Studies towards the isolation of a functional plant dehydratase of fatty acid biosynthesis

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A thesis submitted for degree of Doctor of Philosophy

By

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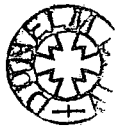

Summary

Fatty acid synthetases have been well studied, in plants such that cDNA clones have been isolated for all of the component enzymes with the exception of β-hydroxyacyl ACP dehydratase. The gene coding for β-hydroxyacyl ACP dehydratase from *E. coli* has been identified (*fabZ*).

The amino acid sequence derived from FabZ was used to interrogate the EST database (dBEST), and a homologous sequence form *Ricinus communis* was identified which showed 45 % homology to the *E. coli fabZ* sequence. The EST was amplified form a developing castor endosperm cDNA library and the amplified product use to screen a developing *Brassica napus* developing embryo library. A number of clones were identified on the primary screen and were purified. One clone (SD4) contained a single open reading frame that showed 40.4 % homology to the EST sequence used as probe and the *E. coli* fabz sequence. Analysis on the amino acid deduced from SD4 revealed that the sequence contains a putative chloroplast target sequence at the N-terminus. Cleavage of the target sequence would leave a mature protein of 19 kDa. This is similar in size to β-hydroxyacyl ACP dehydratase previously purified form *Spinacia oleracea*. A number of other clones from the library screen contained cDNA inserts that were very similar to SD4 but contained significant differences in both the translated and non-translated region Thus indicating that the initial isolated clone SD4 clone to a multigene family in *Brassica napus*.

To confirm that SD4 does indeed code for β-hydroxyacyl ACP dehydratase the mature protein minus the predicted chloroplast target sequence was overexpressed in *E. coli*. The majority of the SD4 protein was insoluble, but a small but significant proportion remained in the soluble phase.

Previously purified β-hydroxyacyl ACP dehydratase has shown an absolute requirement for ACP as its substrate. ACP has been purified form *E .coli* as well as the apo protein being expressed in *E. coli* and then converted to the holo protein *in vitro*. A number of enzymatic and chemical methods were used to try and synthesise the complete substrate for both the forward (β-hydroxyacyl ACP) and reverse (*trans*-2-enoyl ACP) but none were successful.

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Publications

β-hydroxyacyl ACP dehydratase [Acyl Carrier Protein] Dehydratase from *Brassica napus* Simon Doig, Timothy R. Hawkes, and Antoni R. Slabas.(1998). In Advance is Plant Lipid Research. (J. Sanchez, E Cerda-Olmedo and E Martinez-Force). Secretarido De Publicaciones, Seville.

Abbreviations

Amino Acid	Single letter code	three letter code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartate	D	Asp
Cysteine	C	Cys
Glutamate	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenyalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

A	Adenine
ACP	Acyl Carrier Protein
Amp	Ampicillin
ATP	Adenosine triphosphate
Bp,Kb	Base pair, kilobase pairs
BSA	Bovine serum Albumin
C	Cytosine
Cml	Chloramphenicol
CoA	Coenzyme A
Da, Kda	Daltons, Kilodaltons
cDNA	Complementary DNA
DNA	Dioxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetate
EtBr	Ethidium Bromide
G	Guaninie
HAS	Holo ACP synthase
IPTG	Isopropyl- -D-thiogalactoside
Kan	Kanamycin
Mr	Relative molecular mass
mRNA	Messenger RNA
MQ	Double distilled water
NAD, NADH	Nicotinamide adenine dinucleotide (oxidised and reduced)
NEMA	N-ethylmorpholine acetate
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pfu	plaque forming units
RNA	Ribonucleic acid

RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Sodium Saline Citrate
T	Thymine
TBS	Tris Buffered Saline
Tet	Tetracycline
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris.HCl	Tris (Hydroxymethyl)methylamine buffer, pH adjusted with HCl

Chapter 1

Type II Fatty acid synthetases

1.1 What are lipids

Lipids are a heterogeneous class of chemicals that are water-insoluble, oily, greasy organic substances. They are extractable from cells by non-polar solvents, such as chloroform and ether. This definition includes two types of lipid: (a) non-saponifable lipids, which contain no fatty acids and so, cannot form soaps. These include terpene and steroid molecules. (b) saponifiable lipids which when treated with alkali form soaps of their fatty acid components. The fatty acid groups in these types of lipids are long chain organic acids with a single carboxyl group, and a long non-polar hydroxycarbon (acyl) tail, which gives these lipids their oily, greasy nature. The long acyl chain maybe either saturated or unsaturated with one or more double bonds. The saturated fatty acids have free movement around all their single bonds, while the double bonds in the unsaturated fatty acids restrict movement around the particular carbons involved. Most naturally occurring fatty acids are found in the *cis*-configuration, this produces a rigid kink in the acyl chain. This differing degree of saturation leads to differing chemical properties, saturated fatty acids $C_{12\text{-}14}$ are solids with a waxy consistency, while unsaturated fatty acids are oily liquids at 37 °C. Fatty acids are almost never found as the free fatty acid in nature. Instead, they are usually found with the carboxy terminal modified or esterified to another chemical moiety. Fatty acids found in membranes are usually esterified to glycerol. This type of heterogeneous compound is called a glycerolipid. Glycerolipids have fatty acid acyl chains attached at both the *sn*-1 and *sn*-2 position of the glycerol backbone, while at the *sn*-3 position is a polar head group, attached via an ester linkage to phosphoric acid e.g. phosphatidylcholine or phosphatidylserine. These glycerolipids are called phospholipids and it's their amphipathic nature that gives the lipid bi-layer of membranes its characteristics.

1.2 The biological importance of lipids

Phospholipids are the major constituent of all biological membranes. These membranes provide a barrier to the outside of the cell, but also supply internal boundaries that divide the cell up into compartments. Further studies have led to an understanding of

the importance of proteins in membrane function. This is best defined in the fluid mosaic model (Singer and Nicholson, 1972). The biological membrane plays a critical role in the chemiosmotic hypothesis for energy generation. This requires vectoral transport of protons across a membrane by an ATPase (Mitchell, 1979). As well as ATPases membranes provide a fluid environment for other membrane bound enzymes, membranes maybe required for enzyme activity. The bacterial glycerol-3-phosphate acyltransferase, is inactive when homogeneous, but activity is restored by the addition of sonically disrupted *E. coli* membranes (Larson *et al.*, 1980). The degree of saturation of fatty acids contained in the membrane phospholipids determines the temperature at which the membrane remains fluid (disordered), this is important in chilling tolerance of biological organisms (Wada *et al.*, 1990). Products derived from the phospholipid bilayer play an integral role in hormone mediated intra-cellular signalling. Diacylglycerol (DAG) is an activator of protein kinase C. If instead of a polar head group, a third acyl chain is present at the *sn*-3 position, and then you have a triacylglyceride (TAG). TAGs are used as high-energy stores in both plants and animals. Industrially vegetable oils are a major raw resource for both the food and chemical industry. Oil seeds may constitute lipid up to 40 % of the dry weight of the seed. Important oil seeds include soybean, oil seed rape, sunflower and oil palm. Unlike phospholipids TAG contain an enormous variety of acyl chains (Hilditch and Williams, 1964). Some *Cuphea* species accumulate capric (C10) acids while coconut and Californian bay seed accumulate the industrially important lauric acid (C12) in their TAG. Lipids are also involved in the cutin coats of plants, which provide a barrier to help to prevent fungal attack (Kolattukudy, 1987). In bacteria acyl groups are also used to provide hydrophobic groups to hydrophilic molecules, these include (1) Sulphated lipooligosaccharides of *Rhizobium meliloti* which act as signals to elicit root nodule formation (Lerouge *et al.*, 1991), (2) the β-hydroxymyristoyl anchor of lipopolysaccharides in the outer leaflet of enterobacter (Raetz, 1990),

1.3 *de novo* fatty acid synthesis

The synthesis of long chain fatty acids from acetyl CoA and malonyl CoA involves numerous sequential reactions and acyl intermediates (Figure 1). In animal tissues the pre-cursor for cytoplasmic acetyl CoA is derived from mitochondrial citrate (Wakil, *et al.*, 1983), whereas in *E. coli* acetyl CoA is derived directly from pyruvate. The acetyl CoA is converted to malonyl CoA by acetyl CoA carboxylase the first committed step in *de novo* fatty acid biosynthesis.

1.4 Acetyl CoA carboxylase from animals and yeast

Acetyl CoA carboxylase (ACCase) is a soluble class I biotin containing enzyme (Knowles, 1989), that catalyses the ATP dependent formation of malonyl CoA from acetyl CoA and bicarbonate. ACCase in animals is an oligmer of 4-8 million Da (Guchlait *et al.*, 1974) which can dissociate into inactive protomers. Rapid purification procedures lead to the isolation of protomers, which consist of identical subunits of between 230-260 kDa, with each subunit containing one mole of biotin (Lane *et al.*, 1974). This 230 kDa subunit contains the biotin carboxylase, biotin carboxyl carrier protein, carboxyltransferase activities, as well as a regulatory allosteric site. Therefore each subunit contains all the enzyme activities required for ACCase activity. Animals ACCase is regulated by citrate, long chain acyl CoAs (Lane and Moss, 1971) and is post-translationally modified by the action of kinases in response to hormones (Brownsey and Hardie, 1980).

1.5 Fatty acid synthetase

Fatty acid synthetases are made up of six different enzyme activities and one peptide that acts as the substrate for the other six enzymes (Figure 1). These are Malonyl CoA:ACP transacylase (MCAT), β-ketoacyl ACP synthases (KAS), β-ketoacyl ACP reductase (KR), β-hydroxyacyl ACP dehydratase (DH), enoyl ACP reductase (EnR), acyl ACP thioesterase (TE) and as the substrate the acyl carrier protein (ACP). The MCAT transfers the malonyl moiety from CoA to ACP. The KAS acts as a condensing enzyme which adds two carbons from malonyl ACP onto the elongating acyl chain, this condensation produces an internal ketide group, which is removed by a series of enzymes that include KR, DH and EnR which produce the saturated fatty acid. This saturated acyl chain can either be further elongated or removed from the ACP by the action of TE. The FAS of eukaryotes (excluding plants) are complexes of multifunctional proteins that can take two distinct molecular organisations. The animal FAS are multifunctional proteins of two 500 kDA identical subunits which form a α_2 homodimer for function. The yeast synthetase is a complex of two non-identical subunits α (213kDa) and β (203kDa) with an apparent molecular weight of 2.4×10^6 Da, it has a $\alpha_6\beta_6$ molecular structure. The yeast FAS requires FMN for activity and yields palmitoyl and stearoyl CoA as products. In contrast the animal FAS do not utilises FMN as a co-factor but yields palmitate and stearate as the products (Wakil *et al.*, 1983). This type of molecular arrangement is

designated type I multifunctional FAS. *E. coli* FAS has a different molecular arrangement than type I FAS, *E. coli* and plant FAS are arranged as a multi-subunit type II FAS. Here the individual enzyme activities are located on separate polypeptide chains, *E. coli* FAS utilises NADH and NADPH as cofactors and yields palmitoyl ACP, palmitoleoyl-ACP and *cis*-vaccenoyl-ACP as its products. While plant FAS II also utilises NADH and NADPH as cofactors but yields palmitate, stearate and oleate as its product.

