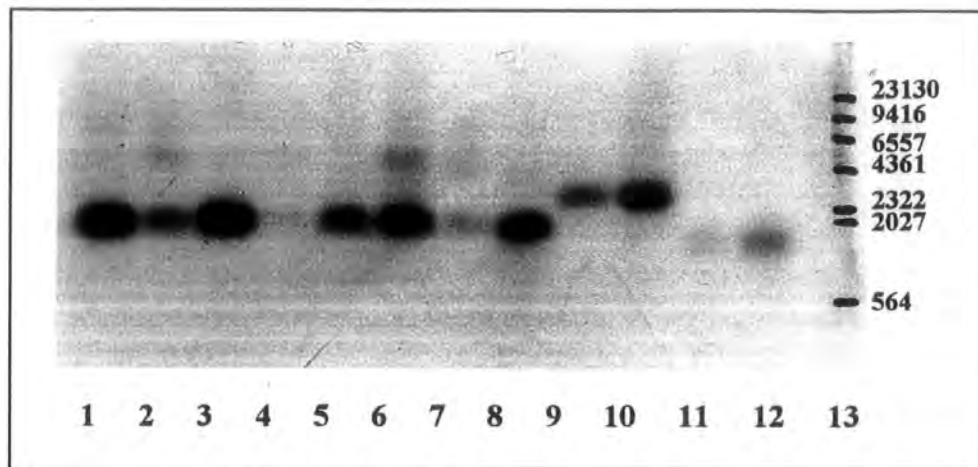
Figure 3.4 Sizing *Brassica napus* leaf cDNA clones by Southern blot analysis.

Each clone was sized by comparison to molecular weight markers. Clone sizes are given in brackets. The lanes are in pairs, e.g. pERL 1 and pERL 2 are from colonies isolated during plasmid excision of one clone.

Lane 1) pERL 1 (1450 bp)   Lane 2) pERL 2 (1450 bp)

Lane 3) pERL 3 (1450 bp)   Lane 4) pERL 4 (1450 bp)

Lane 5) pERL 5 (1450 bp)   Lane 6) pERL 6 (1450 bp)

Lane 7) pERL 7 (1450 bp)   Lane 8) pERL 8 (1450 bp)

Lane 9) pERL 9 (2300 bp)   Lane 10) pERL 10 (2300 bp)

Lane 11) pERL 11 (1450 bp)   Lane 12) pERL 10 (1450 bp)

Lane 13) Hind III digested 'phage λ DNA molecular weight markers (23130,

9416, 6557, 4361, 2322, 2027, 564 bp)

The sequence for pERL 8 was compared to the seed enoyl-ACP reductase cDNA, using DNA Strider™ 1.2, which is a computer package for DNA and protein sequences analysis (Christian Marck, CEA, France). pERL 8 showed homology but not identity to *Brassica napus* seed enoyl-ACP reductase cDNA at both the 5' and 3' ends, suggesting that it encoded a different enoyl-ACP reductase isoform. Consequently, the complete nucleotide sequence for pERL 8 was obtained. Primers available to the group, that had previously been designed to sequence enoyl-ACP reductase cDNA isolated from an *Arabidopsis thaliana* library were used to obtain partial sequence data. However, to obtain the full sequence, five more specific primers were designed and synthesised. This enabled pERL 8 to be completely sequenced in both directions. The nucleotide and derived amino acid sequences are shown in figure 3.5, and the cDNA sequence has been submitted to the EMBL nucleotide sequence database under the accession number X95462. By comparison to the seed cDNA, it was determined that pERL 8 contained a putative transit sequence, coding region, stop codon, 3' non-translated region and a poly A tail.

The other five clones were sequenced at the 5' and 3' ends only, in order to characterise them. Different cDNA's encoding enoyl-ACP reductase are more likely to vary in the non-translated regions than in the coding region, and so if two clones are identical at the 5' and 3' non-translated regions, it is likely that they would be identical throughout the cDNA. By comparing sequence data it was determined that pERL 2 and pERL 6 were highly homologous to pERL 8 at both ends, yet pERL 9 was only homologous to pERL 8 at the 3' end (figure 3.6). This suggests that pERL 2 and pERL 6 are the same clone as pERL 8. In comparison, pERL 9 is not identical to pERL 8. This is not surprising, though, since pERL 9 is larger than the other clones, and this could be a result of a cloning artefact at the 5' end or due to a membrane binding domain within the clone. However, since at least 400 bp are not homologous to pERL 8 at the 5' end, whereas the 3' non-translated region is

AA TTC GCG GCC GCC TCT CTC TAG GCT CTC TCA GAA AAC ATT AGA AAG GTC GTG AGA ATG
                                                                                M

61/21   91/31
GCG GCG ACA GCA GCT TCA AGC TTG CAA TTT GCT ACA ATA AGG CCA AGC ATC TCT AGC AAA
A   A   T   A   A   S   S   L   Q   F   A   T   I   R   P   S   I   S   S   K

121/41   151/51   646→
GTT GTT AAA GCA GGG ACC TAC ATT GTC GGT GCC AAT CCC AGG AAC GCA TCA TGG GAC AAA
V   V· K   A   G   T   Y   I   V   G   A   N   P   R   N   A   S   W   D   K

181/61   211/71
CTT GCC TGC ACT CGC CAT CTA TCG AAA CAC GGA TGT TTG AGA AAC AAC AGT TCT CTT CCA
L   A   C   T   R   H   L   S   K   H   G   C   L   R   N   N   S   S   L   P
                                                      ⇓

241/81   271/91
ACT TCT AAA AAG AGT TTT TCC TTT TCA ACA AAG GCC ATG TCT GAA TCC AGC GAG AAC AAG
T   S   K   K   S   F   S   F   S   T   K   A   M   S   E   S   S   E   N   K

301/101   331/111
GCT TCT TCT GGA CTT CCT ATT GAT TTG AGA GGG AAA AGG GCT TTC ATT GCT GGT ATA GCT
A   S   S   G   L   P   I   D   L   R   G   K   R   A   F   I   A   G   I   A

361/121   391/131
GAT GAT AAT GGA TAT GGT TGG GCC ATA GCC AAA TCT CTT GCT GCT GCT GCT GCT GAA ATA
D   D   N   G   Y   G   W   A   I   A   K   S   L   A   A   A   A   A   E   I

421/141   648→   451/151
TTG GTT GGG ACA TGG GTT CCT GCA CTT AAC ATT TTC GAG ACG AGC CTG AGA CGT GGA AAA
L   V   G   T   W   V   P   A   L   N   I   F   E   T   S   L   R   R   G   K

481/161   511/171   ←371
TTT GAC CAG TCA CGC GTG CTG CCT GAC GGG TCA TTG ATG GAG ATT AAA AAG GTT TAT CCT
F   D   Q   S   R   V   L   P   D   G   S   L   M   E   I   K   K   V   Y   P

541/181   571/191
TTG GAT GCT GTG TTT GAC AGT CCT GAA GAT GTG CCT GAA GAT GTG AAA GCG AAT AAG CGA
L   D   A   V   F   D   S   P   E   D   V   P   E   D   V   K   A   N   K   R

601/201   631/211
TAT GCT GGA TCA TCA AAC TGG ACA GTA CAG GAA GCT GCT GAA TGT GTT AGA AAA GAT TTT
Y   A   G   S   S   N   W   T   V   Q   E   A   A   E   C   V   R   K   D   F

661/221   691/231   369→
GGA ACC ATT GAC ATT CTT GTC CAC TCA CTT GCA AAT GGG CCC GAG GTT AGC AAA CCT CTT
G   T   I   D   I   L   V   H   S   L   A   N   G   P   E   V   S   K   P   L

721/241   ←651   751/251
CTG GAG ACA TCA AGA AAA GGC TAC CTC GCT GCT ATC TCT GCT TGC AGT TAC TCT TTT GTT
L   E   T   S   R   K   G   Y   L   A   A   I   S   A   C   S   Y   S   F   V

781/261   811/271
TCC CTC CTC AGG CAT TTC CTG CCA ATT ATG AAC CCA GGA GGT GCT TCT ATA TCT CTT ACT
S   L   L   R   H   F   L   P   I   M   N   P   G   G   A   S   I   S   L   T

841/281   871/291
TAC ATT GCT TCT GAA AGG ATC ATT CCT GGG TAT GGT GGG GGT ATG AGT TCT GCC AAA GCC
Y   I   A   S   E   R   I   I   P   G   Y   G   G   G   M   S   S   A   K   A

901/301   ←650 931/311
GCA CTA GAG AGT GAT ACA CGG GTG CTT GTA TTT GAA GCT GGA AGG AAA CAA AAA ATT AGG
A   L   E   S   D   T   R   V   L   V   F   E   A   G   R   K   Q   K   I   R

961/321   991/331   367→
GTC AAC ACC ATC TCT GCA GGT CCT TTG GGA AGC CGA GCA GCG AAA GCA ATT GGC TTC ATA
V   N   T   I   S   A   G   P   L   G   S   R   A   A   K   A   I   G   F   I

1021/341   1051/351
GAC ACC ATG ATT GAG TAT TCA TAC AAT AAT GCG CCT GTT CAG AAA ACA TTG ACC GCA GAT
D   T   M   I   E   Y   S   Y   N   N   A   P   V   Q   K   T   L   T   A   D

1081/361   1111/371
GAA GTT GGG AAT GCA GCA GCC TTC TTG GTA TCT CCA TTG GCC TCT GCC ATA ACC GGT GCA
E   V   G   N   A   A   A   F   L   V   S   P   L   A   S   A   I   T   G   A

1141/381   ←649   1171/391
ACC ATC TAT GTT GAC AAT GGC TTG AAT TCA ATG GGT GTT GCC ATC GAC AGT CCA GTT TTC
T   I   Y   V   D   N   G   L   N   S   M   G   V   A   I   D   S   P   V   F

1201/401   1231/411
AAA GAC CTC AAA TAG AGC CTT TTA AGT AAC TGT AGT AAC TCA CTT TTT CTT GTG CTG CAT
K   D   L   K   *

1261/421   1291/431
TTT TTT CAA CTG AGT GGA TGC TGT TTT TCA AAC TAC TTT GTT TTC TAG AAC AAA ATA AAA

1321/441
TAT TTA AAA CAA AAA AAA AGC GGC CGC GAA TT

Figure 3.5 The complete nucleotide and derived amino acid sequence of the *Brassica napus* cDNA clone pERL 8, encoding leaf enoyl-ACP reductase.

The deduced amino acid sequence is given below the nucleotide sequence. Numbers in the left margin refer to nucleotides and amino acids, respectively. The start of the putative transit peptide is indicated by ↓ and the start of the putative mature protein is indicated by ⇩. The stop codon and consensus of a poly A signal are highlighted in bold. The positions of the primers used for sequencing are underlined; primers 367, 369 and 371 were designed to sequence enoyl-ACP reductase cDNA from *Arabidopsis thaliana*, the other primers were specifically designed to sequence leaf enoyl-ACP reductase cDNA from *Brassica napus*.

```
pERL 8   AATTCGCGGCCGCCTCTCTCTAGGCTCTCTCAGAAAACATTAGAAA
pERL 2   AATTCGCtaCCGCCTCcCTCgAGG-TC-gaCgGAAA-CATaAGctt
pERL 6   AATTCGCGGCCGC----CTCTAGGCTCTCTCAGAAAACATTAGAAA
pERL 9   AATTCGCGGCCGaaagTagtaAtGgctTCctctAtgctcTcctccg
                          ↓
pERL 8   GGTCGTGAGAATGGCGGCGACAGCAGCTTCAAGCTTGCAATTTGCT
pERL 2   GaT-aTGAtAtTGGCGGCGACtt*AGCTTCAtGtTTGCAATaTGCT
pERL 6   GGTCGTGAGAATGGCGGCGACAGCAGCTTCAAGCTTGCAATTTGCT
pERL 9   ctgCtgtgGttacctGaCcggctCAagccaccatggtCgcTccatT


pERL 8   ACAATAAGGCCAAGCATCTCTAGCAAAGTTGTTAAAGCAGGGACCT
pERL 2   AC*AgAAGGgCAAGtATC--TAGgAAAG-TGTcAAAtCAGtaACCT
pERL 6   ACAATAAGGCCAAGCATCTCTAGCAAAGTTGTTAAAGCAGGGACCT
pERL 9   caccggcttgaAgtCtTCctctGCAttcccagTcAccCgcaaggCc


pERL 8   ACATTGTCGGTGCCAATCCCAGGAACGCATCATGGGACAAACTTGC
pERL 2   ACAaTGTCGcTGCCAATgCCAGGAACGCATgATGGGACAtACTTGC
pERL 6   ACATTGTCGGTGCCAATCCCAGGAACGCATCATGGGACAAACTTGC
pERL 9   AacaatgacaTtaCttcCatcGctAgcaAcggaGGaAgAgttagct


pERL 8   CTGCACTCGCCATCTATCGAAACACGGATGTTTGAGAAACAACAGT
pERL 2   CTcCACTCGaCATCTtTCcAAACtCGG-TGTTTGAGgAACAACAcT
pERL 6   CTGCACTCGCCATCTATCGAAACACGGgTGTTTG*GAAACAACAGT
pERL 9   gcatgaagGtgtggccaCcatttggaaagaagaagtttgaccCtcT


pERL 8   TCTCTTCCAACTTCTAAAAAGAGTTTTTCCTTTTCAACAAAGGCCA
pERL 2   TCT*TTC-AACTTgTcAAAAGAGTT*TTCCTTTTgAACcAAGGCCA
pERL 6   TCTCTTCCAACTTCTAAAAAGAGTTTTTCCTTTTCAACAAAGGCCA
pERL 9   TtaaccttcctgaaccttAccgaagTTgaaaTTgggtaAggaagtt
                ⇓
pERL 8   TGTCTGAATCCAGCGAGAACAAGGCTTCTTCTGGACTTCCTATTGA
pERL 2   TG
pERL 6   TG
pERL 9   gactacctTCttcCGcaacaAgtGggatTcCTtGtgTTtgaATTcg


pERL 8   TTTGAGAGGGAAAAGGGCTTTCATTGCTGGTATAGCTGATGATAAT
pERL 9   agTtgGtGcatggAttttTTgtAccGtgaGcAcgGaaGgcaccAcc


pERL 8   GGATATGGTTGGGCCATAGCCAAATCTCTTGCTGCTGCTGCTGC
pERL 9   GGgTActaTgatGgacgttaCtggTCaaTgtggaggttccttTGt
```

85

Figure 3.6 a) Comparison of the 5' end of pERL 8 with the other isolated cDNA clones at the nucleotide level.

The start of the putative transit peptide is indicated by ↓ and the start of the putative mature protein is indicated by ⇓. Unknown nucleotides (a result of sequencing in one direction only) are marked *. Comparisons were made using the gap program, part of the Genetics Computer Group (GCG) package, Daresbury Laboratory, Warrington, which aligns two complete sequences to maximise the number of matches and minimise the number of gaps (-). Nucleotides identical to pERL 8 are indicated by uppercase letters.

```
pERL 8  AATTCGCGGCCGCTTTTTTTTTGTTTTAAATATTTTATTTTGTTCT
pERL 2  AATTCGCGGCCGCTTTTTTTTTGTTTTAAATATTTTATTTTGTTCT
pERL 6  AcTgCGCtGCgGCcTcTcTagTGT---AgATgTaTcATTcTGgTCg
pERL 9  AATTCGCGGCCGCTTTTTT*TTGTTTTAAATATTTaATTTT*T-Cg


pERL 8  AGAAAACAAAGTAGTTTGAAAAACAGCATCCACTCAGTTGAAAAAA
pERL 2  AGAAAACAAAGT--TTTGAAAAACAGCATCCACTCAGTTGAAAAAA
pERL 6  AGAtt-Cgc-GTc*c-TGAAAAACAGCATCCACTCAGTTGAAAAAA
pERL 9  AGAAAACAgAGTAGTTTGAAAAACAGC-TCCACTCAcg*-AAAAA*


pERL 8  ATGCAGCACAAGAAAAAGTGAGTTACTACAGTTACTTAAAAGGCTC
pERL 2  ATGCAGCACAAGAAAAAGTGAGTTACTACAGTTACTTAAAAGGCTC
pERL 6  ATGCAGCACAAGAAAAAGTGgGTTACTACAGTTACTTAAAAGGCTC
pERL 9  -TGCAaCAaAGAAAAAGTGAGTTtCTACAGTTACTTAAAAGGCTC


pERL 8  TA
pERL 2  TA
pERL 6  TA
pERL 9  TA
```

Figure 3.6 b) Comparison of the 3' end of pERL 8 with the other isolated cDNA clones at the nucleotide level.

The stop codon is highlighted in bold. Unknown nucleotides (a result of sequencing in one direction only) are marked *. Comparisons were made using the gap program. Nucleotides identical to pERL 8 are indicated by uppercase letters.

homologous to pERL 8, the data are consistent with the hypothesis that the clone contains a 5' cloning artefact.

Clone pERL 4 had very high homology to the *Brassica napus* seed enoyl-ACP reductase cDNA (λEAR7) at both the 5' and 3' ends (figure 3.7), suggesting that the two clones were the same. This lead to a hypothesis that tissue specific cDNA's do not exist for enoyl-ACP reductase in *Brassica napus*, and evidence for this hypothesis came from the detection of four isoforms for enoyl-ACP reductase in both leaf and seed material, determined from 2-D Western blots (Fawcett *et al.*, 1994).

Clone pERL 12 had no homology to the *Brassica napus* sequences, and was thought to be a false positive.

3.5 Discussion.

Since Southern blot analysis has shown the existence of four enoyl-ACP reductase genes in *Brassica napus*, yet only one cDNA has previously been isolated, a *Brassica napus* leaf library was screened in an attempt to isolate more cDNA's, to aid analysis of gene expression. Six clones were isolated from a *Brassica napus* leaf cDNA library using a probe from λEAR7 (an enoyl-ACP reductase cDNA isolated from a *Brassica napus* seed library) and one clone, pERL 8, was fully sequenced in both directions, so that it could be compared to λEAR7 at the nucleotide and amino acid levels. Comparison of the two enoyl reductase sequences, shown in figure 3.8, reveals that the degree of identity between the two coding regions is extremely high; 91 % at the nucleotide level and 96 % at the amino acid level. However, this high degree of homology is not seen throughout the two clones. The two putative transit peptides only share 84 % homology at the nucleotide level and 76 % homology at the amino acid level. Their difference is also highlighted by their lengths, since the

```
                                                                      ↓
λEAR7   AATTCCAGGATCTATCAGAAAATATTTAAAATATTGTGAGGGATGG
pERL4   AATTCCAGG-TCTATCttcAAATcTaTAAAATATTtaGA-tGATGG


λEAR7   CGGCAACAGCAGCAGCTTCGAGCTTGCAAATGGCTACAACAAGGCCA
pERL4   CGGCAACAtCAGCAGCTTCGAtCTTGCAAATGGCTACAACAAGGCCA


λEAR7   AGCATTTCTGCTGCCTCTAGCAAAGCAAGGACCTACGTTGTCGGTGC
pERL4   AGCATTTCTGCTGCCTCTAcCAAAGCAAGG-CCTACaTTGTCGGTGC


λEAR7   CAATCCCAGGAACGCATACAAAATTGCTTGCACTCCCCATCTATCGA
pERL4   CAATCCCAGGAACGCATACAAAATTGCTTGCACTCCC-ATCTATCGA


λEAR7   ACCTCGGGTGTTTGAGAAACGACAGTGCTCTTCCAGCTTCTAAAAAG
pERL4   ACCTCGGGTGTTT**GAAACGACAGT*tTCTTCCAGCTTCTAAAAAG
                                     ⇓
λEAR7   AGTTTTTCCTTTTCGACAAAGGCCATG
pERL4   --TTT*TCCTTTTCGgCAAAGGCCATG
```

Figure 3.7 a) Comparison of the 5' end of λEAR7 with pERL 4 at the nucleotide level.

The start of the putative transit peptide is indicated by ↓ and the start of the putative mature protein is indicated by ⇓. Unknown nucleotides (a result of sequencing in one direction only) are marked *. Comparisons were made using the gap program. Nucleotides identical to λEAR7 are indicated by uppercase letters.

```
λEAR7  AATTCCGCAAACTTCAATCAATAGAGAAATAGTGCAGCAGAAAT
pERL4  AATTC-GCgggCTTtAATtttTAGA-AAcT-GTcCAGC--AAAT


λEAR7  AGTGCAGCAGTTATTACATACAGCTAAAGTGACAAACTAGAAACAT
pERL4  AaTGCAGCAGTTATTACAcA*tGCTAAAG*GACAAACTAacA*gtT


λEAR7  AACATATATATTCTCTTAAAAAGGGAAAAAAAAAATACAAAGCCGA
pERL4  AACATgTATATTCT*TT-AAAAGGGAAAg*AAAt--ACAAAGCCGA


λEAR7  AGGAGGAACATATGCTATTCGTCGATCACACACCCTTA
pERL4  tGGAGG*-CATAaGCTcTTCGTCGATCAaAaACCCTTA
```

Figure 3.7 b) Comparison of the 3' end of λEAR7 with pERL 4 at the nucleotide level.

The stop codon is highlighted in bold. Unknown nucleotides (a result of sequencing in one direction only) are marked ⋆. Comparisons were made using the gap program. Nucleotides identical to λEAR7 are indicated by uppercase letters.

```
λEAR7   1 ..............AATTCCAGGATCTATCAGAAAATATTTAAAATATT 35
                        ||  |||  |||  |||||||||  |||   |||   |
pERL 8  1 AATTCGCGGCCGCCTCTCTCTAGGCTCTCTCAGAAACATTAGAAAGGTC 50
                      ↓
       36 GTGAGGGATGGCGGCAACAGCAGCAGCTTCGAGCTTGCAAATGGCTACAA 85
          ||||  |  |||||||||  |||       |||||||| |||||||||| |  |||||||
       51 GTGA.GAATGGCGGCGACA....CAGCTTCAAGCTTGCAATTTGCTACAA 95

       86 CAAGGCCAAGCATTTCTGCTGCCTCTAGCAAAGCAAGGACCTACGTTGTC 135
          ||||||||||||| |||                ||||||  |||||||| |||||
       96 TAAGGCCAAGCATCTCTAGCAAAGTGTTTAAAGCAGGGACCTACATTGTC 145

      136 GGTGCCAATCCCAGGAACGCAT......ACAAAATTGCTTGCACTCCCCA 179
          |||||||||||||||||||||||      ||||| |||| ||||||| |||
      146 GGTGCCAATCCCAGGAACGCATCATGGGACAAACTTGCCTGCACTCGCCA 195

      180 TCTATCGAACCTCGGGTGTTTGAGAAACGACAGTGCTCTTCCAGCTTCTA 229
          |||||||| |  |||  |||||||||||||  |||||   ||||||| ||||||
      196 TCTATCGAAACACGGATGTTTGAGAAACAACAGT.TTCTTCCAACTTCTA 244
                                       ⇓
      230 AAAAGAGTTTTTCCTTTTCGACAAAGGCCATGTCTGAATCCAGCGAAAGC 279
          |||||||||||||||||||||  ||||||||||||||||||||||||  | |
      245 AAAAGAGTTTTTCCTTTTCAACAAAGGCCATGTCTGAATCCAGCGAGAAC 294

      280 AAGGCTTCTTCTGGACTTCCCATTGATTTGAGAGGGAAAAGAGCCTTTAT 329
          |||||||||||||||||||||  ||||||||||||||||||||||  || || ||
      295 AAGGCTTCTTCTGGACTTCCTATTGATTTGAGAGGGAAAAGGGCTTTCAT 344

      330 TGCTGGTATAGCTGATGATAATGGATACGGTTGGGCCGTAGCCAAATCTC 379
          |||||||||||||||||||||||||||||  ||||||||||  |||||||||||
      345 TGCTGGTATAGCTGATGATAATGGATATGGTTGGGCCATAGCCAAATCTC 394

      380 TTGCTGCTGCTGGTGCTGAAATTTTGGTTGGGACTTGGGTTCCTGCACTT 429
          ||||||||||||  |||||||||  ||||||||||||  ||  |||||||||||
      395 TTGCTGCTGCTGCTGCTGAAATATTGGTTGGGACATGCCTTCCTGCACTT 444

      430 AACATTTTTGAGACGAGCTTGAGACGTGGAAAATTCGACCAGTCACGCGT 479
          ||||||||  |||||||||  ||||||||||||||||||  ||||||||||||||||
      445 AACATTTTCGAGACGAGCCTGAGACGTGGAAAATTTGACCAGTCACGCGT 494

      480 GTTGCCAGACGGATCATTGATGGAGATTAAAAAGGTTTATCCTTTGGATG 529
          |  ||||  ||||| |||||||||||||||||||||||||||||||||||||||||||||
      495 GCTGCCTGACGGGTCATTGATGGAGATTAAAAAGGTTTATCCTTTGGATG 544

      530 CTGTCTTTGACAATCCTGAAGATGTCCCTGAAGATGTGAAAGCGAATAAG 579
          ||||  |||||||  ||||||||||||| |  |||||||||||||||||||||||||
      545 CTGTGTTTGACACTCCTGAAGATGTGCTCGAAGATGTGAAAGCGAATAAG 594

      580 CGATATGCTGGATCATCAAACTGGACTGTACAGGAAGCTGCAGAATGTGT 629
          |||||||||  ||||  |||||| ||  |||||||||||||  ||||||||
      595 CGATATGCTCCATCACTAAACTGTACAGTACAGGAAGCTGCTGAATGTGT 644

      630 GAGACAAGATTTTGGAAGCATTGACATTCTTGTCCACTCACTTGCAAATG 679
          |||  ||||||||||||  |||||||||||||||||||||||||||||||||||||||||
      645 TAGAAAAGATTTTGGAACCATTGACATTCTTGTCCACTCACTTGCAAATG 694
```

91

```
 680 GACCAGAGGTTAGCAAACCTCTTCTTGAGACATCGAGGAAAGGCTATCTT 729
     |  ||  |||||||||||||||||||||||| ||||||||| || |||||||||| ||
 695 GGCCCGAGGTTAGCAAACCTCTTCTGGAGACATCAAGAAAAGGCTACCTC 744

 730 GCTGCTATCTCTGCTTCCAGCTACTCCTTTGTTTCCCTCCTCAGCCATTT 779
     |||||||||||||||||| ||| |||||| ||||||||||||||||||| |||||
 745 GCTGCTATCTCTGCTTGCAGTTACTCTTTTGTTTCCCTCCTCAGGCATTT 794

 780 TCTCCCAATTATGAACCCAGGAGGTGCTTCTATCTCTCTTACTTACATTG 829
     || ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
 795 CCTGCCAATTATGAACCCAGGAGGTGCTTCTATATCTCTTACTTACATTG 844

 830 CCTCTGAAAGGATCATTCCCGGGTATGGTGGAGGTATGAGTTCTGCCAAG 879
     | |||||||||||||||||||||| |||||||||||| ||||||||||||||||||||
 845 CTTCTGAAAGGATCATTCCTGGGTATGGTGGGGGTATGAGTTCTGCCAAA 894

 880 GCCGCACTAGAGAGTGATACACGTGTGCTTGCATTTGAAGCTGGGAGGAA 929
     ||||||||||||||||||||||||||| ||||||||| ||||||||||||| |||||
 895 GCCGCACTAGAGAGTGATACACGGGTGCTTGTATTTGAAGCTGGAAGGAA 944

 930 ACAAAACATTAGGGTCAACACTATCTCTGCAGGCCCTTTGGGAAGCCGAG 979
     ||||||  ||||||||||||||||| ||||||||||| |||||||||||||||||||
 945 ACAAAAAATTAGGGTCAACACCATCTCTGCAGGTCCTTTGGGAAGCCGAG 994

 980 CAGCTAAAGCAATTGGGTTCATAGACACAATGATCGAGTATTCATACAAT 1029
     ||||  ||||||||||||| ||||||||||| ||||| ||||||||||||||||||
 995 CAGCGAAAGCAATTGGCTTCATAGACACCATGATTGAGTATTCATACAAT 1044

1030 AACGCGCCTATTCAGAAAACACTGACTGCAGATGAAGTTGGGAATGCGGC 1079
     ||  |||  |  |||||||||||  |||| |||||||||||||||||||||||||||  ||
1045 AATGCGAATGTTCAGAAAACATTGACCGCAGATGAAGTTGGGAATGCAGC 1094

1080 AGCCTTCTTGGTATCTCCATTGGCATCTGCCATAACTGGTGCAACCATCT 1129
     |||||||||||||||||||||||||||||||| ||||||||||| |||||||||||||
1095 AGCCTTCTTGGTATCTCCATTGGCCTCTGCCATAACCGGTGCAACCATCT 1144

1130 ATGTGGACAATGGCTTGAATTCAATGGGTGTTGCTCTTGACAGCCCCGTT 1179
     ||||  |||||||||||||||||||||||||||||||||  | |||||| || |||
1145 ATGTTGACAATGGCTTGAATTCAATGGGTGTTGCCATCGACAGTCCAGTT 1194

1180 TTCAAAGACCTCAACAAA**TAA**GGGTCTCTGATCGACGAATAGCATATGTT 1229
     ||||||||||||    || || | || | |    ||| | |
1195 TTCAAAGACCTC....AAA**TAG**AGCCTTTTAAGTAACTGTAGTAACT... 1237

1230 CCTCCTTCGGCTTTGTATTTTTTTTTTCCCTTTTTAAGAGAATATATGTT 1279
          |||| | || | |       |||| || || | ||| |
1238 ........CACTTTTTCTTGTGCTGCATTTTTTTCAACTGAGTGGATGCT 1279

1280 ATGTTTCTAGTTTGTCACTTTAGCTGATATGTAATAACTGCTGCACTATT 1329
     | |||| | | |        || | | || |    | | ||||
1280 GTTTTTCAAACTACT........TTGTTTTCTAGAACAAAATAAATATT 1321

1330 TCTCTATTGATTGAAGTTTGCGGAATTC 1357
     |        |  |||    ||  | |
1322 TAAAACAAAAAAAAGCGGCCGCGAATT 1349
```

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Figure 3.8 a) Comparison of λEAR7 and pERL 8 cDNA's at the nucleotide level.

λEAR7 and pERL 8 cDNA clones were isolated from a seed and a leaf library, respectively. The numbers in the margins refer to nucleotides. The start of the putative transit peptide is indicated by ↓ and the start of the putative mature protein is indicated by ⇓. The stop codon is highlighted in bold. Comparisons were made using the gap program and gaps are shown as ..

```
                                          ↓
λEAR7  1                      MAATAAASSLQMATTRPSISAASSKAR 27

                              |||| ||||||| || |||||      ||

pERL 8 1                      MAAT.AASSLQFATIRPSISSKVVKAG 26
                                                            ⇓

      28 TYVVGANPRNAY..KIACTPHLSNLGCLRNDSALPASKKSFSFSTKAMSE 75

|| |||||||||    |  ||| |||   ||||| | |||||||||||||||||||

      27 TYIVGANPRNASWDKLACTRHLSKHGCLRNNSSLPASKKSFSFSTKAMSE 76


      76 SSESKASSGLPIDLRGKRAFIAGIADDNGYGWAVAKSLAAAGAEILVGTW 125

             |||  |||||||||||||||||||||||||||||| ||||||| |||||||

      77 SSENKASSGLPIDLRGKRAFIAGIADDNGYGWAIAKSLAAAAAEILVGTW 126


     126 VPALNIFETSLRRGKFDQSRVLPDGSLMEIKKVYPLDAVFDNPEDVPEDV 175

         ||||||||||||||||||||||||||||||||||||||||||| |||||||

     127 VPALNIFETSLRRGKFDQSRVLPDGSLMEIKKVYPLDAVFDSPEDVPEDV 176


     176 KANKRYAGSSNWTVQEAAECVRQDFGSIDILVHSLANGPEVSKPLLETSR 225

         ||||||||||||||||||||||||| ||| ||||||||||||||||||||

     177 KANKRYAGSSNWTVQEAAECVRKDFGTIDILVHSLANGPEVSKPLLETSR 226


     226 KGYLAAISASSYSFVSLLSHFLPIMNPGGASISLTYIASERIIPGYGGGM 275

         |||||||||||||||||| |||||||||||||||||||||||||||||||

     228 KGYLAAISASSYSFVSLLRHFLPIMNPGGASISLTYIASERIIPGYGGGM 276


     276 SSAKAALESDTRVLAFEAGRKQNIRVNTISAGPLGSRAAKAIGFIDTMIE 325

         ||||||||||||| ||||||| ||||||||||||||||||||||||||||

     277 SSAKAALESDTRVLVFEAGRKQKIRVNTISAGPLGSRAAKAIGFIDTMIE 326


     326 YSYNNAPIQKTLTADEVGNAAAFLVSPLASAITGATIYVDNGLNSMGVAL 375

         ||||||| |||||||||||||||||||||||||||||||||||||||||

     327 YSYNNAPVQKTLTADEVGNAAAFLVSPLASAITGATIYVDNGLNSMGVAI 376


     376 DSPVFKDLNKZ 486

         ||||||||

     377 DSPVFKDLKZ 486
```

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Figure 3.8 b) Comparison of λEAR7 and pERL 8 cDNA's at the amino acid level.

λEAR7 and pERL 8 cDNA clones were isolated from a seed and a leaf library, respectively. The numbers in the margins refer to amino acids. The start of the putative transit peptide is indicated by ↓ and the start of the putative mature protein is indicated by ⇓. The stop codon is highlighted in bold. Comparisons were made using the gap program.

transit peptide in λEAR7 is 73 amino acids long but in pERL 8 it is 74 amino acids long. Nevertheless, both transit peptides conform to the consensus for stromal-targetting plastid transit peptides, namely they are positively charged and rich in serine and threonine residues. The two sequences also differ in the 3' non-translated region; they share only 40 % homology. Therefore, despite virtual identity in the coding region, the two transit peptides and 3' non-translated regions are clearly not the same, and so the clones must be different.

The other clones were sequenced at the 5' and 3' ends, so they could be characterised. pERL 2 and pERL 6 were identical to pERL 8 at both ends, whereas pERL 9, which is larger than the other clones, showed homology to pERL 8 at the 3' end only. pERL 4 had very high homology to λEAR7, the clone isolated from a seed library, suggesting that this clone is not tissue specific. Tissue specificity of enoyl-ACP reductase has since been investigated by 2-D Western blotting and the results propose the existence of four isoforms present in leaf and seed material, which is consistent with the expression of all four genes in both tissues (Fawcett et al., 1994). pERL 12 was not homologous to the other sequences and was thought to be a false positive.

Since two different cDNA clones have now been isolated that differ in the 3' untranslated region (UTR), the 3' UTR's can be used as a tool to differentiate between the clones and allow differential gene expression to be investigated.

Unfortunately, a probe isolated from soluble enoyl-ACP reductase did not hybridise to a clone containing a membrane binding domain, so further studies to characterise

membrane-associated enoyl reductase will have to be performed at the biochemical level.

To locate blocks of local similarity and identify conserved regions in enoyl-ACP reductase, the derived amino acid sequence of pERL 8 (*Brassica napus* 'leaf' enoyl-ACP reductase) was compared to the coding region amino acid sequences of enoyl reductases from *Brassica napus* 'seed' (λEAR7), *Arabidopsis thaliana, E. coli* and *Anabaena* spp. The sequences were aligned using a Multiple Alignment Construction and Analysis program (MACAW) and the alignment, figure 3.9, shows approximately 70 % similarity across all five sequences.

Comparison of enoyl reductase sequences and identification of conserved domains, aids location of active sites in the proteins. For example, most nucleotide binding enzymes contain a signature motif GxGxxG, where x is any amino acid, however, this sequence is not present in any of the enoyl reductase sequences. However, X-ray analysis of crystallised enoyl reductase encoded by *Brassica napus* 'seed' cDNA, shows that Gly25 and Gly33 have close structural correspondence to the glycines at the first and last positions of the motif (Rafferty *et al.*, 1995). Gly25 (marked * on figure 3.9) is present in all of the sequences compared and is in a domain that is highly conserved, suggesting the importance of this domain in enoyl reductase. From the location of a bound NADH cofactor molecule which has been co-crystallised with enoyl reductase, it has been proposed that Try198 and Lys206 are key active site residues (Rafferty *et al.*, 1995), and this is supported by the multiple sequence alignment, since these two residues (marked + on figure 3.9) are conserved in all the sequences.

```
Leaf        sessenkassglpic LRGKRAFIAGIADDNGYGWAIAKSLAAAAAEI lvgtwvpalnife   60
Seed        sesseskassglpic LRGKRAFIAGIADDNGYGWAVAKSLAAAGAEI lvgtwvpalnife   60
Arabidopsis sessenkapsglpic LRGKRAFIAGIADDNGYGWAIAKSLAAAGAEI lvgtwvpalnife   60
Ecoli       mgf----------- LSGKRILVTGVASKLSIAYGIAQAMHREGAEL aftyqndklkgrv   48
Anabaena    mttkismln------ LTGKNALVTGIANNRSIAWGIAQQLHAAGANL gitylpdergkfe   54


Leaf        tslrrgkfdqsrvlpdgslmeikkvypldavfdspedvpedvkankryaqssnwtvqeaa   120
Seed        tslrrgkfdqsrvlpdgslmeikkvypldavfdnpedvpedvkankryaqssnwtvqeaa   120
Arabidopsis tslrrgkfdqsrvlpdgslmeikkvyaldavfdnpedvpedvktnkryaqssnwtvqeaa   120
Ecoli       eefaaqlgssivlpcdvaedasidamfaelgnvwpkfdgfvhsi---------------   92
Anabaena    kkvselveplnpslflpcnvqndeqiqstfdtirdkwgrldilihcl------------   101


Leaf        ecvrkdfgtidilvh SLANGPEVSKPL let- SRKGYLAAISASSYSFVSLLRHFLPIMNP   179
Seed        ecvrqdfgsidilvh SLANGPEVSKPL let- SRKGYLAAISASSYSFVSLLSHFLPIMNP   179
Arabidopsis ecvkkdfgsidilvh SLAIGPEVSKPL let- SRKDYLAVISASSYSFVSLLRHFLPIMNP   179
Ecoli       --------------- GFAPGDQLDGDY vnav TREGFKVAHDISSYSFVAMAKAIRTMLNP   137
Anabaena    --------------- AFANRDDLTGDF sqt- SRAGFATALDISTFSLVLLGAAKPLNTE   145


Leaf        GGASISLTYIASERIIPGY ggg MSSAKAALESDTRVLVFEAGRKLAIRVNIISAGPLGSR   239
Seed        GGASISLTYIASERIIPGY ggg MSSAKAALESDTRVLAFEAGRRQNIRVNIISAGPLGSR   239
Arabidopsis GGASISLTYIASERIIPGY ggg MSSAKAALESDTRVLAYEAGRKSNIRVNIISAGPLGSR   239
Ecoli       GSALLTLSYLGAERAIPNY nv- MGLAKASLEANVRYMANAMGPEG VRVNAISAGPIRTL   195
Anabaena    GGSIITLSYLGGVRAVPNY nv- MGVAKAGLEASVRYLASELGSQN IRVNAISAGPIRTL   203


Leaf        AAKAIGFIDTMIEYSYNNAPVQKTLTADEVGNAAAFLVSPLASAITGATIYVDNGLNSMG   299
Seed        AAKAIGFIDTMIEYSYNNAPIQKTLTADEVGNAAAFLVSPLASAITGATIYVDNGLNSMG   299
Arabidopsis AAKAIGFIDTMIEYSYNNGPIQKTLTADEVGNAAAFLASPLASAITGATIYVDNGLNAMG   299
Ecoli       AASGIKDFRKMLAHCEAVTPIRRTVTIEDVGNSAAFLCSDLSAGISGEVHHVNGFSIAA   255
Anabaena    ASSAVGGILDMIHHVEQVAPLRRTVTQLEVGNTAAFLASDLASGITGQVIYVAIVEIMD   263


Leaf        Vaidspvfkdlk---   311
Seed        Valdspvfkdlnk--   312
Arabidopsis Valdspvfkdlnskn   314
Ecoli       Vnelelk--------   262
Anabaena    V--------------   264
```

Figure 3.9 Multiple sequence alignment (MACAW) of pERL 8 *Brassica napus* "leaf" enoyl-ACP reductase and enoyl reductase sequences from plant, bacteria and cyanobacteria.