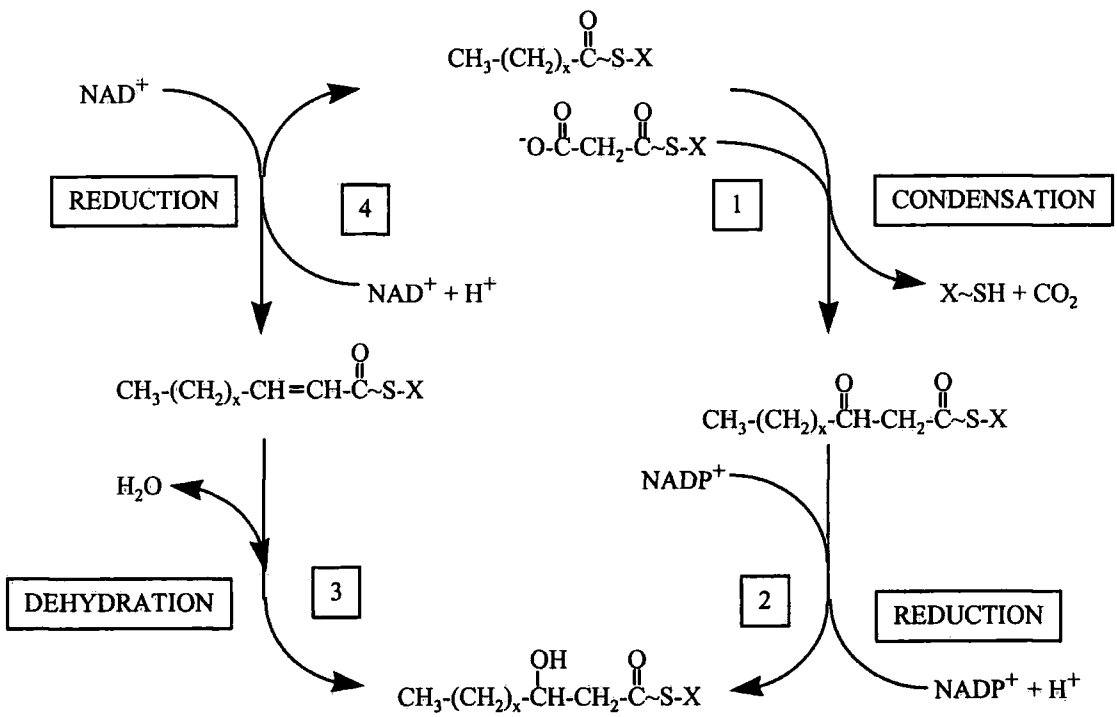


Figure 1: Elongation cycle of fatty acid biosynthesis. Each round of fatty acid elongation is initiated by the condensation of a malonyl moiety with an acyl moiety carried out by β-ketoacyl ACP synthase [1]. This introduces an internal ketide group, this is removed by a series of enzymatic steps catalysed by β-ketoacyl ACP reductase [2], β-hydroxyacyl ACP dehydratase [3] and enoyl ACP reductase [4]. During the rounds of elongation the elongating acyl chain is bound through a thioester linkage through a 4'-phosphopantethene prosthetic group to a protein moiety [X]. In type I FAS this is the FAS enzyme itself, while in type II FAS, it is bound to the acyl carrier protein (ACP).

1.6 *E. coli* fatty acid biosynthesis

E. coli FAS has provided a paradigm for predicting roles of other bacteria and plant FAS systems. For example the polyketide antibiotic synthesis in *streptomyces* is

catalysed by a mechanism analogous to fatty acid synthesis, this conservation of mechanism is highlighted in the strong homologies between genes of the polyketide pathway and those of *E. coli* FAS (Hopwood and Sherman., 1990). A second example is the *nod* genes that determine host specificity of the *Rhizobia*. Sequence similarities between the *nod* genes and those of *E. coli* FAS predict that the species determinants would be acylated molecules, a prediction which has been confirmed (Lerouge *et al.*, 1990; Nap and Bisseling, 1990).

The individual reactions of *E. coli* FAS are catalysed by separate proteins, which can be purified individually and are encoded by unique genes. There are often multiple proteins that catalyse the same chemical reaction. However, because of the differences in substrate specificity each enzyme plays a unique role in determining the products of the pathway and hence the biochemical properties of the membrane bilayer. The first committed step of *E. coli* FAS is the conversion of acetyl-CoA to malonyl CoA catalysed by acetyl CoA carboxylase.

1.6.1 Control of intracellular CoA levels

Coenzyme A (CoA) is an essential co-factor involved in fatty acid biosynthesis and a number of other metabolic pathways, CoA levels inside the *E. coli* cell are tightly regulated. CoA is synthesized by a sequence of five reactions from pantothenate (Brown, 1959). *E. coli* regulates pantothenate synthesis, such that the intracellular concentration remains constant at around 1 μM. The intracellular pantothenate is controlled by the action of pantothenate permase (*panF*). PanF exports excess pantothenate from the cell as well as importing pantothenate from the extracellular medium (Jackowski and Alix, 1990; Vallari and Rock, 1985). Pantothenate permase is an inner membrane protein that catalyses the sodium dependent uptake and export of pantothenate, but the rate of uptake is not the controlling factor in CoA biosynthesis.

CoA synthesis is governed by a feedback inhibition of pantothenate kinase (*coaA*), the first reaction in the CoA synthesis pathway. Pantothenate kinase activity is mediated by the intracellular concentration of non-esterified CoA, and the total CoA acyl-thioester pool (Vallari and Jackowski, 1988; Vallari *et al.*, 1987). CoA kinetically competes for the ATP binding site on the pantothenate kinase. Therefore the product of the synthesis pathway controls the flux through the pathway (Figure 2).

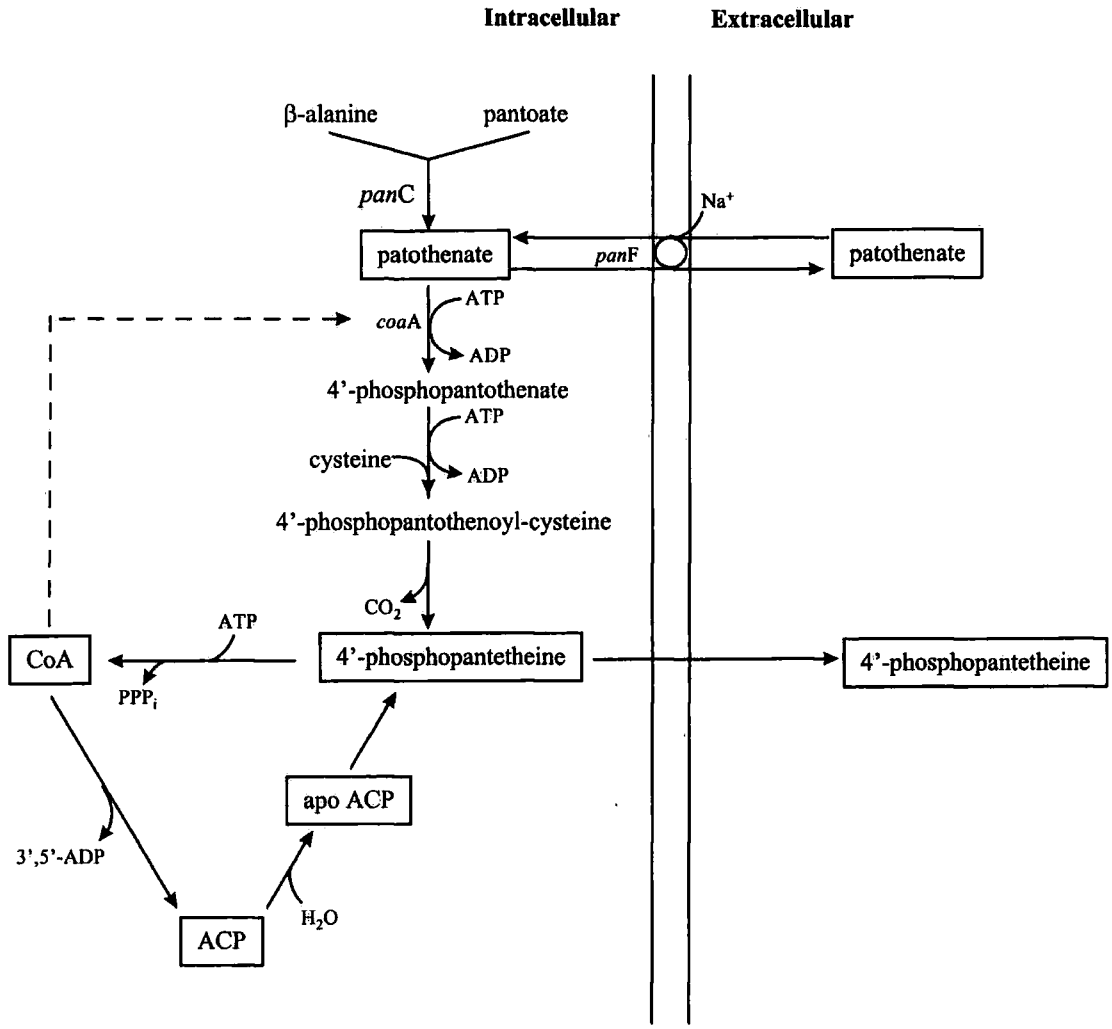


Figure 2: Pathway for biosynthesis of CoA. Pantothenate in *E. coli* is synthesised from β-alanine and pantoate catalysed by pantothenate synthetase (*panC*). Alternatively pantothenate can be taken up into the cell from the medium by the action of a sodium-dependent transporter pantothenate permease (*pan*F). The rate-controlling step in CoA biosynthesis is pantothenate kinase (*coa*A), which is regulated by feedback inhibition by CoA.

1.6.2 Structure and function of Acetyl CoA carboxylase from *E. coli*

The overall reaction catalysed by ACCase is composed of two distinct half reactions: (1) the ATP dependent carboxylation of biotin to form carboxybiotin, (ii) transfer of the carboxy group from carboxybiotin to acetyl CoA to form malonyl CoA. Biotin is covalently coupled to a small 16.7 kDa protein called biotin carboxy carrier protein (BCCP) (Sutton *et al.*, 1977). The biotin is coupled to BCCP via an Σ-amino

group of a lysine residue. The carboxylation of biotin is catalysed by a homodimeric enzyme composed of two 55 kDa subunits, biotin carboxylase (BC) (Gucchait *et al.*, 1974). The enzyme that transfers the carboxy group from biotin to acetyl CoA is the carboxyltransferase component, this is a heterodimer composed of two copies of two dissimilar subunits α and β (Li and Cronan, 1992). In cell extracts the acetyl CoA carboxylase activity cannot be measured, but the activity can be re-constituted *in vitro* by using high concentrations of the purified components from *E. coli* (Polakis *et al.*, 1974). This suggests that the subunits are not tightly bound *in vivo*. The acetyl CoA carboxylase of *Pseudomonas citronellolis* has been isolated as an intact active complex in the presence of high salt (Fall, 1976). This complex has an apparent molecular weight of 280 kDa.

1.6.3 *E. coli* Acyl Carrier Protein (ACP)

The substrate for all of the reactions of fatty acid biosynthesis is the acyl carrier protein (ACP). ACP is one of the most abundant proteins in *E. coli* and can constitute up to 0.25 % (~$6x10^4$ molecules per cell) of the total soluble protein (Rock and Cronan, 1979). The complete amino acid sequence of the homogeneous protein (Vanaman *et al.*, 1968), along with high resolution magnetic resonance spectroscopy has defined the solution structure of ACP (Rock and Cronan, 1979; Holack *et al.*, 1988; Kim and Prestegard, 1991). This data has revealed that ACP is a rod shape protein, which contains 3 α-helices which determine the major axis of the structure. Acyl intermediates are bound to ACP via a thioester bond to a 4'-phosphopantetheine prosthetic group which in turn is covalently linked to ACP through a phosphodiester bond to serine 36, serine 36 is located within a conserved sequence situated in a β-turn located between the second and third α-helices. The fatty acid intermediate extends up along the second α-helix, and makes contact with both isoleucine 54 and alanine 59 (Rock and Cronan, 1979). The acyl intermediate lies within a pocket which corresponds to the length of six carbons, the pocket undergoes a conformational change in response to the presence of charged acyl groups, this increases the accessibility of the thioester bond to the fatty acid biosynthesis enzymes (Rock and Cronan, 1979).

The 4'-phosphopantetheine prosthetic group undergoes metabolic turnover, with the apo-protein being functionally inactive. The 4'-phosphopantetheine group is transferred to ACP from CoA by holo ACP synthase (Elvoson and Vagelos, 1968; Lambalot and Walsh, 1995) and is removed by holo ACP hydrolase (Vagelos and

Larrabee, 1967). The 4'-phosphopantetheine group is recycled through CoA biosynthesis via phosphopantetheine adenyltransferase or is excreted from the cell (Jackowski and Rock, 1983). In cells growing in the absence of stress the CoA pool is eight times higher than the ACP pool, and virtually all of the ACP is maintained as holo ACP. (Jackowski and Rock, 1983). This suggests the supply of the prosthetic group does not limit fatty acid biosynthesis, with the function of the prosthetic turnover cycle being control of intracellular CoA levels (Jackowski and Rock, 1984).

ACP is coded for by the *acpP* gene that is located within a cluster of other fatty acid biosynthesis genes (Rawlings and Cronan, 1992), but little is known about how this gene cluster is regulated. Overproduction of the *acpS* gene in *E. coli* is toxic (Rawlings and Cronan, 1992; Jones *et al.*, 1993), as the majority of the expressed ACP is found in the apo form which is a potent inhibitor of glycerol-3-phosphate transferase (Keating *et al.*, 1995)

1.6.4 Condensing enzymes of *E. coli*

E. coli contains three different condensing enzymes KAS I, KAS II and KAS III. KAS I is composed of two identical subunits and has a molecular mass of 66 kDa (Garwin *et al.*, 1980). Both subunits contain both malonyl ACP and fatty acyl ACP binding sites (D'Agnolo *et al.*, 1975). During the condensation reaction the acyl group is covalently linked to a cysteine residue on the enzyme (D'Agnolo *et al.*, 1975). This covalently bound acyl ACP intermediate then undergoes condensation with malonyl ACP to form β-ketoacyl ACP, CO_2, holo ACP, plus the releasing the free enzyme.

KAS II (76kDa) has a higher molecular weight than KAS I (D'Agnolo *et al.*, 1975) and KAS II also has a lower K_m and higher V_{max} than KAS I for palmitoleyl ACP suggesting that KAS II functions specifically in the elongation of palmitoleyl ACP to form *cis*-vaccenoyl ACP (D'Agnolo *et al.*, 1975). When KAS II is overexpressed in *E. coli*, it is toxic, and this toxicity is partially relieved by the overproduction of MCAT (Subrahmanyam and Cronan, 1998). This suggests that the overexpression of KAS II blocks the accessibility of MCAT to the KAS isoforms and so indicates that the KAS enzymes and MCAT closely associate *in vivo*.

Both KAS I And KAS II are inhibited by the antibiotic cerulenin (D'Agnolo *et al.*, 1973, Kauppinen *et al.*, 1988). Prior incubation of both KAS I and KAS II with acyl ACP protects both enzymes from subsequent inhibition with cerulenin, indicating that inhibition by cerulenin occurs because the antibiotic binds to the acyl ACP binding sites

on both KAS I and KAS II. Cells treated with cerulenin accumulate short chain acyl ACPs (4-8 carbons), indicating the presence of a short chain condensing enzyme that does not contain an acyl ACP binding site. This observation lead to the identification novel condensing enzyme KAS III (Jackowski and Rock, 1987).

Thiolactomycin inhibits all three condensing enzymes, and this inhibition can be relieved by prior incubation of the enzymes with malonyl ACP, suggesting that thiolactomycin interacts with the malonyl ACP binding site rather than the acyl ACP binding site. These results have produced a picture for the different condensation reactions which take place in *E. coli* with KAS III condensing the initial rounds of elongation, KAS I condensing the longer chain fatty acid intermediates and with KAS II playing a specific role in the elongation of palmitoleoyl ACP to *cis*-vaccenoyl ACP (Figure 3).

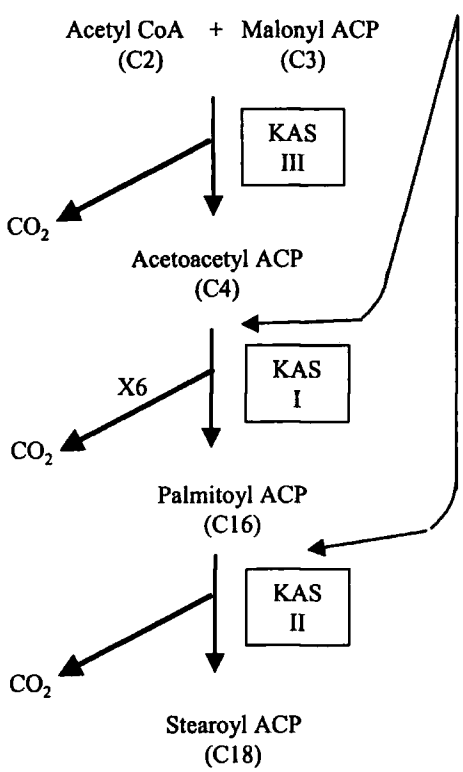


Figure 3: Roles of the three condensing enzymes in type II fatty acid synthetase. The role of the three condensing enzymes in fatty acid biosynthesis in E. coli.

1.6.5 Initiation of fatty acid biosynthesis in *E. coli*

The acyl CoA pool acts as a source of precursors for fatty acid biosynthesis. Acetyl CoA is a substrate for KAS III and acts as a primer for the initiation of fatty acid biosynthesis (Jackowski and Rock, 1987; Jackowski *et al.* 1989). Acetyl CoA may also be converted into acetyl ACP by a transacylase activity, this can serve as a primer for KAS I. The acetyl CoA:ACP transacylase (ACAT) activity was considered to be a discrete protein (Alberts *et al.*, 1969; Williamson and Wakil, 1966). However recently however the ACAT activity was shown to be a partial activity of KAS III (Tsay *et al.*, 1992).

Malonyl CoA is first loaded onto ACP by the action of MCAT. The MCAT activity has been purified to homogeneity from *E. coli* and is associated with a 32 kDa monomeric protein (Joishi and Wakil, 1971; Ruch and Vagelos, 1973). MCAT accepts the malonyl group from malonyl CoA to form a stable enzyme intermediate (Prescott and Vagelos, 1972; Ruch and Vagelos, 1973). Mutants deficient in MCAT activity have been isolated (Harder *et al.*, 1974) and the MCAT gene cloned by complementation of this mutation (Magnusson *et al.*, 1992; Verwoert *et al.*, 1992). The MCAT protein has been overexpressed and crystallised (Serre *et al.*, 1994).

Both KAS I and KAS II are capable of initiating fatty acid synthesis in the absence of acetyl ACP by utilising a side reaction involving the decarboxylation of malonyl ACP (Alberts *et al.*, 1972). Evidence that this pathway may play an important role in the initiation of fatty acid biosynthesis came from the observation that overproduction of KAS I renders *E. coli* resistant to thiolactomycin inhibition (Tsay *et al.*, 1992). This lead to a model being proposed in which acetyl ACP is a by-product of fatty acid biosynthesis rather than an active participant in chain elongation (Heath and Rock, 1995). Both KAS I and KAS II show malonyl CoA decarboxylase activity which is stimulated by the binding of acyl ACP to the enzyme (Figure 4).

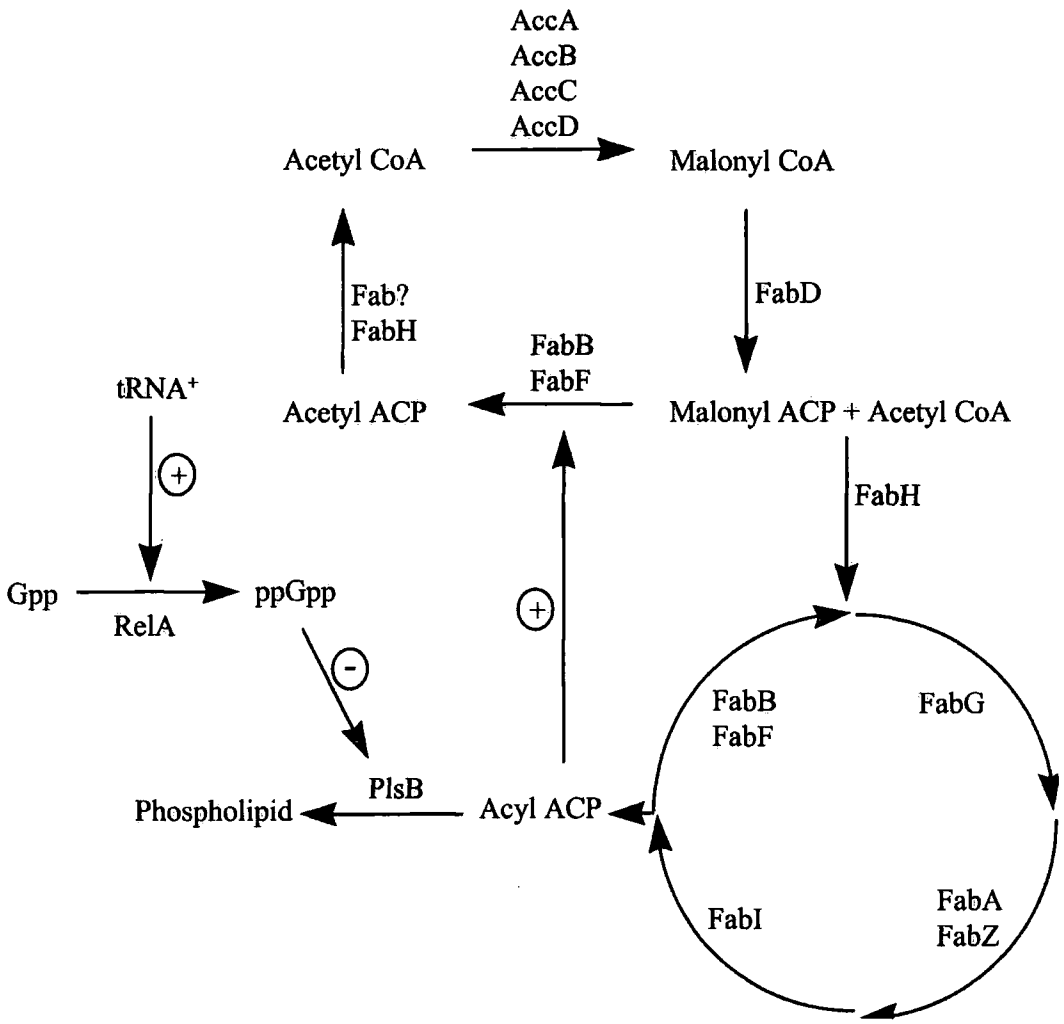


Figure 4: Model for regulation of fatty acid synthesis in *E. coli*. Fatty acid synthesis is co-ordinated with other macromolecule synthesis in response to environmental conditions. If fatty acid synthesis overtakes phospholipid biosynthesis, then long chain acyl ACP's build up in the cytoplasm stimulating the malonyl decarboxylation futile cycle, which reduces the flux of malonyl ACP into *de novo* fatty acid biosynthesis. If *E. coli* enters starvation conditions then amino acids become limiting and charged tRNA proliferate in the cytosol, this stimulates the formation of the signal molecule ppGpp, which down regulates PlsB, leading to a build up of long chain fatty acids which stimulates the malonyl ACP decarboxylation cycle again leading to a reduction in fatty acid biosynthesis.

1.6.6 β-ketoacyl ACP reductase form *E. coli*

An open reading frame encoding a protein which is homologous to several β-ketoacyl ACP reductases (*fab*G) from other species is located in the fatty acid biosynthesis cluster between *fab*D and *acp*P genes (Rawlings and Cronan, 1992). This is the only β-ketoacyl ACP reductase required for fatty acid biosynthesis, as the recombinant FabG protein has been functionally reconstituted into *E. coli* FAS *in vitro* (Heath and Rock, 1996). Using this reconstituted FAS system it was demonstrated that

FabG can participate in rounds of elongation of both saturated and unsaturated fatty acids of any chain length.

1.6.7 β-hydroxyacyl ACP dehydratase from *E. coli*

The *fab*A mutant of *E. coli* (Silbert and Vagelos, 1967) and 3-decenoyl-*N*-acetylcysteamine inhibited *E. coli* (Helmkamp *et al.*, 1968), are unable to synthesise unsaturated fatty acids. They are able to produce saturated fatty acid indicating the existence of at least one other dehydratase capable of elongating saturated fatty acids. From the FabA mutants an active β-hydroxyacyl ACP dehydratase enzyme was partially purified (Birge *et al.*, 1967). This purified β-hydroxyacyl ACP dehydratase differed from FabA in that the enzyme has a shorter chain length substrate specificity and was inactive with the substrate analogs *N*-acetylcysteamine and CoA.

The gene encoding this second dehydratase (*fabZ*) has recently been identified in *E. coli* (Mohan *et al.*, 1995). The gene does not lie within the cluster of fatty acid biosynthestic genes which contains *acpS* and *fabG*, but instead lies in a cluster of genes involved in lipid A biosynthesis (Raetz, 1990). The *fabz* gene was identified as potentially coding for a dehydratase based upon significant homology between the *fabZ* gene product and the FabA protein (Raetz, 1990). The FabZ protein was overexpressed in *E. coli* and the gene product demonstrated to be able to dehydrate (3*R*)-hydroxymyristoyl ACP (Mohan *et al.*, 1995). Definitive proof that *fabZ* encodes for β-hydroxyacyl ACP dehydratase came from the reconstitution of *E. coli* FAS *in vitro* (Heath and Rock, 1996). Using this reconstituted FAS, it was shown that FabZ could participate in the rounds of elongation of both saturated and unsaturated fatty acids. The reaction catalysed by FabZ marks a branch point between *de novo* fatty acid biosynthesis and lipid A biosynthesis. Mutants carrying point mutations in the *lpxA* locus (*lpxA2*) have a reduced lipid A content (Mohan *et al.*, 1995). Revertants of the *lpxA* phenotype did not accumulate mutations in the *lpxA* gene but instead accumulated mutations in the adjacent *fabZ* gene. The model proposed indicated that the accumulation of new mutations in the *fabZ* gene cause changes in the FabZ protein which decrease the affinity of the enzyme for its substrate and so decrease the flux through the fatty acid biosynthesis pathway. This leads to an increased pool of (3*R*)-hydroxymyristoyl ACP which is sufficient to overcome the Km mutants in LpxA producing wild type levels of lipid A (Mohan *et al.*, 1995).