Sequences were obtained from the Swissprot database. Conserved blocks within the five sequences are indicated by uppercase letters and are highlighted. The numbers in the margin refer to amino acids.

Interestingly, though, comparison of the derived amino acid sequence of 'leaf' enoyl reductase with the other sequences reveals that at residue 44 (marked ° on figure 3.9), glycine cannot be essential for activity, because even though it is conserved in four of the sequences, it is not present in 'leaf' enoyl reductase. Similarly, at position 217 (marked o on figure 3.9), alanine is replaced by valine in 'leaf' enoyl reductase.

Therefore, the isolation of a second enoyl-ACP reductase from a *Brassica napus* library aids determination of the residues necessary for enzyme activity.

Chapter 4: Development and optimisation of an assay for membrane-associated enoyl reductase activity in *Brassica napus* seeds.

4.1 Introduction.

In plants, enoyl reductase exists as both soluble and membrane-associated activities. Soluble NADH-specific enoyl-ACP reductase is involved in the synthesis of fatty acids up to 18 carbons in length and has been well characterised. By comparison, membrane-associated enoyl reductase, which is involved in the elongation of VLCFA's, has been investigated to a lesser extent. Research previously performed on these enzymes is summarised in section 1.4.1 and 1.4.3, respectively.

The activity of membrane-associated enoyl-ACP reductase has been measured in *Allium porrum* (leek) microsomes, where it is involved in the synthesis of saturated VLCFA's, which play an important role in wax biosynthesis (Cassagne and Lessire, 1978). Activity was measured using C18:1-CoA[*t*2] and C20:1-CoA[*t*2] substrates (Lessire *et al.*, 1992 and Spinner *et al.*, 1995, respectively). In comparison to *Allium porrum*, high erucic acid rape (HEAR) accumulates the unsaturated VLCFA, erucic acid (C22:1) in its seeds.

During elongation of fatty acids, the first substrate acted upon by membrane-associated enoyl reductase in *Brassica napus* (oil seed rape) seeds is C20:2-CoA[*t*2, *c*11]. As shown in figure 4.1, C18:1-CoA[*c*9] synthesised by the soluble FAS, is condensed with malonyl-CoA to produce a C20 acyl-CoA, which is reduced by membrane-associated enoyl reductase to form C20:1-CoA[*c*11]. Therefore, to assay membrane-associated enoyl reductase activity in *Brassica napus*, C20:2-CoA[*t*2, *c*11] would have to be synthesised.
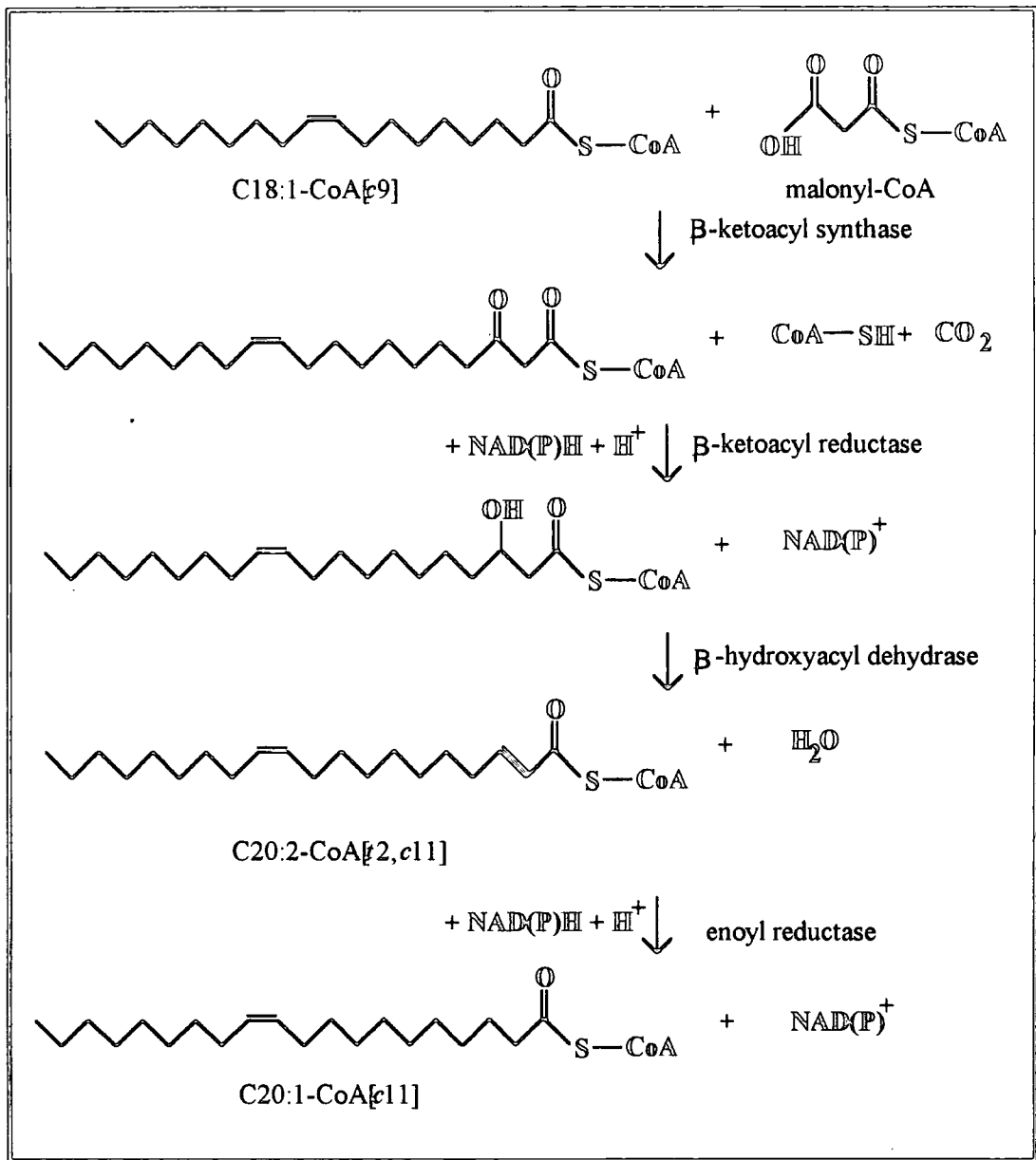
Figure 4.1 Elongation of fatty acids from C18:1[*c*9].

For obvious reasons, HEAR must contain an elongase, yet prior to 1993, determination of its subcellular location had not been determined. However, since elongase activity has been detected in microsomes from *Allium porrum* and *Pisum sativum* (pea) (Lessire *et al.*, 1992 and Macey and Stumpf, 1968, respectively), membrane-associated enoyl reductase activity is probably present in *Brassica napus* microsomes, which consist mainly of membranes from the endoplasmic reticulum. Recently, the presence of elongase activity in microsomal fractions of *Brassica napus* has been confirmed (Whitfield *et al.*, 1993 and Fuhrmann *et al.*, 1994).

Extensive studies have been performed on the substrate specificity of soluble enoyl-ACP reductase using substrates up to C16 in chain length. The published literature (table 4.1) shows that soluble enoyl-ACP reductase is capable of reducing both acyl-ACP's and acyl-CoA's, with a broad chain length specificity. These studies are limited, though, because the use of very long chain substrates was not investigated, and since soluble enoyl-ACP reductase is capable of utilising acyl-CoA's, it may reduce C20:2-CoA[*t*2, *c*11]. Determination of the use of C20:2-CoA[*t*2, *c*11] by soluble enoyl-ACP reductase is necessary because reduction of the acyl-CoA by this enzyme would lead to misinterpretation of membrane-associated enoyl reductase assays, if microsomes were contaminated with the soluble enzyme. The membrane-associated and soluble enoyl reductases could be differentiated, though, if they had different pyridine nucleotide specificities. Soluble enoyl-ACP reductase from *Brassica napus* is specific for NADH with acyl-CoA's (Slabas *et al.*, 1986), and so the requirement of NADPH by membrane-associated enoyl reductase would enable rapid differentiation from the NADH-specific soluble enzyme. Analysis of experiments performed on membrane-associated enoyl reductase activity in *Allium porrum* microsomes failed to consider the effects of contaminating soluble enoyl-ACP reductase, which could seriously affect the interpretation of activity measured

Table 4.1 The substrate specificities of soluble enoyl-ACP reductases.

Source of enoyl-ACP reductase	Pyridine nucleotide specificity	Substrate specificity	Reference
Spinacia oleracea (spinach) leaves	NADH	C6:1-ACP>C8:1-ACP>C4:1-ACP>C10:1-ACP>C12:1-ACP>C16:1-ACP>C4:1-CoA	Shimakata and Stumpf, 1982c
Carthamus tinctorius L. (safflower) seeds	NADH	C4:1-ACP>C10:-ACP>C4:1-CoA	Shimakata and Stumpf, 1982a
	NADPH > NADH	C10:1-ACP	Shimakata and Stumpf, 1982a
Brassica napus (oil seed rape) seed	NADH	C4:1-ACP>C4:1-CoA	Slabas *et al.*, 1986
E. coli	NADH	C10:1-ACP>C4:1-ACP>C4:1-CoA	Weeks and Wakil, 1968
	NADPH	C4:1-ACP>C10:1-ACP>C4:1-CoA	Weeks and Wakil, 1968

with NADH. Therefore, it is vitally important to study the substrate specificity of both the membrane-associated and soluble forms of enoyl reductase.

An end objective of the purification of any enzyme is to obtain enough protein for N-terminal sequencing and antibody production, since these are required for cDNA cloning, which aids enzyme characterisation and determination of protein structure. As a result of this, it is important to estimate the theoretical amount of membrane-associated enoyl reductase present in *Brassica napus* seeds, to see if sufficient pure protein could be isolated from the starting material, before making attempts to purify the enzyme. This can be done by comparing soluble enoyl-ACP reductase activity to that which would be required for elongation reactions. In this calculation, it is assumed that soluble enoyl-ACP reductase is involved in 8 cycles of fatty acid synthesis (from C2 to C18), membrane-associated enoyl reductase is involved in 2 cycles (from C18 to C22), C18 fatty acids are synthesised at the same rate as C22 fatty acids and that the Kcat values of the two enoyl reductases are similar. Under these circumstances, the amount of membrane-associated enoyl reductase present in the seed would be one quarter of soluble enoyl-ACP reductase. Detailed studies have been performed to determine the amount of soluble enoyl-ACP reductase in seeds (Slabas *et al.*, 1990), which is approximately 1.92 mg per 100 g of seed, as determined by quantitative Western blotting. If it is assumed that membrane-associated enoyl reductase has a similar molecular weight to soluble enoyl-ACP reductase, the theoretical calculation above suggests that sequencing of membrane-associated enoyl reductase should be possible, providing the enzyme was successfully solubilised and purified with sufficient yield. Approximately, 100 pmoles of protein are required for sequencing, therefore, 3.2 µg of enzyme would be needed. Since *Brassica napus* seeds theoretically contain approximately 0.48 mg of membrane-associated enoyl reductase per 100 g of seed, obtaining sufficient enzyme

for sequencing following purification seems feasible provided stability of the enzyme and positive identification of the protein is achieved.

Prior to purification of membrane-associated enoyl reductase, detailed knowledge of the properties of both the membrane-associated and soluble enoyl reductases is required. This would allow the development of an assay which will discriminate between the two types of enoyl reductase. Determination of the activities of both forms of the enzyme using C20:2-CoA[t2, c11] as a substrate is critical to this discrimination.

4.2 Synthesis of C20:2-CoA[t2, c11] substrate for membrane-associated enoyl reductase.

Synthesis of the assay substrate, C20:2-CoA[t2, c11], was based on the oxidation of palmitoyl-CoA to trans-hexadec-2-enoyl-CoA by acyl-CoA oxidase from Candida species (Broadway, 1992). Figure 4.2 shows the reaction catalysed by acyl-CoA oxidase to convert C20:1-CoA[c11] to C20:2-CoA[t2, c11].
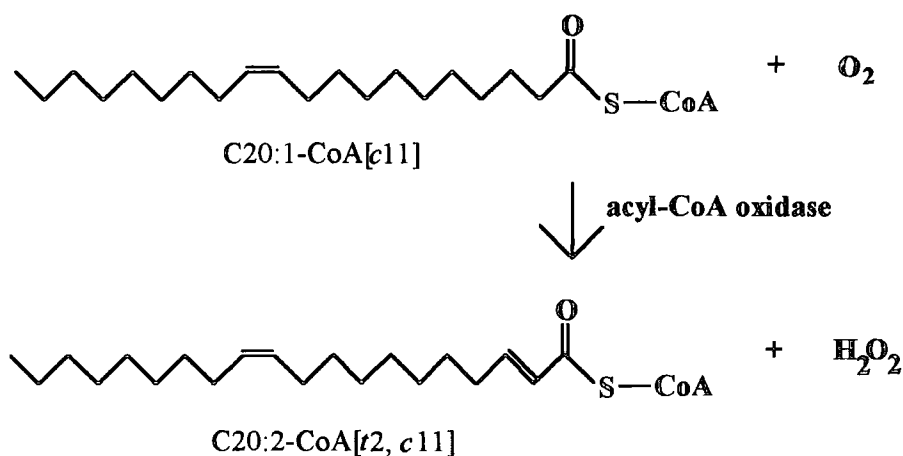
Figure 4.2 Oxidation of C20:1-CoA[c11] by acyl-CoA oxidase.

Prior to C20:2-CoA[t2, c11] synthesis, the preparation and storage of acyl-CoA oxidase and C20:1-CoA[c11] were considered. Acyl-CoA oxidase was dissolved at a concentration of 1 mg ml^{-1} (3.3 U ml^{-1}) in 100 mM potassium phosphate (pH 7.0) containing 9 % v/v glycerol. A unit of acyl-CoA oxidase is defined as the amount of enzyme that forms 1 μmole of product from substrate per minute at pH 8.0 at 30°C. The solution was stable to freezing at -80°C without loss of activity for at least six weeks; the enzyme was stored in small aliquots and only used once. C20:1-CoA[c11] was dissolved at a concentration of 5 mg ml^{-1} (5 mM) in 100 mM potassium phosphate (pH 7.0) containing 0.05 % v/v Triton X-100 to achieve solubilisation. This was also stored in small aliquots and was stable at -80°C for at least six weeks.

The reaction mixture for C20:2-CoA[t2, c11] synthesis contained 10 mM potassium phosphate (pH 8.0), 0.4 units of acyl-CoA oxidase and 30 μM C20:1-CoA[c11] in a total volume of 30 ml. The reaction, performed at 30°C, was initiated by the addition of acyl-CoA and was monitored by the increase in absorbance at 263 nm, due to the formation of the *trans* double bond (Shimizu *et al.*, 1979), until no further increase in absorbance was seen; the reaction lasted approximately 20 minutes. A molar extinction coefficient of 6.7 x 10^3 M^{-1} cm^{-1} at 263 nm was used to calculate the extent of the reaction (Stern and del Campillo, 1956).

Initial experiments gave a low yield of C20:2-CoA[t2, c11], only 43 % of C20:1-CoA[c11] was oxidised (figure 4.3). Therefore, attempts were made to increase the yield by removing hydrogen peroxide (H$_2$O$_2$), by the inclusion of various amounts of catalase to the reaction mixture. Removal of H$_2$O$_2$ would prevent the equilibrium from being obtained, so that more C20:1-CoA[c11] would be converted to C20:2-CoA[t2, c11]. Prior to its use, the biological activity of catalase was established using the method described in section 2.5.1. As shown in figure 4.3, the

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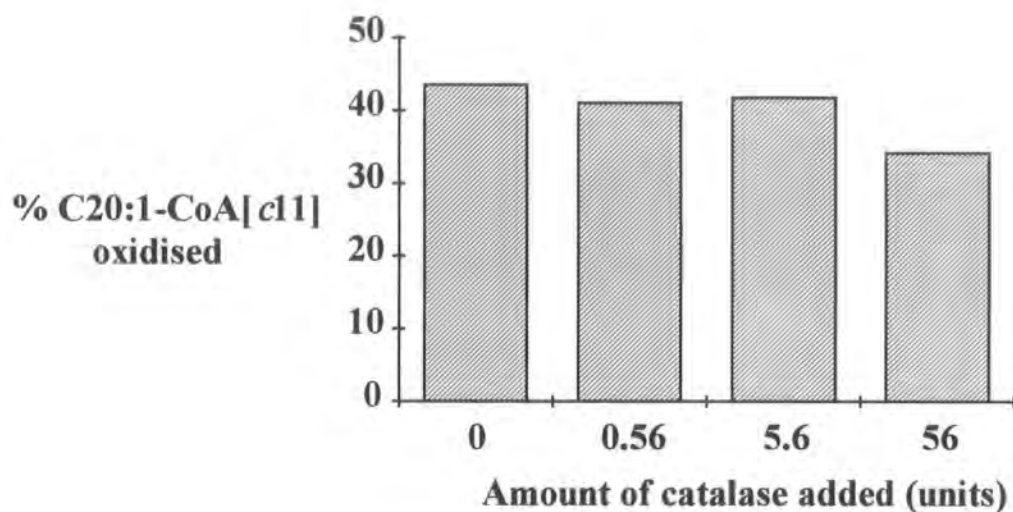
Figure 4.3 Effect of catalase on the acyl-CoA oxidase catalysed oxidation of C20:1-CoA[*c*11] to C20:2-CoA[*t*2, *c*11].

Reaction mixtures were based on the synthesis of C20:2-CoA[*t*2, *c*11] (section 4.2). The reaction mixture, in a total volume of 1 ml, contained 10 mM potassium phosphate (pH 8.0), 30 µM C20:1-CoA[*c*11], 0.01 units of acyl-CoA oxidase and the appropriate amount of catalase. The extent of the reaction was determined by monitoring the increase in absorbance at 263 nm.

addition of catalase failed to increase the amount of C20:1-CoA[c11] oxidised above 43 %, so the acyl-CoA was routinely synthesised in the absence of catalase. As the amount of C20:2-CoA[t2, c11] synthesised was sufficient for subsequent enzyme assays, the cause of the low recovery of C20:2-CoA[t2, c11] was not further investigated and the acyl-CoA was routinely synthesised as described above.

4.2.1 Concentrating C20:2-CoA[t2, c11].

Using the above experimental procedure, the concentration of synthesised C20:2-CoA[t2, c11] was calculated to be 13 µM from the absorbance at 263 nm. This was too dilute to use in an enoyl reductase assay as it is much lower than the 120 µM concentration of C4:1-CoA[t2] routinely used to assay soluble enoyl-ACP reductase (Slabas et al., 1986), and is approximately 1/14 th the apparent K_M for C4:1-CoA[t2], since soluble enoyl-ACP reductase has an apparent K_M of 178 µM for C4:1-CoA[t2] (Slabas et al., 1986). Consequently, C20:2-CoA[t2, c11] was concentrated using two methods; either extraction into n-butanol which could be removed by centrifugal evaporation, or by freeze-drying. Extraction into n-butanol was the first method used to concentrate C20:2-CoA[t2, c11], because during extraction contaminating buffer salts would be removed from the acyl-CoA, as the salts would remain in the aqueous phase. However, the recovery of C20:2-CoA[t2, c11] was calculated to be only approximately 10 %, using the quantification method described later in section 4.4. This low recovery may be due to C20:2-CoA[t2, c11] sticking to the side of the tube during centrifugal evaporation, as seen with some proteins. As an alternative, the method of concentration by freeze-drying was attempted. This method is not ideal as it causes the concentration of all components of the reaction mixture, but produced a recovery of approximately 85-90 % concentrated C20:2-CoA[t2, c11], which is perfectly adequate for further experimental use.

4.3 C20:2-CoA[t2, c11] as a putative substrate for soluble enoyl-ACP reductase.

Since the reduction of C20:2-CoA[t2, c11] by soluble enoyl-ACP reductase would complicate interpretation of membrane-associated enoyl reductase assays, it was important to determine if soluble enoyl-ACP reductase would utilise C20:2-CoA[t2, c11]. Freeze-dried C20:2-CoA[t2, c11] was resuspended in 10 mM sodium acetate/HCl (pH 6.0) and used in a soluble enoyl-ACP reductase assay at 80 μM (section 2.5.1.2). C20:2-CoA[t2, c11] was resuspended in sodium acetate/HCl because acyl-CoA's are generally stable in neutral and moderately acid solutions, but are rapidly hydrolysed in alkaline solutions. The concentration of C20:2-CoA[t2, c11] used was 80 μM rather than 120 μM as previously used in soluble enoyl-ACP reductase assays (Slabas et al., 1986), to make efficient use of the limited amount of substrate. In addition, assays were performed in a total volume of 125 μl using micro-cuvettes rather than in a total volume of 1 ml as described by Slabas et al., 1986, to further conserve substrate. Care was necessary when using micro-cuvettes to ensure that they were properly washed and dried in between reactions and so a washing procedure was developed which involved washing the micro-cuvettes with Milli Q water, then acetone and finally diethyl ether, to ensure that they were completely dry.

C20:2-CoA[t2, c11] was utilised by soluble enoyl-ACP reductase, and so the activity of soluble enoyl-ACP reductase with 80 μM C20:2-CoA[t2, c11] was compared to that obtained with an equal concentration of C4:1-CoA[t2], the usual assay substrate. The data in figure 4.4 clearly show that a greater reaction velocity was seen with C20:2-CoA[t2, c11] than with C4:1-CoA[t2]. With 1 μl of crude soluble enoyl-ACP reductase, the ratio of activities with C20:2-CoA[t2, c11] and C4:1-CoA[t2] is approximately 5:1.
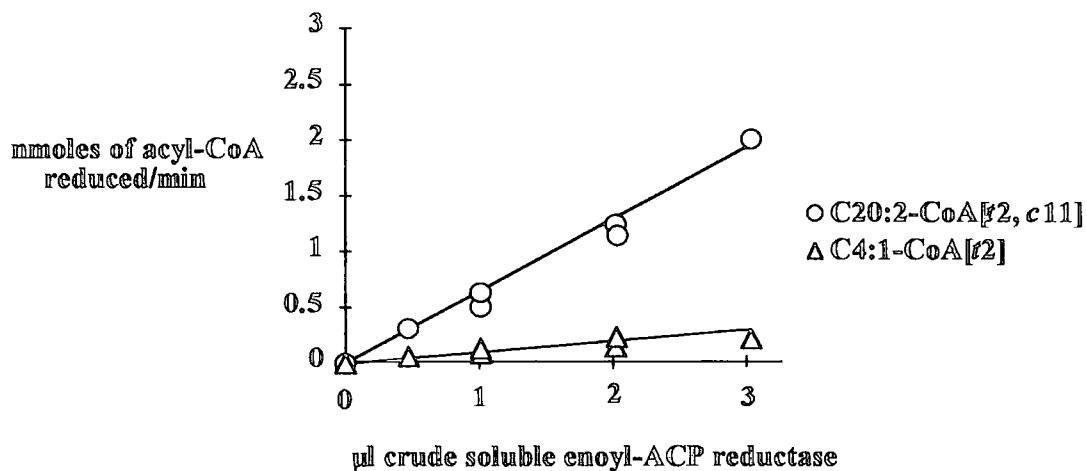
Figure 4.4 Effect of substrate chain length on the activity of crude *Brassica napus* soluble enoyl-ACP reductase.

The source of crude *Brassica napus* soluble enoyl-ACP reductase was the supernatant from a 40 000 to 200 000 x g spin. The results from two identical experiments performed concurrently are plotted.

4.4 Quantification of C20:2-CoA[t2, c11] using soluble enoyl-ACP reductase.

As soluble enoyl-ACP reductase had been shown to use C20:2-CoA[t2, c11], the enzyme was used to quantify the concentrated C20:2-CoA[t2, c11]. This was necessary because losses in biologically active acyl-CoA could have occurred during freeze-drying. Quantification was achieved by determination of the end-point of a soluble enoyl-ACP reductase catalysed reaction with this substrate. Enoyl-ACP reductase from *E. coli* catalyses an essentially irreversible reaction; attempts made to reverse the reaction by either changing the pH of the medium or by increasing the concentrations of the reaction products were unsuccessful (Weeks and Wakil, 1968). As the reaction is essentially irreversible, it has been shown that the stoichiometry of the reaction is consistent with the oxidation of one mole of reduced pyridine nucleotide for every mole of substrate reduced (Weeks and Wakil, 1968). It was assumed that enoyl-ACP reductase from *Brassica napus* would also catalyse an irreversible reaction, and so from the total absorbance change during a reaction, the amount of NADH oxidised, and hence the amount of C20:2-CoA[t2, c11] reduced could be calculated. To calculate the recovery of acyl-CoA after concentration, it was used as the limiting factor in a soluble enoyl-ACP reductase assay. The reaction mixture, in a total volume of 125 µl, contained 10 mM sodium phosphate (pH 6.2), 140 µM NADH, a limiting amount of C20:2-CoA[t2, c11] and 2 x 10^{-3} U of pure recombinant soluble enoyl-ACP reductase. The reaction was initiated by the addition of acyl-CoA and the decrease in absorbance at 340 nm was monitored until no further change was seen. Varying amounts of freeze-dried C20:2-CoA[t2, c11] were used and from the data (figure 4.5) it was shown that the amount of pyridine nucleotide oxidation was directly proportional to the amount of C20:2-CoA[t2, c11] added.
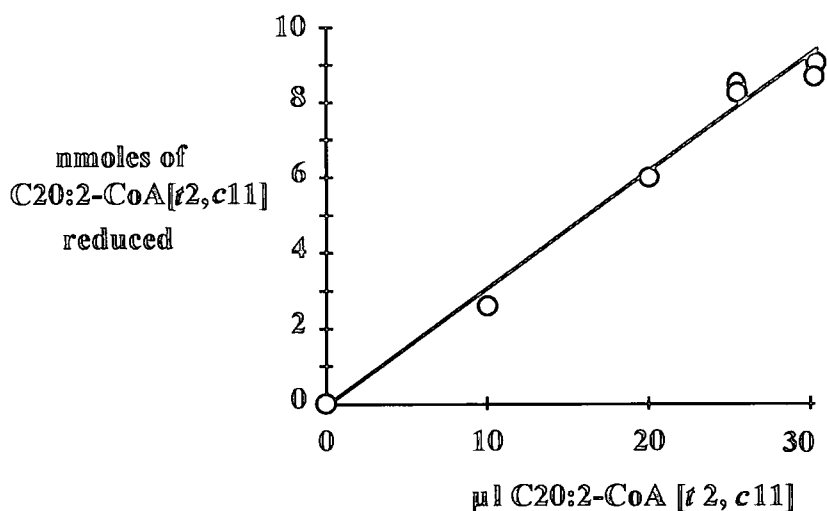
Figure 4.5 Quantification of C20:2-CoA[t2, c11] using purified soluble enoyl-ACP reductase.

Freeze-dried C20:2-CoA[t2, c11] was quantified by determining the end-point of a soluble enoyl-ACP reductase catalysed reaction. As 1 mole of reduced pyridine nucleotide is oxidised for every mole of substrate reduced, the amount of C20:2-CoA[t2, c11] reduced was calculated. The results from two identical experiments performed concurrently are plotted.

After freeze-drying, the acyl-CoA was stored in small aliquots at -20°C over desiccant. When required for an assay, the acyl-CoA was dissolved in 10 mM sodium acetate/HCl (pH 6.0) to a final concentration of 400 μM; the acyl-CoA had been concentrated 29-fold. The stability of freeze-dried C20:2-CoA[t2, c11] at -20°C was determined by quantifying the amount of active C20:2-CoA[t2, c11] remaining after storage and the data in figure 4.6 show that C20:2-CoA[t2, c11] was stable at -20°C for at least two weeks.

4.5 Preparation of microsomes from *Brassica napus* seeds.

Membrane-associated enoyl reductase activity was measured in microsomes prepared by differential centrifugation. During preparation of microsomes the temperature was maintained at 4°C. *Brassica napus* seeds, stored at -80°C, were ground in a coffee grinder for 30 seconds, to break the seeds, before homogenisation buffer was added in a buffer:seed ratio of 2.5:1 v/w. Homogenisation buffer contained 10 mM sodium phosphate (pH 7.0), 4 mM EDTA and 1 mM DTT. The cells were broken using a polytron with a 2 cm diameter probe (Kinematica, Switzerland) for 2 minutes at 20 second intervals, and the temperature, which never exceeded 10°C, was allowed to drop to 4°C between intervals. To maintain a low temperature, the extract was placed in an ice-waterbath. After filtering the extract through four layers of muslin, it was centrifuged at 40 000 x g for 30 minutes, to remove organelles and cellular debris. The fat layer was removed using a spatula, before the supernatant was centrifuged at 200 000 x g, to pellet microsomes. The time of centrifugation was dependent on the type of centrifuge and rotor used. A run time of 30 minutes was used in a Beckman Optima™ TLX ultracentrifuge (Beckman Instruments (UK) Ltd., Progress Road, Sands Industrial Estate, High Wycombe, Buckinghamshire, HP12 4JL) with a 8 x 3.2 ml fixed angle rotor; in a Beckman L-70 ultracentrifuge with a
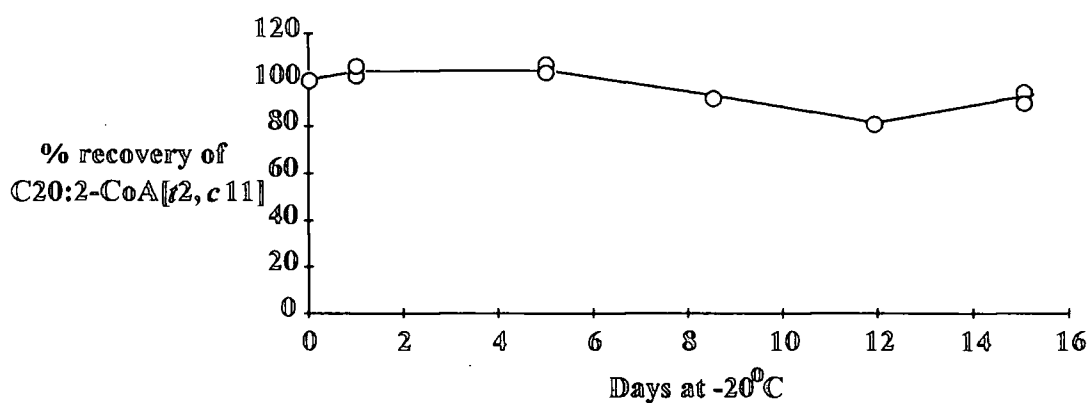
Figure 4.6 Stability of C20:2-CoA[*t*2, *c*11] at -20°C.

The stability of C20:2-CoA[*t*2, *c*11] at -20°C was determined by quantifying the amount of C20:2-CoA[*t*2, *c*11] remaining after storage. C20:2-CoA[*t*2, *c*11] recovery is shown as a percentage of the starting acyl-CoA concentration. The results from two identical experiments are plotted.

6 x 70 ml fixed angle rotor, the run time was 2 hours. This extra time was needed because the 70 ml rotor had a higher pelleting efficiency than the 3.2 ml rotor, due to the increased tube length, which meant that it took longer for microsomes to pellet.

To remove trapped soluble and loosely membrane-associated proteins from microsomes, the efficiency of three different types of salt wash was investigated. The 40 000 - 200 000 x g microsomal pellet was resuspended in homogenisation buffer containing 0.1 M sodium chloride, 0.5 M sodium chloride or 0.1 M sodium carbonate (pH 11.5) and centrifuged at 200 000 x g, as described above. The volume of wash buffer used was equal to the volume of homogenisation buffer used. Sodium chloride was used since it removes trapped soluble proteins from microsomes (Rock and Cronan, 1979), and peripheral membrane proteins by interrupting electrostatic interactions (Thomas and McNamee, 1990). In comparison, sodium carbonate (pH 11.5) removes ribosomes from endoplasmic reticulum membranes, converts closed membrane vesicles to open sheets, thus releasing trapped soluble proteins, and causes peripheral membrane proteins to be released into the supernatant (Fujiki *et al.*, 1982). Washing was important as a purification step, in particular to remove soluble enoyl-ACP reductase from microsomes, since utilisation of C20:2-CoA[*t*2, *c*11] by soluble enoyl-ACP reductase could interfere with membrane-associated enoyl reductase assays.

Final microsomal pellets were resuspended in homogenisation buffer (10 mM sodium phosphate (pH 7.0), 4 mM EDTA and 1 mM DTT) in 1/20 th of the starting volume, frozen in liquid nitrogen and stored in small aliquots at -80°C. The enzyme was never refrozen to prevent any denaturation that might occur due to repetitive freeze-thawing. This enabled the production of a uniform starting material for subsequent experiments.

4.6 Assaying membrane-associated enoyl reductase activity in *Brassica napus* seeds.

The assay for membrane-associated enoyl reductase was a modification of the soluble enoyl-ACP reductase assay. Activity was measured by following the substrate dependent oxidation of pyridine nucleotide at 340 nm. Assays were performed at 25°C and the reaction mixture, in a total volume of 125 μl, contained 10 mM sodium phosphate (pH 6.2), 140 μM NADH, 80 μM C20:2-CoA[*t*2, *c*11] and an appropriate amount of resuspended microsomal pellet (usually 10 μl). The reaction was initiated by the addition of acyl-CoA, after any endogenous pyridine nucleotide oxidase activity had been measured. As the amount of acyl-CoA used in a soluble enoyl-ACP reductase assay had been successfully reduced by limiting its concentration to 80 μM and by performing reactions in a total volume of only 125 μl, this procedure was applied to membrane-associated enoyl reductase assays to conserve both substrate and enzyme extract.

Using microsomes that had been washed with 0.5 M sodium chloride, the assay was linear over the range of enzyme extract measured (figure 4.7). Care had to be taken when measuring the initial velocity of the reactions, though, as the change in absorbance with respect to time, was not linear for long; the reaction velocity would soon decrease, usually after approximately 30 seconds (figure 4.8). This may have been due to the instability of the enzyme in the reaction mixture. Yet, despite this decrease in velocity with time, reproducible initial velocities could be measured as shown by the reasonable agreement in duplicates (figure 4.7).
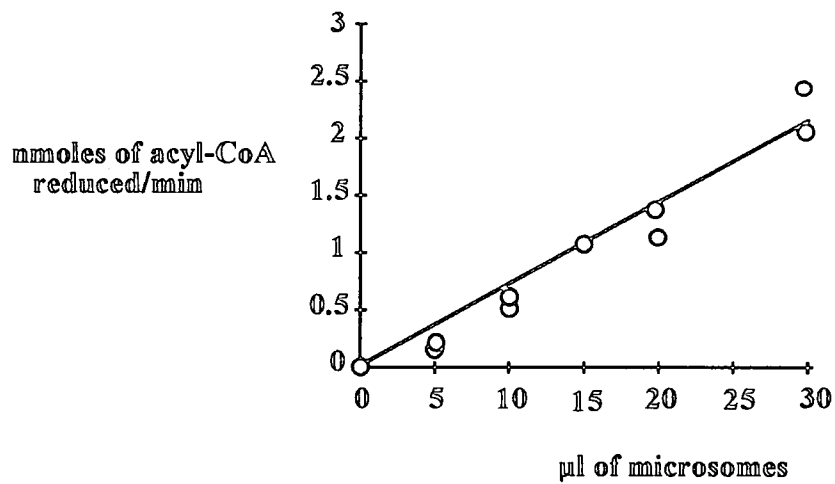
Figure 4.7 Linearity of the assay for NADH utilising membrane-associated enoyl reductase.

The results from two identical experiments are plotted.

Time(mins)

1   2

(nmoles of acyl-CoA
reduced/min)

0.02

change in
absorbance

0.04

1=5 µl microsomes   (0.10)

2=10 µl microsomes   (0.54)

3=15 µl microsomes   (1.03)

4=20 µl microsomes   (1.39)

1

4   3   2
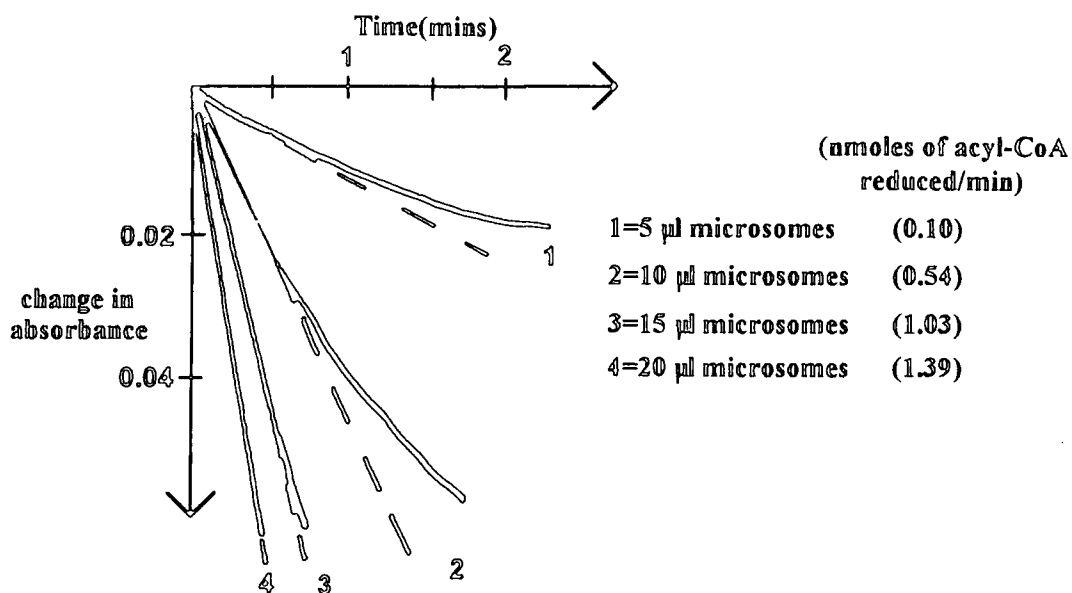
Figure 4.8 Typical traces obtained from membrane-associated enoyl reductase assays highlighting the difficulties involved in determining initial velocities.

To determine the amount of soluble enoyl-ACP reductase removed from microsomes by the three different types of washing, 10 μl of resuspended microsomes were assayed using the method described in section 4.6, with C20:2-CoA[$t2$, $c11$], C4:1-CoA[$t2$] and both pyridine nucleotides. The results are shown in figure 4.9. It has previously been shown that soluble enoyl-ACP reductase reduces both C20:2-CoA[$t2$, $c11$] and C4:1-CoA[$t2$] with NADH (section 4.3). Little was known about the substrate specificity of membrane-associated enoyl reductase, except that it could reduce C20:2-CoA[$t2$, $c11$] in the presence of NADH (section 4.6). Using washed microsomes, activity was also detected in the presence of NADPH and C20:2-CoA[$t2$, $c11$], and since soluble enoyl-ACP reductase cannot use NADPH with acyl-CoA's, activity with this pyridine nucleotide must have been due to membrane-associated enoyl reductase. This was the first evidence to suggest that membrane-associated enoyl reductase from *Brassica napus* seeds could utilise both pyridine nucleotides. As no activity was detected with C4:1-CoA[$t2$] and NADPH, it was assumed that membrane-associated enoyl reductase could not utilise C4:1-CoA[$t2$] and consequently, any activity seen with C4:1-CoA[$t2$] and NADH must be due to trapped soluble enoyl-ACP reductase.

The optimal washing method used 0.5 M sodium chloride, as this removed more trapped soluble enoyl-ACP reductase than both the "no salt" and 0.1 M sodium chloride washes, and produced the highest activities of membrane-associated enoyl reductase with both NADH and NADPH. Consequently, this method was used to wash subsequent microsomes.

By assaying unwashed and 0.5 M sodium chloride washed microsomes with 80 μM C4:1-CoA[$t2$] and NADH, to determine the amount of soluble enoyl-ACP reductase
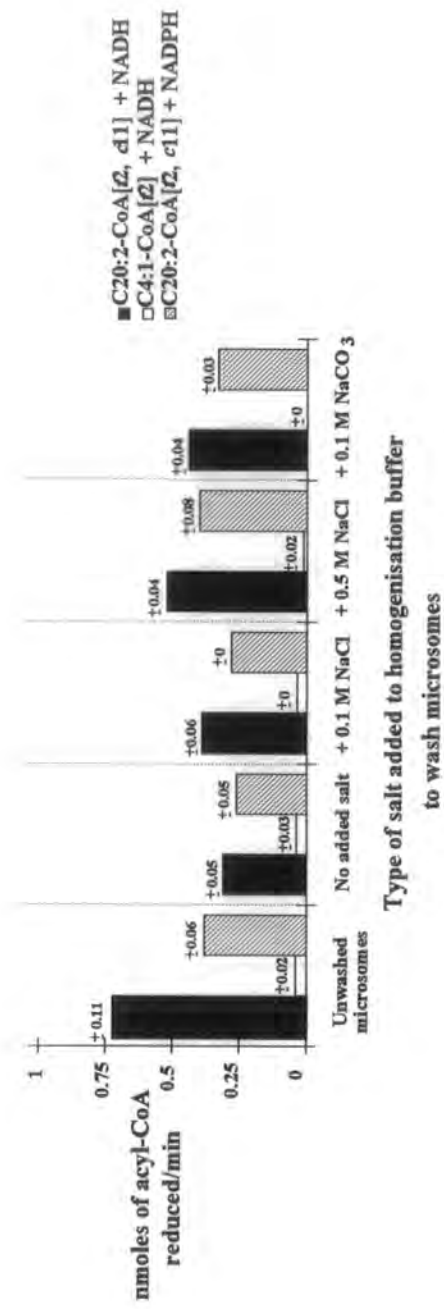
Figure 4.9 Effectiveness of salt washes to remove contaminating soluble enoyl-ACP reductase from microsomes, without loss of membrane-associated enoyl reductase activity.

Microsomes, prepared as described in section 4.5, were washed in homogenisation buffer containing the appropriate salt and the final pellets were resuspended in homogenisation buffer containing no added salt. Assays were done in duplicate, the means are plotted and the standard deviations are shown.

removed by washing, it was observed that 67 % of contaminating soluble enoyl-ACP reductase was removed. The results were not affected by membrane-associated enoyl reductase, as this enzyme is unable to reduce C4:1-CoA[$t2$]. In comparison, it can be seen that no NADPH membrane-associated enoyl reductase activity is lost due to this washing method. The amount of NADH membrane-associated enoyl reductase removed by washing cannot be determined because part of the reduction in activity with C20:2-CoA[$t2$, $c11$] is due to the removal of the soluble enzyme.

To determine the amount of total soluble protein removed by washing microsomes with 0.5 M sodium chloride, fractions were analysed by SDS PAGE (section 2.5.6). It can be clearly seen in figure 4.10 that a large proportion of contaminating proteins are removed from microsomes by this washing step. However, washing does not remove an equal amount of all proteins, they are selectively removed. For example, in lane 1, the band marked A is present at a higher concentration than the band marked B, yet after washing, it can be seen that the ratio has changed in favour of band B. Determination of the total protein concentration by the Bio-Rad protein assay (described in section 2.5.3) showed that the protein concentration of the resuspended microsomes was approximately 2 mg/ml and the 0.5 M sodium chloride wash removed 70 % of total protein from the microsomes (table 4.2).

Table 4.2 Comparison of total protein concentration of unwashed and washed microsomes.

The means and standard deviations of two experiments are shown.

Microsomal pellet	Total Protein (mg/ml)
Unwashed	5.54 ± 0.66
Washed with 0.5 M sodium chloride	1.80 ± 0.30
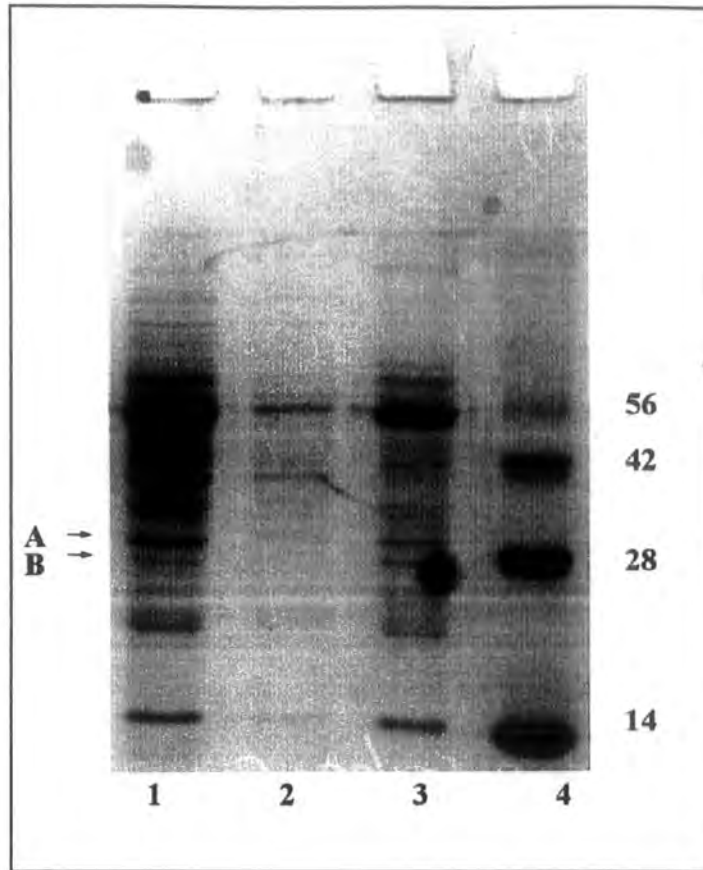
Figure 4.10 SDS PAGE gel showing the protein samples obtained during the preparation of washed microsomes.

The above photograph shows a 10 % w/v polyacrylamide gel stained with Coomassie blue. The ratio of volumes loaded in lanes 1, 2 and 3 is 5:1:25, respectively.

Lane 1) unwashed microsomal pellet

Lane 2) supernatant from a 0.5 M sodium chloride wash

Lane 3) 0.5 M sodium chloride washed microsomal pellet

Lane 4) molecular weight standards (56, 42, 28, 14 kDa)

The specific activities of NADPH membrane-associated enoyl reductase in unwashed and 0.5 M sodium chloride washed microsomes were calculated. The data in table 4.3 show that by this washing procedure, NADPH membrane-associated enoyl reductase was purified approximately 2.6-fold.

Table 4.3 Comparison of the specific activities of NADPH-utilising membrane-associated enoyl reductase in unwashed and washed microsomes.
Assays were done in duplicate; the means and standard deviations are shown.

Microsomal pellet	Specific activity (nmoles/mg/min)	Purification
Unwashed	6.86 ± 0.33	1
Washed with 0.5 M sodium chloride	17.78 ± 0.11	2.59

In summary, this is a useful, rapid purification step, since 67 % soluble enoyl-ACP reductase activity and 70 % total protein are removed by the 0.5 M sodium chloride wash, but no NADPH-utilising membrane-associated enoyl reductase activity is lost.

4.8 Optimisation of the membrane-associated enoyl reductase assay.

Several studies have been reported in the literature to optimise elongase activities *in vitro*. Investigations into the elongation of C16:0-CoA in rat liver microsomes showed that the rate of enoyl-CoA reductase activity was markedly influenced by BSA (Bernert and Sprecher, 1978). The reductase was six times more active when the substrate to albumin molar ratio was 2:1 compared to the rate obtained in the absence of albumin. In addition, studies in *Limnanthes alba* (meadowfoam) showed

that the presence of 1 mM magnesium chloride and 2 mM DTT in the reaction mixture doubled the rate of VLCFA biosynthesis (Lardans and Trémolières, 1992).

The separate and combined effects of BSA, magnesium chloride and DTT on membrane-associated enoyl reductase activity with NADH in *Brassica napus* microsomes were studied to optimise the assay. Assays were performed using C20:2-CoA[*t*2, *c*11], with the addition of BSA, magnesium chloride and DTT as shown in figure 4.11. The data show that BSA, at a molar ratio of 2:1 (substrate: BSA), increased membrane-associated enoyl reductase activity in *Brassica napus* microsomes by 34 % and 0.8 mM magnesium chloride increased activity by 82 %. DTT (1.6 mM) did not affect enzyme activity. However, the effects of BSA and magnesium chloride were both increased in the presence of DTT.

Even though BSA, magnesium chloride and DTT were not essential for membrane-associated enoyl reductase activity, none of them inhibited activity. Consequently, the effect of all three present in an assay was investigated and together they increased the activity measured by approximately 116 %, and therefore, all were included in subsequent reaction mixtures.

Following the above results, the effect of BSA concentration on membrane-associated enoyl reductase activity was further examined. The data in figure 4.12 shows that membrane-associated enoyl reductase was most active in the presence of BSA at a concentration of 3 mg/ml (i.e. 40 μM). At this concentration, the substrate to albumin molar ratio was 2:1. If the concentration of BSA was increased, activity decreased. These results were also observed during investigations into the elongation of C16:0-CoA in rat liver microsomes (Bernert and Sprecher, 1978).

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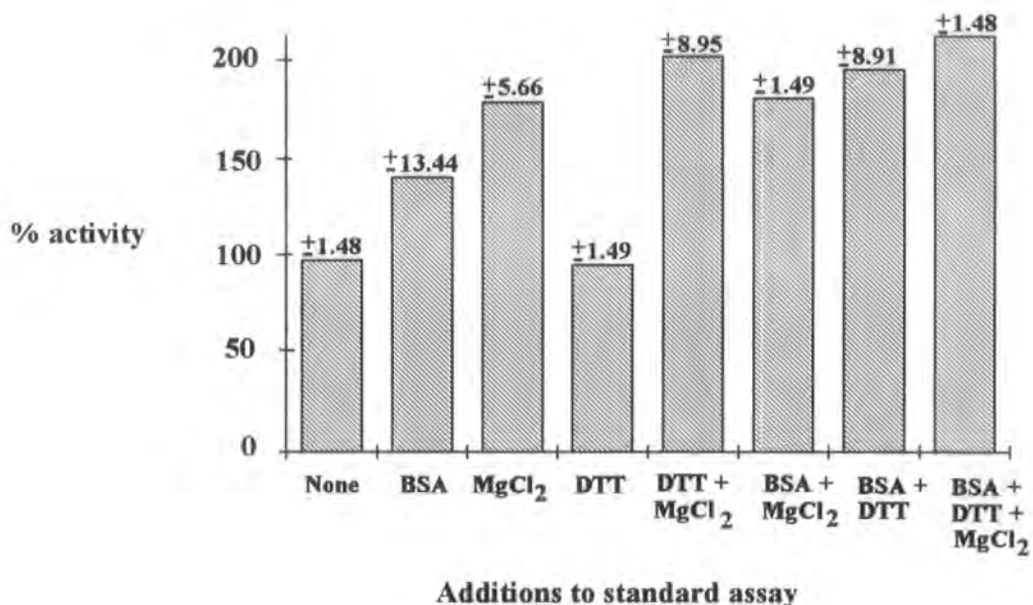
Figure 4.11 Combined effects of BSA, magnesium chloride and DTT on the initial velocity of membrane-associated enoyl reductase activity.

Assays were performed as described in section 4.6, except 40 µM BSA, 0.8 mM MgCl$_2$ and 1.6 mM DTT were included in the reaction mixture as indicated. In the absence of MgCl$_2$, 10 mM EDTA was added to chelate any Mg^{2+} present in the crude microsomes. Assays were done in duplicate and activities are expressed as a percentage of the standard reaction mixture rate. The means are plotted and the standard deviations are shown
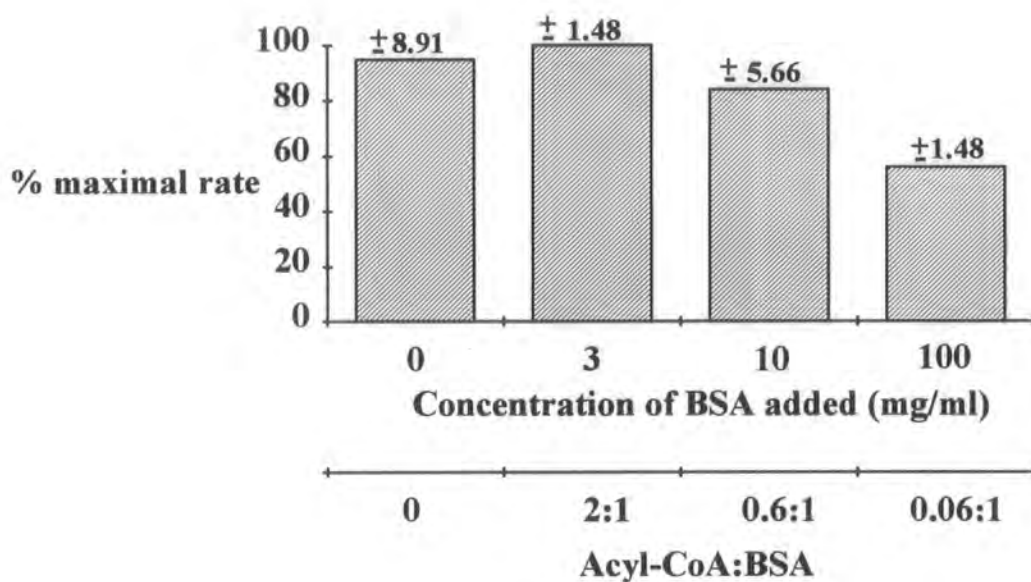
Figure 4.12 Effect of BSA on the initial velocity of membrane-associated enoyl reductase activity.

Assays were performed as described in section 4.6, except 0.8 mM $MgCl_2$, 1.6 mM DTT and the appropriate amount of BSA (as indicated) were included in the reaction mixture. Assays were done in duplicate and activities are expressed as a percentage of the maximum rate. The means are plotted and the standard deviations are shown.

4.9 Initial characterisation of membrane-associated enoyl reductase.

Optimisation of the membrane-associated enoyl reductase assay and preparation of washed microsomes enabled membrane-associated enoyl reductase to be characterised in a more effective way than with totally unrefined systems. Several parameters were then investigated.

4.9.1 Effect of freezing microsomes at -80°C.

Washed microsomes could be stored at -80°C, after rapid freezing in liquid nitrogen, for at least 8 weeks without loss of membrane-associated enoyl reductase activity (table 4.4).

Table 4.4 Effect of freezing microsomes at -80°C.

The means and standard deviations of four experiments are shown.

	Membrane-associated enoyl reductase activity (nmoles of C20:2-CoA[t2, c11] reduced/min)	
	+ NADH	+ NADPH
Freshly prepared microsomes	0.86 ± 0.08	0.50 ± 0.01
Microsomes stored at -80°C	0.87 ± 0.06	0.48 ± 0.05

This is an important consideration as a "stock" of frozen microsomes could be used for future characterisation studies. Microsomes were stored in small aliquots and were never refrozen, to eliminate any problems that might occur due to repeated freeze-thawing.

4.9.2 Pyridine nucleotide specificity of membrane-associated enoyl reductase.

The pyridine nucleotide specificity of membrane-associated enoyl reductase was determined by assaying membrane-associated enoyl reductase with C20:2-CoA[$t2$, $c11$] and either NADH or NADPH. The results, in figure 4.13, show that unlike soluble enoyl-ACP reductase from *Brassica napus*, which can only use NADH with acyl-CoA's, membrane-associated enoyl reductase can utilise both NADH and NADPH with C20:2-CoA[$t2$, $c11$]; NADH being the preferred reductant. The ratio of activities with NADH and NADPH for membrane-associated enoyl reductase was approximately 2:1. Membrane-associated enoyl reductase from *Allium porrum* microsomes can also utilise both pyridine nucleotides; however, NADPH is the preferred reductant (Spinner *et al.*, 1995).

4.9.3 Effect of pH on membrane-associated enoyl reductase.

To determine the optimal pH to assay membrane-associated enoyl reductase, the effect of pH on enzyme activity was investigated using a variety of buffers, over the pH range of 4.5-9.0. This would also aid in discrimination between soluble and membrane-associated enoyl reductases. Assays were performed under optimal conditions with washed microsomal pellets (using both NADH and NADPH) and purified recombinant soluble enoyl-ACP reductase (using NADH). The very long chain substrate C20:2-CoA[$t2$, $c11$] was used for all assays and buffers were used at 80 mM in their optimal pH ranges. As shown in figure 4.14, three distinct pH profiles were obtained; membrane-associated enoyl reductase was most active in MOPS/NaOH (pH 6.0) and MES/NaOH (pH 5.0) when assayed with NADH and NADPH, respectively, whereas soluble enoyl-ACP reductase was most active in Tris/HCl (pH 8.0). Activities measured in MOPS/NaOH with NADH membrane-associated enoyl reductase were clearly affected by pH, but under the same

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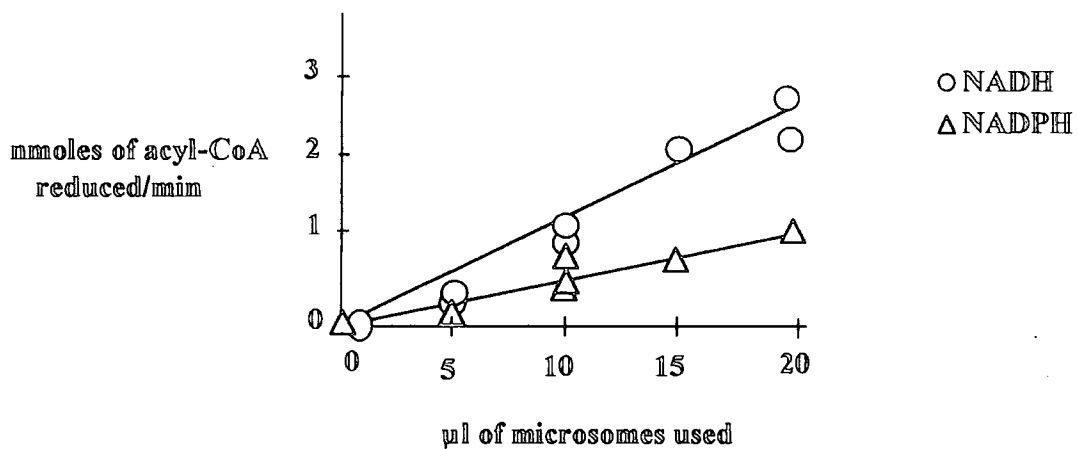
Figure 4.13 Pyridine nucleotide specificity of membrane-associated enoyl reductase.

The results of two identical experiments are shown.
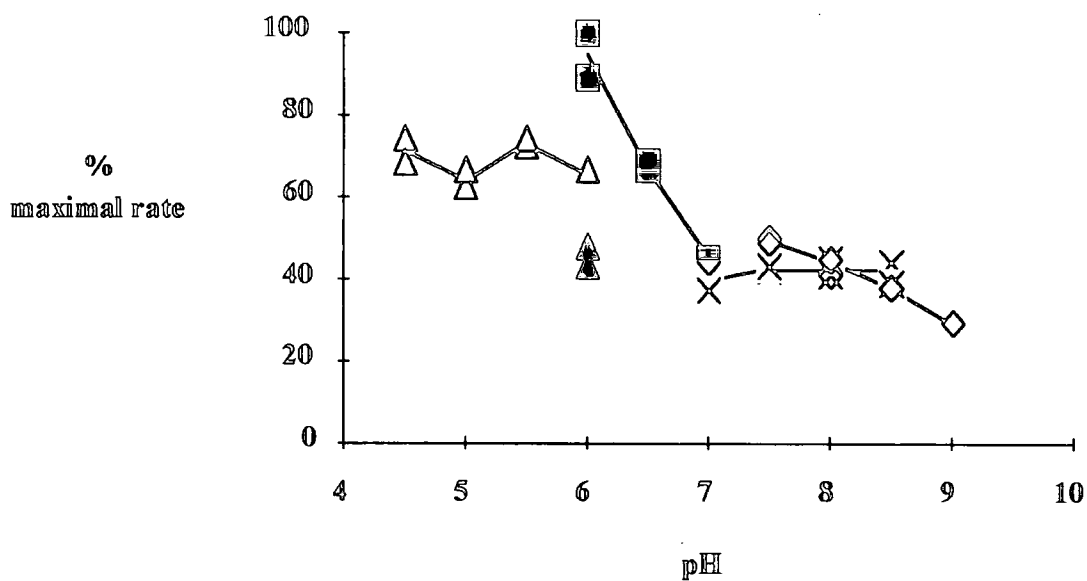
a) membrane-associated enoyl reductase with NADH
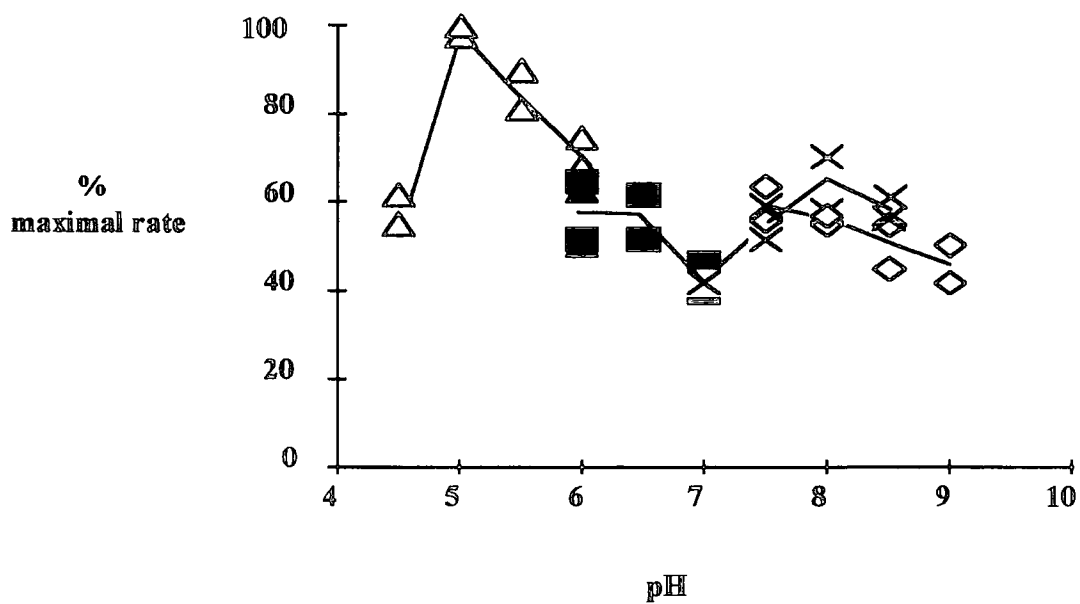
b) membrane-associated enoyl reductase with NADPH

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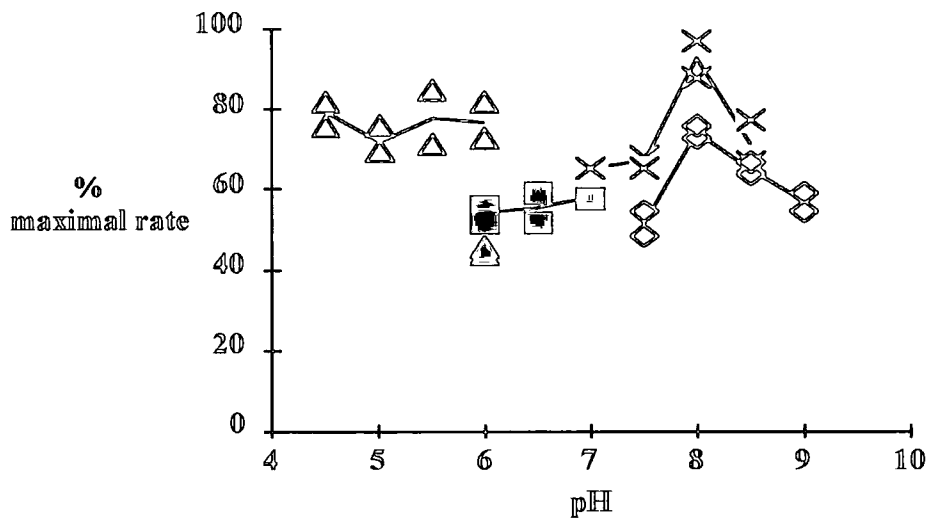
c) soluble enoyl-ACP reductase with NADH

The buffers used were △ MES/NaOH ,
◩ MOPS/NaOH ,
✕ Tris/HCl ,
△ K phosphate ,
◇ Bicine

Figure 4.14 pH profiles of enoyl reductases.

For membrane-associated enoyl reductase assays, 10 µl of a washed microsomal pellet was used, for soluble enoyl-ACP reductase assays, purified recombinant enzyme was used. Activities are expressed as a percentage of the maximum rate (1.29, 0.58 and 0.8 nmoles of acyl-CoA reduced/min for membrane-associated enoyl reductase with NADH, membrane-associated enoyl reductase with NADPH and soluble enoyl reductase with NADH, respectively). The results of two identical experiments are plotted.

conditions, NADPH membrane-associated enoyl reductase and NADH soluble enoyl-ACP reductase were pH independent. By comparison, in MES/NaOH, only NADPH membrane-associated enoyl reductase was markedly affected by pH. At pH 6.0, NADPH membrane-associated enoyl reductase was not affected by the buffer used, but NADH soluble enoyl-ACP reductase was most active in MES/NaOH and NADH membrane-associated enoyl reductase was strongly activated by MOPS/NaOH.

These data clearly suggest that NADH-utilising membrane-associated enoyl reductase is different to soluble enoyl-ACP reductase, and as a result of this, activities previously measured in microsomes in this thesis were due to membrane-associated enoyl reductase and not contaminating soluble enoyl-ACP reductase. The results also suggest that microsomes contain two separate membrane-associated enoyl reductases, one that utilises NADH and a second form that utilises NADPH. This is the first evidence to suggest that two forms of membrane-associated enoyl reductase exist in *Brassica napus* microsomes. Alternatively, only one membrane-associated enoyl reductase enzyme may exist whose active sites are affected differently by changing pH.

Since membrane-associated enoyl reductase was most active in MOPS/NaOH (pH 6.0) and MES/NaOH (pH 5.0) with NADH and NADPH, respectively, subsequent standard assays were performed at these pH's, as described in section 2.5.1.3.

4.9.4 Determination of the K_M for membrane-associated enoyl reductase substrates.

The apparent K_M values for *Brassica napus* membrane-associated enoyl reductase substrates were determined, using the standard reaction mixtures, except the concentration of one of the substrates was varied. The results, in figure 4.15, show

a)

(nmoles of substrate reduced/min)$^{-1}$

X NADH
O NADPH

-0.1 -0.075 -0.05 -0.025   0   0.025   0.05   0.075   0.1

[C20:2-CoA[$\mathit{2}$, c11]]$^{-1}$ (μM)

b)

(nmoles of substrate reduced/min)$^{-1}$

X NADH
O NADPH

-0.1   -0.08   -0.06   -0.04   -0.02   0   0.02   0.04

[pyridine nucleotide]$^{-1}$ (μM)

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c)
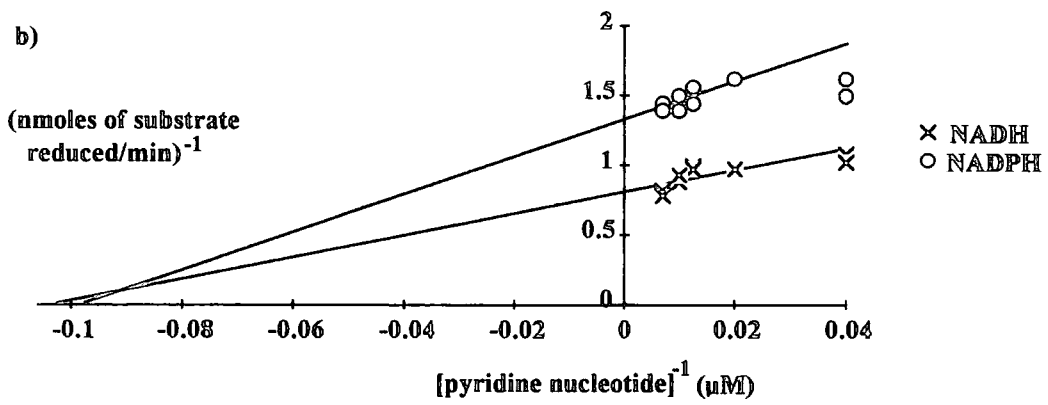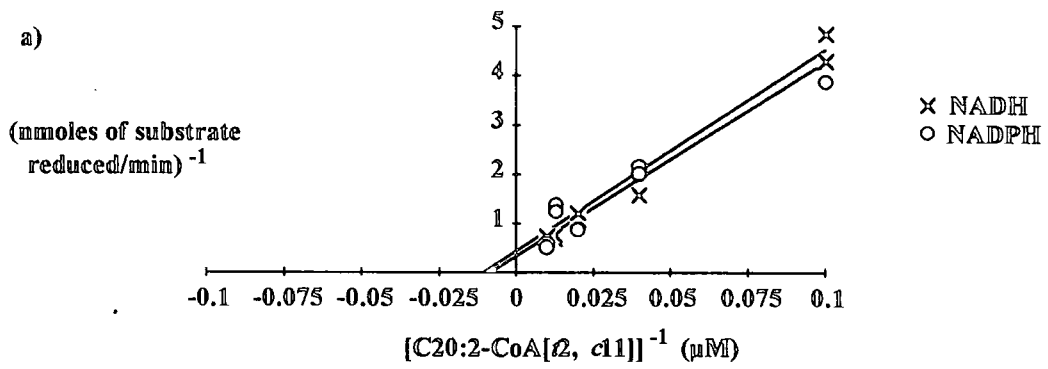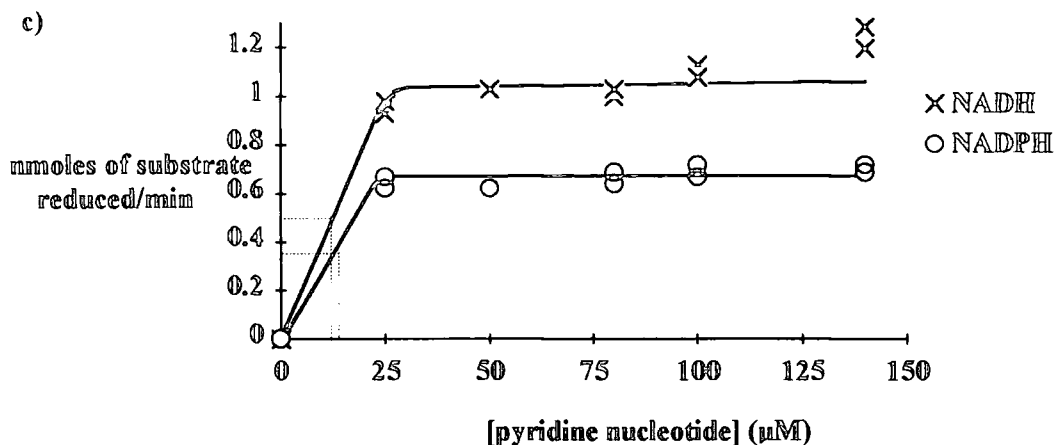
Figure 4.15 Determination of the apparent K_M's for membrane-associated enoyl reductase substrates.

a) Lineweaver-Burk plot of membrane-associated enoyl reductase with C20:2-CoA[$t2$, $c11$] as the variable substrate. The pyridine nucleotide concentration was 140 μM.

b) Lineweaver-Burk plot of membrane-associated enoyl reductase with pyridine nucleotide as the variable substrate. The C20:2-CoA[$t2$, $c11$] concentration was 80 μM.

c) A Michaelis-Menton plot of membrane-associated enoyl reductase with pyridine nucleotide as the variable substrate. The C20:2-CoA[$t2$, $c11$] concentration was 80 μM.

The results of two identical experiments are plotted.

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that the apparent K_M for C20:2-CoA[t2, c11] with NADH and NADPH was approximately 100 μM and 125 μM, respectively. Calculation of the K_M's for NADH and NADPH was more difficult as there was departure from linearity. However, from the Michaelis-Menton plot, which is saturating, it can be determined that the apparent K_M for NADH was approximately 9 μM, whereas for NADPH, it was approximately 11 μM. This is the first determination of K_M values for an enoyl reductase using the C20:2-CoA[t2, c11] substrate.

These values are similar to those for soluble enoyl-ACP reductase which has an apparent K_M for NADH of 7.6 μM and a K_M of 178 μM for C4:1-CoA[t2] (Slabas *et al.*, 1986).

The K_M values for membrane-associated enoyl reductase from *Allium porrum* microsomes have been determined for the pyridine nucleotides and are 9 and 35 μM for NADPH and NADH, respectively (Spinner *et al.*, 1995); a concentration of 9 μM C20:1-CoA[t2] was used in these studies. These values are similar to those obtained for membrane-associated enoyl reductase from *Brassica napus* microsomes.

4.9.5 To determine if the mutation in fatty acid elongation in LEAR is due to loss of membrane-associated enoyl reductase activity.

As previously stated, in the original variety of *Brassica napus*, erucic acid accounted for 44 % of the total fatty acids accumulated in the seed (Hilditch and Williams, 1964). HEAR was replaced by low erucic acid rape (LEAR), developed by classical breeding methods (Stefansson *et al.*, 1961), after studies suggested that oils containing erucic acid had detrimental nutritional effects. However, there is now increased interest in the biosynthesis of erucic acid because of its commercial value.

Since the nature of the mutation that prevents elongation of fatty acids to erucic acid in LEAR has not been investigated, assays were performed to determine if the enoyl reductase component of the elongase had been mutated to produce LEAR. Microsomes were prepared from two varieties of *Brassica napus*; Miranda, a high erucic acid rape (HEAR) variety and Falcon, a low erucic acid rape (LEAR) variety and 10 μl (18 μg total protein) of each were assayed for membrane-associated enoyl reductase activity. Equivalent activities were measured in both types of microsome (table 4.5), suggesting that the mutation did not involve membrane-associated enoyl reductase.

Table 4.5 Membrane-associated enoyl reductase activity in HEAR and LEAR. The means and standard deviations of eight experiments are shown.

Type of *Brassica napus*	Membrane-associated enoyl reductase activity (nmoles/mg/min)	
	+ NADH	+ NADPH
LEAR	37.4 ± 5.0	28.9 ± 5.0
HEAR	47.5 ± 5.0	26.1 ± 3.0

4.10 Comparison of the quantity of soluble and membrane-associated enoyl reductases in *Brassica napus*.

To compare the quantity of soluble and membrane-associated enoyl reductases in *Brassica napus*, the maximum rates of enoyl reductase activities at the optimal pH, under optimal conditions, using C20:2-CoA[*t*2, *c*11] were determined (section 2.5.1.3). To be comparable with membrane-associated enoyl reductase assays,

soluble enoyl-ACP reductase assays (section 2.5.1.2) also contained 40 μM BSA, 0.8 mM magnesium chloride and 1.6 mM DTT in the reaction mixture. 0.5 M sodium chloride washed microsomes were used to assay membrane-associated enoyl reductase and the source of soluble enoyl-ACP reductase was the supernatant from a 40 000 to 200 000 x g spin. The results obtained are shown in table 4.6.