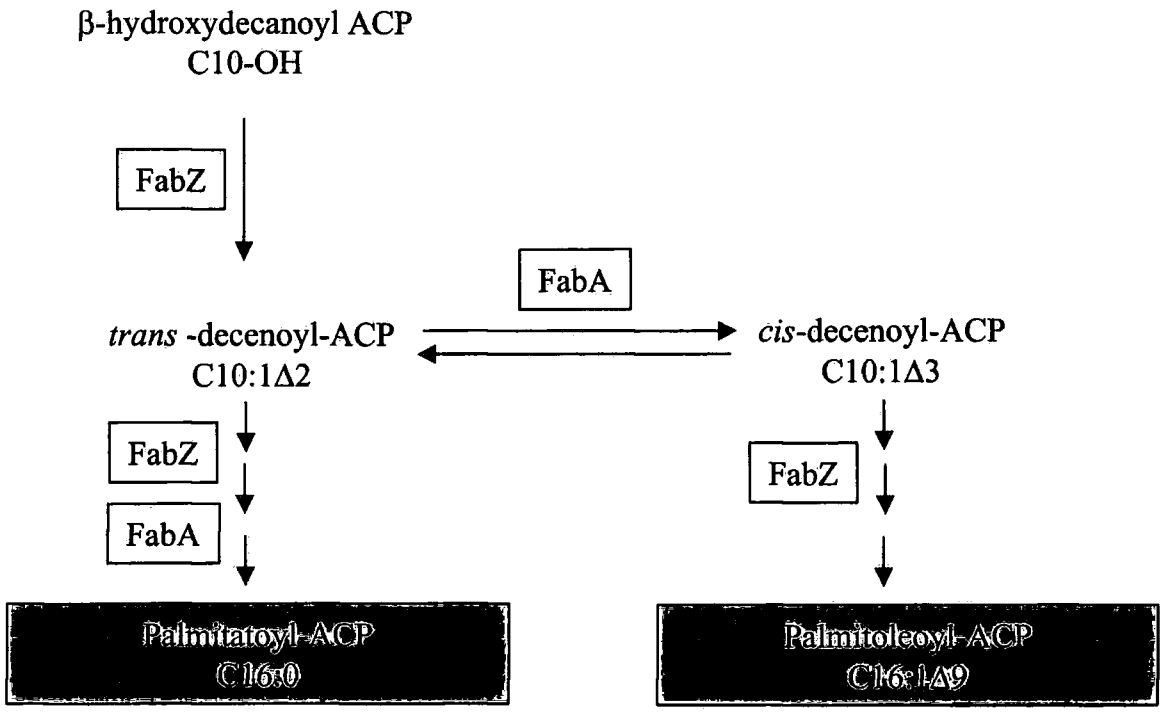


Figure 5: Role of FabZ and FabA in saturated and unsaturated fatty acid biosynthesis in *E. coli* . β-hydroxydecanoyl ACP dehydratase catalyses the key step in production of unsaturated fatty acids. Both FabA and FabZ are involved in the saturated fatty acid biosynthesis while only FabZ can participate in elongation of unsaturated fatty acids (Heath and Rock, 1996).

1.6.8 Role of FabA in unsaturated fatty acid biosynthesis in *E. coli*

Unsaturated and saturated fatty acid biosynthesis diverges at the level of β-hydroxydecanoyl-ACP. Here β-hydroxydecanoyl ACP dehydratase (*fab*A) specifically catalyses the dehydration of β-hydroxydecanoyl ACP to a mixture of *trans*-2-decenoyl-ACP and *cis*-3-decenoyl-ACP (Bloch, 1971). The reaction proceeds via the formation of an enzyme bound intermediate *trans*-2-decenoyl-ACP, which can dissociate from the enzyme. If this intermediate is reduced by enoyl ACP reductase and is then subsequently converted to saturated fatty acids so completing the round of elongation. If the intermediate does not dissociate, the *trans*-2-decenoyl ACP is isomerized to form *cis*-3-decenoyl ACP (Figure 5). The double bond is preserved and the *cis*-3 intermediate is elongated to the unsaturated fatty acids palmitoleic and *cis*-vaccenic acid. The first mutants of isolated in *E. coli,* FAS were called *fab*A mutants. These lack β-hydroxyacyl ACP dehydratase activity (Silbert and Vagelos, 1967). It was found that *in vitro* these

mutant FabA enzymes can neither form *cis*-3 or *trans*-2-decenoyl-ACP. The ratio of unsaturated to saturated fatty acids in *E. coli* is dependent on the levels of β-hydroxydecanoyl-ACP dehydratase and KAS I (*fab*B), if *fab*A is overexpressed in *E. coli* , the levels of unsaturated fatty acids do not increase instead there is a significant increase in the amount of saturated fatty acids incorporated into the membrane phospholipids (Clark *et al.*, 1983). This suggests that FabA is involved in the synthesis of unsaturated fatty acid biosynthesis but is not the controlling factor.

The nucleotide sequence of *fab*A has been determined (Cronan *et al.*, 1988). And the FabA protein has been crystallised and structure determined (Leesong *et al.*, 1996). This has identified the enzyme is composed of two 18 kDa subunits, which has two independent active sites are located in a 6x20 Å tunnel formed by the two subunits. The active site histidine (His-70) which had been previously been predicted to be involved in the initiation of the dehydration reaction (Bloch, 1970) is located halfway down the active site tunnel. The 4'-phosphopantetheine prosthetic group of ACP is predicted to be inserted into this tunnel and is constrained from moving to another active site without first completely extracting itself from the substrate binding tunnel. There is an area of positively charged residues located at the entrance to the tunnel, which have been predicted to be the site of interaction with the negatively charged ACP (Leesong *et al.*, 1996). The FabA has been reconstituted into *E. coli* FAS *in vitro*. Using this reconstituted system it was demonstrated that FabA could participate in rounds of elongation of saturated fatty acids, but suprisingly FabA could not participate in rounds of elongation of unsaturated fatty acids (Heath and Rock, 1996). A potential explanation why this is so maybe derived from the crystal structure as *cis*-unsaturated fatty acids contain a kink in the acyl chain which may prevent the acyl chain from entering the FabA active site tunnel (Leesong *et al.*, 1996).

1.6.9 Enoyl ACP reductase in *E. coli*

E. coli was thought to contain two enoyl ACP reductases, one NADH-dependent and the other NADPH-dependent (Weeks and Wakil, 1968). The gene encoding the NADH dependent enoyl ACP reductase was isolated in an *E. coli* strain resistant to the boron containing heterocyclic antibiotic diazaborine (Bergler *et al.*, 1989). Diazaborine inhibits fatty acid biosynthesis, and resistance is associated with the *envM* gene product (Turnowsky *et al.*, 1989). The *envM* gene was first identified as a temperature sensitive mutant with an osmotically reparable membrane (Egen and Russell, 1973). The *envM*

when overexpressed in *E. coli* showed NADH dependent enoyl ACP reductase activity, and also bound radiolabeled diazaborine. The *envM* gene product has been renamed FabI (Bergler *et al.*, 1989). The *fabI* gene has been sequenced and the diazaborine resistance shown to be conferred by a single point mutation (Bergler *et al.*, 1992). The broad range antimicrobial compound triclosan has been the subject of recent investigations, with tricolosan resistance *E. coli* having the resistance phenotype mapped to the *fabI* locus (Mcmurray *et al.*, 1998). Biochemical evidence that triclosan directly inhibits enoyl ACP reductase has been demonstrated with the *E. coli* enzyme (Heath *et al.*, 1998). The *fabI* analog in *Mycobacterium tuberculosis* the *inh*A gene product, is the target for the antibiotics isoniazid and ethanamide. A single point mutation confers resistance to both antibiotics (Banerjee *et al.*, 1994). *Inh*A has been purified and crystallised and shown to be an NADH dependent enoyl ACP reductase (Dessen *et al.*, 1995). Enoyl reductase has been shown to be the target for a diverse range of antibacterial drugs.

Inhibition of *fab*I by either diazaborine or by raising the temperature to the restrictive temperature in an *env*M temperature sensitive mutant strain is lethal. This suggests that the NADH dependent enoyl ACP reductase activity cannot fully be compensated for by the NADPH dependent enzyme (Bergler *et al.*, 1993). A reconstituted *E. coli* FAS, was used to show that the gene product of *fab*I was the only enoyl ACP reductase required for the synthesis of fatty acids. FabI was able to complete the elongation of both long chain saturated and unsaturated fatty acids (Heath and Rock, 1995). This has been confirmed by the analysis of the complete *E. coli* genome, which does not contain a *fabI* homolog.

1.6.10 Unsaturated fatty acid biosynthesis

Thermal regulation of membrane fluidity is common to all organisms, normal cell functions require a membrane layer in a fluid state. As temperature is lowered the membrane undergoes a change from a fluid (disordered) to non-fluid (ordered) state. The temperature at the point at which the transition occurs depends on the fatty acid composition of the membrane lipids. *E. coli* adjusts it's fatty acid composition in response to lower growth temperatures by increasing the amount of palmitoleoic acid incorporated into the membrane phospholipids. The amount of palmitate incorporated however remains unchanged (Marr and Ingraham, 1962). Lower growth temperatures result in the number of unsaturated fatty acids increasing in the membrane phospholipids. At 37 °C palmitic acid occupies the *sn*-1 position, while palmitoleic acid is found at the

sn-2 position (Cronan and Gelman, 1975; Baldassare *et al.*, 1976). As the growth temperature is lowered *cis*-vaccenic acid competes with palmitic acid for the *sn*-1 position. This mechanism allows *E. coli* to grow over a wide range of temperatures, by regulating the fluidity of it's membranes to optimise membrane function. The original unsaturated fatty acid auxotrophs were placed into two complementation groups (Cronan, 1968). The first group contains the *fab*A mutants. The second group, were shown to have normal *fab*A activity, but still required unsaturated fatty acids for growth. This second class was termed *fab*B. The *fab*B mutants have been shown to be deficient in KAS I (Rosenfield *et al.*, 1973). As KAS I is not involved directly in the introduction of double bond, it must be required for the elongation reaction in unsaturated fatty acid biosynthesis. FabA mutants contain very low levels of *cis*-vaccenoyl in their phosphoholipids. Reversion and transductional analysis showed this low *cis*-vaccenoyl phenotype was independent of the *fab*A mutation (Gelman and Cronan, 1972). This *cvc* strain was found to be deficient in the elongation of palmitoleoyl ACP to *cis*-vaccenoyl ACP and was unable to increase the amount of *cis*-vaccenic acid incorporated into phospholipid upon shift to the lower growth temperature. This suggests that the elongation of palmitoleoyl ACP plays a role in thermal regulation. KAS II (*fab*F) shows greater reactivity with palmitoleyl ACP than does KAS I (D'Agnolo *et al.*, 1975). The increased rate of *cis*-vaccenic acid is evident within thirty seconds of shift to the lower temperature (Garwin and Cronan, 1980). This indicates a pre-existing protein is required for the thermal regulation as there is insufficient time to translate a new protein. The *cvc* and *fabF* phenotype have been mapped to the same locus (Garwin *et al.*, 1980). KAS II has not only been shown to have a preference for palmitoleoyl ACP as a substrate, but the effect is higher at lower temperatures (Garwin *et al.*, 1980). Overproduction of KAS I in a *fab*F background increased the *cis*-vaccenic content of the cells but this increase was found to be temperature independent (deMendoza *et al.*, 1983). Therefore KAS II is solely responsible for thermal modification of fatty acid composition.

1.6.11 Acyl ACP acyltransferases

The first step in membrane phospholipid formation is the transfer of the acyl group from acyl ACP to *sn*-glycerol-3-phosphate (glycerol-P). Glycerol-P is a water-soluble intermediate that forms the scaffold for all phospholipids. *E. coli* mutants with defective acyltransferase activity (*pls*B) (Bell, 1974) are glycerol-P auxotrophs, and show an increased K_m for glycerol-P *in vitro* acyltransferase assays (Bell, 1974; Bell 1975).