Table 4.6 Maximum rates of enoyl reductase activities.

Assays were done in duplicate, and the means and standard deviations are shown.

Enzyme	Activity (μmoles/10 g seed)
Membrane-associated enoyl reductase + NADH	0.108 ± 0.02
Membrane-associated enoyl reductase + NADPH	0.059 ± 0.002
Total membrane-associated enoyl reductase activity	0.167 ± 0.02
Soluble enoyl-ACP reductase + NADH	7.23 ± 0.61

The ratio of membrane-associated enoyl reductase activity to soluble enoyl-ACP reductase was approximately 1:40. As it was expected that membrane-associated enoyl reductase would be one quarter the activity of soluble enoyl-ACP reductase (section 4.1), one tenth of the expected membrane-associated enoyl reductase activity was detected.

4.11 Discussion.

The research in this chapter was aimed at the development of an assay to identify and initially characterise membrane-associated enoyl reductase from *Brassica napus* seeds, which is involved in the synthesis of VLCFA's.

An optical assay was designed based on the decrease in absorbance at 340 nm due to the oxidation of pyridine nucleotide. Development of this assay required the enzymatic synthesis of C20:2-CoA[t2, c11], which was achieved by the oxidation of C20:1-CoA[c11] by acyl-CoA oxidase. As soluble enoyl-ACP reductase could utilise C20:2-CoA[t2, c11], it was used to quantify the synthesised acyl-CoA.

C20:2-CoA[t2, c11] was used to assay membrane-associated enoyl reductase in *Brassica napus* seed microsomes, that had been washed with 0.5 M sodium chloride, to remove contaminating soluble enoyl-ACP reductase and other soluble proteins. Membrane-associated enoyl reductase activity was measured and detected in both HEAR and LEAR varieties, suggesting that the mutation that prevents erucic acid biosynthesis in LEAR is not the membrane-associated enoyl reductase. Therefore, whilst the site of mutation has still to be determined, it is known that is it not the enoyl reductase catalysed step.

Optimisation of the membrane-associated enoyl reductase assay was achieved by the inclusion of BSA, DTT and magnesium chloride in the reaction mixture. Membrane-associated enoyl reductase was most active when the substrate to BSA molar ratio was 2:1 and this is similar to observations with the enoyl-CoA reductase involved in the elongation of C16:0-CoA in rat liver microsomes (Bernert and Sprecher, 1978). At this particular ratio, the reverse reaction in rat liver microsomes, catalysed by the dehydrase, was partially inhibited. However, if the substrate to albumin molar ratio was either increased or decreased, the activity of the enoyl reductase in rat liver microsomes decreased. It was thought that a decrease in BSA concentration favoured dehydrase activity for the reverse reaction, yet an increase in BSA concentration decreased the activities of both the enoyl reductase and dehydrase; BSA may bind to the substrate in such a way that the resulting complex is less accessible to either enzyme. At the optimum ratio of substrate to BSA, it is thought

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that BSA may to bind the substrate and prevent or retard micelle formation, but when the substrate concentration exceeds the critical micelle concentration, the micelles that form may inhibit the enzyme, or alternatively they may bind to the enzyme but be acted upon at a slower rate than the monomeric form (Bernert and Sprecher, 1978).

A linear, reproducible micro-assay was developed to assay membrane-associated enoyl reductase; a micro-assay was necessary to conserve both acyl-CoA and enzyme extract. Assays showed that both NADH and NADPH could be utilised by membrane-associated enoyl reductase, whereas soluble enoyl-ACP reductase could only utilise NADH. In addition, membrane-associated enoyl reductase can only reduce the very long chain acyl-CoA, yet both C4:1-CoA[t2] and C20:2-CoA[t2, c11] are substrates for soluble enoyl-ACP reductase. Consequently, both membrane-associated and soluble enoyl-ACP reductase activities exist in *Brassica napus* seeds.

Further discrimination of soluble and membrane-associated enoyl reductases was obtained from their distinct pH profiles; the data also suggested the existence of two membrane-associated enoyl reductases, based on their pyridine nucleotide requirements. The profile obtained for soluble enoyl-ACP reductase with C20:2-CoA[t2, c11] had an optimum at Tris/HCl pH 8.0 which is different to the profile obtained by Slabas *et al.*, in 1986, using C4:1-CoA[t2], where the profile had two pH optimums, at MOPS/NaOH pH 6.0 and Bicine pH 7.5. The differences seen may be because this study was performed with purified recombinant enzyme, rather than with purified *Brassica napus* seed enzyme (as used by Slabas *et al.*, 1986), or due to the differences in substrate used. Studies on thioesterase-B from Peking duck uropygial glands, showed that the pH dependence of the rate varied with the chain length of the substrate. At pH 9.0, the highest rates were observed with C8, C10, C12 and C14 acyl-CoA; yet at pH 8.0, clear preference was shown for C10-CoA,

whereas C8 and C12 showed slightly over half of the rate observed with C10, and C14 gave a much lower rate (Cheesbrough and Kolattukudy, 1985). Therefore, it is possible that soluble enoyl-ACP reductase has a pH optimum with C20:2-CoA[$t2$, $c11$] that differs from the pH optimum with C4:1-CoA[$t2$]. Since substrate specificity can be altered by pH, care has to be taken when comparing rates with different substrates, as is the case with soluble enoyl-ACP reductase which is more active with C20:2-CoA[$t2$, $c11$] than C4:1-CoA[$t2$] at pH 6.2.

The apparent K_M's for membrane-associated enoyl reductase substrates were determined and were approximately 9 μM and 11 μM for NADH and NADPH, respectively. The K_M for C20:20-CoA[$t2$, $c11$] with NADH and NADPH was approximately 100 μM and 125 μM, respectively. These values are similar to those for soluble enoyl-ACP reductase from Brassica napus (Slabas et al., 1986) and membrane-associated enoyl reductase from Allium porrum microsomes (Spinner et al., 1995).

It was determined that the membrane-associated enoyl reductase activity measured in Brassica napus seed microsomes was one tenth of the calculated expected activity (section 4.1).

In summary, an assay to measure membrane-associated enoyl reductase activity was developed and was used to determine that membrane-associated enoyl reductase can utilise both pyridine nucleotides. And since membrane-associated enoyl reductase, when assayed with NADH, has a different pH profile to NADH-utilising soluble enoyl-ACP reductase, activity measured in microsomes cannot be due to trapped soluble enoyl-ACP reductase.

Chapter 5: Discrimination of the different types of enoyl reductase in *Brassica napus*.

5.1 Introduction.

Brassica napus seeds contain both soluble and membrane-associated forms of enoyl reductase. The substrate specificities of both of these types of enoyl reductase were studied to a limited extent in chapter 4. In summary, data suggest that membrane-associated enoyl reductase is specific for very long chain acyl-CoA's; C20:2-CoA[*t*2, *c*11] is reduced, but C4:1-CoA[t2] is not. In comparison, soluble enoyl-ACP reductase can reduce both very long chain and short chain acyl-CoA's. Therefore, the acyl-CoA substrate specificities of membrane-associated and soluble enoyl reductases suggest that the enzymes are distinct. Yet, since both enoyl reductases utilised the pyridine nucleotide, NADH, further studies were required to ensure that activity measured in microsomes with NADH were due to membrane-associated enoyl reductase and not contaminating NADH soluble enoyl-ACP reductase. This was achieved by determining the effect pH has on activity since membrane-associated enoyl reductase with NADH is most active in MOPS/NaOH (pH 6.0), whereas NADH soluble enoyl-ACP reductase is optimal in Tris-HCl (pH 8.0) (section 4.9.3).

In comparison to soluble enoyl-ACP reductase, it has been shown that membrane-associated enoyl reductase can utilise both NADH and NADPH (section 4.9.2), but it is not known whether there is one enzyme present in microsomes that can use both pyridine nucleotides, or whether two distinct enzymes exist. The pyridine nucleotide specificities of other enoyl reductases are summarised in table 5.1; enoyl reductases from *Brassica napus* and *Chlorella vulgaris* are specific for NADH, whereas the enzyme from *Streptomyces collinus* is specific for NADPH. From other sources, the

Table 5.1 Pyridine nucleotide specificities of enoyl reductases.

Source of enoyl reductase	Type of enoyl reductase	Pyridine nucleotide	Substrate used	Reference
Rat liver microsomes	Membrane-associated	NADPH	C4:1-CoA[t2] and C6:1-CoA[t2]	Prasad et al., 1983
		NAD(P)H	C16:1-CoA[t2]	
Allium porrum (leek) seedling microsomes	Membrane-associated	NADPH > NADH	C20:1-CoA[t2] > C18:1-CoA[t2] > C16:1-CoA[t2]	Spinner et al., 1995
Brassica napus (oil seed rape) seed	Soluble	NADH	C4:1-ACP> C4:1-CoA	Slabas et al., 1986
Chlorella vulgaris	Soluble	NADH	C4:1-ACP[t2]	Saito et al., 1980
Carthamus tinctorius L. (safflower) seeds	Soluble	NADH	C4:1-ACP> C10:-ACP> C4:1-CoA	Shimakata and Stumpf, 1982a
		NADPH > NADH	C10:1-ACP	Shimakata and Stumpf, 1982a
E. coli	Soluble	NADH	C10:1-ACP> C4:1-ACP> C4:1-CoA	Weeks and Wakil, 1968
		NADPH	C4:1-ACP> C10:1-ACP> C4:1-CoA	Weeks and Wakil, 1968
Streptomyces collinus	Soluble	NADPH	C4:1-CoA	Wallace et al., 1995

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situation is more complex, as there is evidence of enzyme activity with both NADH and NADPH. This requires further research, since to date, there is no evidence of a purified enoyl reductase utilising both pyridine nucleotides; most pyridine nucleotide requiring enzymes are specific for one particular pyridine nucleotide (Boyer, 1970). There are, however, exceptions to this, such as glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* that can utilise NAD^+ and $NADP^+$ equally well (de Moss, 1955). During purification of the enzyme, the ratio of activities with NAD^+ and $NADP^+$ remains at 0.67, suggesting the existence of a single enzyme that is non-specific in pyridine nucleotide requirement. This hypothesis is supported by the fact that NAD^+ reduction is inhibited by NADPH.

Further studies are clearly needed to determine the number of enoyl reductases present in microsomes. In addition to this, it would also be interesting to compare the properties of NADH membrane-associated and soluble enoyl reductases so that an assay that discriminates between the two could be designed.

The soluble enoyl-ACP reductase used in these studies was a purified recombinant protein, which is available in milligram quantities. A cDNA for soluble enoyl-ACP reductase has been isolated from a *Brassica napus* seed library (Kater *et al.*, 1991) and expression of enoyl-ACP reductase cDNA in *E. coli* enables large amounts of protein to be easily purified.

5.2 Discrimination of soluble and membrane-associated enoyl reductases.

5.2.1 Effect of diflufenican on enoyl reductases.

Diflufenican is a herbicide used for the selective control of broad leaf and grass weeds in winter cereals. Its primary mode of action is on carotenoid biosynthesis,

but it also inhibits plant fatty acid synthesis (Ashton *et al.*, 1994b). As diflufenican has structural similarities to pyridine nucleotides, the effect of its action on *Persea americana* (avocado) β-ketoacyl-ACP reductase and enoyl-ACP reductase was studied. The herbicide had no effect on β-ketoacyl ACP reductase activity, but competitively inhibited enoyl-ACP reductase activity; the K_M for NADH was increased in the presence of diflufenican (Ashton *et al.*, 1994b).

Since diflufenican inhibits soluble enoyl-ACP reductase from *Persea americana*, studies were performed to determine the effect of 25 μM diflufenican (in 2.5 % v/v ethanol) on both soluble and membrane-associated forms of enoyl reductase from *Brassica napus* seeds to determine whether a differential assay could be developed. Membrane-associated enoyl reductase was assayed with C20:2-CoA[*t*2, *c*11] and NADH, using the standard method described in section 2.5.1.3, except varying amounts of NADH (100, 50 and 25 μM) were used and the reaction mixture contained 25 μM diflufenican. Soluble enoyl-ACP reductase activity was assayed with C20:2-CoA[*t*2, *c*11] or C4:1-CoA[*t*2] and NADH (section 2.5.1.2), except varying amounts of NADH were used, as in membrane-associated enoyl reductase assays, and the reaction mixture also contained 25 μM diflufenican. Different amounts of NADH were used because it had previously been shown that diflufenican increases the K_M for NADH (Ashton *et al.*, 1994b), so any inhibition caused by diflufenican is more likely to be detected at lower pyridine nucleotide concentrations. Since diflufenican was dissolved in 2.5 % v/v ethanol, control reactions contained 2.5 % v/v ethanol. During assays, diflufenican or ethanol were added to the reaction mixture after the enzyme, but before the acyl-CoA was added to initiate the reaction; the reaction mixture was not preincubated before the addition of acyl-CoA. Table 5.2 shows the results obtained.

Table 5.2 Effect of 25 μM diflufenican on enoyl reductase activity.

Activities are expressed as a percentage of the rates obtained in the presence of 2.5 % v/v ethanol. Assays were done in duplicate and the means and standard deviations are shown.

	% activity remaining		
	Soluble enoyl-ACP reductase + C4:1-CoA[$t2$]	Soluble enoyl-ACP reductase + C20:2-CoA[$t2$, $c11$]	Membrane-associated enoyl reductase + C20:2-CoA[$t2$, $c11$]
100 μM NADH	81 ± 1.5	86 ± 3.0	110 ± 2.9
50 μM NADH	56 ± 5.5	85 ± 1.6	103 ± 0.0
25 μM NADH	63 ± 4.1	99 ± 2.1	104 ± 14.3

When assayed with C20:2-CoA[$t2$, $c11$], neither soluble enoyl-ACP reductase nor membrane-associated enoyl reductase were significantly inhibited by diflufenican, so consequently, this method cannot be used to discriminate between soluble and membrane-associated forms of enoyl reductase. Interestingly, though, soluble enoyl-ACP reductase was inhibited by diflufenican when assayed with C4:1-CoA[$t2$]. The differing effects of diflufenican on soluble enoyl-ACP reductase when assayed with C4:1-CoA[$t2$] and C20:2-CoA[$t2$, $c11$] may be due to different rate limiting steps depending on the substrate used. When C4:1-CoA[$t2$] is used as substrate, the rate limiting step probably involves pyridine nucleotide binding or releasing, as diflufenican increases the K_M for NADH (Ashton et al., 1994b). Yet, when C20:2-CoA[$t2$, $c11$] is used as substrate, diflufenican does not increase the K_M for NADH, so a different part of the reaction may be rate limiting. This could be release of the acyl-CoA from the enzyme complex, which might be seen if the very long chain acyl-

CoA was a better substrate than C4:1-CoA[t2] and, as a result bound more strongly to the enzyme.

It is not uncommon for enzymes to have different rate limiting steps for different substrates. For example, chymotrypsin is a protein-hydrolysing enzyme, which catalyses the hydrolysis of amide and ester bonds via acyl enzyme intermediates (Price and Stevens, 1989). During the hydrolysis of esters, the rate of the deacylation step is pH dependent and can be slowed down to such an extent that at low pH the acyl enzyme can be isolated. In comparison, when amides are hydrolysed, it would appear that the deacylation step is more rapid than the formation of the acyl enzyme (Price and Stevens, 1989).

5.2.2 Effect of salt on enoyl reductases.

The effect of sodium chloride on the enoyl reductases was studied in an attempt to determine whether three enoyl reductases are present in *Brassica napus* seeds. Membrane-associated enoyl reductase assays were performed under standard conditions with NADH and NADPH (section 2.5.1.3), except 0 - 0.8 M sodium chloride was present in the reaction mixture. Soluble enoyl reductase assays (section 2.5.1.2) were performed with C20:2-CoA[t2, c11] and NADH, including sodium chloride (0 - 0.8 M); and to ensure proper buffering, due to the high concentration of salt present, the usual 10 mM sodium phosphate (pH 6.2) was replaced by 80 mM buffer. In addition, soluble enoyl-ACP reductase was assayed in the presence of 40 μM BSA, 0.8 mM DTT and 1.6 mM magnesium chloride to be comparable to membrane-associated enoyl reductase assays. The data in figure 5.1 show that activities were affected differently by sodium chloride. For example, in the presence of 0.08 M sodium chloride, membrane-associated enoyl reductase activity with NADH and NADPH was reduced by approximately 33 and 59 %, respectively. In

Figure 5.1 Effect of sodium chloride on enoyl reductase activity.

Activities are expressed as a percentage of the maximum rate. Assays were done in duplicate; the means are plotted and the standard deviations are shown.

comparison, soluble enoyl-ACP reductase activity was not inhibited, suggesting the existence of three forms of enoyl reductase; one soluble and two membrane-associated. For maximal activity, membrane-associated enoyl reductase required no added sodium chloride and 0.4 M sodium chloride, when assayed with NADH and NADPH, respectively. However, soluble enoyl-ACP reductase required the presence of 0.08 M sodium chloride in the reaction mixture. This again suggests three types of enoyl reductase in *Brassica napus* seeds as proposed by the three distinct pH profiles (section 4.9.3), suggesting the existence of two enoyl reductases in microsomes, differing by their pyridine nucleotide requirement.

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5.2.3 Effect of the detergent, Triton X-100, on enoyl reductases.

The effect of Triton X-100 on enoyl reductase activity was also investigated to further discriminate between the NADH and NADPH utilising membrane-associated enoyl reductases. Standard membrane-associated enoyl reductase assays were performed, except the reaction mixture also contained 0 - 0.08 % v/v Triton X-100. Soluble enoyl-ACP reductase assays were performed with C20:2-CoA[*t*2, *c*11] and NADH, in the presence of Triton X-100 (0 - 0.08 % v/v) and, to be comparable to membrane-associated enoyl reductase assays, 40 μM BSA, 0.8 mM DTT and 1.6 mM magnesium chloride. In all assays, Triton X-100 was added after the enzyme, but before the reaction was initiated with C20:2-CoA[*t*2, *c*11]. As shown in figure 5.2, membrane-associated enoyl reductase was inhibited more than the soluble enzyme in the presence of 0.04 % v/v Triton X-100; membrane-associated enoyl reductase was inhibited by approximately 82 % and 77 % when assayed with NADH and NADPH, respectively, whereas soluble enoyl-ACP reductase was only inhibited by approximately 53 %. It is not unexpected that a detergent inhibits a membrane-associated enzyme more than a soluble one, because the detergent could partially solubilise the membrane-associated enzyme, resulting in possible loss of activity.
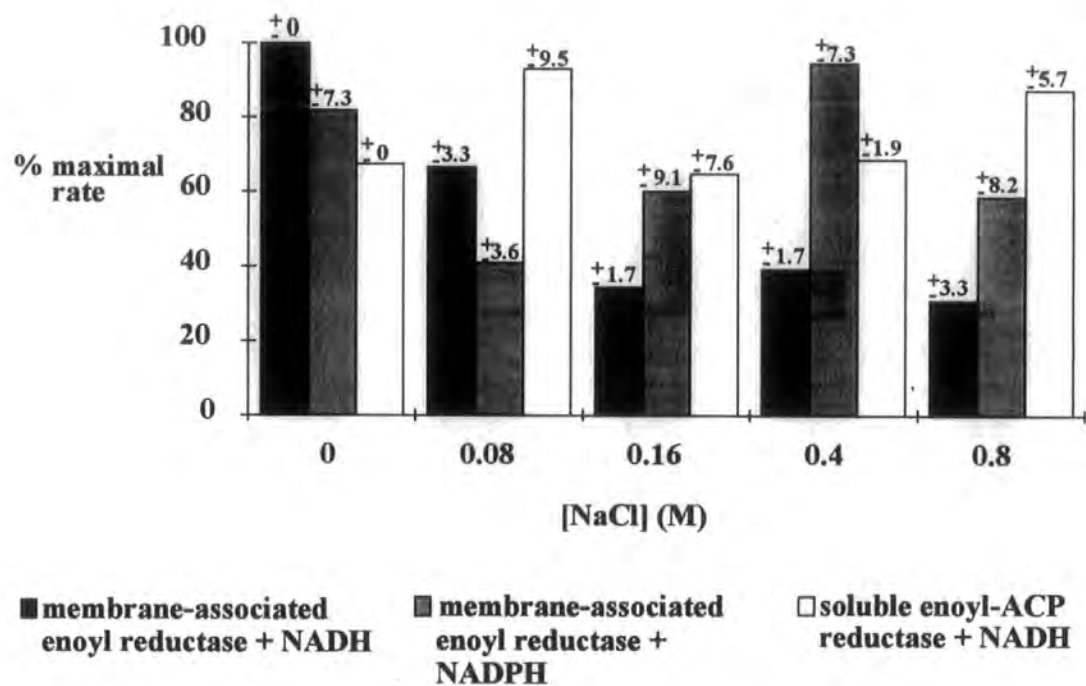
Figure 5.2 Effect of Triton X-100 on enoyl reductases.

Activities are expressed as a percentage of the maximum rate. Assays were done in duplicate; the means are plotted and standard deviations are shown.

Even though these data show that NADH membrane-associated and soluble enoyl reductases are distinct, it is not clear whether the utilisation of both pyridine nucleotides by membrane-associated enoyl reductase is due to the presence of one or two enzymes, and therefore, further studies are required to clarify this.

5.3 Differentiation of two membrane-associated enoyl reductases.

5.3.1 Thermal stability of membrane-associated enoyl reductases.

The thermal stability of membrane-associated enoyl reductase was investigated by incubating microsomes at either 50°C or 60°C (for up to 30 minutes) prior to assaying for activity. The results show that activity detected with NADPH is more thermally stable than activity assayed with NADH at both temperatures (figure 5.3). After incubation at 60°C for 30 minutes, membrane-associated enoyl reductase was still active with NADPH, but the NADH utilising form was inactive. This strongly indicates that the NADH and NADPH membrane-associated enoyl reductases are probably present in *Brassica napus* seeds as separate enzymes.

5.3.2 Effect of detergents on membrane-associated enoyl reductases.

The effects of octyl glucoside, CHAPS and LDAO on the two membrane-associated enoyl reductases were investigated as a prelude to solubilisation attempts, as the concentration of detergent in the assay mixture might prevent solubilised enzyme from being detected even if it was there. Assays were performed with the appropriate amount of detergent: 0 - 3 % w/v octyl glucoside, 0 - 0.5 % w/v CHAPS and 0 - 0.1 % v/v LDAO, and the results in figure 5.4 support the hypothesis of the existence of two membrane-associated enzymes because the activities with both pyridine nucleotides were affected differently by all three detergents. At high levels of octyl glucoside (1 % w/v) and LDAO (0.05 % v/v)

150
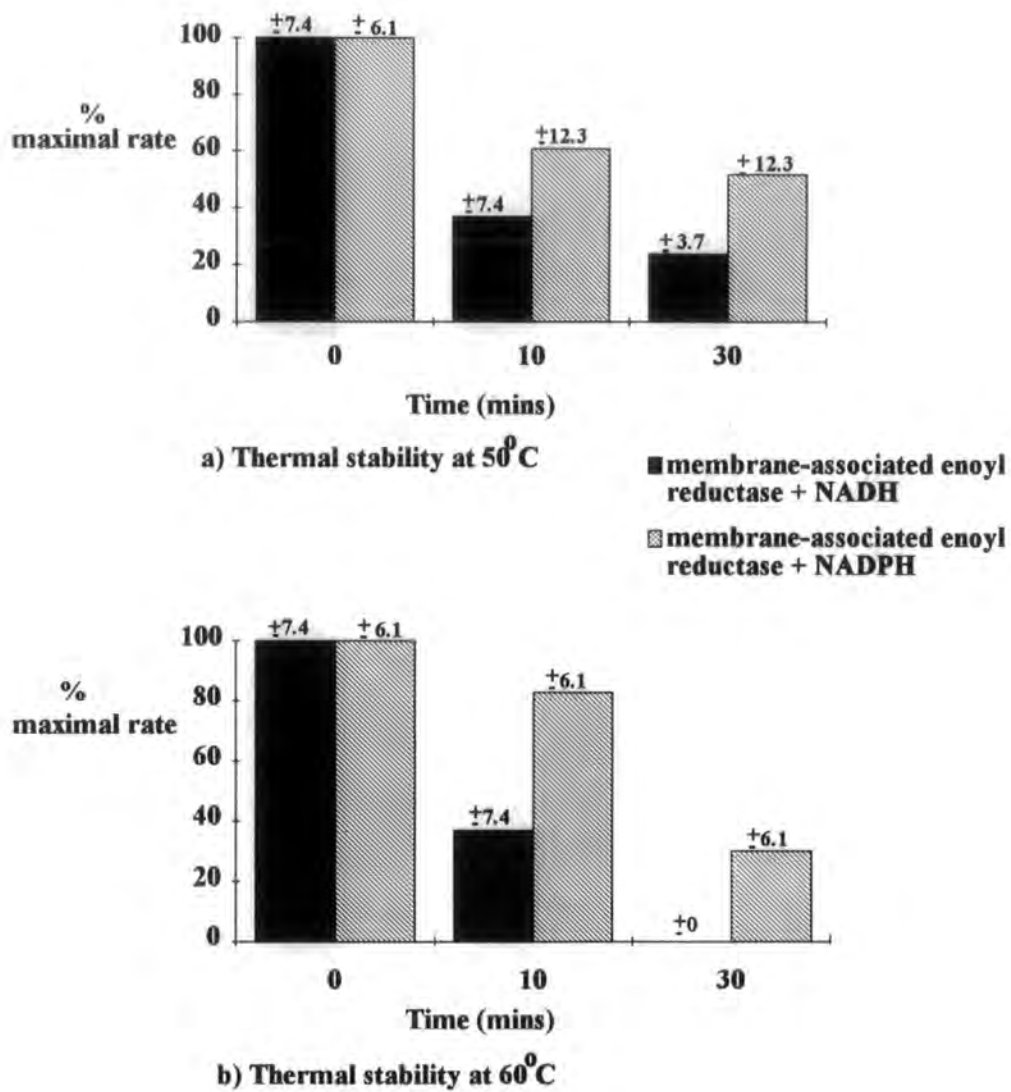
Figure 5.3 Thermal stability of membrane-associated enoyl reductases.

Activities are expressed as a percentage of the maximum rate. Assays were done in duplicate; the means are plotted and standard deviations are shown.

% maximal rate

% w/v octyl glucoside

% maximal rate

% w/v CHAPS
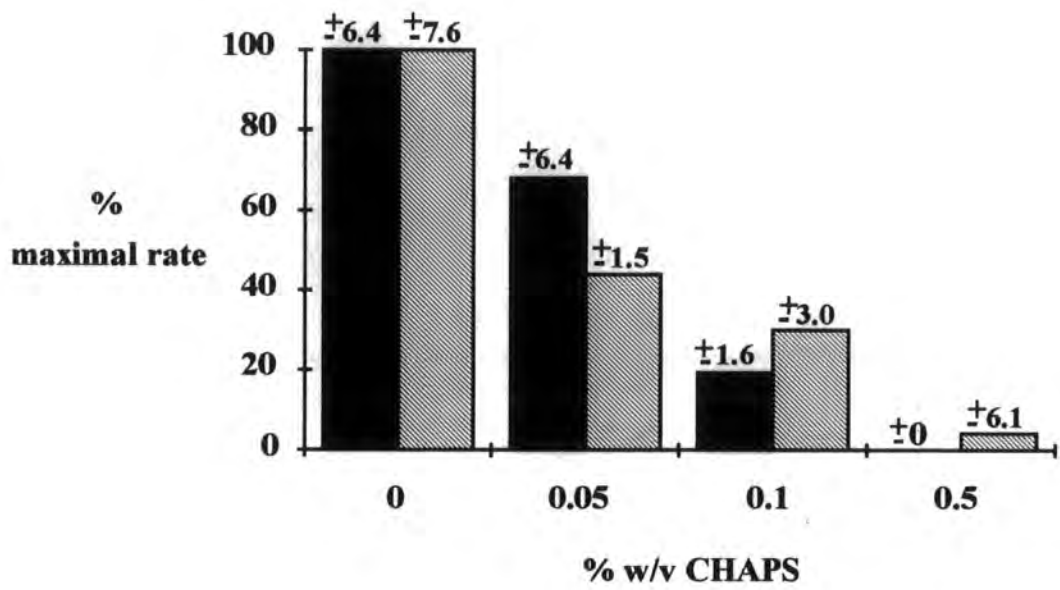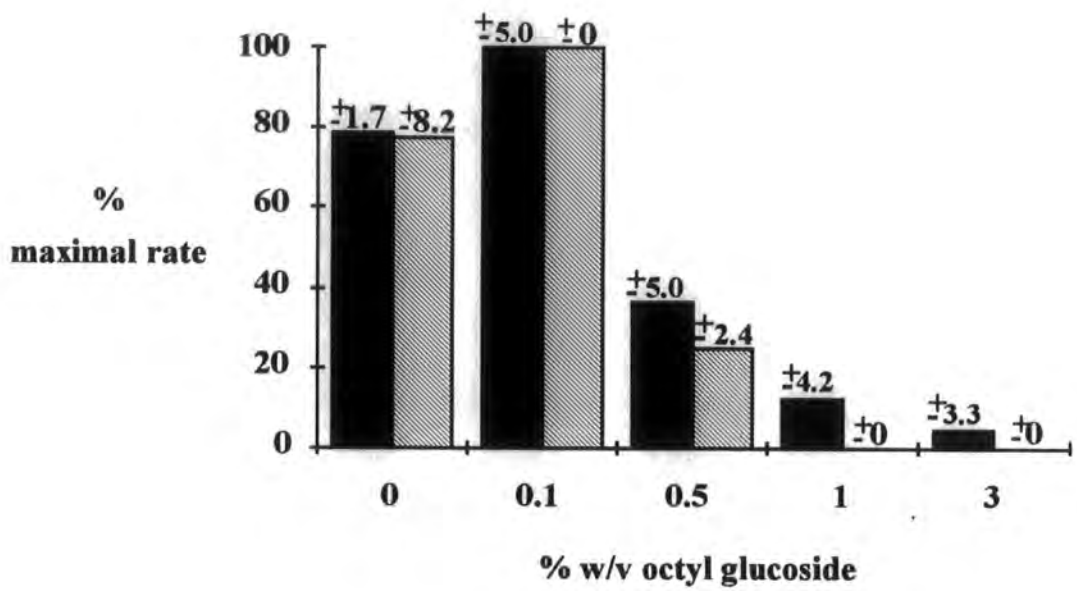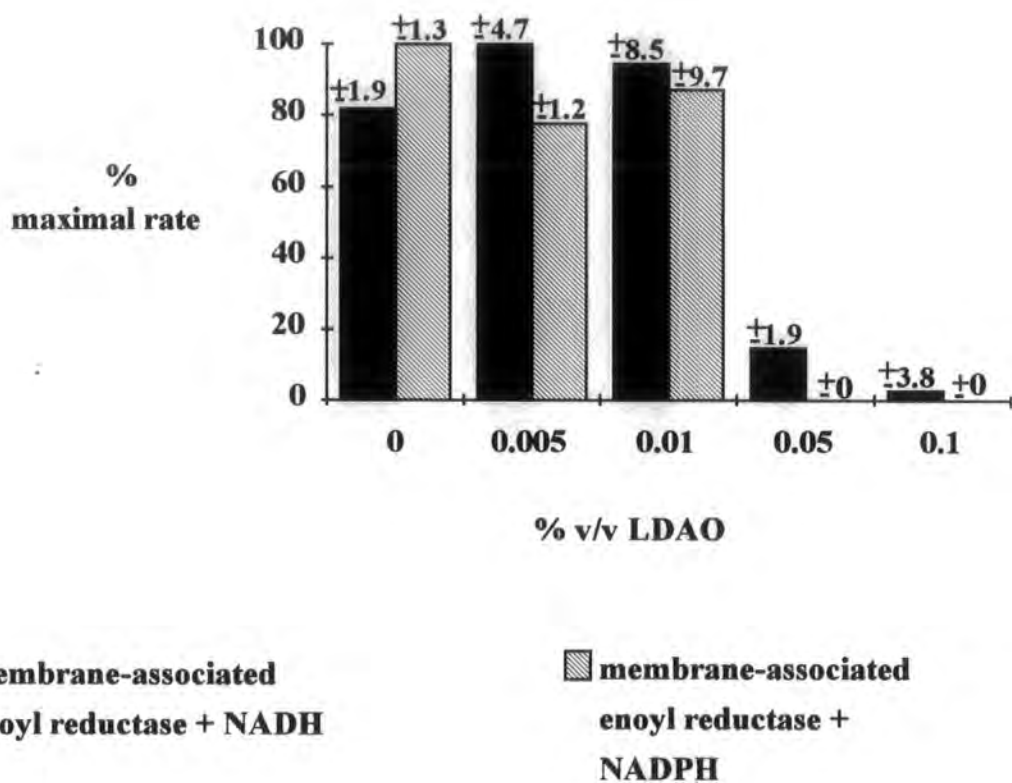
Figure 5.4 Effect of detergents on membrane-associated enoyl reductases.
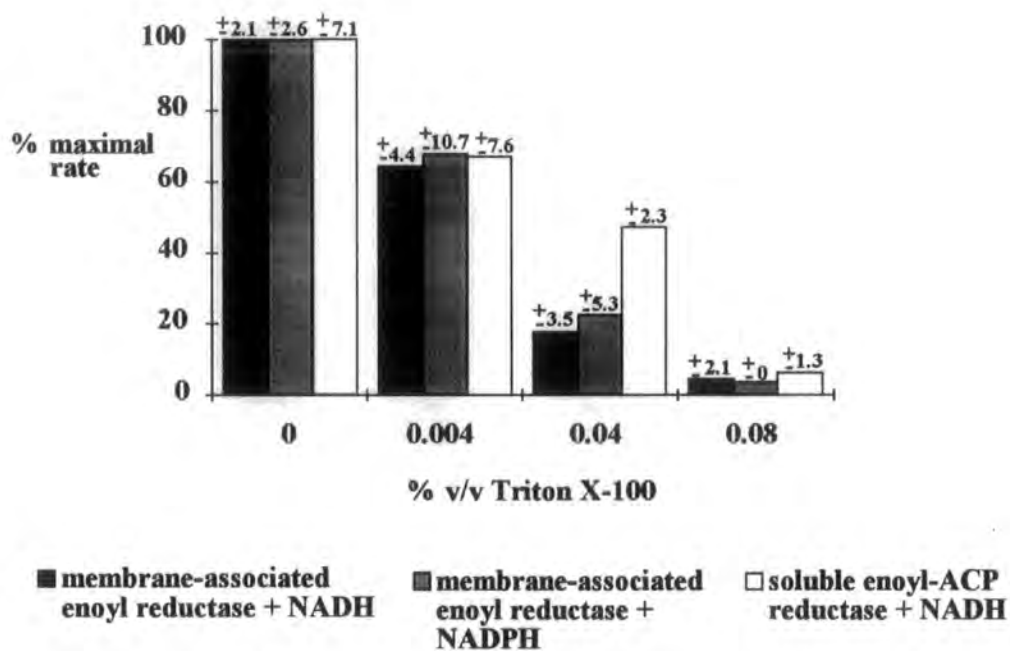
Activities are expressed as a percentage of the maximum rate. Assays were done in duplicate; the means are plotted and standard deviations are shown.

membrane-associated enoyl reductase was still active with NADH (even though the rates were severely inhibited), however, no activity was detected with NADPH. In the presence of 0.05 % w/v CHAPS, membrane-associated enoyl reductase activity measured with NADH was inhibited by approximately 32 %, whereas, when assayed with NADPH, activity was inhibited by approximately 56 %. These studies also show that it would be unwise to attempt solubilisation of membrane-associated enoyl reductases with concentrations of octyl glucoside, CHAPS and LDAO above 0.1 % w/v, 0.05 % w/v and 0.01 % v/v, respectively, since even if solubilisation had occurred, detection of activity in the supernatant would be difficult.

5.4 Discussion.

Since both soluble and membrane-associated forms of enoyl reductase can utilise C20:2-CoA[$t2$, $c11$], it was important to determine properties of the two forms, to be certain that activity measured in microsomes with NADH was not due to trapped soluble enoyl-ACP reductase. No similar in-depth study has been performed in any other system.

As stated in chapter 4, the existence of two forms of enoyl reductase was suggested from their substrate specificities; membrane-associated enoyl reductase can utilise either pyridine nucleotides with C20:2-CoA[$t2$, $c11$], but soluble enoyl-ACP reductase can only use NADH. Additionally, soluble enoyl-ACP reductase can reduce both short and very long chain substrates, yet membrane-associated enoyl reductase can only reduce C20:2-CoA[$t2$, $c11$]. On account of this, the enzymes may have different catalytic mechanisms, since they recognise different chain length acyl-CoA's.

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Studies of the effect of the herbicide diflufenican on enoyl reductase activity did not discriminate between soluble and membrane-associated forms when the same substrate, C20:2-CoA[f2, c11], was used, and so diflufenican cannot be used in an assay to differentiate between the forms. Nevertheless, soluble enoyl-ACP reductase was inhibited by diflufenican when assayed with C4:1-CoA[f2], suggesting that soluble enoyl-ACP reductase has a different rate limiting step dependent on the acyl-CoA used.

The effect of sodium chloride and Triton X-100 on enoyl reductase activity were determined in an attempt to discriminate between the soluble and membrane-associated forms, and since the enzymes had different sensitivities to both sodium chloride and Triton X-100, it is concluded that NADH-utilising membrane-associated enoyl reductase is a distinct enzyme from the soluble enoyl-ACP reductase. Assays performed in the presence of sodium chloride suggested the existence of two forms of membrane-associated enoyl reductase, differing in their pyridine nucleotide specificity, but from the data obtained from assays performed in the presence of Triton X-100, it could not be concluded whether one or two enzymes were present. However, the determination of differential thermal stabilities for activities assays with NADH and NADPH, is consistent with the hypothesis that two separate membrane-associated enoyl reductase enzymes exist in *Brassica napus* microsomes.