Complementation of these mutants facilitated the cloning and biochemical characterisation of the glycerol-P acyltransferase (Lightner *et al.*, 1983; Green *et al.*, 1981; Larson *et al.*, 1980). The nucleotide sequence of *pls*B predicts a protein of 92 kDa (Lightner *et al.*, 1983) which can catalyse the formation of 1-acyl glycerol-P from either acyl CoA or acyl ACP as acyl donors (Green *et al.*, 1981). The enzyme has a preference for saturated acyl CoA's over unsaturated CoA's *in vitro* (Green *et al.*, 1981). This correlates with the *in vivo* position as *E. coli* phospholipids are predominately acylated with saturated fatty acids at the *sn*-1 position (Raetz, 1978). The next step in phospholipid biosynthesis is acylation of the 1-acyl glycerol-P to form phosphatidic acid which is catalysed by 1-acyl glycerol-P acyltransferase (*pls*C). Temperature sensitive mutants that accumulate 1-acyl glycerol-P at the restrictive temperature also possess temperature sensitive *pls*C activity *in vitro* (Coleman, 1990). The enzyme utilises either acyl CoAs or acyl ACP's as acyl donors and is thought to selectively transfer unsaturated fatty acids to the *sn*-2 position (Coleman, 1990). In wild type strains of *E. coli* inhibition of protein synthesis bought about by amino acid starvation results in a strong inhibition of stable RNA synthesis. This inhibition of RNA synthesis correlates with accumulation of the nucleotide guanosine-5'-diphosphate-3'-diphosphate (ppGpp), this is called the stringency response (Cashel *et al.*, 1996). The product of the *rel*A gene ppGpp synthase I is a ribosomal protein that produces ppGpp in response to charged tRNA. The accumulation of ppGpp is also correlated with the accumulation of long chain acyl ACPs (Heath *et al.*, 1994). Overexpression of acyltransferases prevents the accumulation of acyl ACP, and alleviates the inhibition of both fatty acid and phospholipid synthesis from occurring. This suggests that PlsB is a proximal target for ppGpp and decreases the rate of phosphololipid biosynthesis, increases the intracellular concentration of long chain fatty acids which stimulate the malonyl decarboxylation futile cycle which decreases the flux through *de novo* fatty acid biosynthesis.

1.6.12 Acyl ACP thioesterases

E. coli contains two thioesterases, which cleave the thioester bond of acyl CoA molecules. Both enzymes are less active on acyl ACP than acyl CoA (Spencer *et al.*, 1978). Thioesterase I is a 20.5 kDa protein encoded by the *tes*A gene, it cleaves acyl CoA's of greater than 12 carbons, and is unable to cleave β-hyoxyacyl CoA (Barnes and Wakil, 1968; Cho and Cronan, 1992). The predicted amino acid sequence of *tes*A has active site residues homologous to those found in mammalian thioesterase (Cho and

Cronan, 1992). The TesA protein has an amino terminal extension consistent with a periplasmic targeting sequence, and thioesterase I is released from the cell upon osmotic shock (Cho and Cronan, 1992). In contrast thioesterase II encoded by the *tes*B gene is a cytosolic tetrameric protein composed of 32kDa subunits (Naggert *et al*, 1991; Bonner and Bloch, 1972). TesB lacks the active site motif found in TesA, and shows no homology to other known proteins (Naggert *et al.*, 1991). Both *tes*A and *tes*B have been disrupted to give null mutants (Cho and Cronan, 1992; Naggert *et al.*, 1991), but neither effect cell growth. A *tes*AB double mutant strain grows normally (Cho and Cronan, 1994; Cho and Cronan, 1995), indicating that neither is essential for growth. Overexpression of either of the thioesterases allows continued fatty acid synthesis, in *pls*B mutant strains (Jiang and Cronan, 1994).

1.6.13 Regulation of fatty acid biosynthesis

There are three points in the fatty acid biosynthesis pathway that are potentially sites of regulation by acyl ACP, (a) ACCase, this controls the supply of malonyl moieties that are required for each round of elongation, stopping the flow of malonyl CoA to the pathway would lead to a rapid reduction in fatty acid synthesis; (b) KAS III, which catalyses the first condensation reaction in the pathway, and is ideally placed to control the initiation of fatty acid synthesis; (c) Enoyl ACP reductase, which plays a determinant role in each round of elongation, inhibition of this enzyme would lead to a general reduction in the rate of fatty acid elongation. The products of the pathway acyl ACP also regulate fatty acid biosynthesis. This is not a negative feedback inhibition but instead acyl ACP stimulates a futile cycle that reduces the flux through the pathway by reducing the supply of one of the precursors (Figure 3). The glycerol-P acyltransferase (*pls*B) acts as a receptor for intracellular signal responses that co-ordinate regulation of membrane phospholipid formation, with the synthesis of other macromolecules. Inhibition of the acyltransferase leads to a rise in intracellular acyl ACP, which in turn act as feedback inhibitors of fatty acid elongation, and a positive inducer of the futile malony ACP decarboxylation cycle reducing the flux. So glycerol-P acyltransferase can be viewed as the sensor, and acyl ACP as an effector, in the regulation of fatty acid biosynthesis.

1.7 Fatty acid synthesis in plants

Avocado mesocarps extracts can be resolved into two components that have no FAS activity alone, but when combined can synthesise palmitate and stearate (Overath

and Stumpf, 1964). One of these components is heat and acid stable and can be substituted for *E. coli* ACP in a isolated fatty acid synthetase *in vitro* (Overath and Stumpf, 1964). This was the first indication that plants have a type II dissociable FAS. The molecular nature was confirmed by the partial purification of the individual catalytic components from barley (Hoj and Mikkelsen, 1982) and safflower (Shimakata and Stumpf, 1982) and avocado (Caughey and Kekwick, 1982). Conclusive proof came with the purification to homogeneity of the individual components of the reductive part of the pathway from spinach (Shimakata and Stumpf, 1982).

1.7.1 Acetyl CoA Carboxylase

ACCase in leaves has been shown to be located in the chloroplast (Mohan *et al.*, 1980; Nikolau *et al.*, 1984; Thompson *et al.*, 1981). Initial purification data suggested that the structure of ACCase from plants was similar to the arrangement in *E. coli* (Stumpf, 1980). But these initial ACCase preparations were thought to be degraded by proteases as ACCase purified using a rapid purification scheme using proteases inhibitors yielded ACCase with a molecular weight of between 210-240 kDa (Egin-Buhler and Ebel, 1983; Slabas and Hellyer, 1985). This is a similar molecular weight to the ACCase found in animals and yeast. A full length clone for a multifunctional ACCase was isolated form *Cytotella cryptica* (Roessler and Ohlrogge, 1993). The gene showed strong homology to the biotin carboxylase and carboxytransferase domains of mammalian ACCase but the homology was less pronounced in the BCCP domain. The ACCase from *Cytotella cryptica* (Roessler and Ohlrogge, 1993) contained a predicted chloroplast target sequence at the N-terminus, suggesting that the ACCase was located in the choroplast. Two full length cDNAs have been isolated for multifunctional ACCase from plants one from Arabidopsis (Elborough *et al.*, 1993) and one from alfalfa (Shorrosh *et al.*, 1994) both clones showed significant homology to each other as well as strong homology to mammalian ACCase, but neither clone contained a predicted chloroplast target sequence at the N-terminus suggesting that they were both located in the cytosol. A multigene family for multifunction ACCase has been isolated from *Brassica napus*, one of the clones contained a predicted chloroplast target sequence at the N-terminus. This predicted chloroplast target sequence was fused to green fluorescent protein (GFP) and was sufficient to direct GFP in to the chloroplast *in vitro*.

A multisubunit ACCase similar to the *E. coli* ACCase has been identified in pea leaves (Sasaki *et al.*, 1993; Alban *et al.*, 1994). The biotin carboxylase subunit is a 50

kDa nuclear encoded polypeptide (Shorrosh *et al.*, 1995). The BCCP is a 34 kDa nuclear encoded protein (Choi *et al.*, 1995), and a third subunit has been identified in the plastid genome based upon it's homology to *acc*D from *E. coli* (Sasaki *et al.*, 1993). Immunoprecipitation of this subunit leads to co-precipitation of the other subunits suggesting the separate subunits associate in a complex which is tightly bound (Sasaki *et al.*, 1993; Roessler *et al.*, 1996). The exact arrangement of this multisubunit form of ACCase is unclear, as the molecular weights of the individual subunits (including possible dimers) does not add up to 700 kDa. All the dicots and some monocots studied to date, contain a multisubunit form in the chloroplast (Konishi *et al.*, 1996). The chloroplast ACCases of the *Graminaceae* have been shown to be multifunctional proteins (Ashton *et al.*, 1994; Egli *et al.*, 1993; Gornicki and Haselkorn, 1993; Konishi and Sasaki, 1994). Multifunctional ACCase is sensitive to several important herbicides of the aryloxyphenoxypropionic acid and cyclohexane-1,3-dione classes. In barley leaves treated with the inhibitor fluazifop, labelling of very long chain fatty acids is not inhibited despite the rapid inhibition of *de novo* fatty acid biosynthesis (Walker *et al.*, 1988). As synthesis of very long chain fatty acids occurs outside the plastid there is a requirement for generation of malonyl CoA outside the plastid. Antisense of the multifunctional form of ACCase in oil seed rape, leads to plants that are more sensitive to UV damage and contain reduced amounts of flavonoids (Personal communication Dr. Andrew White). This suggests the role of the cytoplasmic multifunctional ACCase is in supplying malonyl CoA for the fatty acid biosynthesis outside the plastid. The two forms of ACCase differ in their biochemical properties. The multifunctional ACCase can carboxylate other straight chain acyl CoA's especially propinoyl CoA albeit at lower rates (Dehaye *et al.*, 1994; Egin-Buhler and Ebel, 1983; Lichethaler *et al.*, 1992), whilst the multisubunit ACCase isolated from pea and that of *E. coli* have a strict requirement for acetyl CoA.

1.7.2 ACP

Plant ACP has been purified from spinach, avocado (Simoni *et al.*, 1967) and oil seed rape (Slabas *et al.*, 1987). They have a molecular mass of ~11 kDa, containing a single 4'-phosphopantetheine group. They are characterised by a predominance of acidic amino acids, and a lack of aromatic and hydrophobic residues. A partial sequence of 17 amino acids from around the 4'-phosphopantetheine attachment site from spinach ACP was obtained and was found to be identical to the same conserved sequence in *Arthobacter* and *E. coli* (Matsumura and Stumpf, 1968). Complete amino acid sequences

for ACP have been obtained from barley (Hoj and Svendsen, 1983), spinach (Kuo and Ohlrogge, 1984) and *Brassica napus* (Slabas *et al.*, 1987). The amino acid sequences of isolated plant ACP show strong sequence homology with the sequences of bacterial ACP. It is likely that many of the structural features of *E. coli* ACP (Rock and Cronan, 1979) are conserved in plant ACP. β-turns analogous to those found in *E. coli* ACP have been predicted to occur in spinach ACP (Kuo and Ohlrogge, 1984). Spinach ACP is a poor substrate for the dehydratase components of *E. coli* FAS (Simoni *et al.*, 1967). In contrast *E. coli* ACP when used with barley (Hoj and Svendsen, 1983) or spinach (Simoni *et al.*, 1967) FAS the same products are formed, but the overall rate of fatty acid synthesis is higher. This may represent an evolutionary difference between the two FAS systems. During the amino acid sequencing of ACP, the amino acid composition of some residues were found to be heterogeneous (Matsumura and Stumpf, 1968). This suggests ACP might exist in different isoforms. Isoforms of ACP have been demonstrated in barley (Hoj and Svendsen, 1983), spinach, castor, soya bean (Ohlrogge and Kuo, 1985) and oil seed rape (Safford *et al.*, 1988). The isoforms of spinach are so closely related they can only be separated by HPLC (Ohlrogge and Kuo, 1985). Three isoforms have been found in barley (Hoj and Svendsen, 1984) and two of them ACP-I and ACP-II have been purified to homogeneity. Both isoforms of barley are capable of supporting *in vitro* fatty acid biosynthesis (Hoj and Svendsen, 1984) ruling out the possibility that only one isoform is involved in fatty acid biosynthesis. In a cDNA library prepared from developing *Brassica napus* embryos ten different cDNAs have been isolated for ACP. Differences in the transit peptide allowed two distinct classes of ACP to be categorised (Safford *et al.*,1988). This complexity in *Brassica napus* probably arose from its origin as a cross between *Brassica campestris* and *Brassica oleracea* as this produces a an equivalent of a six arabidopsis genome. ACP isoforms have different tissue distributions, in spinach seeds only ACP-II is present, while in leaves both isoforms are present (Ohlrogge and Kuo, 1985). In castor leaves two distinct isoforms are found which can be resolved on SDS-PAGE, while only one isoform is present in seeds (Ohlrogge and Kuo, 1985). *in vitro* transcription/translation data together with cDNA cloning, have demonstrated that ACP is synthesised as a pre-cursor with a N-terminal leader sequence which is consistent with a transit peptide necessary for transport into the plastid (Hoj and Svendsen, 1984; Ohlrogge and Kuo, 1984; Safford *et al.*, 1988). It was initially suggested that ACP was post translationally modified in the cytosol by the action of holo ACP synthetase (HAS) (Elhussein *et al.*, 1988). However experiments with an ACP mutant

lacking the prosthetic group attachment site, demonstrated that apo-ACP can be imported into chloroplasts (Fernandez and Lamppa, 1990). A chloroplast located holo ACP synthase has been identified which can catalyse the attachment of the prosthetic group before cleavage of the transit peptide had taken place. It was concluded that removal of the transit peptide, is a faster reaction than the pantethenylation of apo-ACP. Suggesting the apo-ACP is the primary substrate for the chloroplast holo ACP synthase (Fernandez and Lamppa, 1991). In experiments where pre-apo-ACP and pre-holo-ACP were presented to spinach chloroplasts pre-holo-ACP was imported more efficiently (Savage and Post-Beittenmuller, 1994). This may suggest that post-translational modification of ACP occurs in the cytosol, and the plastid holo ACP synthase is required to for the addition of the 4'-phosphopantetheine group to ACP which lost the prosthetic group during import into the chloroplast. Holo ACP that has been stripped of it's prosthetic group. The highest level of ACP activity in developing rape seed occurs prior to the onset of storage lipid biosynthesis (Slabas *et al.*, 1987). Expression of an arabidopsis ACP-I/GUS fusion, shows promoter activity correlates with the requirements for fatty acid synthesis in distinct cell types and during different stages of organgenesis (Baerson and Lamppa, 1993).