By studying the effects of three detergents; octyl glucoside, CHAPS and LDAO, on membrane-associated enoyl reductase activities, the maximum concentrations that could be used to solubilise the enzymes were determined. Higher concentrations would prevent the detection of any solubilised activity.

Thus, for the first time, proof is presented that there are at least three different enoyl reductases present in *Brassica napus* seeds and that there are two different forms of the membrane-associated enzyme, one that utilises NADH and the other NADPH. It will be interesting in the future to solubilise and purify both membrane-associated forms of enoyl reductase to determine their individual roles *in vivo* since at present, it is not known why two membrane-associated enoyl reductases are needed in the cell.

Chapter 6: Solubilisation of membrane-associated enoyl reductase from *Brassica napus* seeds.

6.1 Introduction.

Prior to the purification of NADH and NADPH-utilising membrane-associated enoyl reductases, for further characterisation, the proteins have to be solubilised from the membrane. As with soluble proteins, there is not a single, precise set of methods for the purification of all membrane-associated enzymes, nor is there a single method for their solubilisation. Each membrane protein possesses a unique set of physical characteristics, such that conditions which are suitable for the solubilisation and purification of one protein may not be suitable for others. Since biological activity is necessary to monitor the membrane-associated enoyl reductases during purification, the choice of solubilisation methods is limited to techniques that do not cause irreversible denaturation. Therefore, even though various solubilisation methods are available it is preferable to use the mildest conditions possible.

Cells in general contain two types of membrane proteins. Peripheral membrane proteins, which are predominantly hydrophilic, are associated with the membrane surface through electrostatic interactions. They are usually easily solubilised and afterwards can be purified by conventional chromatographic methods (Thomas and M\[c\]Namee, 1990). In comparison, integral membrane proteins are predominantly amphiphilic molecules with hydrophilic regions exposed to the aqueous environment and hydrophobic regions embedded in the lipid matrix. Consequently, these proteins usually need to be associated with lipids to maintain functional activity. Prior to their

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purification, they need to be extracted from the membrane, usually by amphiphilic detergents (Thomas and McNamee, 1990).

The exact association of the membrane-associated enoyl reductases with membranes is unknown, consequently a range of solubilisation methods would have to be tested for optimum solubility. The methods involve detergent solubilisation, washing with salts or chelating agent, extraction using acetone and washing with a chaotropic agent. Once membrane-associated enoyl reductase has been solubilised, it could be purified using standard chromatographic techniques, which would resolve the enoyl reductases associated to membranes in *Brassica napus* seeds and would allow their individual roles to be studied. Purification would determine whether the elongase in *Brassica napus* is a type I or a type II FAS. Evidence suggests that the elongase in *Arabidopsis thaliana*, a highly related species, is a dissociable type II FAS, since FAE1 has been isolated and is thought to encode the condensing enzyme for the elongase (James *et al.*, 1995). As other components of the elongase are solubilised and purified, interaction between them can be studied. Even if the elongase is a type II FAS, the separate components may associate with each other *in vivo*.

Plant membrane-associated elongases have been solubilised from several sources using detergents (table 6.1), suggesting that the elongases are integral membrane proteins. Purification of these elongases is now underway, however, it is proving to be a difficult task. Nevertheless, some elongases e.g. the C18:0-CoA elongase from *Allium porrum* and the C18:1-CoA[*c*9] elongase from *Brassica napus* have been partially purified to three major bands on SDS PAGE, but the biological function of these individual proteins is yet to be determined and elongase activity could be in a minor component.

Table 6.1 Solubilisation of plant elongases.

Enzyme	Source	Detergent used	% recovery of activity	Reference
C18:0-CoA elongase	*Limnanthes alba* (meadowfoam) seeds	Triton X-100	18	Lardens and Trémolières, 1992
C18:0-CoA elongase	*Allium porrum* (leek) leaves	Triton X-100	96	Bessoule *et al.*, 1989
C18:1-CoA[c9] elongase	*Lunaria annua* (honesty) seeds	Triton X-100	42	Fehling *et al.*, 1992
C18:1-CoA[c9] elongase	*Brassica napus* (oil seed rape) seeds	Zwittergent 3 - 10	85	Imai *et al.*, 1995
C18:1-CoA[c9] elongase	*Brassica napus* (oil seed rape) seeds	Triton X-100	65	Creach and Lessire, 1993
C20:1-CoA[c11] elongase	*Brassica napus* (oil seed rape) seeds	Triton X-100	80	Creach and Lessire, 1993

6.2 Solubilisation of the NADH-utilising form of membrane-associated enoyl reductase.

Membrane-associated enoyl reductase can utilise both NADH and NADPH to reduce C20:2-CoA[*t*2, *c*11] and as the ratio of activities with NADH and NADPH is approximately 2:1, (section 4.9.2), the solubilisation of the NADH-utilising enzyme was attempted first. Proof of solubilisation of membrane-associated enoyl reductase was taken to be the retention of biological activity in the soluble phase after centrifugation at 200 000 x g for 30 minutes. Once a successful method had been designed, it was hoped it could be adapted to solubilise the NADPH form.

6.2.1 Detergent solubilisation.

Since detergents are most commonly used to solubilise membrane-associated proteins, a range of different detergents was used to determine their effectiveness at solubilising NADH-utilising membrane-associated enoyl reductase. Routinely, detergents were used at their critical micelle concentration (cmc), since protein solubilisation occurs at or near the cmc for most detergents (Hjelmeland and Chrambach, 1984). Detergents were added to resuspended, 0.5 M sodium chloride washed, microsomal pellets (section 4.7) and incubated for 2 hours at 4°C with gentle shaking, before centrifugation at 200 000 x g for 30 minutes at 4°C. NADH membrane-associated enoyl reductase activity was measured using the standard method (section 2.5.1.3) and assays were performed with resuspended microsomal pellets prior to treatment, the detergent treated microsomes prior to incubation, the supernatant containing solubilised proteins and the resuspended pellet, in order to account for any losses in biological activity.

Initially, LDAO, a nonionic detergent, (0.03 % v/v and 0.1 % v/v) was used to determine if it would solubilise membrane-associated enoyl reductase because its

presence should not interfere with subsequent ionic or affinity chromatography. These concentrations corresponded to a detergent:protein ratio of 0.17:1 and 0.5:1 w/w, respectively. LDAO has a cmc of 0.02 - 0.05 % v/v (Neugebauer, 1994). As 10 µl of enzyme was assayed in a total volume of 125 µl, the concentrations of LDAO in the reaction mixture were 0.0024 % v/v and 0.008 % v/v, respectively, and as it had previously been determined that 0.01 % v/v LDAO inhibited NADH-utilising membrane-associated enoyl reductase by approximately only 10 % (section 5.3.2), the concentrations used for solubilisation should not severely inhibit enzyme activity. The results obtained during attempts to solubilise membrane-associated enoyl reductase with LDAO are shown in table 6.2.

Table 6.2 Solubilisation of NADH-utilising membrane-associated enoyl reductase using LDAO.

Activities are expressed as a percentage of membrane-associated enoyl reductase activity in the absence of LDAO. The means and standard deviations of duplicate experiments are shown. (N. D. = not determined.)

Amount of LDAO in solubilisation mixture	% activity in solubilisation mixture	% activity solubilised (in 200 000 x g supernatant)	% activity in resuspended 200 000 x g pellet	Modification to microsomal preparation (section 4.5)
0.03 % v/v LDAO	81 ± 1.9	33 ± 3.9	N. D.	Centrifugation in 3.2 ml tubes for 30 mins
0.1 % v/v LDAO	66 ± 4.3	26 ± 3.2	16 ± 2.4	Centrifugation in 3.2 ml tubes for 30 mins
0.1 % v/v LDAO	39 ± 3.8	0.0 ± 0.0	N. D.	Centrifugation in 70 ml tubes for 2 hours
Control (no detergent)	100 ± 2.4	33 ± 7.0	67 ± 5.2	Centrifugation in 3.2 ml tubes for 30 mins

Initial studies using 0.03 % v/v and 0.1 % v/v LDAO suggested that NADH-utilising membrane-associated enoyl reductase had been solubilised, since enzyme activity was detected in the solubilised protein fraction. This result, performed using microsomes prepared on a small scale and centrifuged in 3.2 ml tubes for 30 minutes, was reproducible, but despite repeated attempts to scale up the experiment by using larger volumes, and consequently centrifugation for 2 hours in 70 ml tubes, no activity was detected in the solubilised protein fraction when the experiment was performed on a large scale.

As this observation could not be explained, a control experiment containing no detergent was performed, using microsomes prepared on a small scale. In the absence of LDAO, the recovery of enzyme activity in the supernatant containing solubilised proteins was 33 %. This was similar to the results obtained with 0.03 % and 0.1 % v/v LDAO, using microsomes prepared on a small scale, and therefore, it was thought that this activity was unlikely to be due to membrane-associated enoyl reductase that had been solubilised, but was more likely to be due to trapped soluble enoyl-ACP reductase that had been released from microsomes. Even though microsomes had previously been washed with 0.5 M sodium chloride to remove trapped soluble enoyl-ACP reductase, not all of the contamination was removed (figure 4.9). Using C4:1-CoA[t2] as substrate, the rate measured was 0.011 nmoles/min (figure 4.9), yet it can be expected that this would be higher if measured with C20:2-CoA[t2, c11], since C20:2-CoA[t2, c11] is the preferred substrate for soluble enoyl-ACP reductase (figure 4.4).

As a result of this observation, microsomes that had been washed twice with 0.5 M sodium chloride were used in a control experiment. After this extra wash, no activity was detected in the control supernatant, and 96 % of the activity was recovered in

the resuspended pellet. Consequently, microsomes that had been washed twice with 0.5 M sodium chloride were used in all subsequent solubilisation attempts.

This clearly highlights the vital importance of adequate controls when performing solubilisation experiments. No such controls were reported in the solubilisation experiments summarised in table 6.1, hence, some of the activity detected in the soluble fraction were probably due to contaminating soluble FAS enzymes that can utilise elongase substrates.

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As a result of no activity being detected in the supernatant containing solubilised proteins produced using 0.1 % v/v LDAO and microsomes prepared on a large scale, it can be concluded that under these conditions membrane-associated enoyl reductase cannot be solubilised by LDAO. It is thought that no soluble enoyl-ACP reductase activity was detected in the microsomes prepared on a larger scale because the membranes were pelleted more slowly in the larger tubes, so soluble enzymes were less likely to get trapped in the microsomes.

Since membrane-associated enoyl reductase could not be solubilised using the nonionic detergent, LDAO, a range of different types of detergents was used, at their individual cmc's, to try and solubilise NADH-utilising membrane-associated enoyl reductase. As a result of earlier studies using 0.1 % w/v octyl glucoside, which slightly stimulated membrane-associated enoyl reductase activity, and 0.05 % w/v CHAPS, which reduced activity by approximately 35 % (section 5.3.2), the concentrations of these detergents used for solubilisation should not significantly

inhibit enzyme activity, since in the solubilisation assay reaction mixtures they would be 0.048 % and 0.04 % for octyl glucoside and CHAPS, respectively.

The results are shown in table 6.3 and it can be seen that 0.2 % w/v SDS and 0.02 % w/v sodium deoxycholate could not be used for solubilisation because they both completely inhibited membrane-associated enoyl reductase activity. They may have solubilised the membrane-associated enoyl reductases, but without activity, the enzymes can not be detected. Using the other detergents, no activity was measured in the supernatant containing solubilised proteins, and in most cases, enzyme activity was detected in the resuspended 200 000 x g pellet, suggesting that membrane-associated enoyl reductase had not been solubilised.

It is interesting that Triton X-100, which was used to solubilise most of the elongases summarised in table 6.1, did not solubilise membrane-associated enoyl reductase. However, it is important to note that the conditions used in this experiment were not identical to those used previously by researchers to solubilise the elongases. The amount of detergent used, protein concentration, detergent to protein ratio and the presence of any salts are all important factors that have to be considered when solubilising membrane proteins. In addition, appropriate controls were not reported in the experiments summarised in table 6.1 to ensure that results were not affected by contaminating soluble FAS enzymes which could seriously affect the interpretation of results.

Solubilisation of NADH-utilising membrane-associated enoyl reductase was also attempted using 0.1 % v/v LDAO with different protein concentrations of microsomes, 0.1 % v/v LDAO plus sodium acetate (0.5 - 2 M) or sodium chloride (1.5 M) and other concentrations of other detergents. However, no activity was

Table 6.3. Detergent solubilisation of NADH-utilising membrane-associated enoyl reductase.

Activities are expressed as a percentage of membrane-associated enoyl reductase activity in the absence of detergent. The means and standard deviations of duplicate experiments are shown.

Detergent	Amount of detergent used	cmc (Neugebauer, 1994)	% activity in solubilisation mixture	% activity solubilised (in 200 000 x g supernatant)	% activity in resuspended 200 000 x g pellet
anionic SDS	0.2 % w/v	0.2 % w/v	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
anionic sodium cholate	0.5 % w/v	0.39 - 0.65 % w/v	71 ± 10.2	0.0 ± 0.0	86 ± 6.7
anionic sodium deoxycholate	0.2 % w/v	0.08 - 0.25 % w/v	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
nonionic octyl glucoside	0.6 % w/v	0.58 - 0.61 % w/v	76 ± 13.4	0.0 ± 0.0	67 ± 6.7
nonionic Triton X-100	0.015 % v/v	0.015 % v/v	123 ± 43.0	0.0 ± 0.0	38 ± 10.9
nonionic Tween 20	0.007 % v/v	0.007 % v/v	69 ± 10.9	0.0 ± 0.0	31 ± 10.8
zwitterionic CHAPS	0.5 % w/v	0.37 - 0.61 % w/v	100 ± 10.8	0.0 ± 0.0	92 ± 10.8

detected in any of the supernatants, but instead it was recovered in the resuspended pellet. Consequently, membrane-associated enoyl reductase had not been inactivated by the treatments and could not be solubilised under these conditions.

In summary, after paying careful attention to avoid misinterpretation of results due to soluble enoyl-ACP reductase, solubilisation of NADH-utilising membrane-associated enoyl reductase could not be obtained using detergents under these given conditions. The reports of solubilisation by others could well be artefacts due to contaminating soluble enzymatic activity which was not accounted for in the controls they used.

6.2.2 Solubilisation by addition of salts.

Since membrane-associated enoyl reductase could not be solubilised using detergents, microsomes were washed with salts to try and solubilise the enzyme. The addition of salts to membrane proteins affects the ionic interaction between membranes and associated proteins, and can cause solubilisation of peripheral membrane proteins (Thomas and McNamee, 1990). Both sodium chloride (0 - 4 M) and lithium chloride (0 - 2 M) were used in an attempt to solubilise NADH membrane-associated enoyl reductase. Microsomes were centrifuged at 200 000 x g for 30 minutes at 4°C to pellet membrane-associated enoyl reductase, then the pellet was resuspended in homogenisation buffer (10 mM sodium phosphate [pH 7.0], 4 mM EDTA and 1 mM DTT) containing a range of salt concentrations. After incubation at 4°C for 1 hour with gentle shaking, the mixture was centrifuged at 200 000 x g and membrane-associated enoyl reductase activity was measured in the non-treated resuspended microsomal pellet, the salt treated microsomes prior to incubation, the supernatant containing solubilised proteins and resuspended pellet. Table 6.4 shows the results obtained.

Table 6.4. Salt solubilisation of NADH-utilising membrane-associated enoyl reductase.

Activities are expressed as a percentage of membrane-associated enoyl reductase activity in the absence of salt. The means and standard deviations of duplicate experiments are shown.

Amount of salt used	% activity in solubilisation mixture	% activity solubilised (in 200 000 x g supernatant)	% activity in resuspended 200 000 x g pellet
0 M	100 ± 3.8	0.0 ± 0.0	43 ± 1.0
1 M NaCl	68 ± 4.1	0.0 ± 0.0	20 ± 2.4
2 M NaCl	68 ± 2.9	0.0 ± 0.0	42 ± 2.9
4 M NaCl	40 ±1.4	0.0 ± 0.0	29 ± 5.8
0.5 M LiCl	86 ±2.8	0.0 ± 0.0	58 ± 2.8
1 M LiCl	100 ± 11.1	0.0 ± 0.0	89 ± 0.0
2 M LiCl	102 ±2.8	0.0 ± 0.0	78 ± 0.0

Even though membrane-associated enoyl reductase activity was not detected in the fractions containing soluble proteins, it was detected in the resuspended pellet, indicating that total loss of activity had not occurred during solubilisation. As a result of this, it can be concluded that neither salt was successful at solubilising NADH membrane-associated enoyl reductase.

6.2.3 Solubilisation by addition of a chelating agent.

As neither detergents nor salts were effective at solubilising NADH-utilising membrane-associated enoyl reductase, the effect of a chelating agent, EDTA, was

investigated. It is thought that Ca^{2+} and Mg^{2+} may be involved in binding proteins to membranes, so the addition of EDTA, which binds to Ca^{2+} and Mg^{2+}, may release proteins from membranes (Thomas and McNamee, 1990). Pelleted microsomes were resuspended in homogenisation buffer containing EDTA (10 and 50 mM) and after incubation at 4°C for 1 hour with gentle shaking, the mixture was centrifuged at 200 000 x g for 30 minutes at 4°C and assayed for solubilisation. Membrane-associated enoyl reductase activity was not detected in the supernatant containing soluble proteins, but approximately 88 and 67 % activity was recovered in the resuspended pellets after treatment with 10 and 50 mM EDTA, respectively, showing that complete loss of biological activity had not occurred and in fact, the enzyme was still attached to membranes, so consequently had not been solubilised by EDTA.

6.3 Solubilisation of NADH and NADPH-utilising forms of membrane-associated enoyl reductase.

Due to the fact that NADH-utilising membrane-associated enoyl reductase could not be solubilised using routine methods, the solubilisation of both NADH and NADPH-utilising forms of membrane-associated enoyl reductase was attempted using more drastic conditions. The advantage of trying to solubilise the NADPH-utilising membrane-associated enoyl reductase was that any activity detected with NADPH had to be due to membrane-associated enoyl reductase and not soluble enoyl-ACP reductase, since the soluble form will not use NADPH with acyl-CoA's (Slabas *et al.*, 1986).

6.3.1 Solubilisation by treatment with acetone.

Acetone treatment is not generally effective for the solubilisation of most membrane proteins (Akao and Kusaka, 1976); however, because NADH membrane-associated enoyl reductase activity was not removed from membranes using detergents, salts or a chelating agent, it was used in an attempt to solubilise the enzymes. During extraction with acetone, solubilised proteins are recovered from an acetone powder (Penefsky and Tzagoloff, 1971), but most proteins are denatured in organic solvents and only a small proportion is soluble in an organic phase (Thomas and M^{C}Namee, 1990). In spite of this, the method has been successfully used to solubilise diglyceride acyltransferase from the membrane of *Mycobacterium smegmatis*; the bulk of diglyceride acyltransferase activity (70 - 90 %) and 10 % of total protein was recovered in the final supernatant (Akao and Kusaka, 1976).

To try and solubilise membrane-associated enoyl reductase, cold acetone (-20°C) was gradually added to microsomes in a ratio of 9:1. After vortexing, the mixture was centrifuged at 2 000 x g for 10 minutes at 4°C and the pellet obtained was washed in 1 volume of acetone (-20°C). After drying, the pellet was resuspended in 1/10 th volume of homogenisation buffer and centrifuged at 200 000 x g for 30 minutes at 4°C (Akao and Kusaka, 1976). As proteins are more easily denatured in organic solvents at temperatures above 10°C (Bollag and Edelstein, 1991), special care was taken to work with solutions and rotors chilled to 4°C. The supernatant, microsomes and resuspended pellet were assayed for membrane-associated enoyl reductase activity and the results are shown in table 6.5.

Table 6.5 Solubilisation of NADH and NADPH-utilising forms of membrane-associated enoyl reductase by treatment with acetone.

The means and standard deviations of duplicate experiments are shown.

Protein fraction	Membrane-associated enoyl reductase activity (nmoles of C20:2-CoA[t2, c11] reduced/min)	
	+ NADH	+ NADPH
microsomes	0.84 ± 0.14	0.50 ± 0.08
supernatant	0.0 ± 0.0	0.0 ± 0.0
resuspended pellet	0.0 ± 0.0	0.0 ± 0.0

No membrane-associated enoyl reductase activity was detected with either pyridine nucleotide as substrate, in the supernatant or the resuspended pellet; presumably the enzyme had been denatured by -20°C acetone. Acetone may have solubilised the membrane-associated enoyl reductases, but due to loss of biological activity, the enzymes could not be detected. Consequently, this method cannot be used to solubilise membrane-associated enoyl reductase, unless an alternative detection method is designed.

6.3.2 Solubilisation using a chaotropic agent.

Chaotropic agents, which disorder the structure of water, have previously been used to solubilise membrane-associated enzymes. They act by disrupting hydrophobic bonds near the surface of membrane structures and promote the transfer of hydrophobic groups from an apolar environment to the aqueous phase (van Renswoude and Kempf, 1984). Apolar groups form hydrophobic bonds mainly as a result of their thermodynamically unfavourable interaction with water, rather than as a consequence of attraction for each other; therefore, if the structure of water is

disordered, the transfer of apolar groups to the aqueous phase is facilitated, hydrophobic interactions become weaker, and membrane and biological macromolecules are destabilised (Hatefi and Hanstein, 1974).

An example of the solubilisation of a membrane protein using a chaotropic agent is the solubilisation of succinate dehydrogenase from *Rhodospirillum rubrum* using 0.85 M $NaClO_4$, which resulted in 40 % of enzyme activity being solubilised (Hatefi *et al.*, 1972).

Prior to the attempted solubilisation of membrane-associated enoyl reductase using the chaotropic agent, guanidinium thiocyanate, its effect on enzyme activity was determined. Pelleted microsomes were resuspended in homogenisation buffer containing 2 M guanidinium thiocyanate, and membrane-associated enoyl reductase activity was determined using both pyridine nucleotides. The effect of 2 M guanidinium thiocyanate on soluble enoyl-ACP reductase activity was also examined, using C20:2-CoA[*t*2, *c*11] and NADH. The results, in figure 6.1, show that 2 M guanidinium thiocyanate completely inhibits soluble enoyl-ACP reductase, but inhibits NADH-utilising membrane-associated enoyl reductases by approximately only 25 % and stimulates NADPH-utilising membrane-associated enoyl reductase by approximately 10 %. The differing effects of guanidinium thiocyanate on NADH membrane-associated and soluble enoyl reductases support the hypothesis that they are two distinct enzymes. As a result of this observation, future studies on membrane-associated enoyl reductase could be performed in the presence of 2 M guanidinium thiocyanate, thereby preventing any anomalies due to contaminating soluble enoyl-ACP reductase. Guanidinium thiocyanate is thus an inhibitor that inactivates soluble enoyl-ACP reductase and not membrane-associated enoyl reductase and could be used as a tool to differentiate between the two different forms of enoyl reductase.
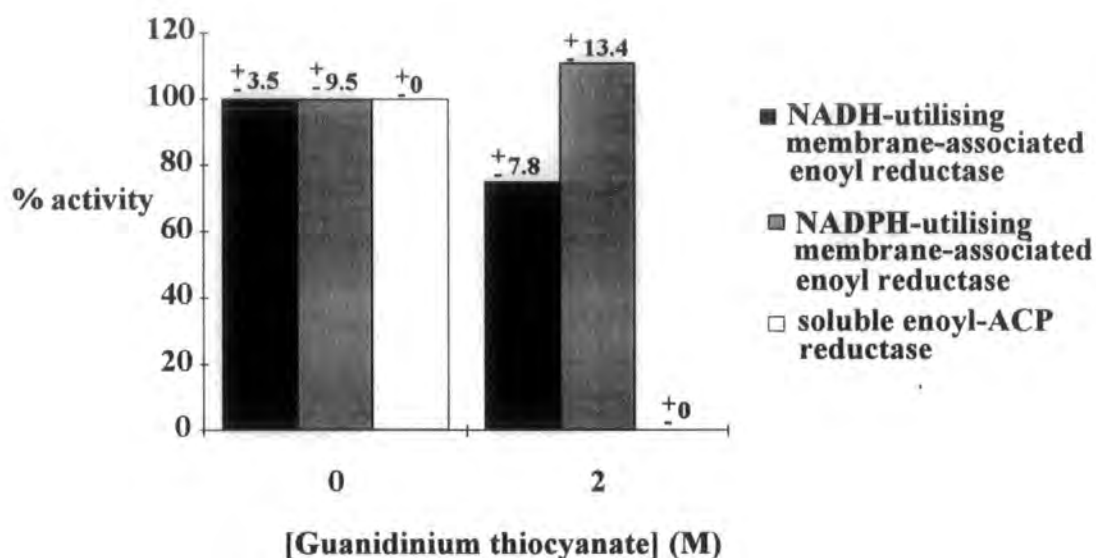
Figure 6.1 Effect of guanidinium thiocyanate on enoyl reductase activity.

Activities are expressed as a percentage of control reactions containing no guanidinium thiocyanate. Assays were done in duplicate; the means are plotted and the standard deviations are shown.

As guanidinium thiocyanate did not significantly inhibit membrane-associated enoyl reductase activity, it was used in an attempt to solubilise both NADH and NADPH-utilising forms. Pelleted microsomes were resuspended in homogenisation buffer containing a range of guanidinium thiocyanate concentrations and solubilised proteins were recovered from membranes by centrifugation at 200 000 x g for 30 minutes at 4°C. Membrane-associated enoyl reductase activity was measured in the non-treated resuspended microsomal pellet, the solubilisation mixture (enzyme and guanidinium thiocyanate), supernatant containing solubilised protein and the final resuspended pellet and the results are shown in figure 6.2.

The presence of 0.5 M or 1 M guanidinium thiocyanate did not solubilise membrane-associated enoyl reductase activity. However, when 2 M or greater guanidinium thiocyanate was added to microsomes, activity was detected in the fraction containing solubilised proteins. 2 M guanidinium thiocyanate gave the highest activities; 3 and 4 M guanidinium thiocyanate inhibited NADH-utilising membrane-associated enoyl reductase activity. In the presence of 2 M guanidinium thiocyanate, approximately 40 % and 80 % of membrane-associated enoyl reductase, assayed with NADH and NADPH respectively, was solubilised, but no remaining activity was detected in the resuspended pellet. This suggests that all membrane-associated enoyl reductase may have been solubilised, but removal of membranes reduced enzyme activity.

The activity measured in the supernatant with NADH as reductant, could not be due to soluble enoyl-ACP reductase that had been released from microsomes because 1) the control, containing no guanidinium thiocyanate, did not contain any activity, and 2) 2 M guanidinium thiocyanate completely inhibits soluble enoyl-ACP reductase (figure 6.1). As the presence of 2 M guanidinium thiocyanate alone in the reaction mixture (without any solubilised membrane-associated enoyl reductase) did

a) Enzyme activity assayed with NADH

- ■ Solubilisation mixture
- □ Supernatant
- ▨ Resuspended pellet
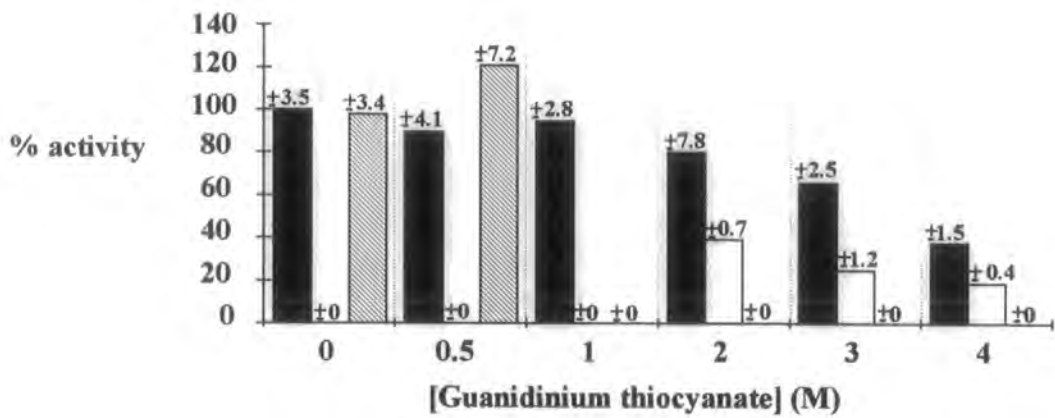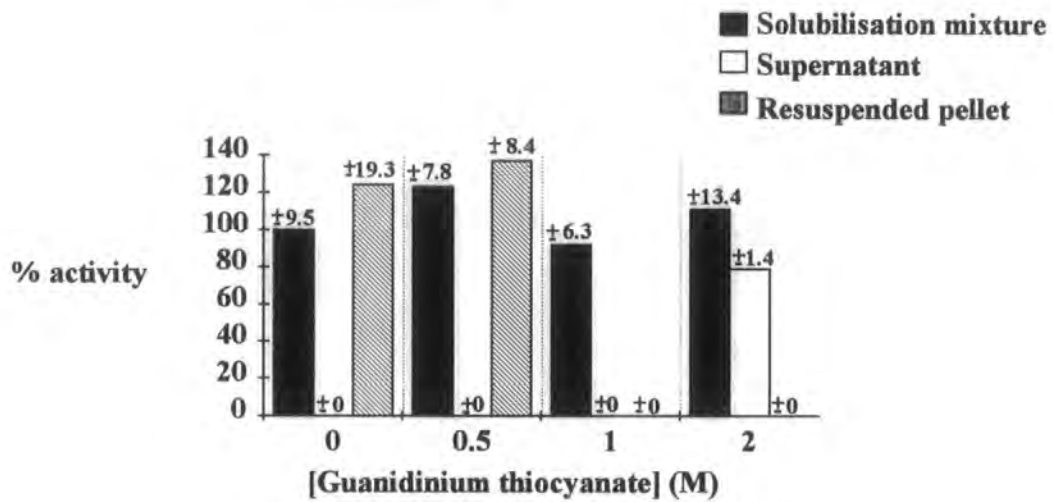
b) Enzyme activity assayed with NADPH

Figure 6.2 Guanidinium thiocyanate solubilisation of membrane-associated enoyl reductase.

The amount of guanidinium thiocyanate illustrated is the concentration added to resuspended microsomes. Activities are expressed as a percentage of control reactions containing no guanidinium thiocyanate. The means and standard deviations of duplicate experiments are shown.

not cause a decrease in absorbance at 340 nm, this suggests that the change in absorbance monitored must be due to enzyme activity.

The results show that both forms of membrane-associated enoyl reductase had been solubilised using a novel procedure. This is the first reported solubilisation of both forms of membrane-associated enoyl reductase where it has been conclusively shown that results are not affected by contaminating soluble enoyl-ACP reductase. A photograph of a Coomassie blue stained gel of the 2 M guanidinium thiocyanate solubilised proteins is shown in figure 6.3. In order to overcome artefacts due to high salt, which may prevent SDS PAGE running effectively, protein samples were concentrated by precipitation with chloroform and methanol, using the method described in section 2.5.5, prior to loading. It can be clearly seen that guanidinium thiocyanate removes the majority of proteins from microsomes, whereas when no guanidinium thiocyanate was present, most proteins remained attached to membranes. As the band pattern is similar in each lane, it appears that guanidinium thiocyanate solubilises all proteins equally, rather than selectively solubilising certain proteins.

6.4 Further characterisation of NADH-utilising membrane-associated enoyl reductase.

6.4.1 Effect of washing microsomes with 0.5 M sodium chloride.

In section 4.7, 0.5 M sodium chloride was used to remove contaminating soluble proteins from microsomes, and it was determined that 67 % soluble enoyl-ACP reductase and 70 % total protein were removed by washing microsomes with
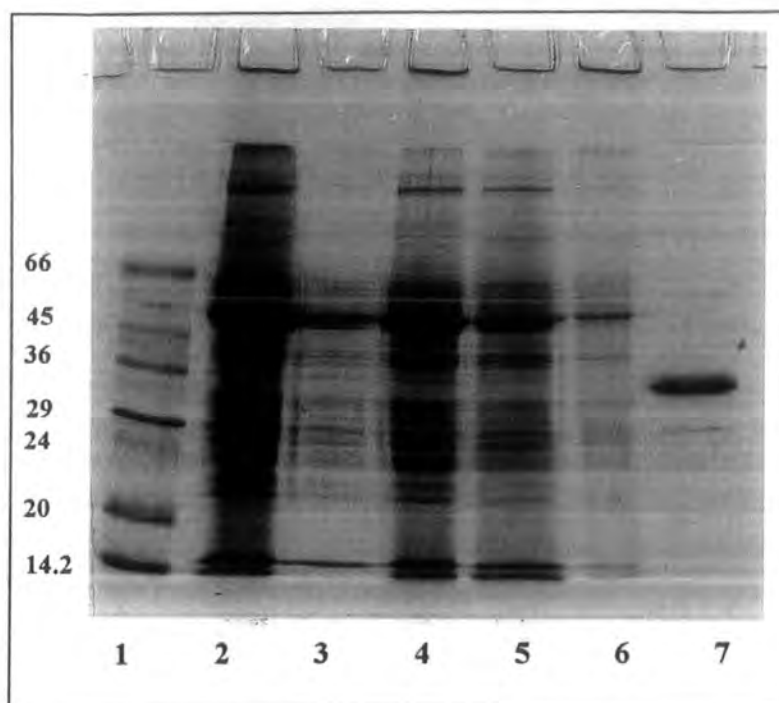
Figure 6.3 SDS PAGE gel showing the protein samples obtained during guanidinium thiocyanate solubilisation.

The above photograph shows a 10 % w/v polyacrylamide gel stained with Coomassie blue. Equal volumes (50 μl) are loaded in lanes 2-7.

Lane 1) molecular weight standards (66, 45, 36, 29, 24, 20, 14.2 kDa)

Lane 2) resuspended microsomal pellet

Lane 3) supernatant from a 0 M guanidinium thiocyanate solubilisation

Lane 4) supernatant from a 2 M guanidinium thiocyanate solubilisation

Lane 5) pellet from a 0 M guanidinium thiocyanate solubilisation

Lane 6) pellet from a 2 M guanidinium thiocyanate solubilisation

Lane 7) pure recombinant soluble enoyl-ACP reductase

0.5 M sodium chloride, but no NADPH membrane-associated enoyl reductase activity was removed. At the time, the amount of NADH-utilising membrane-associated enoyl reductase activity removed by washing could not be determined because contaminating NADH-utilising soluble enoyl-ACP reductase would affect the results. Since it has been determined that 2 M guanidinium thiocyanate completely inhibits soluble enoyl-ACP reductase, the amount of NADH-utilising membrane-associated enoyl reductase removed during microsomal washing could be accurately determined if 2 M guanidinium thiocyanate was included in the reaction mixture. Therefore, standard membrane-associated enoyl reductase assays were performed with washed and unwashed microsomes, in the presence of 2 M guanidinium thiocyanate (table 6.6).

Table 6.6 Comparison of NADH-utilising membrane-associated enoyl reductase activity in unwashed and washed microsomes.

Assays were done in duplicate; the means and standard deviations are shown.

Microsomal pellet	Membrane-associated enoyl reductase activity (nmoles/min)	Specific activity (nmoles/mg/min)
Unwashed (assayed in the absence of 2 M guanidinium thiocyanate)	1.07 ± 0.11	19.3
Unwashed (assayed in the presence of 2 M guanidinium thiocyanate)	0.61 ± 0.03	11.0
Washed twice with 0.5 M sodium chloride (assayed in the presence of 2 M guanidinium thiocyanate)	0.57 ± 0.02	31.7

The data clearly show that, as for NADPH membrane-associated enoyl reductase, no NADH-utilising membrane-associated enoyl reductase activity was removed during washing, and therefore, this is an effective purification step.

6.4.2 Determination of the acyl-CoA specificity of membrane-associated enoyl reductase.

It had previously been determined that membrane-associated enoyl reductase cannot reduce C4:1-CoA[$t2$] in the presence of NADPH and since membrane-associated enoyl reductase utilises both NADH and NADPH, it was assumed that any activity with NADH and C4:1-CoA[$t2$] was due to soluble enoyl-ACP reductase contamination (section 4.7). This situation could now be clarified if 2 M guanidinium thiocyanate, which inhibits soluble enoyl-ACP reductase, was present when the utilisation of C4:1-CoA[$t2$] by membrane-associated enoyl reductase in the presence of NADH was further investigated. The results are shown below in table 6.7.

Table 6.7 Utilisation of C4:1-CoA[$t2$] by membrane-associated enoyl reductase in the presence of NADH.

The microsomes used had been washed once with 0.5 M sodium chloride.

Substrate	Activity (nmoles/min)	
	C20:2-CoA[$t2$, $c11$]	C4:1-CoA[$t2$]
membrane-associated enoyl reductase	0.74 ± 0.08	0.013 ± 0.002
membrane-associated enoyl reductase in the presence of 2 M guanidinium thiocyanate	0.55 ± 0.03	0.0 ± 0.0

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By inhibiting contaminating soluble enoyl-ACP reductase with 2 M guanidinium thiocyanate, it is concluded that C4:1-CoA[$t2$] cannot be utilised as a substrate by membrane-associated enoyl reductase, and therefore, membrane-associated enoyl reductase can reduce C20:2-CoA[$t2$, c11], but not C4:1-CoA[$t2$]. In comparison, soluble enoyl-ACP reductase can utilise both acyl-CoA's.

6.5 Extensive washing of microsomes.

During attempts to solubilise membrane-associated enoyl reductase, it was noticed that under certain conditions, e.g. using 0.03 % v/v LDAO, 100 % enzyme activity was recovered in the resuspended 200 000 x g pellet. Using this information, microsomes were excessively washed, prior to solubilisation with guanidinium thiocyanate, to partially purify membrane-associated enoyl reductase.

Proteins were washed from microsomal pellets using 0.03 % v/v LDAO followed by 0.5 M sodium chloride, before membrane-associated enoyl reductase was solubilised with 2 M guanidinium thiocyanate. Samples were assayed for biological activity, using the standard assay procedure, and total protein concentrations were determined using the Bio-Rad protein assay (section 2.5.3). As shown in table 6.8, a 0.03 % v/v LDAO wash, followed by a 0.5 M sodium chloride wash before solubilisation with 2 M guanidinium thiocyanate results in a final supernatant containing 0.25 mg/ml total protein, with a specific activity of 72 nmol/mg/min for NADH membrane-associated enoyl reductase and 164.8 nmol/mg/min for the NADPH enzyme.

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Table 6.8 Extensive washing of microsomes.