1.7.3 Initiation of fatty acid biosynthesis

The first condensation reaction of plants was thought to be catalysed by KAS I using malonyl ACP and acetyl ACP (Stumpf, 1987). The two substrates are produced by acetyl CoA:ACP transacylase (ACAT) and malonyl CoA:ACP transacylase (MCAT) respectively. ACAT activity was purified from spinach leaves (Shimakata and Stumpf, 1983) and barley chloroplasts (Hoj and Mikkelsen, 1982), but the validity of the existence of this enzyme was cast into doubt when a new cerulenin insensitive condensing enzyme KAS III was identified in *E. coli* (Jackowski and Rock, 1987). The KAS III isolated from *E. coli* can use acetyl CoA as a substrate. An equivalent enzyme activity has been measured in spinach leaves (Jaworski *et al.*, 1989). The purified KAS III from spinach (Clough *et al.*, 1992) and *E. coli* (Tsay *et al.* 1992) both contain ACAT activity, as a partial activity casting doubt onto which enzyme had been purified previously. The KAS III activity in spinach has higher activity than ACAT, and initial rates of acyl ACP formation are not inhibited by cerulenin, suggesting the enzyme does not bind long acyl chains. This suggests that the plant KAS III is analogous to the *E. coli* enzyme and as such catalyses the initial reaction of plant FAS (Waspish *et al.*, 1990). The involvement

of a separate ACAT protein is cast further into doubt as the ratio of ACAT to KAS III activity remains constant during enzyme purification (Clough *et al.*, 1992). There is however clear evidence that an independent ACAT activity exists in plants. The ratio of ACAT to KAS III activity changes during the development of barley leaves (Walsh *et al.*, 1990). The ratio would remain constant if the activities were due to the same enzyme. Purification of KAS III from avocado has separated the ACAT activity away from the KAS III activity (Gulliver and Slabas, 1994). This makes the physiological role of ACAT unclear, but it possibly has no direct role in fatty acid biosynthesis. MCAT has been purified from a number of plant sources (Caughey and Kekwick, 1982; Hoj and Mikkelsen, 1982; Stapelton and Jaworski, 1984; Guerra and Ohlrogge, 1986; Lessire and Stumpf. 1983), as well as the cyanobacterium *Anabaena viriabulus* (Stapleton and Jaworski, 1984). The enzyme has a molecular mass of around 40 kDa. In soybean leaves two isoforms are present, but only one is found in seeds (Guerra and Ohlrogge, 1986). An EST from maize that was homologous to the *E. coli fabD* gene was used to clone a cDNA from *Brassica napus* (Simon and Slabas, 1998). A genomic clone from arapidopsis has been sequenced through the efforts of the arabidopsis genome project (Simon and Slabas, 1998).

1.7.4 Condensing enzymes in plants

Two forms of β-ketoacyl ACP synthetase were initially identified in spinach leaves (Shimakata and Stumpf, 1983). These condensing enzymes differ in their substrate specificity, KAS I utilises acyl ACP in the range C_2-C_{14}, while KAS II was only active with myristoyl ACP and palmitoyl ACP (Shimakata and Stumpf, 1983). KAS I was cerulenin-sensitive and arsenite-resistant, while KAS II was inhibited severely by arsenite, but was less sensitive to cerulenin (Shimakata and Stumpf, 1983). This may explain why arsenite inhibits stearate, but not palmitate synthesis (Harwood and Stumpf, 1980). KAS I has been purified to homogeneity from rape seed (Mackintosh *et al.*, 1989) and barley (Siggaard-Andersen *et al.*, 1991). Gel filtration experiments suggest KAS I is a homodimer with a subunit size of 43 kDa. Radiolabeled cerulenin tagging experiments on barley chloroplasts identified three dimeric forms of KAS I αα, αβ, and ββ (Siggaard-Andersen *et al.*, 1991). The amino acid sequence of the β-subunit was used to clone a cDNA which showed 49 % similarity and 35 % identity with the *E. coli fab*B gene (Siggaard-Andersen *et al.*, 1991). When a similar radiolabeled tagging experiment was performed on spinach, only a single dimeric protein was labeled (Siggaard-Andersen *et*

al., 1991). KAS II has been partially purified from rape (Mackintosh *et al.*, 1989), and clones with homology to the *fab*F gene have been isolated from barley, castor (Genex, 1993) and *Cuphea wrightii* (Slaubaugh *et al.*, 1993). An arabidopsis fatty acid biosynthesis (fab) mutant that is unable to elongate palmitic acid to oleic acid (Browse and Sommerville. 1991) has been identified which has a lesion in the KAS II locus. KAS III has been purified from spinach (Clough *et al.*, 1992) and avocado (Gulliver and Slabas, 1994). Gel filtration experiments suggest the enzyme exist as a homodimer with a subunit size of around 40 kDa. Purified KAS III is sensitive to thiolactomycin (Clough *et al.*, 1992). The amino acid data obtained from the purified spinach KAS III was compared to that of FabH (Tsay *et al.*, 1992), this information was used to isolate a cDNA clone from spinach (Jaworski *et al.*, 1994). The proposed active site residues when compared to that of *E. coli* and a putative KAS III from the red algae *Porphyra umbilicalis* (Reith, 1993) showed nine residues which are conserved in all three KAS IIIs but are only partially conserved in the other condensing enzymes. A cDNA clone has been isolated from arabidopsis, which is 60 % and 72 % homologous at the nucleotide and amino acid level with the spinach KAS III (Tai *et al.*, 1994). KAS III has also been cloned from *Brassica napus*, and antisense data suggest down regulation has an effect on the fatty acid content of seeds (Clarke *et al.*, 1999). FAS preparations from *Cuphea lanceolata* (a plant that accumulates up to 90 % capric (C10) acid in triacylglycerides), showed that KAS III is inhibited by decanoyl ACP (Bruck *et al.*, 1996) and 3-oxoacyl ACP (Winter *et al.*, 1997). The inhibition of KAS III by 3-oxoacyl ACP was accompanied by induction of decarboxylation of malonyl ACP to acetyl ACP. A model based on this observation has been proposed for the large build up of capric acid in *Cuphea lanceolata* (Winter *et al.*, 1997). Here a build up of decanoyl ACP causes the inhibition of the initial condensation reaction catalysed by KAS III. A second response is stimulation of the decarboxylation of malonyl ACP to acetyl ACP, so reducing the flow of substrate into the pathway. Both of these mechanism act to slow FAS down allowing for the interaction of a medium-chained thioesterase. An analogous system exists in *E. coli* , but no similar system has been found in any other plant species.

1.7.5 β-ketoacyl ACP reductase

β-ketoacyl ACP reductase has been reported in two forms in avocado, one NADPH-dependent and the other a NADH-dependent. The NADH-dependent enzyme has been purified to homogeneity from spinach (Shimakata and Stumpf, 1982), *Brassica*

napus (Schuz *et al.*, 1982) and avocado mesocarp (Sheldon *et al.*, 1990). In spinach the purified enzyme can use both nucleotides but has a preference for NADPH (Shimakata and Stumpf, 1983). Gel filtration experiments suggest the enzyme is a tetramer, with a subunit size of 28 kDa. β-ketoacyl-ACP-reductase from avocado mesocarp, is sensitivve to dilution and this dilution sensitivity can be protected by incubation with NADPH. Antibodies raised to the purified avocado protein localised the protein in the plastid (Sheldon *et al.*, 1990). N-terminal amino acid sequencing shows similarities with the *nod*G gene from *Rhizobium melloti*, and the *graIII* and *actII* genes from *streptomyces*. cDNA clones have been isolated from developing rape seed and arabidopsis (Martinez-Rivas *et al.*, 1993).

1.7.6 β-ketoacyl ACP dehydratase

β-ketoacyl ACP dehydratase has been purified to homogeneity from spinach (Shimakata and Stumpf, 1982). It has activity with chain lengths C_4 to C_{16}, and has a requirement for acyl ACP as a substrate. Gel filtration studies suggest it is tetrameric with a subunit size of 19 kDa (Shimakata and Stumpf, 1982). No corresponding cDNA clone has been isolated from any plant source.

1.7.7 Enoyl ACP Reductase

Two forms of enoyl ACP reductase have been detected in plants, type I which is NADH-dependent and type II which is NADPH-dependent (Shimakata and Stumpf, 1982). Both enzymes have been identified in safflower, castor and rape seeds (Slabas *et al.*, 1979), although only one type I enzyme appears in leaf (Shimakata and Stumpf, 1983). The type I enoyl ACP reductase has been purified to homogeneity from spinach leaves (Shimakata and Stumpf, 1982) and rape seed (Slabas *et al.*, 1986). Gel filtration studies suggest it's a tetramer with a subunit size 35 kDa (Shimakata and Stumpf, 1983; Slabas *et al.*, 1986). The rape seed enoyl ACP reductase is inhibited by phenylglyoxal, suggesting a role for arginine residues at the active site (Cottingham *et al.*, 1988). A cDNA for enoyl ACP reductase has been cloned from rape (Kater *et al.*, 1991) and the predicted translated protein contains a chloroplast target peptide at the N-terminus. Southern blot analysis shows that *Brassica napus* contains two pairs of related genes derived from it's two ancestors *Brassica oleracea* and *Brassica rapas* (Kater *et al.*, 1991). Western blot analysis showed all four isoforms are present in both seed and leaf (Fawcett *et al.*, 1994). The rape enoyl ACP reductase is not inhibited by diazaborine, and

is able to functionally replace the *fabI* enoyl reductase of *E. coli* FAS (Kater *et al.*, 1994). The *Brassica napus* enzyme has been crystallized, the crystals belong to the tetragonal system and have been resolved to beyond 1.9 A resolution (Rafferty *et al.*, 1994).

1.7.8 Acyl ACP thioesterases

The chloroplast stroma contains medium and long chain acyl ACP thioesterases, which are involved in chain termination. Acyl ACP thioesterases have been purified from soybean seed (Kinney *et al.*, 1990) and oil seed rape (Hellyer *et al.*, 1990). Gel filtration studies indicate the protein is dimeric with a subunit size of 38 kDa. Two cDNA's have been isolated from safflower, one clone encodes a 389 amino acid protein, which includes a 60 amino acid transit peptide. The second clone codes for a 385 amino acid protein that is 80 % homologous to the first. Both thioesterases were active with oleoyl ACP (Knutzen *et al.*, 1992). Two cDNAs have also been isolated from *Brassica napus* embryos (Loader *et al.*, 1993). The cDNA clones were highly homologous to each other, showing 95.7 % identity at the DNA level and 98.4 % identity at the amino acid level. A cDNA clone has also been identified from coriander, using the safflower cDNA as a probe. When overexpressed in *E. coli*, the safflower thioesterase had higher activity towards oleoyl ACP compared to other acyl ACP's (Dormann *et al.*, 1994). The seed oils from the Californian bay (*Umbellularia californica*) and from *Cuphea* species accumulate medium chain fatty acid in their triacylglycerides. Accumulation of these medium chained fatty acids is correlated with induction of a lauroyl ACP specific thioesterase in Californian bay (Davies *et al.*, 1991; Pollard *et al.*, 1991). When this thioesterase was expressed in plants that do not normally produce medium chained fatty acids, then they also accumulate medium chained fatty acids in their seed oil (Voelker *et al.*, 1992). Transgenic rape expressing a medium chained thioesterase, activity is found in leaves as well as seed, but the phospholipids in leaf do not accumulate laurate (Eccleston *et al.*, 1996) suggesting there is a distinction between fatty acids synthesized for phospholipid biosynthesis and those for triacylglyceride biosynthesis. A number of medium chain length synthesizing plants were studied, all contained a medium chain length thioesterase (Davies, 1993), as well as the ubitiquous oleoyl ACP thioesterase (Dormann *et al.*, 1993). This indicates that premature chain termination requires a specific thioesterase. A partial cDNA from arabidopsis that shows sequence similarity to the lauroyl ACP thioesterase from Californian bay was isolated (Grellet *et al.*, 1993). This was used to isolate a full length cDNA, the deduced amino acid was 51 % identical

to the lauroyl ACP thioesterase and 39 % identical to safflower oleoyl ACP thioesterase (Dormann *et al.*, 1995). Antibodies raised to the over-expressed protein detected a 40 kDa band in all tissue (Dormann *et al.*, 1995). When the antibody was used to probe a time course on developing rape seed, maximal expression was detected 18-31 days after flowering, which coincides with rapid oil accumulation (Dormann *et al.*, 1995).

1.7.9 Stearoyl Δ9 desaturase

Plant unsaturated fatty acids are made by the sequential aerobic desaturation of stearate to oleate then linoleate and finally α-linolenate (James, 1963). The first desaturation reaction introduces a double bond at the Δ9 position using stearoyl ACP as a substrate. The enzyme activity was first identified in *Chlorella* and spinach chloroplasts (Nagi and Bloch, 1968), and later in developing safflower seeds (Jaworski and Stumpf, 1974). Stearoyl ACP desaturase was first purified from safflower, where it was found to be a dimer with a subunit of 38 kDa which utilizes ferrodoxin as an electron donor (McKeon and Stumpf, 1982). The enzyme has since been purified from soybean (Cho and Cheesbrough, 1990; Kinney, 1990) and avocado (Shanklin and *et al.*, 1991). cDNA clones have been isolated from castor, cucumber (Shanklin and Sommerville, 1991), safflower (Thompson *et al.*, 1991), soybean (Kinney, 1990), spinach (Nishida *et al.*, 1992) and *Thumbergia alata* (Cahoon *et al.*, 1994). The homology of stearoyl ACP desaturase between castor and cucumber is approximately 90 % at the amino acid level. All of the sequences contain a predicted chloroplast targeting sequence. *In vivo* stearoyl ACP desaturase shows little activity towards palmitoyl ACP, while the recombinant enzyme does (Gibson, 1993). The recombinant enzyme from castor has been crystallized (Schneider *et al.*, 1992). The enzyme contains four atoms of iron per homodimer (Fox *et al.*, 1993). The enzyme has properties consistent with other O_2^- activating proteins, with di-iron centers. Comparison of the primary sequence of this class of proteins has led to the identification of a conserved pair of histidine motifs, these are separated by around 100 amino acids. It has been proposed that stearoyl ACP desaturase proceeds by a reaction mechanism that generates high valent iron-oxo species derived from the di-iron cluster (Fox *et al.*, 1993).

1.7.10 Regulation of fatty acid synthesis

ACCase in animals and yeast has a major regulatory role in fatty acid production. Several lines of evidence suggest that ACCase in plants is also a regulated step. Both

acetate and pyruvate are incorporated into acetyl CoA by isolated chloroplasts in the dark, but malonyl CoA and fatty acids are formed in the light (Nakamura and Yamada, 1979). ACCase is inhibited by ADP (Eastwell and Stumpf, 1983), and changes in pH/Mg^{2+}, ATP/ADP ratio during light and dark conditions could account for increased ACCase activity in the light (Nikolau and Hawke, 1984). Recently it has been shown that a major redox cascade including thioredoxin interacts with ACCase, this might provide the link between light and fatty acid synthesis (Sasaki *et al.*, 1997). This all suggests that the light dependent step of fatty acid synthesis is catalysed by ACCase. Further evidence comes from analyzing the acyl CoA and acyl ACP pools in light and dark incubated chloroplasts. The rate of spinach leaf fatty acid synthesis in the dark is only one sixth the rate seen in the light (Browse *et al.*, 1981). In the light the pre-dominant form of ACP, is the free non-acylated form (Post-Beittenmiller *et al.*, 1991). In the dark the level of acetyl ACP increases substantially with a corresponding decrease in free ACP. In similar experiments when chloroplasts were shifted to the dark, malonyl ACP and malonyl CoA disappear and acetyl ACP levels rise over several minutes. This indicates ACCase activity is a major determinant in light dark control of fatty acid synthesis rates in leaves. When the multifunctional ACCase of maize and barley were incubated with ACCase inhibiting herbicides and radiolabeled acetate, a flux control co-efficient of between 0.5 and 0.6 was calculated for acetate incorporation into lipids (Page *et al.*, 1994). A flux control of this magnitude suggests a strong control (Crabtree and Newsholme, 1987; Kacser and Porteous, 1987) of fatty acid synthesis by ACCase. Thus both forms of ACCase have a major influence over plant fatty acid biosynthesis.

1.7.11 Utilization of the products of plant fatty acid synthetase

The usual products of plant FAS are palmitate and oleate. These fatty acids have two fates, they may be used directly in the chloroplast lipids via the prokaryotic pathway. (Douce and Joyard, 1979; Heemskerk *et al.*, 1990). Alternatively they can exported from the chloroplast as CoA esters (Block *et al.*, 1983) to enter the eukaryotic pathway at the endoplasmic reticulum (Moore, 1982). The first enzyme of the prokaryotic pathway is the stromal acyl ACP:glycerol-3-phosphate acyltransferase. This enzyme is highly specific for 18:1 ACP (Frentzen *et al.*, 1983), the second acylation step is catalyzed by a membrane bound acyl ACP lysophosphatidic acyltransferase, which specifically uses 16:0 ACP to yield diacylglyceol (DAG) with 18:1 at the *sn*-1 position and 16:0 at the *sn*-2 position. Chloroplast phosphatidylglycerol (PG) is synthesized from the prokaryotic PA

in all higher plants (Roughan and Slack, 1982). In 16:3 plants a prokaryotic DAG pool is formed from PA by the action of phosphatidic acid phosphatase (Douce and Joyard, 1979; Joyard and Douce, 1977). In angiosperms (18:3) plants such a DAG pool is not formed, so PG is the only prokaryotic lipid in these plants. A DAG pool derived exclusively from the eukaryotic pathway is used for the synthesis of other chloroplast lipids (Gardiner and Roughan, 1983; Heinz and Roughan, 1983).

1.8 Aims

Type II Fatty acid synthetase from plants and cDNA clones have been identified for each of the different enzyme activities, with the exception of β-hydroxyacyl ACP dehydratase. The aim of the thesis is to identify a cDNA clone for β-hydroxyacyl ACP dehydratase from a plant source. This would fill a significant gap in the understanding of plant type II fatty acid synthetase.

All of the genes responsible for fatty acid synthetase have been identified in *E. coli* with last to be identified being the gene responsible for encoding β-hydroxyacyl ACP dehydratase (*fabZ*) (Mohan *et al.*, 1995). Each of these genes has been histidine tagged, overexpressed and purified (Heath and Rock, 1995). This has facilitated the reconstitution of the whole of the *E. coli* type II fatty acid synthetase *in vitro* (Heath and Rock, 1995). Using this reconstituted system it has been possible to gain a greater understanding how each of the individual components interact to produce an active type II fatty acid synthetase. The cloning and overexpression of all the plant type II fatty acid synthetase will also allow the reconstitution of the plan type II fatty acid synthetase *in vitro.* The reconstituted plant fatty acid synthetase system as well as being of academic interest for the study of protein-protein interactions, is also relevant industrially as type II fatty acid synthetase is known to be the target of a range of herbicides and anti-microbials. A reconstituted fatty acid synthetase system would allow the interactions between these inhibitors and their targets to be studied in isolation away from the complex metabolic environment that is the plant cell. The FAS systems from *E. coli* and plant contain a lot of similarities as well as containing a number of significant differences, these differences may have important implications in how the components interact as well as the specific interactions with different inhibitors.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All Chemicals were analytical grade or higher, and were purchased from BDH, Sigma or Bio-Rad.

2.1.2 DNA modification enzymes

Taq DNA polymerase was purchased from Bioline along with the relevant buffers. T4 DNA ligase was purchased from Promega.

2.1.3 Restriction endonucleases

All restriction endonucleases were purchased from Boehringer, and used as per the manufacturers instructions.

2.1.4 Microbiological media

All media components were purchased from Difco or Oxoid. Media were prepared as described in Sambrook *et al.*, 1989. All media was sterilized by autoclaving at 121 °C and 20 pounds per square inch for 20 min.. Media was solidified with 1.5 % (w/v) bacto-agar prior to autoclaving.

2.1.5 Antibiotics

Antibiotics were prepared as described in Table 1. Antibiotics that were dissolved in water were filter sterilized by passing slowly through a 0.2 μm filter (Millipore) prior to addition to the media.

Antibiotic	Mode of Action	Uses	Working Strength
Ampicillin Made as a 100 mg mL^{-1}stock in water.	Gram Positive bactericidal. Inhibits cell wall peptidoglycan synthesis at the transpeptidation step	Selection and maintenance of *E. coli* strains carrying the β-lactamase gene	100 μg mL^{-1} of medium
Chloramphenicol Made as a 30 mg mL^{-1} in Ethanol	Bacteriostatic (originally from *Strep. venezuelae*); inhibits 50S ribosomal elongation	Selection and maintenance of *E. coli* strains carrying the cat gene	25 μg mL^{-1} of medium.
Kanamycin Made as a 25 mg mL^{-1} stock in water	Bactericidal amino glycoside; binds 70S ribosome, causing mRNA misreading	Selection and maintenance of *E. coli* strains carrying the *kan* resistance gene.	25 μg mL^{-1} of medium
Tetracyclin Made as a 12.5 mg mL^{-1} in 75 % Ethanol	A Bacteriostatic agent that binds to the 30S sub-unit of ribosomes, preventing protein synthesis	Selection and maintenance of *E. coli* strains carrying the Tn10 transposon	12.5 μg mL^{-1} of medium.

Table 1: Selectable markers. All concentrations were taken from Sambrook *et al.*, 1989

2.1.6 *E. coli* Strains

The following strains were used in this work. Genotype information is from the supplier.

2.1.6.1 XL1Blue

This proprietary bacterium is provided with all Stratagene kits. It has been engineered, so it does not produce large quantities of carbohydrates, which would otherwise interfere with plasmid DNA isolation. The strain does not express endonucleases, allowing the introduction of foreign DNA via transformation. The *lac* operon has been disabled and a modified version is present on an F' plasmid, which is maintained by tetracycline selection. The F' *lac* operon has a deleted *lacZ* (β-galactosidase) function, preventing lactose utilisation. These properties make XL1Blue an ideal cloning strain with *lacZ* complementing vectors. The strain was also used as a host to propagate lambda phage carrying cDNA libraries.

Genotype:

Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F' *proAB, lacI*^{q}*ZΔM15,* Tn109tet^{r})]

2.1.6.2 BL21(DE3)

This proprietary bacterium is provided with all pET bacterial overexpression systems (Novagen). It is an *E. coli* strain B that has been engineered to lack the soluble protease *lon* protease and the outer membrane protease *ompT* that can degrade proteins during purification (Grodberg and Dunn, 1998). This strain of *BL21* contains the DE3 lysogen. DE3 is a lambda derivative that has the immunity region of phage 21 and carries a DNA insert which contains the *lacI* (*lac* repressor) and the T7 RNA polymerase under the control of the *lacUV* promoter (Studier and Moffat, 1986). The insert disrupts the *int* gene of lambda preventing the DE3 from integrating or excising from the chromosome without helper phage. The *lacUV* promoter is inducible by addition of IPTG to the growth medium. This induces the T7 RNA polymerase, which in turn translates the target DNA of the plasmid.

Genotype

F$^-$ *ompT hsdS* $_B(r_B^- m_B^-)$ *gal dcm* (DE3)

2.1.6.3 BL21(DE3) pLysS

This is the same *E. coli* strain as above, but contains a compatible plasmid that provides a small amount of T7 lysozyme, an inhibitor of T7 RNA polymerase (Moffat and Studier 1987; Studier, 1991). T7 lysozyme is a bi-functional protein it digests a specific bond in the peptidoglycan layer of *E. coli* cell wall (Inouye *et al.*, 1973), it also binds to T7 RNA polymerase therefore inhibiting transcription. The pLysS plasmid is maintained under chloramphenicol selection.

Genotype:

F$^-$ *ompT hsdS* $_B(r_B^- m_B^-)$ *gal dcm* (DE3) pLysS

2.1.6.4 DH5α

This strain of *E. coli* was used for general cloning purposes. Like XL1Blue it contains a low levels of carbohydrate and endonucleases plus it also contains a disrupted *lac* operon.