Prior to assaying, pellets were resuspended in homogenisation buffer (10 mM sodium phosphate [pH 7.0], 4 mM EDTA and 1 mM DTT). The means and standard deviations of two experiments are shown.

a) NADH-utilising membrane-associated enoyl reductase.

Treatment	Sample assayed	Activity (nmol/ml/min)	Total protein (mg/ml)	Specific activity (nmol/mg/min)	Purification
	Unwashed microsomes	61.0 ± 3.1	5.54	11.0	1
	Microsomes washed twice with 0.5 M NaCl	57.0 ± 2.4	1.06	53.8	4.89
0.03 % v/v LDAO wash	LDAO wash pellet	51.4 ± 6.1	0.68	75.6	6.87
	LDAO wash supernatant	0 ± 0	0.23	0 ± 0	0 ± 0
0.5 M NaCl wash	NaCl wash pellet	43.7 ± 1.7	0.45	97.1	8.8
	NaCl wash supernatant	0 ± 0	0.10	0 ± 0	0 ± 0
Guanidinium thiocyanate solubilisation	Guanidinium thiocyanate pellet	7.7 ± 0.9	0.10	77	7
	Guanidinium thiocyanate supernatant	18.0 ± 0.0	0.25	72	6.5
	Guanidinium thiocyanate supernatant from microsomes only washed twice with 0.5 M NaCl	22.8 ± 4.2	0.51	44.7	4.1

b) NADPH-utilising membrane-associated enoyl reductase.

Treatment	Sample assayed	Activity (nmol/ml/min)	Total protein (mg/ml)	Specific activity (nmol/mg/min)	Purification
	Unwashed microsomes	38.0 ± 2.6	5.54	6.86	1
	Microsomes washed twice with 0.5 M NaCl	32.0 ± 3.4	1.06	30.2	4.4
0.03 % v/v LDAO wash	LDAO wash pellet	43.7 ± 3.6	0.68	64.3	9.36
	LDAO wash supernatant	0 ± 0	0.23	0 ± 0	0 ± 0
0.5 M NaCl wash	NaCl wash pellet	46.0 ± 4.7	0.45	102.2	14.9
	NaCl wash supernatant	0 ± 0	0.10	0 ± 0	0 ± 0
Guanidinium thiocyanate solubilisation	Guanidinium thiocyanate pellet	0 ± 0	0.10	0 ± 0	0 ± 0
	Guanidinium thiocyanate supernatant	41.20 ± 1.6	0.25	164.8	24
	Guanidinium thiocyanate supernatant from microsomes only washed twice with 0.5 M NaCl	37.88 ± 2.4	0.51	74.1	10.8

In comparison, a 2 M guanidinium thiocyanate supernatant containing equivalent amounts of enzyme activity, which had not been washed with LDAO and sodium chloride, contained 0.51 mg/ml total protein, with specific activities of 44.7 and 74.1 nmol/mg/min for the NADH and NADPH enzymes, respectively. On account of the extra two washes removing 51 % of total protein with no loss of enzyme activity, purification is increased by approximately two fold. Thus, this is a rapid, effective method to aid enzyme purification.

6.6 Characterisation of membrane-associated enoyl reductase after solubilisation.

6.6.1 Effect of guanidinium thiocyanate on enzyme activity.

After solubilisation of membrane-associated enoyl reductase, the linearity of the assay was determined, using varying amounts of guanidinium thiocyanate solubilised enzyme, to show whether the rate monitored was directly proportional to the quantity of enzyme. Assays were performed with both NADH and NADPH and the results are shown in figure 6.4a.

The assay was not linear with respect to enzyme concentration, suggesting that increasing amounts of guanidinium thiocyanate inhibit enzyme activity. On account of this, the effect of varying amounts of guanidinium thiocyanate on a constant amount (10 µl) of solubilised enzyme was studied. As seen in figure 6.4b, a concentration of greater than 0.16 M guanidinium thiocyanate in the reaction
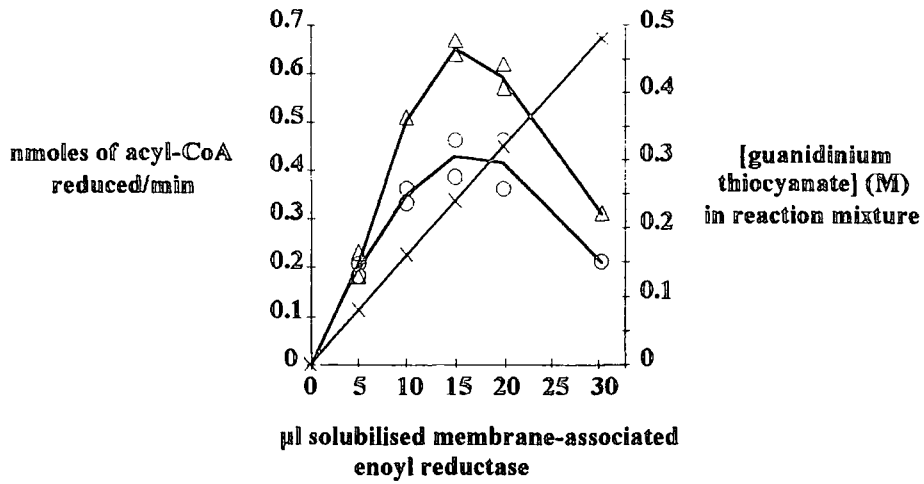
a) Effect of varying the amount of enzyme and the concentration of guanidinium thiocyanate
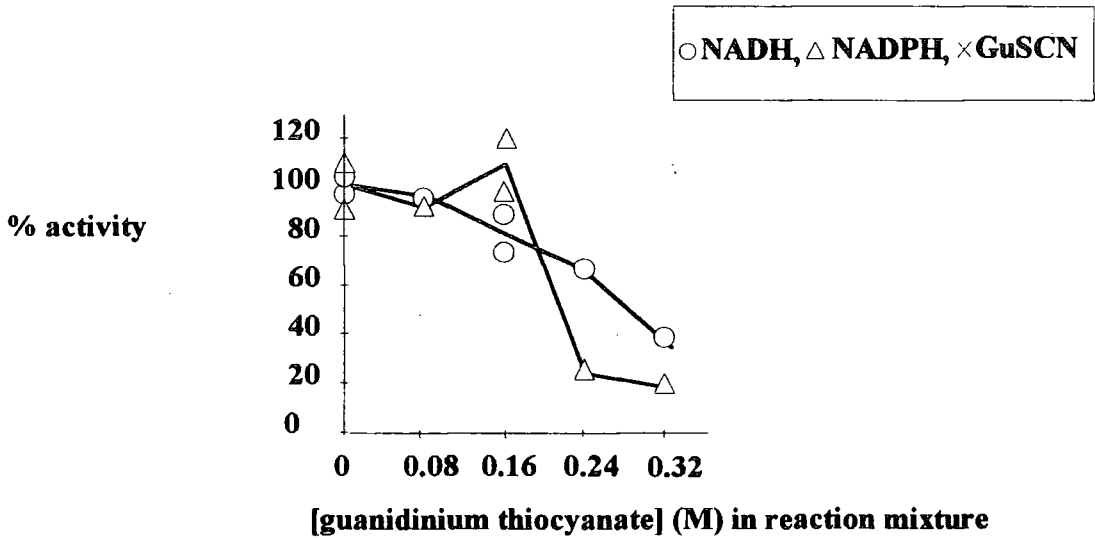
b) Effect of varying the concentration of guanidinium thiocyanate with a constant amount of enzyme (10 µl)

Activities are expressed as a percentage of control reactions containing no quanidinium thiocyanate

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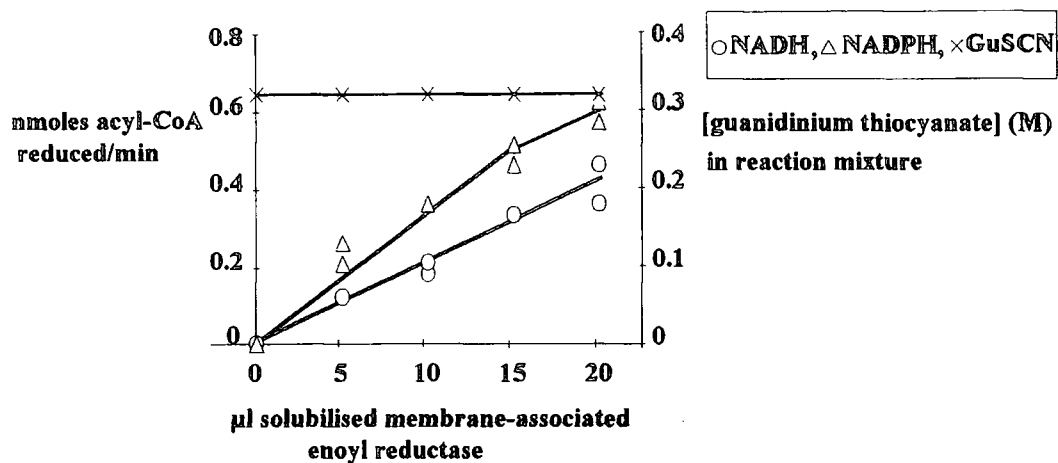
c) Effect of varying the amount of enzyme at a constant guanidinium thiocyanate concentration

Figure 6.4 Effect of guanidinium thiocyanate on membrane-associated enoyl reductase activity.

The amount of guanidinium thiocyanate illustrated is the final concentration in the assay mixture. The results of two identical experiments are plotted for each graph.

mixture caused inhibition of solubilised membrane-associated enoyl reductase. Therefore, for a linear assay to be obtained, the concentration of guanidinium thiocyanate had to be maintained constant while the amount of enzyme was varied. As shown in figure 6.4c, this produced a linear assay, which is consistent with the rate being directly proportional to enzyme activity and not some other factor, such as a changing guanidinium thiocyanate concentration.

6.6.2 Removal of guanidinium thiocyanate by dialysis.

The presence of guanidinium thiocyanate in crude solubilised membrane-associated enoyl reductase extract would prevent purification of the enzyme by ion exchange and affinity chromatography, due to the high ionic charges of guanidinium thiocyanate. As a consequence of this, it is necessary to remove (or at least lower the concentration of) guanidinium thiocyanate prior to chromatography and attempts were, therefore, made to achieve this by dialysis.

Solubilised membrane-associated enoyl reductase (100 μl) was dialysed against 250 ml homogenisation buffer (10 mM sodium phosphate [pH 7.0], 4 mM EDTA and 1 mM DTT) containing 0 - 30 % v/v glycerol. Dialysis tubing was prepared as previously described in section 2.5.2 and dialysis was performed in apparatus specifically designed for the dialysis of small volumes (500 μl or less) at 4°C for 2 hours. The apparatus, shown in figure 6.5, was manufactured by Mr. J. Hodgeson, Mechanical Workshop, Department of Chemistry, University of Durham.
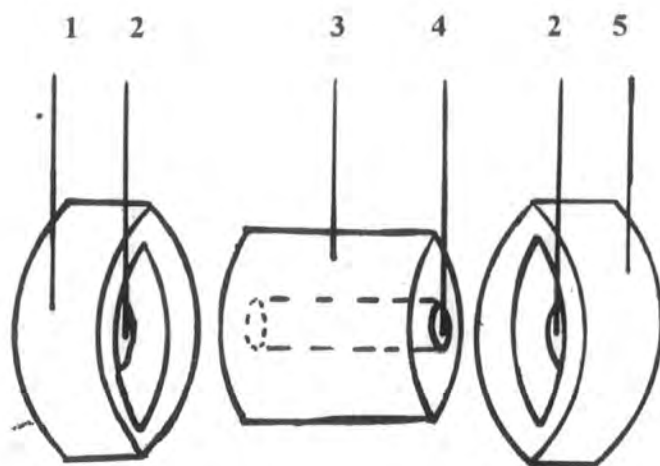
(Not to scale, actual height is 2.5 cm)

1) screw on teflon lid with silicone gasket 4) hole for sample

2) hole covered with dialysis membrane 5) screw on teflon base with silicone gask

3) teflon sample holder

Figure 6.5. Apparatus used to dialyse small volumes.

The sample to be dialysed is enclosed in the sample holder by dialysis membrane in the lid and base. Once the apparatus has been assembled, it is clipped to a magnet so that dialysis can occur with mixing.

After dialysis, assays were performed using the standard method to calculate recovery of enzyme activity (table 6.9).

Table 6.9 Effect of the presence of glycerol during dialysis on solubilised membrane-associated enoyl reductase activity.

Activities are expressed as a percentage of activity prior to dialysis. The means and standard deviations of duplicate experiments are shown.

% v/v glycerol present	% activity recovered after dialysis	
	+ NADH	+ NADPH
0	0.0 ± 0.0	0.0 ± 0.0
5	63 ± 4.7	0.0 ± 0.0
10	38 ± 3.2	0.0 ± 0.0
20	89.5 ± 6.4	33 ± 2.2
30	75 ± 5.7	45 ± 4.2

In the absence of glycerol, all enzyme activity was lost after removal of guanidinium thiocyanate; yet, in the presence of glycerol, activity with NADH was recovered. In comparison, at least 20 % v/v glycerol was necessary to recover activity with NADPH as substrate. Consequently, the optimum concentration of glycerol for maximum recovery of activity was 20 %. The presence of 30 % glycerol gave comparable results, but for future chromatography steps, it was advisable to use the lower concentration of glycerol. It is thought that glycerol may help to maintain membrane-associated enoyl reductase activity by stabilising the enzyme in an enzymatically active conformation.

Before attempts were made to purify guanidinium thiocyanate solubilised membrane-associated enoyl reductase by chromatography, its stability at room temperature and 4°C was investigated. To determine the optimal storage conditions for solubilised membrane-associated enoyl reductase, stability at -20°C and -80°C was also examined. The enzyme source used was guanidinium thiocyanate solubilised membrane-associated enoyl reductase, before and after dialysis against homogenisation buffer containing 20 % glycerol. The results, figure 6.6, show that in all cases, enzyme activity was more stable prior to dialysis. Consequently, the solubilised extract was stored in the presence of guanidinium thiocyanate, rather than in the presence of glycerol. Despite this, once dialysed, the enzymes could still be assayed after storage at room temperature or 4°C for at least 24 hours, therefore, stability should not be a problem during chromatography.

6.6.4 Determination of the molecular weight of membrane-associated enoyl reductase by gel filtration.

To prove that membrane-associated enoyl reductase had been solubilised, it was subjected to gel filtration. Membranes would be contained in the void volume, whereas the included volume of a gel filtration column would contain membrane-associated enoyl reductase if it had been solubilised. A Superose 12 (Pharmacia) gel filtration column (300 mm x 3.2 mm) was used on a Pharmacia Smart system to determine the molecular weight of membrane-associated enoyl reductase. The molecular weight of membrane-associated enoyl reductase involved in elongating VLCFA's has not yet been reported for any plant species. Gel filtration may also be sufficient to separate the NADH and NADPH activities. Initially, the column was equilibrated in 10 mM sodium phosphate (pH 7.0) and 2 M guanidinium thiocyanate.

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% recovery of enzymatic activity

Time (hours)
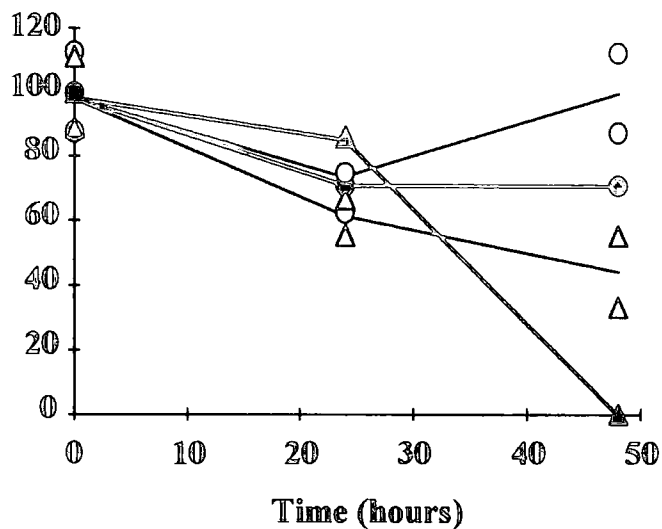
a) room temperature

% recovery of enzymatic activity

Time (hours)
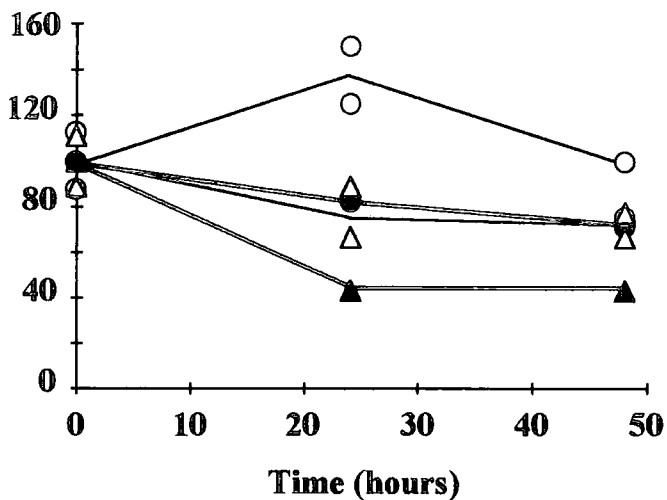
b) 4 $^\circ$C

- ● Dialysed, then assayed with NADH
- ▲ Dialysed, then assayed with NADPH
- ○ Non dialysed, then assayed with NADH
- △ Non dialysed, then assayed with NADPH

c) -20°C
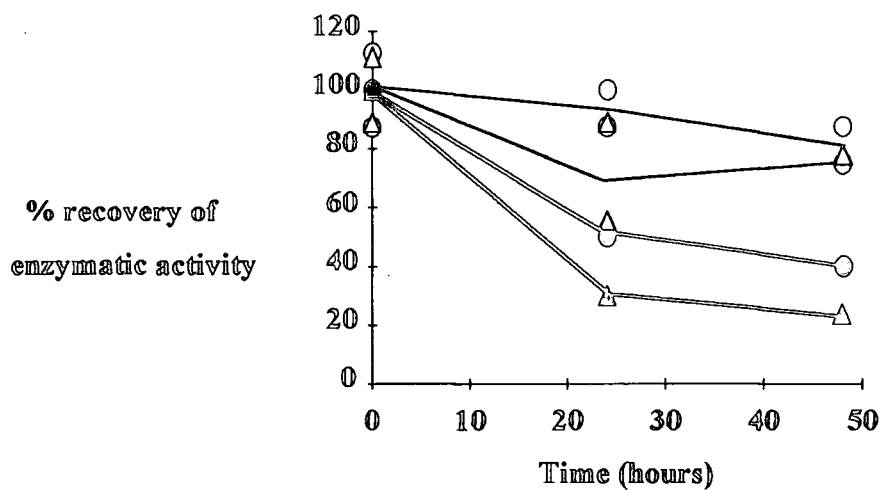
d) -80°C

Legend:
- ○ Dialysed, then assayed with NADH
- △ Dialysed, then assayed with NADPH
- ○ Non dialysed, then assayed with NADH
- △ Non dialysed, then assayed with NADPH

% recovery of enzymatic activity

Time (hours)

Figure 6.6 Stability of solubilised membrane-associated enoyl reductase.

Activities are expressed as a percentage of the activities at time zero. The results of duplicate experiments are plotted.

This was because enzyme activity was more stable in 2 M guanidinium thiocyanate, than in 20 % v/v glycerol (figure 6.6), and the presence of guanidinium thiocyanate would not have a detrimental effect on gel filtration chromatography. Crude solubilised membrane-associated enoyl reductase (50 μl) was loaded on to the column and separation occurred at a flow rate of 40 μl/min. Elution was monitored by following the absorbance at 280 nm, due to the absorption of tryptophan and tyrosine, and 100 μl fractions were collected throughout. An aliquot (20 μl) of each fraction was assayed for solubilised membrane-associated enoyl reductase activity, but no activity was detected with either NADH or NADPH as substrate. As the solubilised enzyme extract may have been diluted too much to detect enzyme activity, gel filtration was repeated using extract that had been concentrated 4-fold using an Ultrafree®-MC filter with a 30 kDa cut off. Unfortunately, sensitivity could not be increased by assaying 30 μl of each fraction, because this would increase the final concentration of guanidinium thiocyanate in the reaction mixture to 0.48 M which, as was shown in figure 6.4, causes inhibition of enzyme activity. As a result, 10 μl of each fraction was assayed to maintain the concentration of guanidinium thiocyanate in the reaction mixture below inhibitory levels, since the final concentration would be 0.16 M. Even so, no activity was detected in any of the fractions.

The column was then equilibrated in 10 mM sodium phosphate (pH 7.0) and 0.7 M guanidinium thiocyanate, so that assaying 30 μl of each fraction would give a final concentration of guanidinium thiocyanate in the reaction mixture of 0.168 M, which does not inhibit membrane-associated enoyl reductase activity (figure 6.4). Again, the enzyme extract was concentrated 4-fold before loading; but no activity was detected after gel filtration. As the stability of solubilised membrane-associated enoyl reductase in 0.7 M guanidinium thiocyanate was unknown, the column was equilibrated in 50 mM sodium phosphate (pH 7.0), 4 mM EDTA, 1 mM DTT,

100 mM sodium chloride and 20 % v/v glycerol (buffer A). It has previously been shown that membrane-associated enoyl reductase dialysed into buffer containing 20 % v/v glycerol is at least 60 % active with both NADH and NADPH after storage at room temperature for 24 hours (figure 6.6); therefore, the enzyme should not lose activity during a gel filtration chromatography run which lasts for approximately 1 hour. 50 mM sodium phosphate and 100 mM sodium chloride were used to minimise ionic interactions between the sample and the Superose 12 matrix and the other components were added to aid enzyme stability. Solubilised membrane-associated enoyl reductase was dialysed into buffer A before it was loaded on to the column. Aliquots (30 μl) of each fraction were assayed, but no enzyme activity was detected.

In conclusion, after several attempts, using different conditions to favour enzyme stability, the molecular weight of solubilised membrane-associated enoyl reductase could not be determined. As an explanation, it is thought that the enzyme may be too dilute to detect any activity. Alternatively, the enzyme may interact with the column matrix and irreversibly bind to it, or membrane-associated enoyl reductase may be part of a loosely associated protein complex that dissociates during gel filtration thereby losing activity.

6.7 Proof that the rates measured are due to enzymatic activity.

Since a molecular weight of membrane-associated enoyl reductase could not be determined by gel filtration, there were concerns that the rates previously measured were actually due to enzymatic activity. Therefore, several studies were performed to determine this.

6.7.1 Effect of boiling membrane-associated enoyl reductase.

To determine whether the rates measured were genuinely due to enzyme activity, the effect of boiling crude membrane-associated enoyl reductase was investigated because this treatment would denature and inactivate the majority of enzymes. Enzymes were boiled for 30 minutes prior to activity being assayed and the results of this study are shown in table 6.10.

Table 6.10 Effect of boiling membrane-associated enoyl reductase.

Activities obtained after boiling are expressed as a percentage of activities prior to boiling. The means and standard deviations of two experiments are shown.

Enzyme source	% enzymatic activity before boiling		% enzymatic activity after boiling for 30 mins	
	+ NADH	+ NADPH	+ NADH	+ NADPH
Microsomal pellet	100 ± 12.9	100 ± 10.2	0.0 ± 0.0	0.0 ± 0.0
Solubilised membrane-associated enoyl reductase in 2 M guanidinium thiocyanate	100 ± 0.0	100 ± 11.0	79.5 ± 3.2	104 ± 11.3
Microsomal pellet in 2 M guanidinium thiocyanate	100 ± 7.4	100 ± 15.7	100 ± 37.2	100 ± 8.8
Solubilised membrane-associated enoyl reductase dialysed into 20 % v/v glycerol	100 ± 12.9	100 ± 13.7	72.7 ± 0	55.5 ± 8.7

Membrane-associated enoyl reductase activity was completely inhibited by boiling when the enzyme was still attached to the membrane. However, in the presence of 2 M guanidinium thiocyanate (either when the enzyme was still attached to the membrane, or after it had been solubilised) it appeared that enzyme activity was no longer severely inhibited by boiling. From these results, it can be concluded that either the solubilised enzyme was resistant to heat denaturation, or the decrease in absorbance measured was due to a non-enzymatic reaction. However, 2 M guanidinium thiocyanate without membrane-associated enoyl reductase in the reaction mixture did not cause a decrease in absorbance, therefore, a non-enzymatic reaction was unlikely. This suggests that guanidinium thiocyanate somehow protects the enzyme from inactivation due to heat, and maintains activity.

As guanidinium thiocyanate is a denaturant, it may alter the structure of the enzyme so that it is not further affected by boiling. Prions also show unusual thermal stability under certain conditions (Brown et al., 1990). Prions are proteins that probably cause spongiform encephalopathy; the most common form of which is scrapie, found in sheep and goats, but prions also affect mink, deer, elk, cats, cows and humans (Prusiner, 1995). Using scrapie-infected hamster brains that were subjected to formaldehyde and steam autoclaving, alone or in combination, it was found that autoclaving caused inactivation or reduced infectivity. However, treatment with formaldehyde before autoclaving stabilised infectivity. Formaldehyde denatures proteins by reacting primarily with the numerous amino groups found in proteins, forming stable bridges between and within polypeptide chains and it was thought that this molecular cross-linking effect of formaldehyde causes the degraded prion protein to acquire sufficient rigidity to resist the subsequent destruction of hydrogen bonds by heat (Brown et al., 1990). On account of this, it is possible that guanidinium thiocyanate, which is also a denaturant, stabilises membrane-associated enoyl reductase so that it is not further affected by heat treatment.

Nevertheless, even when solubilised membrane-associated enoyl reductase was dialysed against 20 % v/v glycerol, enzyme activity was still detected after boiling for 30 minutes, suggesting that, like guanidinium thiocyanate, glycerol also stabilises membrane-associated enoyl reductase. As stated previously, glycerol may somehow stabilise membrane-associated enoyl reductase in a conformation that is enzymatically active, even during extensive heat treatment.

As membrane-associated enoyl reductase appeared to have unusual properties, further studies were performed to determine whether the decreases in absorbance seen during assays were due to enzymatic activity.

6.7.2 Effect of proteinase K on membrane-associated enoyl reductase activity.

The effect of proteinase K from *Tritirachium album* on solubilised membrane-associated enoyl reductase was investigated to see if the component required for a decrease in absorbance at 340 nm was proteinaceous. Prior to the experiment, the effect of proteinase K on BSA in the presence of 2 M guanidinium thiocyanate was investigated, to determine whether proteinase K was active in the presence of the chaotropic agent. BSA (0.3 mg/ml) in 2 M guanidinium thiocyanate, was digested in a reaction mixture containing 5 mM EDTA, 10 mM Tris/HCl (pH 8.0) and 180 μg/ml proteinase K in a total volume of 110 μl. Digestion was performed at 40°C for 1 hour. BSA samples incubated at 40°C with and without proteinase K were concentrated by precipitation with chloroform and methanol prior to analysis by SDS PAGE (section 2.5.6). A photograph of a Coomassie blue stained gel of the samples is shown in figure 6.7. BSA and BSA incubated at 40°C without proteinase K can be seen on the gel, but BSA incubated at 40°C in the presence of proteinase K is not visible. Thus, proteinase K digested BSA in the presence of 2 M guanidinium
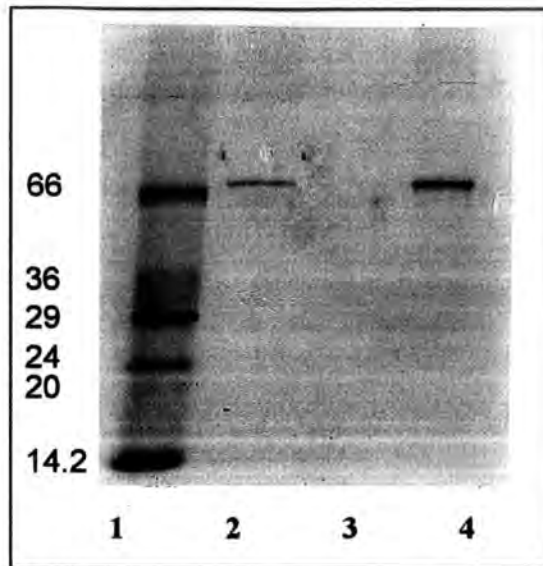
Figure 6.7 SDS PAGE gel showing the effect of proteinase K on BSA in 2 M guanidinium thiocyanate.

The above photograph shows a 10 % w/v polyacrylamide gel stained with Coomassie blue.

Lane 1) molecular weight standards (66, 36, 29, 24, 20, 14.2 kDa)

Lane 2) 2 μg BSA in 2 M guanidinium thiocyanate

Lane 3) 2 μg BSA in 2 M guanidinium thiocyanate incubated in proteinase K at
    40°C for 1 hour

Lane 4) 2 μg BSA in 2 M guanidinium thiocyanate incubated at 40°C for 1 hour

thiocyanate after incubation at 40°C for 1 hour and the peptides produced were not precipitated with chloroform and methanol. Consequently, proteinase K is active in 2 M guanidinium thiocyanate.

Digestion of solubilised membrane-associated enoyl reductase (0.3 mg/ml) was performed in 5 mM EDTA, 10 mM Tris/HCl (pH 8.0) and 180 μg/ml proteinase K in a total volume of 110 μl and the reaction mixture was incubated at 40°C for 1 hour. The controls used were; incubation of enzyme at 40°C for 1 hour in the absence of proteinase K (to check that incubation at 40°C did not inhibit enzyme activity) and solubilised membrane-associated enoyl reductase in proteinase K without incubation (to check that the presence of extra proteins did not cause enzyme inhibition). The results are shown in table 6.11.

Table 6.11 Effect of proteinase K on solubilised membrane-associated enoyl reductase.

Activities are expressed as a percentage of enzyme activity prior to treatment. The means and standard deviations of two independent experiments are shown.

Treatment	% recovery of enzyme activity	
	+ NADH	+ NADPH
Solubilised membrane-associated enoyl reductase	100 ± 0.0	100 ± 0.0
Solubilised membrane-associated enoyl reductase, incubated with proteinase K at 40°C for 1 hour	0.0 ± 0.0	0.0 ± 0.0
Solubilised membrane-associated enoyl reductase, incubated at 40°C for 1 hour	88.9 ± 7.6	125 ± 11.8
Solubilised membrane-associated enoyl reductase containing proteinase K, but not incubated	66.7 ± 4.5	75 ± 10.8

Complete inhibition of solubilised membrane-associated enoyl reductase activity is only achieved by incubation at 40°C for 1 hour in the presence of proteinase K, and so it can be assumed that the component required for reduction of C20:2-CoA[t2, c11] is a protein.

6.7.3 Approximate determination of the molecular weight of solubilised membrane-associated enoyl reductase activity.

Since determination of the molecular weight of the membrane-associated enoyl reductases by gel filtration was unsuccessful, an Ultrafree®-MC filter with a 30 kDa cut off was used to determine whether the component responsible for the decrease in absorbance at 340 nm in the assay was greater or smaller than 30 kDa, since any molecules greater than 30 kDa would be retained by the filter. As the proteins do not pass through a matrix, this method eliminated any possible binding between proteins and the matrix.

Solubilised membrane-associated enoyl reductase (100 μl) was centrifuged through an Ultrafree®-MC filter at 4 000 x g at 4°C in a Beckman Avanti™ 30 centrifuge with a F2402 eppendorf rotor for 20 minutes. Proteins retained by the membrane were resuspended in 100 μl of homogenisation buffer (10 mM sodium phosphate [pH 7.0], 4 mM EDTA and 1 mM DTT) and 2 M guanidinium thiocyanate. Assays were performed on solubilised membrane-associated enoyl reductase, the fraction that went through the membrane and the fraction that was retained by the 30 kDa cut off membrane, and the results are shown in table 6.12.

Table 6.12 Approximate determination of the molecular weight of solubilised membrane-associated enoyl reductase activity.

Activities are expressed as a percentage of the initial enzyme activity. The means and standard deviations of two identical experiments are shown.

Fraction	% recovery of enzyme activity	
	+ NADH	+ NADPH
Solubilised membrane-associated enoyl reductase	100 ± 0.0	100 ± 8.3
Fraction that went through the 30 kDa cut off membrane	0.0 ± 0.0	0.0 ± 0.0
Fraction that was retained by the 30 kDa cut off membrane	112.5 ± 15.7	88.2 ± 8.3

The component that is essential for a decrease in absorbance at 340 nm was retained by the filter, and so, is greater than 30 kDa.

In summary, solubilised membrane-associated enoyl reductase has a molecular weight of greater than 30 kDa, is a proteinase K sensitive protein and is resistant to denaturation by boiling in the presence of 2 M guanidinium thiocyanate.

6.8 Discussion.

Several techniques have been used in an attempt to solubilise membrane-associated enoyl reductase. Initially, studies concentrated on solubilising the more active NADH-utilising membrane-associated enoyl reductase using detergents. Unfortunately, activities recovered in fractions containing soluble proteins were later

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found to be due to soluble enoyl-ACP reductase that had been released from the microsomes rather than solubilised membrane-associated enoyl reductase, since an additional 0.5 M sodium chloride wash was sufficient to remove this soluble enoyl-ACP reductase contamination from microsomes. Solubilisation of membrane-associated enoyl reductase was not achieved using detergents, salts or a chelating agent.

Further attempts involved harsher methods to solubilise both forms of membrane-associated enoyl reductase. Extraction with acetone was unsuccessful as it resulted in complete loss of enzyme activity. Conversely, the chaotropic agent, guanidinium thiocyanate, was able to solubilise both forms of membrane-associated enoyl reductase. The optimum concentration of guanidinium thiocyanate for solubilisation is 2 M; if the concentration is exceeded, enzyme activity is partially inhibited. The membrane-associated enoyl reductase activities detected were not affected by soluble enoyl-ACP reductase as the soluble form is unable to utilise NADPH with acyl-CoA substrates, and its activity is completely inhibited by 2 M guanidinium thiocyanate. Previous reports in the literature detail the solubilisation of elongase complexes, detected by assaying total elongation activity (table 6.1). However, the use of what is recognised by this research as adequate controls, was not reported. Therefore, it is possible that the results reported by others could be the consequence of artefacts due to soluble FAS components, which the previous studies failed to adequately consider.

Before solubilised membrane-associated enoyl reductase can be purified using standard chromatographic techniques, the concentration of guanidinium thiocyanate needs to be reduced; this was achieved by dialysis. Maximal recoveries required the presence of 20 % v/v glycerol in the dialysis buffer.

Determination of the molecular weight of solubilised membrane-associated enoyl reductase was attempted by gel filtration. After several attempts, though, this proved unsuccessful suggesting that either the enzyme recovered from the gel filtration column was too dilute to be detected or the enzyme interacted with the column matrix and irreversibly bound to it. Alternatively, membrane-associated enoyl reductase may be part of a complex that dissociates during gel filtration, thereby losing activity. This hypothesis does not agree with previous results, though, since Schöpker et al., in 1992, reported that the solubilised elongase from Lunaria annua has a molecular weight of greater than 700 kDa determined by gel filtration. As all components of the elongase were detected, it is either a multifunctional protein, or a complex that does not associate during gel filtration.

The effect of boiling membrane-associated enoyl reductase in the presence of guanidinium thiocyanate produced unexpected results, as enzyme activity was not inhibited. Guanidinium thiocyanate seems to protect the enzyme from further denaturation by heat treatment and this is similar to when prion proteins are heat treated in the presence of formaldehyde. Despite this, it was determined that the component required for membrane-associated enoyl reductase activity is proteinase K sensitive and greater than 30 kDa, which is consistent with it being a protein.

Chapter 7: Immunological detection of membrane-associated and soluble enoyl reductases.

7.1 Introduction.

Solubilisation of both NADH and NADPH-utilising forms of membrane-associated enoyl reductase has been achieved using the chaotropic agent, guanidinium thiocyanate; approximately 40 % and 80 % activity was solubilised, respectively (section 6.3.2). A rapid purification method for these enzymes could be designed if the enzymes cross-react with an antibody, since immobilisation of this antibody to agarose beads via protein A, would enable production of an affinity column.

Additionally, cross-reacting antibodies could be used to develop a more sensitive assay that detects active and inactive enzymes. This is necessary because losses in enzyme activity occurred during solubilisation and dialysis into buffer containing 20 % v/v glycerol; the activities remaining after solubilisation and dialysis were 36 % and 26 % for NADH and NADPH membrane-associated enoyl reductases, respectively. Dialysis is required because the high charges on guanidinium thiocyanate will severely affect ion exchange and affinity chromatography. Since losses of biological activity would be expected during purification attempts using chromatography, and due to the limitation of the assay based on measuring enzyme activity, which would prevent low amounts of enzyme activity to be detected, development of a more sensitive method for detecting membrane-associated enoyl reductase is required to aid enzyme purification. Previous attempts to determine the molecular weights of membrane-associated enoyl reductases gave a minimal molecular weight of 30 kDa (section 6.7.3) but attempts to obtain a more precise molecular weight by gel filtration were not successful and

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this may have been due to dilution of enzyme activity thus preventing its detection (section 6.6.4).

Alternative methods to detect membrane-associated enoyl reductase include the use of cross-reacting affinity tags or antibodies. Affinity tags have previously been used to aid enzyme purification and characterisation. For example, a radiolabelled tag was successfully used to purify ACP from *Brassica napus* seeds. The enzyme rapidly lost activity after chromatography, therefore, using ACP synthase from *E. coli*, partially purified ACP was labelled with [^{14}C] palmitic acid. Not only did this introduce a tag to follow ACP, but it also converted the protein from a hydrophilic species to a hydrophobic one, and this was used as the basis for purification (Slabas *et al.*, 1987).

To aid characterisation of acyl-CoA: alcohol acyltransferase from jojoba, an enzyme involved in liquid wax biosynthesis, it has been labelled with a radioiodinated, photoreactive analogue of acyl-CoA, 12-[*N*-(4-azidosalicyl)]amindodecanoyl-CoA (ASD-CoA), which is an irreversible inhibitor of the enzyme upon exposure to U.V. light. Analysis of microsomal membranes with labelled ASD-CoA resulted in labelling of three proteins. Labelling of one, a 57 kDa polypeptide, was reduced in the presence of C18:1-CoA[*c*9], but increased in the presence of dodecanol, suggesting that this 57 kDa polypeptide may be the acyl-CoA: alcohol acyltransferase (Shockey *et al.*, 1995). Consequently, it can be seen that enzyme activity is not an absolute requirement for protein purification.

Since a large quantity of antibodies are available to this research group, a range of antibodies could be tested for cross-reactivity with membrane-associated enoyl reductase for future production of an immobilised affinity column. Cross-reacting antibodies could also be used to determine the molecular weight of the enzymes and in subcellular immunolocalisation studies. Antibodies have been raised against native NADH soluble enoyl-ACP reductase and if they recognise the membrane-associated

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forms, it would indicate that the two types of enoyl reductase share regions of homology. When using antibodies, though, it is important to minimise non-specific interactions by the use of affinity-purified antibodies, to prevent misinterpretation of results.

Since it is unknown whether the elongase in *Brassica napus* forms a multi-enzyme type I FAS or a dissociable type II FAS, an anti-denatured animal FAS antibody could be used to determine if it will cross-react with a component in *Brassica napus* microsomes; cross-reactivity would suggest the existence of a type I FAS in *Brassica napus* microsomes.

An immobilised anti-soluble enoyl-ACP reductase antibody column may enable the development of a one-step purification method for soluble enoyl-ACP reductase, and by paying careful attention to the elution procedure used, separation of the four isoforms may be achieved. It has previously been determined that this antibody will cross-react to all four of the isoforms (Fawcett *et al.*, 1994). The reason for the existence of four isoforms of this soluble enzyme is unknown and purification would enable sequence data to be obtained, so they could be correlated to the isolated cDNA's and genes to aid determination of their expression and regulation. Also, their individual substrate specificities could be determined.

Previously, antibodies have seldom been used to study components of elongases; however, an antibody has been raised against partially purified acyl-CoA elongase from *Allium porrum* (leek) leaves which recognises fractions with elongating activities. It also allows the formation of an immune complex that is immunoprecipitable with protein A sepharose and this was used to determine that the elongase accounted for 1 % of proteins solubilised from epidermal cells by Triton X-100 (Bessoule *et al.*, 1992).

7.2 Western blot analysis of microsomes using affinity-purified anti-native soluble enoyl-ACP reductase antibody.

7.2.1 Affinity-purification of anti-native soluble enoyl-ACP reductase antibody.

Since polyclonal sera are often found to react with a number of proteins, even when purified antigens are used for immunisation, anti-native soluble enoyl-ACP reductase antibody was affinity-purified before it was used to determine whether it would cross-react with the membrane-associated enoyl reductases. Affinity-purification would minimise cross-reactivity with proteins other than enoyl reductase, and therefore, prevent misinterpretation of the result.

Anti-native soluble enoyl-ACP reductase antibody was purified using an immobilised (Affigel 15) soluble enoyl-ACP reductase column, which had been made by Mr. J. W. Simon, Department of Biological Sciences, University of Durham. Soluble enoyl-ACP reductase was immobilised to 5 ml of Affigel 15 matrix at a concentration of 4 mg/ml. All chromatography steps were performed at room temperature. The column was equilibrated with 4 column volumes of 50 mM Tris/HCl (pH 7.0) at 700 µl/min before serum containing anti-native enoyl-ACP reductase IgG (from sheep or rabbit) was loaded onto the column three times at 1 ml/min, to allow maximum binding. Unbound proteins were washed from the matrix with 50 mM Tris/HCl (pH 7.0) at 1 ml/min until no more protein could be detected by measuring their absorbance at 280 nm. The antibody was eluted in 4 M magnesium chloride containing 20 mM Tris base (the pH was not adjusted and was 6.8) at 350 µl/min. A typical elution profile is shown in figure 7.1. Fractions (1.5 ml) were collected and those containing protein were pooled together and dialysed twice against 2 litres of TBS at 4°C for 2.5 hours. TBS contains 137 mM sodium chloride, 3 mM potassium chloride, 25 mM Tris/HCl (pH 7.4). After dialysis, the sample was centrifuged at

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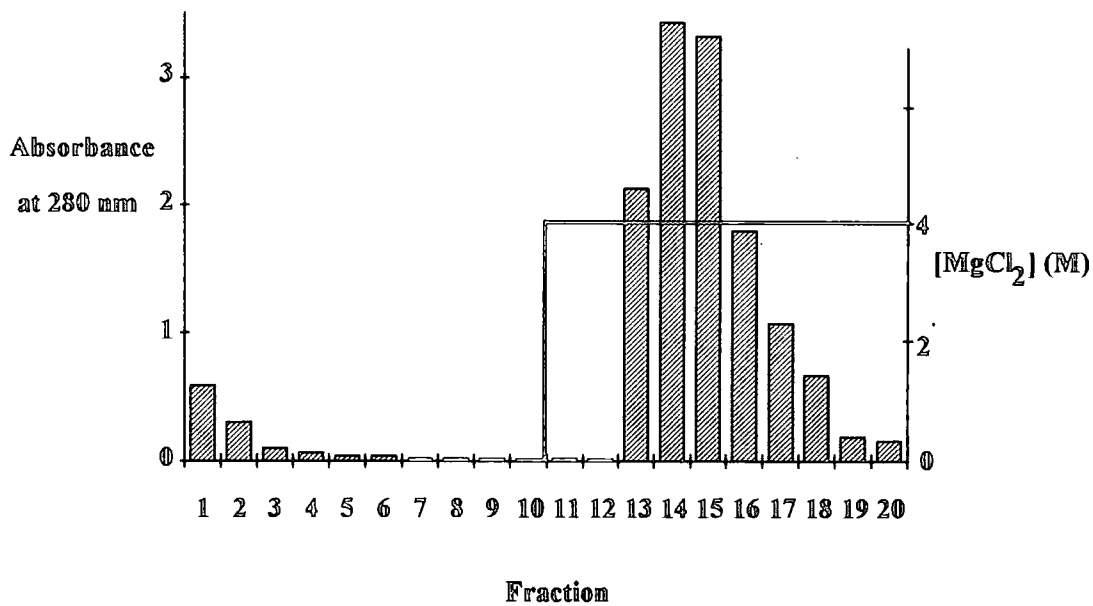
Figure 7.1 A typical elution profile obtained during affinity-purification of anti-native soluble enoyl-ACP reductase antibody.
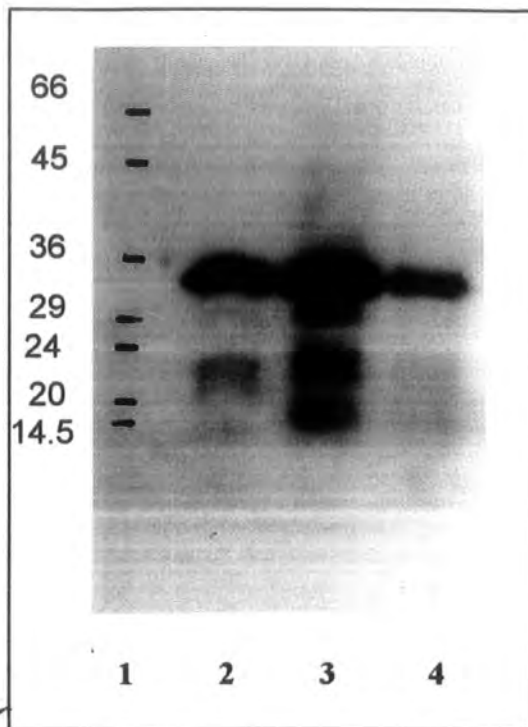
Non-purified anti-native soluble enoyl-ACP reductase antibody
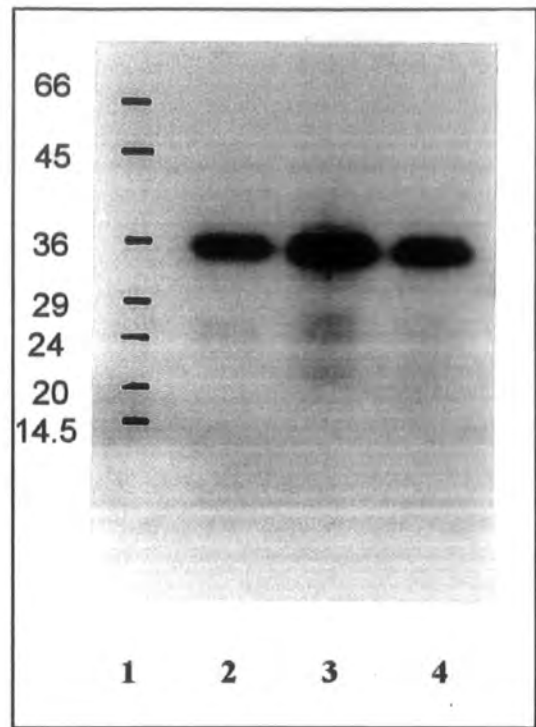
Affinity-purified anti-native soluble enoyl-ACP reductase antibody

Figure 7.2 Western blot analysis of the effect of affinity-purifying anti-native soluble enoyl-ACP reductase antibody.

Lane 1) molecular weight markers (66, 45, 36, 29, 24, 20, 14.2 kDa)

Lane 2) 15 ng of pure recombinant soluble enoyl-ACP reductase

Lane 3) 5 µl of crude *Brassica napus* seed extract

Lane 4) 1 µl of crude *Brassica napus* seed extract

Affinity-purified anti-native soluble enoyl-ACP reductase antibody was tested for cross-reactivity with crude membrane-associated enoyl reductase. Prior to electrophoresis, protein samples were concentrated using chloroform and methanol (section 2.5.5) and Western blotting was performed using 10 % and 5 % w/v polyacrylamide resolving gels, with ^{125}I detection, as described above. The results are shown in figure 7.3. Affinity-purified anti-native soluble enoyl-ACP reductase antibody cross-reacted with a species in the resuspended microsomal pellet extract and the 2 M guanidinium thiocyanate solubilised membrane-associated enoyl reductase extract which had the same molecular weight as soluble enoyl-ACP reductase. From this experiment, though, it was not possible to conclude whether membrane-associated enoyl reductase has the same molecular weight as the soluble enzyme, or whether the membrane-associated enoyl reductase extracts were contaminated with soluble enoyl-ACP reductase. However, it can be seen that the antibody did not cross-react with any high molecular weight species, suggesting that if membrane-associated enoyl reductase was part of a multi-enzyme complex, the complex shared no structural similarity with native soluble enoyl-ACP reductase. The possible existence of a 32 kDa membrane-associated enoyl reductase in *Brassica napus* microsomes was investigated using two-dimensional electrophoresis.

7.2.3 Western blot analysis of *Brassica napus* microsomes separated by two-dimensional electrophoresis.

Two-dimensional electrophoresis separates proteins according to isoelectric point by isoelectric focusing in the first dimension, and according to molecular weight by SDS electrophoresis in the second dimension. Since all four isoforms of soluble enoyl-ACP reductase have previously been identified by two-dimensional

10 % w/v acrylamide resolving gel with
5 % w/v acrylamide stacking gel
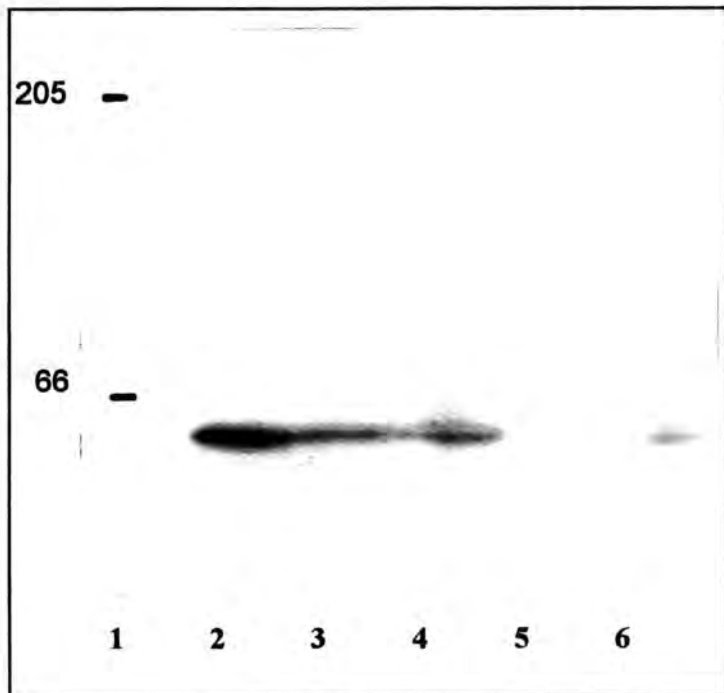
5 % w/v acrylamide resolving gel with
3 % w/v acrylamide stacking gel

Figure 7.3 Western blot analysis of microsomes separated by SDS PAGE using affinity-purified anti-native soluble enoyl-ACP reductase antibody.

Lane 1) molecular weight markers

Lane 2) 15 ng of pure recombinant soluble enoyl-ACP reductase

Lane 3) 1 μl of crude *Brassica napus* seed extract

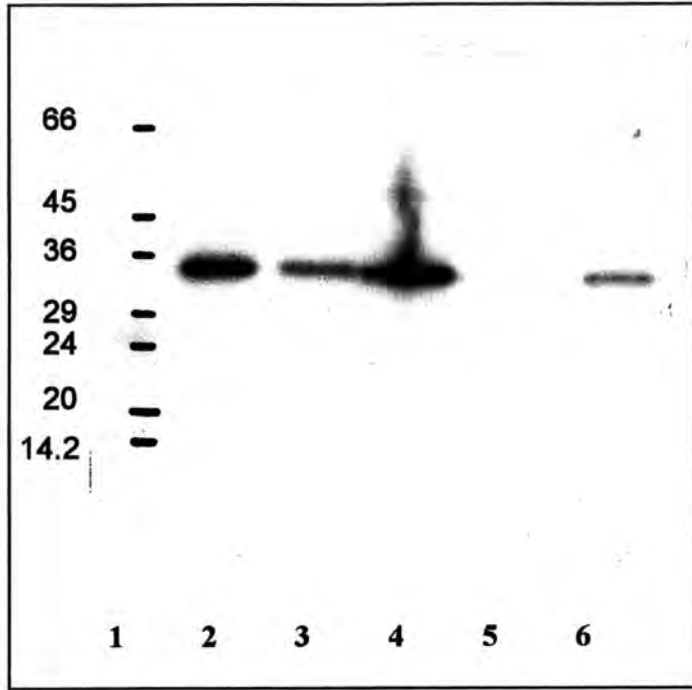Lane 4) 50 μl of resuspended microsomal pellet (washed twice with 0.5 M NaCl)

Lane 6) 50 μl of 2 M guanidinium thiocyanate solubilised membrane-associated
   enoyl reductase (washed with 0.03 % v/v LDAO and 0.5 M NaCl prior to
   solubilisation)

electrophoresis, a comparison of this profile with that obtained using microsomes would determine whether the cross-reactivity between the anti-native soluble enoyl-ACP reductase antibody and protein in microsomes, is due to contaminating soluble enoyl-ACP reductase or the membrane-associated forms.

Two-dimensional electrophoresis was performed (section 2.5.8) on crude *Brassica napus* seed extract (40 000 - 200 000 x g supernatant from a microsomal preparation), resuspended microsomal pellets and 2 M guanidinium thiocyanate solubilised membrane-associated enoyl reductase. Crude soluble enoyl-ACP reductase and microsomes were both prepared from *Brassica napus* var. falcon, to eliminate varietal differences. Prior to electrophoresis, protein samples were concentrated using methanol and chloroform and dried protein pellets were resuspended in 50 µl sample buffer (8 M urea, 268 mM 2-mercaptoethanol, 2 % v/v Pharmalyte 3-10 and 0.5 % v/v Triton X-100) and incubated at room temperature for 1 hour. Immuno-screening was detected using enhanced chemiluminescence (ECL) (section 2.5.11.2), since detection of the low levels of isoforms was not possible using an ^{125}I-labelled antibody. Unlike detection using an ^{125}I-labelled antibody, ECL detection was performed in blocking solution containing 5 % w/v skimmed milk instead of 1 % w/v haemoglobin, since haemoglobin contains endogenous peroxidase activity which would interfere with the detection mechanism. Anti-native soluble enoyl-ACP reductase antibody, raised in sheep, was used at a 1:1000 dilution for 2 hours, rabbit anti-sheep IgG antibody was used at a dilution of 1:1000 for 1 hour and horseradish peroxidase labelled anti-rabbit antibody was used at a 1:4000 dilution in blocking solution containing no sodium azide for 1 hour. Sodium azide was not present because it inhibits horseradish peroxidase. The blot was exposed to Fuji X-ray film for 15 seconds and the results obtained are shown in figure 7.4.

a) Crude soluble enoyl-ACP reductase

18 %

SDS PAGE

8 %

- ─────────────→ +

Isoelectric focusing

b) Crude microsomes

18 %

SDS PAGE

8 %

- ─────────────→ +

Isoelectric focusing

c) 2 M guanidinium thiocyanate solubilised membrane-associated enoyl reductase

18 %

SDS PAGE

8 %

- ─────────────→ +

Isoelectric focusing
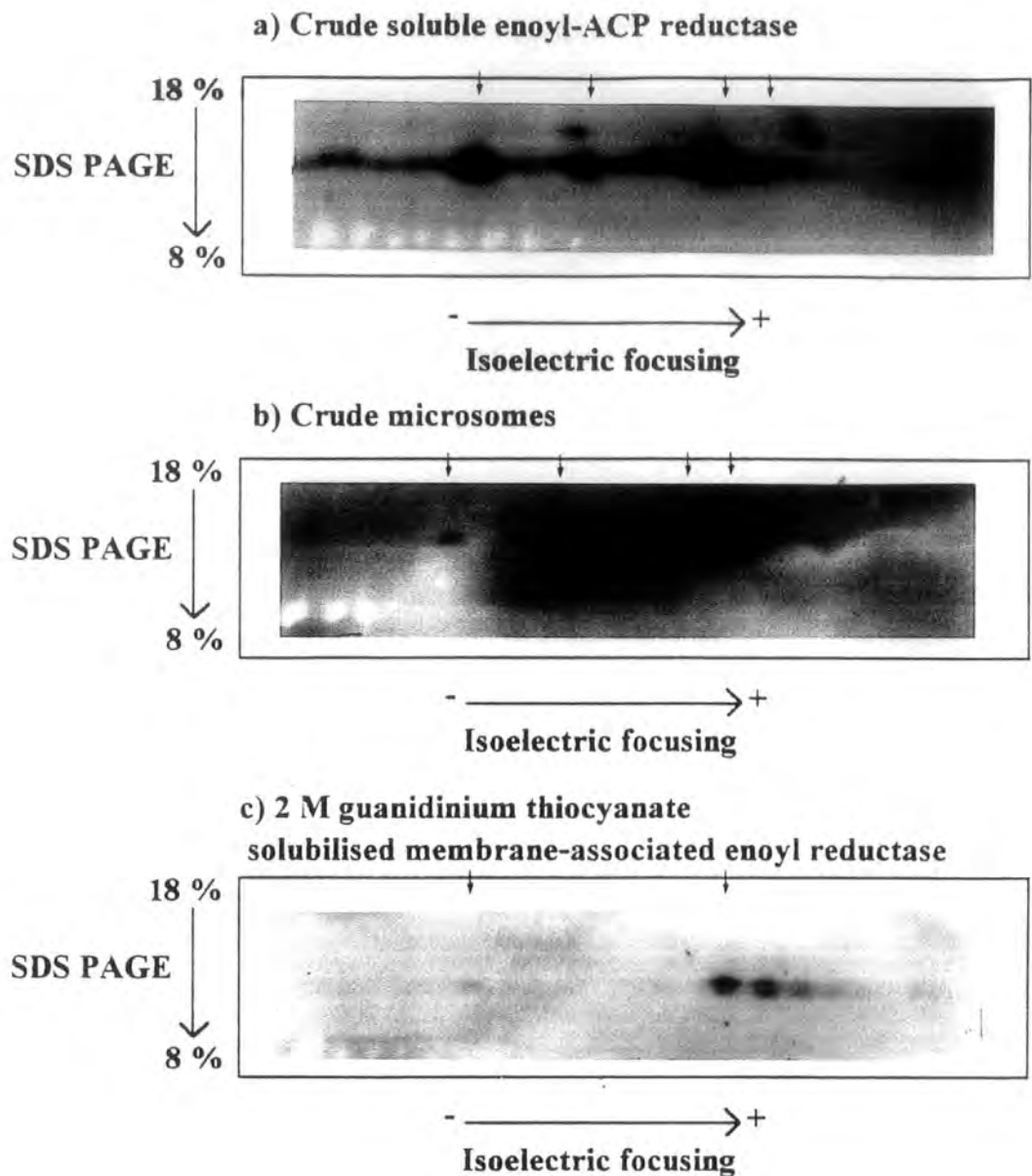
Figure 7.4 Western blot analysis of enoyl reductases separated by two-dimensional electrophoresis using affinity-purified anti-native soluble enoyl-ACP reductase antibody.

Position of spots are marked ↓

a) 5 µl of crude soluble enoyl-ACP reductase

b) 50 µl of crude microsomes

c) 50 µl of 2 M guanidinium thiocyanate solubilised membrane-associated enoyl

reductase

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Crude soluble enoyl-ACP reductase extract contained four isoforms (I to IV from left to right on figure 7.4) which are identical to the isoforms identified in both *Brassica napus* seed and leaf material by Fawcett *et al.* in 1994. Crude microsomes contained four isoforms that were identical to the isoforms in the crude soluble enoyl-ACP reductase extract and since no more isoforms were identified, it was concluded that anti-native enoyl-ACP reductase antibody only recognised soluble enoyl-ACP reductase that was trapped in microsomes, and did not cross-react with the membrane-associated enzyme. In the 2 M guanidinium thiocyanate solubilised membrane-associated enoyl reductase extract, the two most abundant isoforms found in soluble enoyl-ACP reductase were identified, but presumably there was not enough soluble enzyme present to identify the other two isoforms. This is because the microsomes had been extensively washed with 0.03 % v/v LDAO and 0.5 M sodium chloride prior to solubilisation, to remove trapped soluble enoyl-ACP reductase (section 6.5). Carbamylation was detected in the solubilised protein sample, resulting in a characteristic protein trail towards the anode, which is caused when amine groups are progressively converted (carbamylated) into amide groups, resulting in unit changes of the molecule which becomes more acidic. The carbamylation process is shown in figure 7.5.

a) $O = C \underset{NH_2}{\overset{NH_2}{<}} \rightleftharpoons NH_4^+ + NCO^-$

urea   cyanate ion

b) $NCO^- + R - NH_3^+ \longrightarrow R - NH - \overset{O}{\overset{||}{C}} - NH_2$

amine group   conversion of amine group
                                in to amide group
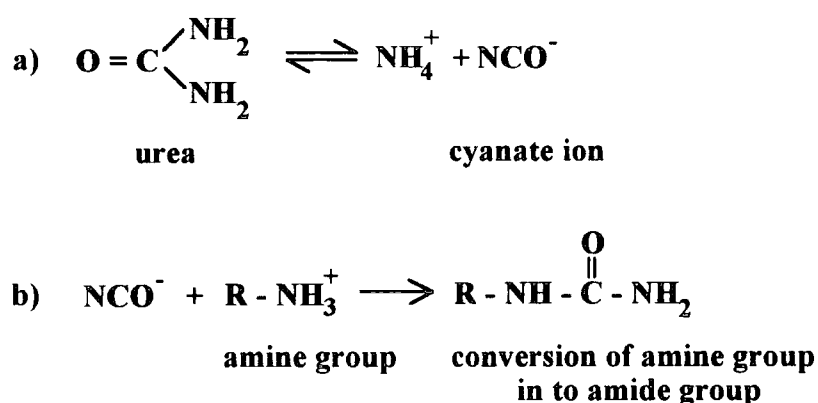
Figure 7.5 The carbamylation process.

Since carbamylation was only detected in the guanidinium thiocyanate solubilised membrane-associated enoyl reductase extract, it suggests that the cause was the presence of the chaotropic agent. It is thought that these extra spots cannot be membrane-associated enoyl reductase, since they are not present in the microsomal extract.

As the only isoforms detected in the membrane-associated enoyl reductase samples were shown to be identical to soluble enoyl-ACP reductase isoforms, it can be concluded that even after extensive washing, not all soluble enoyl-ACP reductase is removed from microsomes and that the anti-native soluble enoyl-ACP reductase antibody does not cross-react with membrane-associated enoyl reductase, and so cannot be used to detect the membrane-associated form. Therefore, an immobilised affinity column cannot be produced to purify membrane-associated enoyl reductase using this antibody. However, the immobilised antibody could be used to remove soluble enoyl-ACP reductase from microsomes.

7.3 Western blot analysis of microsomes using an antibody raised against denatured soluble enoyl-ACP reductase.

Since anti-native soluble enoyl-ACP reductase antibody did not cross-react with membrane-associated enoyl reductase, the cross-reactivity of anti-denatured soluble enoyl-ACP reductase antibody was determined. An anti-denatured antibody will recognise domains in the primary sequence, whereas an anti-native antibody will only recognise domains in the 3-D structure, which are less likely to be conserved between soluble and membrane-associated forms of enoyl reductase.

Anti-denatured soluble enoyl-ACP reductase antibodies, raised in mouse, were prepared by Mr. J. W. Simon, Department of Biological Sciences, University of Durham. Prior to use in a Western blot with microsomes, the sensitivity and specificity of the antibody were determined. To determine the sensitivity of the

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antibody, various amounts of pure recombinant enoyl-ACP reductase (1 - 200 ng) were exposed to the antibody; to examine the specificity of the antibody the cross-reactivity with 20 µl of crude *Brassica napus* seed extract was determined. Western blotting was performed using a 10 % w/v polyacrylamide gel with ECL detection. Anti-denatured soluble enoyl-ACP reductase antibody, raised in mouse, was used at a 1:1000 dilution for 2 hours and horseradish peroxidase labelled anti-mouse IgG antibody was used at a 1:2000 dilution in blocking solution containing no sodium azide for 1 hour. The blot was exposed to Fuji X-ray film for 30 seconds and the results are shown in figure 7.6. Antibody raised against denatured soluble enoyl-ACP reductase was not as sensitive as expected since the minimum amount of antigen recognised was 50 ng whereas routinely, an antibody should be able to detect 1 - 10 ng of antigen. Nevertheless, the antibody was specific for soluble enoyl-ACP reductase; even though proteins with molecular weights greater than soluble enoyl-ACP reductase did cross-react with the antibody, their intensities were much less than soluble enoyl-ACP reductase.

The titre of the antibody that could be used to detect enoyl reductase was determined by examining the ability of different antibody dilutions to detect 100 ng of pure recombinant enoyl-ACP reductase. Enoyl-ACP reductase was directly transferred to nitrocellulose using the Bio-Rad Bio-Dot SF apparatus (section 2.5.10) and immuno-screening of proteins was detected with an ^{125}I-labelled antibody. Anti-denatured enoyl-ACP reductase antibody, raised in mouse, was used at the illustrated dilution (1:250 - 1:10 000) for 2 hours, rabbit anti-mouse IgG antibody was used at a dilution of 1:500 for 1 hour and 5 µCi ^{125}I-labelled donkey anti-rabbit IgG antibody was used for 1 hour. The results, in figure 7.7 shown that detection of antigen was still clearly visible at a dilution of 1:10 000.

After characterisation of the antibody, cross-reactivity of anti-denatured soluble enoyl-ACP reductase antibody with microsomes was determined. The protein
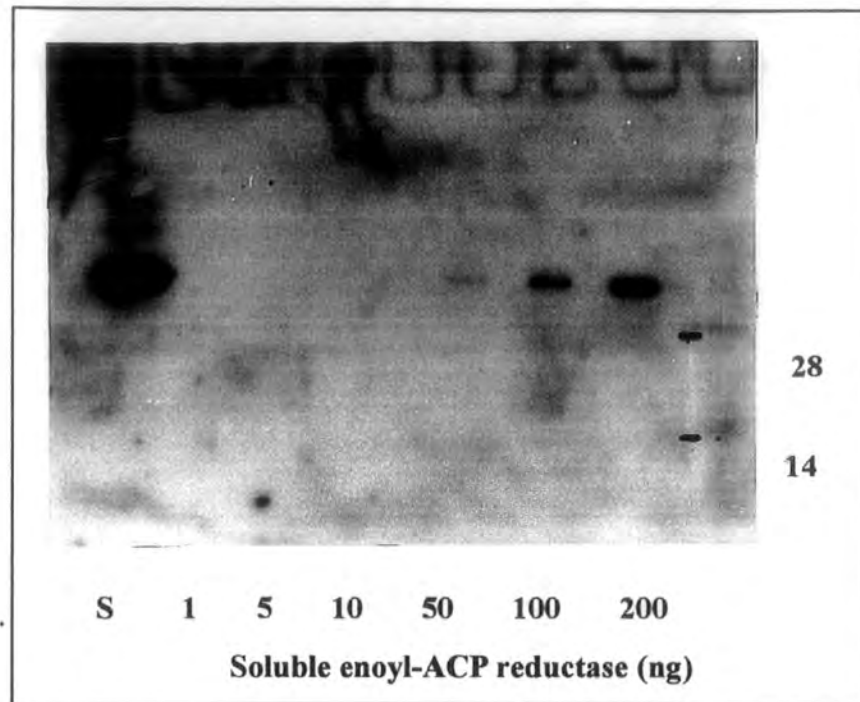
Figure 7.6 Evaluation of the specificity and sensitivity of anti-denatured soluble enoyl-ACP reductase antibody by Western blot analysis.

S = 20 μl of crude *Brassica napus* seed extract

The source of soluble enoyl-ACP reductase used was pure recombinant enzyme
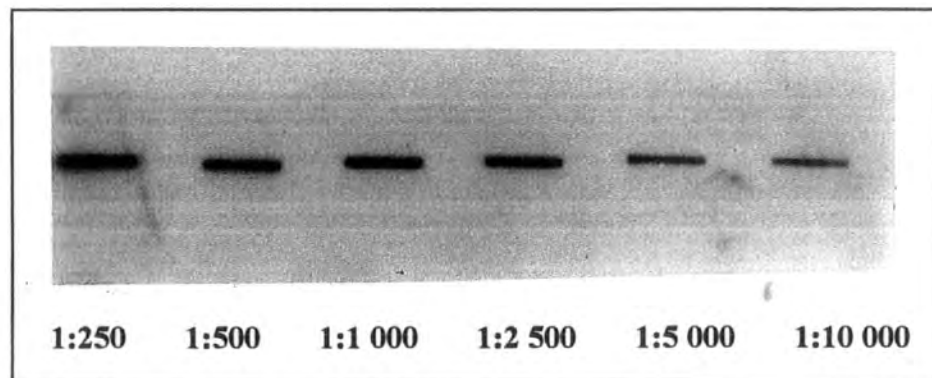
Figure 7.7 Estimation of the titre of anti-denatured soluble enoyl-ACP reductase antibody using slot blotting.

samples used were crude soluble enoyl-ACP reductase (a 40 000 - 200 000 x g supernatant from a microsomal preparation) and resuspended washed microsomal pellets, and Western blotting was performed using a 10 % w/v polyacrylamide resolving gel with ECL detection. ECL detection was used since cross-reactivity between antibody and membrane-associated enoyl reductase may be slight, and so a sensitive detection method was required. Anti-denatured enoyl-ACP reductase antibody, raised in mice, was used at a 1:1000 dilution for 2 hours, even though it had previously been shown that the antibody will recognise antigen at a 1:10 000 dilution, to maximise the cross-reactivity between antibody and membrane-associated enoyl reductase. Horseradish peroxidase labelled anti-mouse antibody was used at a 1:2000 dilution for 1 hour and the blot was exposed to Fuji X-ray film for 30 seconds.

The results, in figure 7.8, show that as with anti-native soluble enoyl-ACP reductase antibody, anti-denatured soluble enoyl-ACP reductase antibody only recognises a species in microsomes that has the same molecular weight as soluble enoyl-ACP reductase. Using 2-dimensional electrophoresis, it has been shown that the anti-native enoyl-ACP reductase antibody cross-reacted with soluble enoyl-ACP reductase only and not membrane-associated enoyl reductase. Due to this contamination of microsomes, it was decided that all soluble enoyl-ACP reductase should be removed from microsomes, using an immobilised anti-native enoyl-ACP reductase antibody column, prior to further investigations to determine whether the anti-denatured soluble enoyl-ACP reductase antibody can cross-react with membrane-associated enoyl reductase.

Figure 7.8 Western blot analysis of microsomes separated by SDS PAGE using anti-denatured soluble enoyl-ACP reductase antibody.

Lane 1) 10 μl of crude soluble enoyl-ACP reductase

Lane 2) 10 μl of resuspended microsomal pellets

Lane 3) molecular weight markers (56, 28, 14 kDa)

Figure 7.9 Western blot analysis of microsomes separated by SDS PAGE using anti-denatured animal FAS antibody.

Lane 1) 50 μl of resuspended microsomal pellets

Lane 2) 5 μl of crude animal FAS

Lane 3) molecular weight markers (56, 200 kDa)

7.4 Western blot analysis of *Brassica napus* microsomes using an anti-animal FAS antibody.

Since antibodies raised against plant soluble enoyl-ACP reductase did not cross-react with membrane-associated enoyl reductase, the cross-reactivity of an anti-denatured animal FAS antibody with *Brassica napus* microsomes was examined. This was to investigate whether plants contain a elongase that has structural similarity to the animal type I FAS as it is unknown whether the *Brassica napus* elongase is a multi-enzyme type I FAS or a dissociable type II FAS.

The antibody used was raised in pig against denatured animal FAS from a lactating rat mammary gland and was prepared by Professor A. R. Slabas, Department of Biological Sciences, University of Durham. An antibody against denatured antigen was used as it is more likely to recognise plant elongase than an antibody raised against native antigen, since an antibody against a denatured antigen recognises domains in the primary amino acid sequence, where there is most likely to be homology with a similar enzyme complex. Protein samples, crude animal FAS from a lactating rat mammary gland (section 2.5.12) and microsomal pellets from *Brassica napus*, were separated by SDS PAGE using a 5 % w/v polyacrylamide resolving gel and a 3 % w/v polyacrylamide stacking gel. A 5 % w/v polyacrylamide resolving gel was required because animal FAS has a subunit molecular weight of 263 kDa (Wakil *et al.*, 1983). Immuno-screening of proteins was detected using an ^{125}I-labelled antibody; anti-denatured animal FAS antibody was used at a 1:500 dilution and incubated with the nitrocellulose for 2 hours, rabbit anti-pig IgG antibody was used at a 1:500 dilution for 1 hour, and 5 μCi ^{125}I-labelled donkey anti-rabbit IgG antibody was incubated for 1 hour. The results are shown in figure 7.9.

The primary antibody recognised animal FAS isolated from a lactating rat mammary gland, but did not cross-react with any proteins in microsomal pellets from *Brassica*

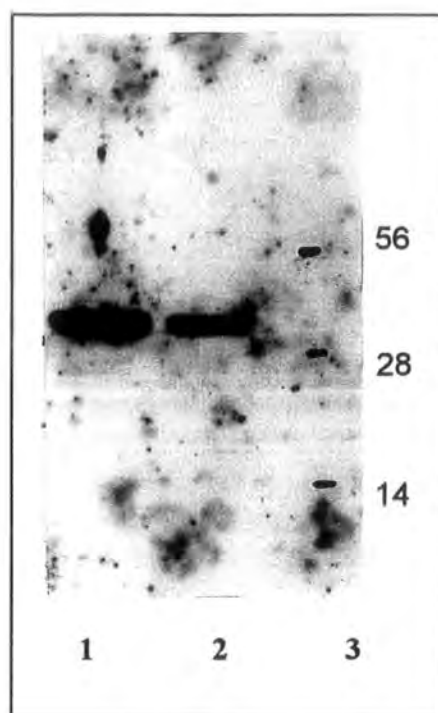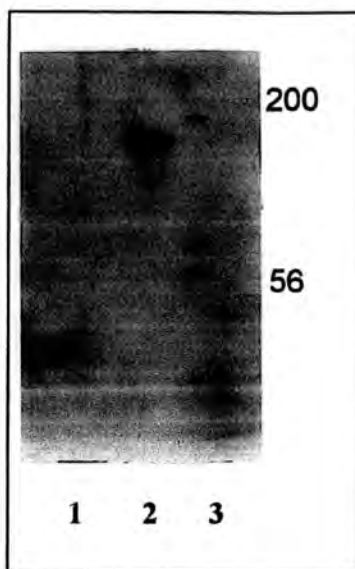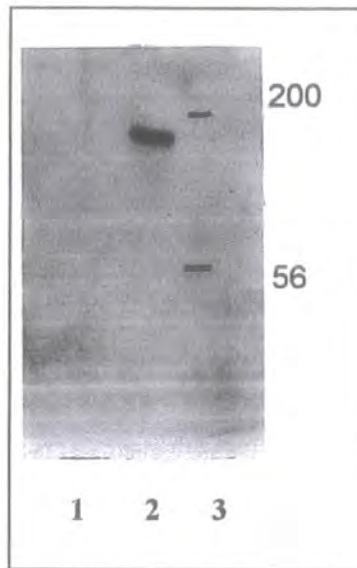**Figure 7.9** Western blot analysis of microsomes separated by SDS PAGE using anti-denatured animal FAS antibody.

Lane 1) 50 μl of resuspended microsomal pellets

Lane 2) 5 μl of crude animal FAS

Lane 3) molecular weight markers (56, 200 kDa)

napus. This result does not directly rule out the possibility of a type I FAS in plants, but if one does exist, it does not have immunological cross-reactivity to the animal type I FAS.

7.5 Western blot analysis of a crude *Brassica napus* extract separated by native gel electrophoresis.

Even though antibodies against soluble enoyl-ACP reductase did not cross-react with membrane-associated enoyl reductase, they could be used to investigate the molecular assembly of soluble enoyl-ACP reductase. Two-dimensional electrophoresis, under denaturing conditions, has shown that four isoforms of soluble enoyl-ACP reductase are present in *Brassica napus* (Fawcett *et al.*, 1994) and studies have shown that the active form of soluble enoyl-ACP reductase is a tetramer (Slabas *et al.*, 1990), but it is not known how the isoforms join to form the tetramer. This could be investigated by determining the number of isoforms present under native conditions, since under these conditions, the isoforms would exist as tetramers. Native PAGE separates components on the basis of their size, net charge and conformation, therefore, if a tetramer was only composed of one isoform, four species would be detected on the Western blot, however, if heterotetramers exist many more combinations of isoforms are possible (e.g. isoforms 1,1,1,1; 1,1,1,2; 1,1,2,2; etc.).

Therefore, Western blot analysis of a crude *Brassica napus* extract separated by native gel electrophoresis using anti-native soluble enoyl-ACP reductase was performed to determine the number of enoyl-ACP reductase species present. Pure recombinant enoyl-ACP reductase and crude soluble enoyl-ACP reductase (a 40 000 - 200 000 x g supernatant from a microsomal preparation) were separated by native gel electrophoresis using a 7.5 % w/v polyacrylamide resolving gel (section 2.5.7) and Western blotting was detected with an ^{125}I-labelled antibody. The

antibody incubations were: affinity-purified anti-native soluble enoyl-ACP reductase antibody, raised in sheep, at a 1:1000 dilution for 2 hours, rabbit anti-sheep IgG antibody at a dilution of 1:1000 for 1 hour and 5 μCi ^{125}I-labelled donkey anti-rabbit IgG antibody for 1 hour. The Fuji X-ray film was exposed at -80°C for 4 hours and cross-reactivity between affinity-purified anti-native soluble enoyl-ACP reductase antibody and the separated proteins is shown in figure 7.10.

Pure recombinant enoyl-ACP reductase contained one main band which was not unexpected since it was formed by expressing one cDNA clone in *E. coli*; in comparison, crude extract contains four major bands. Since only one band is seen when the proteins are separated by SDS PAGE, they all have the same molecular weight, therefore, to be separated by native PAGE, they must differ in net charge and conformation. From the results, it can not be determined exactly how many species the antibody recognises in crude soluble enoyl-ACP reductase, due to the high background caused by smearing. However, since four major bands can be seen, this suggests that the soluble enoyl-ACP reductase isoforms associate into four dominant tetramers in *Brassica napus*.

7.6 Cross-linking of an antibody to an agarose matrix to produce an immobilised antibody column.

Since antibodies raised against soluble enoyl-ACP reductase recognised the soluble enzyme but not the membrane-associated form, immobilised antibodies cannot be used to purify membrane-associated enoyl reductase. However, they could be used to remove contaminating soluble enoyl-ACP reductase from crude membrane-associated enoyl reductase extracts. Microsomes containing no immunologically detectable soluble enoyl-ACP reductase could then be used to determine whether anti-denatured soluble enoyl-ACP reductase antibody cross-reacts with membrane-associated enoyl reductase. Since all soluble enoyl-ACP reductase would have been
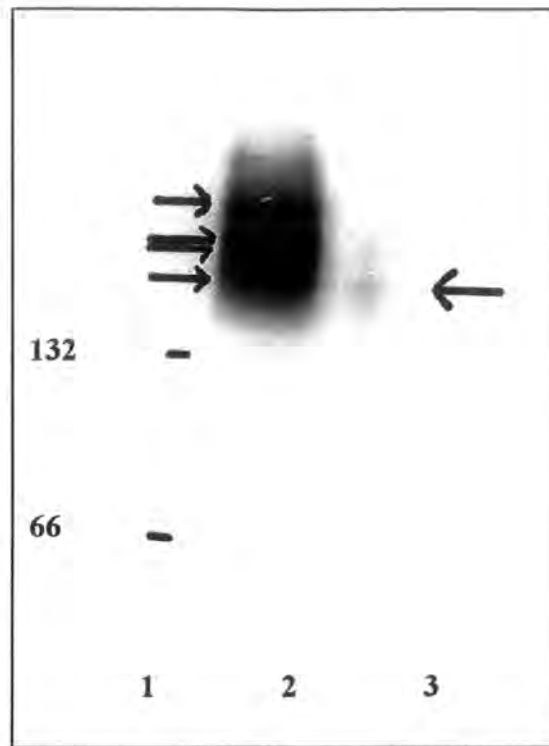
Figure 7.10 Western blot analysis of crude *Brassica napus* extract separated by native gel electrophoresis using affinity-purified anti-native soluble enoyl-ACP reductase antibody.

The positions of the main bands are highlighted with arrows.

Lane 1) molecular weight markers (132, 66 kDa)

Lane 2) 10 µl of crude soluble enoyl-ACP reductase

Lane 3) 15 ng of pure recombinant soluble enoyl-ACP reductase

removed from microsomes, any cross-reactivity would be due to the antibody recognising membrane-associated enoyl reductase. Removal of soluble enoyl-ACP reductase from microsomes could be achieved by immobilising anti-native soluble enoyl-ACP reductase antibodies to a matrix, so that when resuspended microsomal pellets, contaminated with soluble enoyl-ACP reductase, were loaded onto the column, membrane-associated enoyl reductase would be washed through the column, but soluble enoyl-ACP reductase would bind to the antibodies, and so be removed from the extract.

This immobilised antibody column could also be used to easily purify soluble enoyl-ACP reductase so allowing for further characterisation of the isoforms.

7.6.1 Immobilisation of affinity-purified rabbit anti-native soluble enoyl-ACP reductase antibody to protein A agarose.

Affinity-purified rabbit anti-native soluble enoyl-ACP reductase antibody was immobilised to agarose beads via protein A, a 42 kDa cell wall protein from *Staphylococcus aureus*, which binds to the Fc portion of IgG molecules from most mammalian species. Protein A has four potential binding sites for antibodies, yet only two of them can be used at one time. Therefore, since protein A is bifunctional, multimeric complexes are formed. An advantage of coupling antibodies to protein A beads, is that protein A binds to the heavy chain polypeptides of antibody molecules so that the antigen binding site is oriented correctly for maximal interaction with the antigens (Harlow and Lane, 1988). The interaction between protein A and antibodies is shown schematically in figure 7.11.
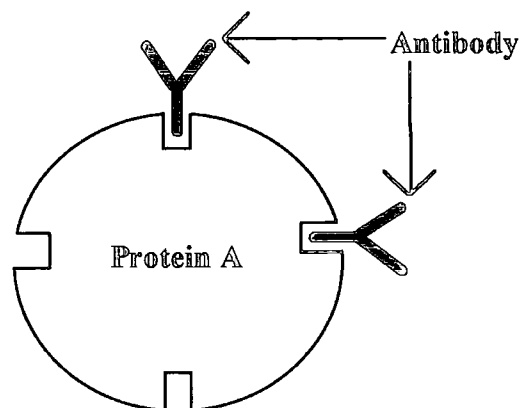
Figure 7.11 Schematic interactions between protein A and antibodies.

The affinity of protein A varies with the species of IgG because the Fc domains vary, and since protein A has a much higher affinity with IgG molecules from rabbit than from sheep (Harlow and Lane, 1988), affinity-purified rabbit anti-native soluble enoyl-ACP reductase antibody was immobilised.

To stabilise the immobilised antibody column, IgG could be cross-linked to protein A via a bifunctional coupling reagent, to overcome the problem of antibody leakage, using the method of Schneider et al., (1982). Dimethyl pimelimidate dihydrochloride is routinely used as a coupling reagent because it is cheap and easy to handle. Both binding groups of dimethyl pimelimidate dihydrochloride bind to free amino groups, and because the carbon backbone has a great deal of flexibility, most antibody-protein A pairs will have reactive sites within a suitable distance to allow efficient coupling (Harlow and Lane, 1988).

Rabbit anti-native soluble enoyl-ACP reductase antibody was immobilised to protein A using a Bio-Rad Affi-Gel® Protein A MAPS® II (monoclonal antibody purification system) kit (section 2.5.13). The optical densities of all supernatants collected were measured at 280 nm, and it was calculated that 1.4 mg of antibody bound to 1 ml of matrix. A molar extinction coefficient of 2.1×10^5 M^{-1} cm^{-1} at

280 nm was used for IgG. In addition, the total protein concentration of each supernatant was calculated using the Bio-Rad protein assay (section 2.5.3) and from these results it was determined that 1.1 mg of IgG bound. This was lower than expected, since according to manufacturer's instructions, protein A is capable of binding 6 - 8 mg IgG per ml protein A. Nevertheless, it was decided that this column could be used since at least 1 mg of antibody had bound which should be capable of binding 2 mg of enoyl-ACP reductase. Since an antibody molecule has two antigen binding sites, it should be capable of binding two antigen molecules at a time, and as the molecular weight of an antibody (150 kDa; Harlow and Lane, 1988) is similar to the molecular weight of enoyl-ACP reductase (140 kDa; Slabas et al., 1990), it can be expected that 2 mg of enoyl-ACP reductase will bind to 1 mg of IgG.

7.6.2 Labelling soluble enoyl-ACP reductase with ^{35}S-methionine.

Before the immobilised antibody column could be used in an attempt to purify crude soluble enoyl-ACP reductase, the binding capacity and recovery of the column were assessed using pure recombinant enoyl-ACP reductase that had been spiked with ^{35}S-methionine labelled enoyl-ACP reductase. ^{35}S-methionine labelled enoyl-ACP reductase was used as it enabled easy detection of antigen. Therefore, prior to calculating the binding capacity and recovery of the column, ^{35}S-methionine labelled enoyl-ACP reductase had to be produced and this was achieved using two different methods. Enoyl-ACP reductase cDNA, which had been cloned into a pET 11-d vector (pEAR2) (Kater et al., 1991) was transformed into calcium chloride competent BL21 E. coli (section 2.4.12), prior to labelling in either LB or minimal media (sections 2.5.14.1 and 2.5.14.2, respectively).

To calculate the amount of radioactivity incorporated into enoyl-ACP reductase by both methods, 5 µl of each supernatant was counted in a scintillation counter. The final supernatant from a 10 ml culture grown in LB media contained approximately 6×10^5 cpm. A similar culture grown in minimal media contained approximately 4×10^6 cpm, and consequently, this was the preferred method.

To identify the labelled proteins, 20 µl of each supernatant were analysed by SDS polyacrylamide electrophoresis, using a 10 % w/v polyacrylamide resolving gel. The gel was soaked in Amplify (from Amersham International Plc.) for 15 minutes for fluorography, dried and exposed to Fuji X-ray film at -80°C overnight. The results, in figure 7.12, show that two major proteins were labelled. The higher band is expected to be enoyl-ACP reductase since it runs in the same position as pure enoyl-ACP reductase; the lower band has previously been sequenced and was shown to be β-lactamase (Dr. T. Fawcett, Department of Biological Science, University of Durham; personal communication). β-lactamase is produced because the β-lactamase gene is orientated in the same direction as the T7 promoter, and therefore, is expressed (Ausubel et $al.$, 1992). Unincorporated ^{35}S-methionine can be seen at the dye front.

Since the ^{35}S-labelled enoyl-ACP reductase extract was contaminated with labelled β-lactamase, it needed to be partially purified, and so radiolabelled enoyl-ACP reductase, produced by growth in minimal media, was partially purified by ion exchange chromatography using a Pharmacia Smart system. A Pharmacia Mono Q anion exchange column (50 mm x 1.6 mm) was equilibrated in 10 mM sodium phosphate (pH 6.2) before a gradient (100 µl/min) was created with 10 mM sodium phosphate (pH 6.2) containing 1 M sodium chloride; 300 µl fractions were collected throughout the run. Elution was monitored by following the absorbance at
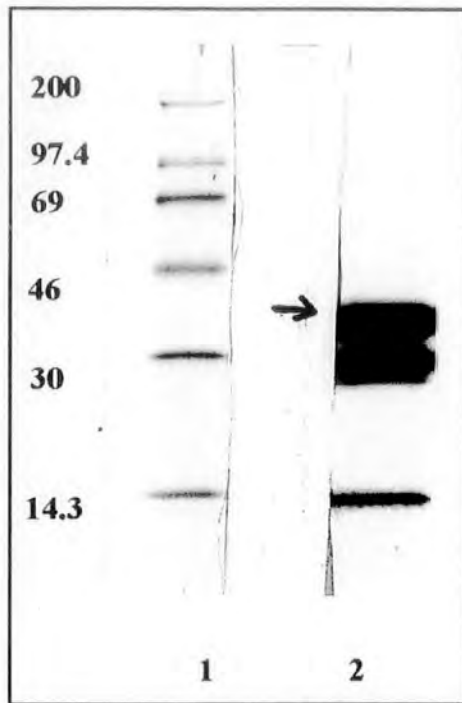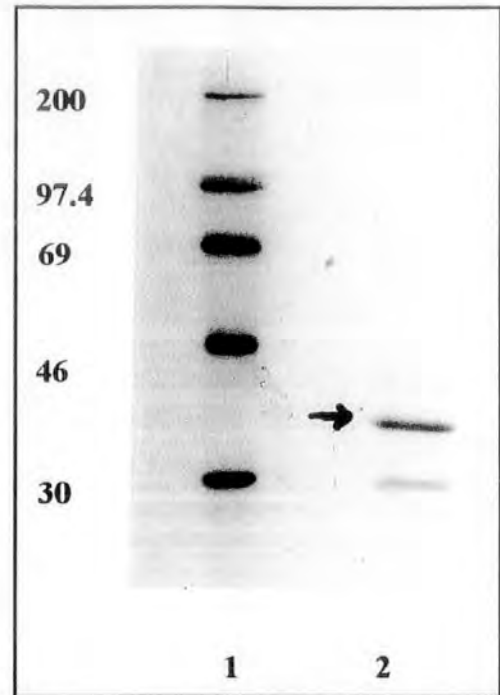
a) Growth in minimal media   **b) Growth in LB media**

Figure 7.12 Analysis of proteins radiolabelled with ^{35}S-methionine.

Radiolabelled proteins were separated by SDS PAGE using a 10 % w/v polyacrylamide resolving gel and subjected to fluorography. The position of pure recombinant enoyl-ACP reductase on the gel is highlighted with an arrow.

Lane 1) ^{14}C-labelled molecular weight markers (200, 97.4, 69, 46, 30 and 14.3 kDa)

Lane 2) 20 µl of supernatant after cell lysis

280 nm and fractions were assayed for soluble enoyl-ACP reductase activity, using the method described in section 2.5.1.2.

The fractions containing enzyme activity were pooled and analysed by SDS PAGE and fluorography, and the results are shown in figure 7.13. After ion exchange chromatography, the only radio-labelled protein detected was enoyl-ACP reductase, therefore, this sample could be used to spike pure recombinant enoyl-ACP reductase. The specific activity of purified radiolabelled enoyl-ACP reductase was approximately 70 μCi/mg.

7.6.3 Calculation of the binding capacity and recovery of an anti-native soluble enoyl-ACP reductase antibody immobilised column.

Before the immobilised antibody column could be used to remove soluble enoyl-ACP reductase from microsomes or to purify soluble enoyl-ACP reductase, the column's binding capacity and recovery need to be calculated. As previously stated, it is expected that 2 mg of enoyl-ACP reductase will bind to 1 mg of IgG, and so the binding capacity of an immobilised anti-native soluble enoyl-ACP reductase antibody column was determined, using radiolabelled enoyl-ACP reductase. All chromatography steps were performed at room temperature. Initially, the column was equilibrated with 5 ml of 4 M magnesium chloride containing 20 mM Tris base (the pH was not adjusted and was 6.8) at 350 μl/min; 1.5 ml fractions were collected and the optical density at 280 nm was measured. This was to check that 4 M magnesium chloride containing 20 mM Tris base did not cause IgG to be eluted from protein A. As no protein was eluted, the column was equilibrated with 4 column volumes of 50 mM Tris/HCl (pH 7.0) at 700 μl/min; 1.5 ml fractions were collected and the optical density at 280 nm was measured. This was done to check that 50 mM Tris/HCl (pH 7.0) did not cause IgG to be eluted from protein A. Again, no
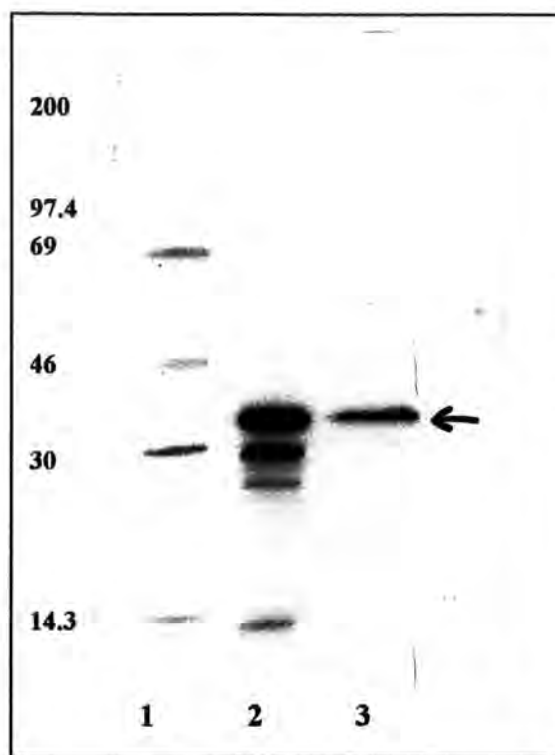
Figure 7.13 Purification of ^{35}S-labelled enoyl-ACP reductase by ion exchange chromatography.

Radiolabelled proteins were separated by SDS PAGE using a 10 % w/v polyacrylamide resolving gel and subjected to fluorography. The position of pure recombinant enoyl-ACP reductase on the gel is highlighted with an arrow.

Lane 1) ^{14}C-labelled molecular weight markers (200, 97.4, 69, 46, 30 and 14.3 kDa)

Lane 2) 5 µl of supernatant after cell lysis

Lane 3) 20 µl of pooled active fractions after ion exchange chromatography

protein was eluted, and therefore, pure recombinant enoyl-ACP reductase (6 mg), containing ^{35}S-labelled enoyl-ACP reductase (35 000 cpm), was loaded onto the column at 350 μl/min, to allow maximum binding. Unbound proteins were washed from the matrix with 50 mM Tris/HCl (pH 7.0) at 1 ml/min until no more protein could be detected at 280 nm. Enoyl reductase in the wash was measured by detecting ^{35}S, measuring enzyme activity and calculating the total protein concentration, and it was determined that approximately 50 % of the enoyl-ACP reductase (3 mg) bound to the column. Considering at least 1 mg of antibody bound to the column, it is expected that approximately 2 - 3 mg of enoyl reductase would bind to the column.

The procedure to elute antigen used 4 M magnesium chloride containing 20 mM Tris base (the pH was not adjusted and was 6.8) at 350 μl/min; 1.5 ml fractions were collected and monitored for the presence of antigen. Recovery of antigen after magnesium chloride elution was; 14 % determined by monitoring ^{35}S, 0 % determined by measuring enzyme activity and 2 % determined from the total protein concentration. From these results, it appeared that enoyl-ACP reductase had not been efficiently eluted, but it is unknown why ^{35}S was detected in the eluted fractions when enoyl reductase activity and total protein were not detected.

Since ^{35}S-labelled enoyl-ACP reductase had not been detected in the eluant, an alternative elution procedure, acid/base elution, was tried to elute the remaining enoyl-ACP reductase (Harlow and Lane, 1988). Elution was performed with 8 ml of 100 mM glycine (pH 2.5) at 350 μl/min and 1.5 ml fractions were collected into tubes containing 200 μl of 1 M Tris/HCl (pH 8.0), to neutralise the acid. Then the column was equilibrated with 5 ml of 50 mM Tris/HCl (pH 7.0), prior to elution

with 8 ml of 100 mM triethanolamine (pH 11.5) at 350 μl/min. Again, 1.5 ml fractions were collected into tubes containing 200 μl of 1 M Tris/HCl (pH 8.0), to neutralise the base. The amount of antigen eluted was determined by monitoring ^{35}S and measuring protein by the absorbance at 280 nm; however, no antigen was eluted using either glycine (pH 2.5) or triethanolamine (pH 11.5).

Therefore, as two elution methods could not elute enoyl-ACP reductase from the column, it was decided to determine whether the antigen had bound irreversibly to the protein A agarose matrix, rather than to the antibody. In this control experiment, a new column was made as described above expect no antibody was used. Labelled enoyl-ACP reductase (11.64 mg, 70 380 cpm) was loaded on to the column, but only 2 % of the total protein (0.23 mg) and 10 % of the radioactivity bound, and since this was much less than that bound to the antibody immobilised column (i.e. 0.23 mg instead of 3 mg), the component responsible for antigen binding must be the antibody.

Since the control experiment showed that most of the antigen can only bind to immobilised antibody, elution of the antigen from the original antibody immobilised column was tried using a range of elutants that were stronger than magnesium chloride, based on the elution series described in Harlow and Lane, 1988. The elutants used were 5 M lithium chloride in 10 mM Tris/HCl (pH 7.2) - the weakest elutant, water, 50 % v/v ethylene glycol (pH 8.0) and 50 % v/v ethylene glycol (pH 11.5) - the strongest elutant. The recoveries obtained are shown in table 7.1.

Table 7.1 Recoveries of ^{35}S-labelled enoyl-ACP reductase from an anti-native soluble enoyl-ACP reductase antibody immobilised column using a variety of elutants.

Prior to elution, 11.11 mg of enoyl-ACP reductase (64 845 cpm) were bound to the column.

Elutant	% enoyl-ACP reductase eluted	
	Determined from cpm	Determined from total protein
5 M lithium chloride in 10 mM Tris/HCl (pH 7.2)	0	0
water	0	0
50 % v/v ethylene glycol (pH 8.0)	0	0
50 % v/v ethylene glycol (pH 11.5)	8	13

Only 50 % v/v ethylene glycol (pH 11.5) caused elution of antigen, the recovery was approximately 10 %; using the other elutants, the recovery was 0 %. Obviously, these recoveries are not good enough to use routinely.

Considering the antibody immobilised to protein A agarose matrix had been affinity-purified by elution from an immobilised antigen column using 4 M magnesium chloride containing 20 mM Tris base, it was decided to repeat magnesium chloride elution of the antigen from the antibody column. A second antibody immobilised column was produced, using the method described above, and 6 mg (125 400 cpm) of enoyl-ACP reductase was loaded onto it. Forty per cent (2.4 mg) of antigen bound (determined by monitoring ^{35}S) and 7.5 % of it was eluted with magnesium chloride.

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It was assumed that no more of this antigen could be removed, so enoyl reductase with a higher specific activity was loaded (0.5 mg, 636 800 cpm). More counts were used to reduce errors caused by background counts. Fifty per cent (0.25 mg) of the label bound and 10 % was eluted by magnesium chloride.

Therefore, using 4 M magnesium chloride or 50 % v/v ethylene glycol (pH 11.5) approximately only 10 % of the label was eluted, but it is not known why the recovery was so low. To confirm that the antigen had bound to the column, a harsh elution method was used to see if under extreme conditions, the antigen could be eluted. An aliquot of matrix (100 μl) was boiled in 1 % w/v SDS for 2 minutes, centrifuged for 1 minute and the supernatant removed. Forty per cent of the label was measured in the supernatant, i.e. 40 % of the antigen had been eluted. Therefore, enoyl-ACP reductase had bound to the column, and this harsh method removed antigen that the magnesium chloride method did not.

Problems with magnesium chloride elution were not anticipated because this method had been used during affinity-purification of anti-native enoyl-ACP reductase antibody. However, it is possible that the method used to affinity-purify the antibody somehow changed the avidity of the antibody, so that it still bound to enoyl-ACP reductase, but irreversibly. In addition, as large amounts of antibody were affinity-purified, there was no need to calculate the recovery, but it is possible that elution with magnesium chloride may not be an efficient method. As a result of this, when small amounts of antigen are bound to the antibody column, losses during elution are clearly detected. Alternatively, the antigen may have bound to the top of the antibody column, so that during elution, it re-bound to the antibody further down the column.

Clearly, this method cannot be used to purify soluble enoyl-ACP reductase from a crude extract; the methods of antibody preparation, and binding and elution of antigen need to be optimised first. Additionally, to determine if the avidity of the antibody was altered by magnesium chloride, less harsh elutants (e.g. acid [pH 1.5 - 3] or base [pH 10 - 12.5]) could be used to affinity-purify the antibody, which could then be immobilised to protein A agarose matrix.

7.7 Discussion.

Several antibodies were used in Western blots with crude *Brassica napus* microsomes, to identify either membrane-associated enoyl reductase or the whole elongase complex. Affinity-purified anti-native soluble enoyl-ACP reductase recognised a species in microsomes, but this was shown to be due to the presence of contaminating soluble enoyl-ACP reductase by 2-dimensional electrophoresis. Since the antibody failed to cross-react with membrane-associated enoyl reductase, it suggests that there is little structural homology between the two types of enzyme and that they are immunologically distinct. As this antibody recognised all four isoforms of soluble enoyl-ACP reductase, it could be a useful tool, but not to study membrane-associated enoyl reductase. However, the antibody could be used to remove contaminating soluble enoyl-ACP reductase from membrane-associated enoyl reductase samples.

Anti-denatured soluble enoyl-ACP reductase antibody cross-reacted with a species in microsomes which has the same molecular weight as soluble enoyl-ACP reductase. Investigations to determine whether this cross-reactivity was due to contaminating soluble enoyl-ACP reductase or the membrane-associated form could easily be

performed once soluble enoyl-ACP reductase had been removed from microsomes using the anti-native soluble enoyl-ACP reductase antibody.

An anti-denatured animal FAS antibody was used to try and identify a type I FAS in plant material but it did not cross-react with any species in *Brassica napus* microsomes. This does not rule out the possible existence of a type I FAS in plants, yet if one does exist, it does not have similarity with the structural domains recognised by an anti-animal FAS antibody. However, the fatty acid elongation 1 (FAE1) gene from *Arabidopsis thaliana* has recently been cloned and the gene product, which is required for the synthesis of very long chain acids, is presumed to be the condensing enzyme that extends the chain length of fatty acids from C18 to C22 (James *et al.*, 1995), suggesting that the *Arabidopsis thaliana* elongase is a type II FAS, composed of dissociable enzymes. It will be interesting to determine if the *Brassica napus* elongase is also a Type II FAS, as the species are highly related.

In summary, membrane-associated enoyl reductase appears to be a distinct enzyme that does not share structural homology with either animal type I FAS or plant type II soluble enoyl-ACP reductase, so to identify membrane-associated enoyl reductase, alternative methods, such as the use of photoreactive substrate analogues may have to be used.

Native gel electrophoresis was used to determine whether the four isoforms of enoyl-ACP reductase form homo- or heterotetramers, since native PAGE separates components on the basis of their size, net charge and conformation, so will separate tetramers that differ by charge. If a tetramer was composed of one isoform only, four species would be detected on the Western blot. However, if heterotetramers existed many more combinations of isoforms are possible. Native PAGE failed to show if the four isoforms of soluble enoyl-ACP reductase form homo- or heterotetramers, since

it could not be determined whether more than four bands were present on the Western blot. However, it did show that four dominant enoyl-ACP reductase tetramers are formed in *Brassica napus* which differ in net charge and conformation and they can be partially separated by native PAGE.

An immobilised antibody column was produced by cross-linking anti-native soluble enoyl-ACP reductase to protein A agarose and it was hoped that this column could be used to easily purify soluble enoyl-ACP reductase and maybe resolve the isoforms, and to remove contaminating soluble enoyl-ACP reductase from crude membrane-associated enoyl reductase extracts, since this antibody does not cross-react with the membrane-associated form. However, using radiolabelled enoyl-ACP reductase, the recovery from the column was very low, and consequently, the procedure needs to be optimised before it can be used to purify soluble enoyl-ACP reductase from a crude extract. Despite this, the column could be a useful tool to remove contaminating soluble enoyl-ACP reductase from crude microsomes.

Chapter 8. Discussion.

8.1 Achievements of this research and suggestions for future work.

The objectives of this research were to characterise membrane-associated enoyl reductase and to compare it to soluble enoyl-ACP reductase.

Previous research has shown that four genes encoding enoyl-ACP reductase exist in *Brassica napus*, yet prior to this research, only one cDNA had been isolated (Kater *et al.*, 1991). Using this cDNA to screen a *Brassica napus* leaf library, a clone was isolated that despite virtual identity to the original cDNA in the coding region, differs in the transit peptide and 3' non-translated region. Clearly, this isolated clone is not the same as the original cDNA and represents a second cDNA encoding enoyl-ACP reductase. Due to the lack of similarity between the two clones at the 3' non-translated region, they only share 40 % homology, these regions can be used as a tool to differentiate between the clones. Since the commencement of the work described in this thesis, 2-D Western blot analysis has identified four isoforms of enoyl-ACP reductase in both leaf and seed material (Fawcett *et al.*, 1994). Future isolation of the remaining two cDNA's would aid subsequent promoter analysis to study regulation and expression of the four genes.

Comparison of the deduced amino acid sequences of the two enoyl-ACP reductase cDNA's with other enoyl reductase sequences highlights conserved regions and aids the determination of the residues required for enzyme activity. For example, Gly25 is conserved in all of the sequences compared, and from X-ray analysis of crystallised enoyl reductase, it is thought that this residue is involved in nucleotide binding. Comparison of the deduced amino acid sequence of the clone isolated from a *Brassica napus* leaf library with the other sequences also highlighted residues that are

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not essential for enzyme activity. For example, Gly44, which is conserved in all the other sequences, is not present in this 'leaf' sequence.

As the probe used to screen the *Brassica napus* leaf library did not hybridise to an enoyl reductase containing a membrane binding domain, biochemical studies were performed to characterise membrane-associated enoyl reductase. This enzyme is involved in the elongation of VLCFA's, and since it is a long term objective to increase the amount of industrially important VLCFA's, the enzymes involved in their biosynthesis need to be well characterised. Prior to this research, little was known about membrane-associated enoyl reductase, as previous work was centred on either the elongating KAS or the complete elongase. This research was aimed at characterising a different component of the elongase and is the first reported detailed study of both soluble and membrane-associated forms of one FAS enzyme.

Using knowledge of soluble enoyl-ACP reductase, an optical assay was developed to measure membrane-associated enoyl reductase activity; this involved the enzymatic synthesis of the appropriate very long chain acyl-CoA, C20:2-CoA[*t*2, *c*11]. Using this acyl-CoA, it was determined that membrane-associated enoyl reductase can utilise both pyridine nucleotides. However, the enzyme was inactive with the short chain substrate, C4:1-CoA[*t*2]. Since soluble enoyl-ACP reductase can also utilise C20:2-CoA[*t*2, *c*11] in the presence of NADH, contamination due to the soluble enzyme caused problems when membrane-associated enoyl reductase activity was detected with NADH. As a result of this, microsomes were washed with 0.5 M sodium chloride to minimise soluble enoyl-ACP reductase contamination. Without this washing step, misinterpretation of results would have occurred, yet the importance of washing microsomes to remove contaminating soluble FAS enzymes has been overlooked by other researchers.

Since membrane-associated enoyl reductase activity was detected in both LEAR and HEAR, it can be concluded that the elongating enoyl reductase was not mutated to produce LEAR. Therefore, while the mutated step still has to be identified, it is known that the enoyl reductase can be ruled out.

Optimisation of the membrane-associated enoyl reductase assay was achieved by the inclusion of BSA, DTT and magnesium chloride in the reaction mixture, and by altering the pH at which assays were performed. The inclusion of 40 µM BSA, 0.8 mM magnesium chloride and 1.6 mM DTT increased NADH membrane-associated enoyl reductase activity by 116 %. Studying the effect pH had on membrane-associated and soluble enoyl reductases discriminated between the two NADH-utilising enzymes and also suggested the existence of two enoyl reductases in microsomes that differ by their pyridine nucleotide specificity.

The effects of the herbicide diflufenican, sodium chloride and Triton X-100 on membrane-associated and soluble enoyl reductase activities were investigated in an attempt to further discriminate between the two NADH utilising enzymes. A discriminating assay could not be designed, but the results confirmed the hypothesis that they are distinct enzymes. Even though studies using diflufenican failed to discriminate between the NADH-utilising soluble and membrane-associated enoyl reductases, the effect it had on soluble enoyl-ACP reductase was dependent on the acyl-CoA used, since activity was only inhibited when the substrate used was C4:1-CoA[t2] and not C20:2-CoA[t2, c11]. This suggests that soluble enoyl-ACP reductase has a different rate limiting step dependent on the acyl-CoA used, and therefore, further studies are required to determine how substrates are recognised and bound to enoyl-ACP reductase. Co-crystallisation of the enzyme with substrates may aid this investigation since similar studies have already been performed with NADH (Rafferty *et al.*, 1995).

Results from studying the effects of sodium chloride, octyl glucoside, LDAO and CHAPS on membrane-associated enoyl reductase activity, along with the differing thermal stability of membrane-associated enoyl reductase when assayed with each pyridine nucleotide, confirmed that two separate membrane-associated enoyl reductases exist in *Brassica napus* seeds. Membrane-associated enoyl reductase in *Allium porrum* microsomes can also utilise NADH and NADPH (Spinner *et al.*, 1995), however, no studies on whether this activity is due to one or two enzymes have been reported. At present, it is not known why two membrane-associated enoyl reductase are involved in VLCFA biosynthesis.

Kinetic data was determined for the two membrane-associated enoyl reductases. The apparent K_M's were approximately 9 μM and 11 μM for NADH and NADPH, respectively and the K_M for C20:20-CoA[$t2$, $c11$] with NADH and NADPH was approximately 100 μM and 125 μM, respectively.

Solubilisation of both forms of membrane-associated enoyl reductase was necessary for future purification attempts. Prior to attempts to solubilise the membrane-associated enoyl reductases, it was important to determine the maximum concentration of a detergent that allows enzyme activity to be detected. The use of higher concentrations of detergent would mean that even if an enzyme had been solubilised, its activity would not be detected in the soluble fraction. Initial studies using the detergent LDAO, suggested that NADH membrane-associated enoyl reductase had been solubilised. However, further studies determined that the activity measured was actually due to contaminating soluble enoyl-ACP reductase. This result highlights the importance of appropriate control experiments when performing solubilisation experiments. Such controls have not been reported in previous elongase solubilisation experiments.

After contaminating soluble enoyl-ACP reductase had been removed from microsomes by washing with sodium chloride, several different solubilisation methods, including use of salts or a chelating agent were used but only 2 M guanidinium thiocyanate solubilised both membrane-associated enoyl reductases. This chaotropic agent is also a soluble enoyl-ACP reductase inhibitor, proving that the solubilised enzyme activity cannot be attributed to contaminating soluble enoyl-ACP reductase. Thus guanidinium thiocyanate can be used as a useful tool to differentiate between NADH-utilising soluble and membrane-associated enoyl reductases. Partial purification of membrane-associated enoyl reductases was achieved by washing microsomes with sodium chloride and LDAO prior to solubilisation with guanidinium thiocyanate and this resulted in a purification of the NADH and NADPH enzymes of 6.5 and 24-fold, respectively.

The solubilised enzymes showed unusual properties because activities were not inhibited by boiling, raising doubts as to whether the activities monitored were actually due to the action of enzymes. This was resolved, though, by the determination that the components required for activity were greater than 30 kDa and were sensitive to proteinase K. It is thought that guanidinium thiocyanate protects the enzyme from denaturation by heat treatment, which is similar to the effect formaldehyde has on prions.

In order to characterise the solubilised membrane-associated enoyl reductases, a variety of antibodies were used to see if immunological cross-reactivity could circumvent extensive purification procedures. In addition, it was desirable to design an alternative method of membrane-associated enoyl reductase detection, prior to purification attempts, since after solubilisation and dialysis against 20 % v/v glycerol (to remove guanidinium thiocyanate for subsequent enzyme purification), only 36 % and 26 % activities remain for the NADH and NADPH enzymes, respectively.

However, even though activity has been lost through these methods, inactive protein may also be present.

Cross-reactivity of an affinity-purified anti-native soluble enoyl-ACP reductase antibody with a species present in microsomes was later found, by 2-D Western blotting, to be due to contaminating soluble enoyl-ACP reductase. Again, the problem of soluble enzyme contamination is highlighted. Since the antibody recognised soluble enoyl-ACP reductase, but not the membrane-associated forms, an affinity column to rapidly purify the membrane-associated enoyl reductases could not be produced, but it is hoped that synthesis of an immobilised antibody column could be used to remove all soluble enoyl-ACP reductase from microsomes. Since an anti-denatured animal FAS antibody also did not cross-react with a species in microsomes, the results are consistent with the membrane-associated enoyl reductases being immunologically distinct to soluble enoyl-ACP reductase and a type I FAS.

Attempts were made to produce an immobilised antibody column that could be used to easily purify soluble enoyl-ACP reductase and by paying careful attention to the elution conditions may separate the four isoforms. Even though soluble enoyl-ACP reductase did bind to the affinity column, the method of elution needs to be optimised before the column is used to purify crude enzyme. Despite this, the column could be used to remove soluble enoyl-ACP reductase from microsomes.

As the available antibodies failed to cross-react with membrane-associated enoyl reductase, they could not be utilised to develop an assay. An alternative detection system which could be used to assay membrane-associated enoyl reductase involves the use of photoreactive substrate analogues. Such analogues have previously been used to study acyl-CoA:alcohol acyltransferase from jojoba (Shockey *et al.*, 1995). A similarly labelled very long chain saturated fatty acyl-CoA would resemble the reaction

product of membrane-associated enoyl reductase and hence may bind to the enzyme. However, care has to be taken when using such an analogue since it may not be specific for one enzyme, and as a result, would bind to several enzymes, such as soluble enoyl-ACP reductase and the condensing enzymes. But by competing binding of the analogue with the true reaction product acyl-CoA, the membrane-associated enoyl reductase should be identified.

Thus some insight into the nature of enoyl reductase in *Brassica napus* has been achieved. Clearly there are three forms of the enzyme, one soluble and two membrane-associated forms. However, the level of membrane-associate enoyl reductase within seeds is lower than anticipated and so elucidation of the structure of the enzymes may require a genetic approach involving mutants. Such an approach has been used to clone the elongase condensing enzyme from *Arabidopsis thaliana* (James *et al.*, 1995). Alternatively, if two probes could be designed from soluble enoyl-ACP reductase, one that represents a catalytic region likely to be present on all enoyl reductases and a second probe representing a region specific to soluble enoyl-ACP reductases, a library could be screened to isolate soluble enoyl-ACP reductase clones, that would hybridise to both probes, and putative membrane-associated enoyl reductase clones, that would only hybridise to the probe encoding a catalytic region.

Despite the fact that the level of membrane-associated enoyl reductase within the seed is lower than anticipated, this study has clearly shown that the membrane-associated enoyl reductases are distinct from the soluble enzyme and by their differing substrate specificities, they must have different roles within the cell.

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