Genotype:

ϕ80d*lacZ*Δ*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsd*R17 (r_k^-,m_k^+), *supE*44, *relA*1, *deoR*, Δ(*lacZ*YA-*argF*) U169

2.1.6.5 INVαF'

This proprietary strain is supplied with all Invitrogen kits. The strain is similar to XL1Blue. The bacterium has been engineered so it does not express the *lac* repressor, so addition of IPTG will have no effect.

Genotype:

F'. *endA*1, *recA*1, *hsdR*17 (r_k^-,m_k^+), *supE*44, *thi*-1, *gyrA*96, *relA*1, ϕ80*LacZ*ΔM15, Δ*lacZ*YA-*arg*F) U169, λ^-

2.1.6.6 SOLR

This proprietary strain was used in the conversion of the λZapII phagemid library into plasmid. This non-suppressing strain prevents replication of the ExAssist helper phage during library plasmid excision.

Genotype:

e14$^-$(*mcrA*), Δ(*mcrCB-hsdSMR-mrr*)*171*, *sbcC*, *recB*, *recJ*, *umuC*::Tn *5*(kan^r),

2.1.7 Phage lambda strains

2.1.7.1 ExAssist Interference-Resistant Helper Phage

The ExAssist interference resistance helper phage is used for efficient excision of the pBluescript phagemid from λZAP vectors (Stratagene), while preventing co-infection with the helper phage. The replication of the phage genome in a non-suppressing *E. coli* strain such as SOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of co-infection with Ex-Assist helper phage.

2.1.8 DNA Vectors

Vector	Supplier	Selectable Marker	Blue/White Selection	Use
pGEM-T and pGEM-T Easy	Promega	Ampicillin	Yes	PCR Cloning vector
pCR2.1	Invitrogen	Ampicillin Kanamycin	Yes	PCR Cloning Vector
pBluescript	Stratagene	Ampicillin	Yes	General cloning vector
pET24a	Novagen	Kanamycin	No	*E. coli* overexpression vector
pET11d	Novagen	Ampicillin	No	*E. coli* overexpression vector

Table 2: Commercially available plasmid vectors

2.1.9 cDNA libraries

A *Ricinus communis* developing endosperm stage V cDNA library was obtained from Pioneer Hybrid (Coughlan *et al.*, 1996). A developing *Brassica napus* (cv. Jet Neuf) cDNA library was obtained from Johan Kroon (Durham). The library was constructed with pooled mRNA isolated from different developmental stages of embryogenesis. The cDNA inserts were cloned into pBluescript (λZapII; Stratagene) using *Eco*RI linkers and packaged into lambda phage.

2.1.10 DNA Markers

A *Hin*dIII restriction digestion of phage lambda genomic DNA (Pharmacia) was used to estimate size and quantity of DNA following agarose gel electrophoresis (using UV-induced fluorescence in the presence of ethidium bromide). A second marker set of DNA markers a *Hae*III restriction digest of ϕX174 genomic DNA (Pharmacia) was used to estimate size of small DNA molecules.

λ/HindIII	ϕX174/HaeIII
23130bp	1353bp
9416bp	1078bp
6557bp	872bp
4361bp	603bp
2322bp	310bp
2027bp	281bp
564bp	271bp
126bp	234bp
	194bp
	118bp
	72bp

Table 3: DNA markers

2.1.11 Protein Standards

The protein standards used throughout were the SDS 7 markers from Sigma. These Standards contained a range of proteins from 14.2-66 kDa (Table 4).

Protein Standard	Daltons
Bovine serum albumin	66,000
Albumin, egg	45,000
Glyceradehyde-3-phosphate dehydrogenase from Rabbit muscle	36,000
Carbonic anhydride from Bovine Erythrocytes	29,000
Trypsinogen, PMSF treated from Bovine pancreas	24,000
Trypsin Inhibitor form Soybean	20,100
α-lactalbumin from Bovine milk	14,200

Table 4: Protein Standards

2.2 Methods

2.2.1 Growth and maintenance of *E. coli* strains

E. coli strains were maintained on 1.5% (w/v) bacto agar plates containing Luria-Bertrani (LB) nutrients (Sambrook *et al.*, 1989). This basal medium contains tryptone (a pancreatic digest of a high molecular weight milk protein) as a source of amino acids, yeast extract (a source of essential vitamins and co-factors) and sodium chloride. Antibiotics were added as selective agents were applicable. Overnight liquid cultures were grown at 37 °C and were shaken at 150-220 rpm in an orbital shaker. Cell stocks were generated by inoculating an overnight liquid culture along with the relevant selectable marker and mixing 1:1 (v/v) with LB containing 20 % glycerol (v/v) without the antibiotic, and stored at -80 °C.

2.2.2 Harvesting of *E. coli* cells

Bacterial cells were harvested by centrifugation at 3,000 rpm for 10 min in a Beckman 2-HC refrigerated centrifuge (using either a J-Lite, J-14, or J-20 rotors depending on the volume of culture). Small volumes of culture (< 2 mL) were harvested in a microfuge tube by centrifuging for 5 min at 13,000 rpm in a micro-centrifuge (Sanyo).

2.2.3 Transformation Protocols

Three types of chemically competent *E. coli* were used. $CaCl_2$ competent Epicurian XL1Blue cells were purchased from Stratgene. These cells were used when important difficult cloning was undertaken.

2.2.3.1 Chemically competent *E. coli*

(a) $CaCl_2$ competent *E. coli* were prepared by diluting an overnight culture of *E. coli* grown under antibiotic selection if possible (e.g. Tetracycline for XL1Blue). This was diluted 1/100 into fresh medium that does not contain any antibiotic. The cells were grown until they reached an OD_{600} of 0.6. The cells were harvested by centrifugation, and resuspended in 0.5 volumes of ice cold 50 mM $CaCl_2$. The cells were kept on ice for 1 h. The cells were harvested as before, and resuspended in 0.1 volumes of ice cold 50 mM

$CaCl_2$ / 20 % Glycerol. The cells were stored in 100 µL aliquots at -80 °C for up to 6 months.

(b) RbCl competent *E. coli* were prepared similar as above, except the cells after harvesting were re-suspended in 0.4 volumes ice cold TFB I solution (30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % Glycerol (v/v)), and then incubated on ice for 5 min. The cells are then harvested and resuspended in 0.04 volumes of ice cold TFB II solution (10 mM MOPS pH 6.5, 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol (v/v)). The cells were stored in 100 µL aliquots at -80°C for up to 6 months.

2.2.3.2 Transformation of *E. coli*

Plasmid DNA (up to 10 µg in 10 µL) was added to 100 µL of competent cells and incubated on ice for 30 min to allow binding of the DNA to the outer membrane of the *E. coli* cells. The cells were then 'heat shocked' at 42 °C for 90 sec to facilitate the uptake of the DNA into the cells. The cells were allowed to recover by incubation in 900 µL of pre-warmed LB at 37 °C for 60 min. This allows the membrane integrity of the cells to reform as well as time for the cells to start synthesising antibiotic resistance proteins required prior to plating on selective media. The cells were harvested and the majority of the medium removed, the cells were resuspended in the remaining medium (~200 µL) the transformation mixture was then spread on selective LB plates and incubated at 37 °C overnight.

2.2.4 Preparation of plasmid DNA

Cultures for plasmid preparations were grown overnight at 37 °C in LB containing the relevant antibiotic. A number of different methods were used depending on the quantity of DNA and the application for which the DNA was required.

2.2.4.1 Preparation of plasmid DNA for sequencing

Either a Wizard™ (Promega) or Hybaid mini-prep kit, was used according to the manufacture's instructions.

2.2.4.2 Large scale plasmid isolation method for sequencing

A Qiagen midi prep kit was used according to the manufacture's instructions.

2.2.4.3 Chemical isolation of plasmid DNA

This method is used for the crude purification of plasmid DNA for use as stocks for transforming DNA or for sub-cloning. A single *E. coli* colony was inoculated in 100 mL of LB broth containing the relevant antibiotic overnight at 37 °C. The cells were harvested and resuspended in 3 mL of 50 mM Tris.Cl pH 8.0, 10 mM EDTA. To the cell suspension 3 mL of lysis solution (0.2 M NaOH, 1 % SDS) was added. To the lysed cells 3 mL of 3 M potassium acetate pH 5.2 was added, and the cells centrifuged at 10,000 g for 15 min in a Beckman 2-HC refrigerated centrifuge. After centrifugation of the potassium acetate pellet, the supernatant is filtered through two layers of muslin. The plasmid DNA is removed from the salt solution and concentrated by the addition of 0.6 volumes isopropanol at 10,000 g for 30 min at room temperature. The DNA pellet was resuspended in a 3 mL of ice cold 10 M LiCl and incubated on ice for 10 min, the high molecular weight RNA is removed by centrifugation at 10,000 g for 15 min at 4 °C. The supernatant was decanted to a fresh centrifuge tube and the DNA concentrated by repeating the isopropanol precipitation step. The precipitated DNA was resuspended in 400 µL of water and the remaining RNA removed by incubating at 37 °C for 15 min with 4 µL of RNAase A (100 mg/mL). The RNAase A was removed by phenol chloroform extraction, and the plasmid DNA concentrated by ethanol/sodium acetate precipitation and the salt removed by a 70% (v/v) ethanol wash. The DNA is resuspended in a 100 µL of water.

2.2.5 DNA methods

2.2.5.1 DNA digestion with restriction endonuclease

DNA digestion was carried out using enzymes according to the manufacturer's instructions with the appropriate supplied buffer. Approximately 5 units of enzyme per µg of DNA were incubated for 1-3 h.

2.2.5.2 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments, and was performed using the Bio-Rad Mini/Midi-Sub Cell. Agarose gels were

0.8% agarose / low melting agarose in 1 x TAE buffer containing 0.5 μg/mL ethidium bromide. Samples were prepared by the addition of loading buffer (5X) and electrophoresied for 1-2 h at constant current in 1 x TAE buffer. Agarose gels were visualised under UV illumination provided by a short wavelength transilluminator.

2.2.5.3 Isolation of DNA fragments from high melting point agarose gels

DNA was extracted from high melting point gels using the Qiaex II gel extraction kit from Qiagen using the manufactures instructions with the exception that the DNA was eluted from the column using water and not the provided elution buffer.

2.2.5.4 Isolation of DNA fragments from low melting point agarose gels

DNA was extracted from low melting point gels using the Wizard™ PCR purification kit (Promega) using the manufactures instructions.

2.2.5.5 Ligation of DNA

Ligations were performed in a 10 μL final volume. A 1:1 and a 3:1 insert to vector molar ratio were used with a maximum of 50 ng of insert DNA. Ligase buffer 10 x (2 μL) and 4 units T4 DNA ligase from Bohringer were added and the ligation mixture incubated at 15 °C for 16 h.

2.2.5.6 Phenol extraction

This method was used to purify nucleic acid. To the DNA an equal volume of phenol: chloroform: isoamyl alchol (25:24:1, v:v). pH 7.6 was added to denature and extract protein and the mixture was vortexed to form an emulsion. The two phases were separated by centrifugation at full speed in a microfuge for 2 min. The upper aqueous phase was transferred to a fresh microfuge tube.

2.2.5.7 Ethanol precipitation

Ethanol precipitation was used to recover and concentrate DNA. The volume of solution was estimated and 0.1 volumes of sodium acetate (3 M, pH 5.5) and 2.5 volumes of ice cold ethanol were added. The solution was mixed and the DNA was precipitated by

incubation at -80 °C for 15 min. After pelleting by centrifugation at full speed in a microfuge at room temperature for 10 min, the supernatant was carefully removed and the pellet washed with 1 mL of ice cold 70 % ethanol. The pellet was aspirated and resuspended in an appropriate volume of water.

2.2.6. Polymerase chain reaction (PCR) methods

2.2.6.1 Standard PCR reaction

All PCR reactions were performed using Biotaq (Bioline) and the relevant supplied buffers. PCR reactions were set up to contain 10 pmoles of each oligonucleotide, 0.4 mM DNTP set (dATP, dCTP, dGTP, dTTP), plus the template, either 30 ng of plasmid, or a single XL1Blue *E. coli* colony or 10 μL of a phage population. All standard PCR reactions were performed in a Perkin-Elmer thermocycler. The amplification was performed using the following standar thermoprofile. 94 °C for 1 min, optimal annealing temperature (for the primer set) for 1 min followed by an extension of 2 min at 72 °C. The amplification was performed over 30 cycles. When the amplified PCR product was to be cloned then after the completion the amplification cycle was followed by a 6 min hold at 72 °C. The optimal annealing temperature for each set of primers was determined prior to their experimental use.

2.2.6.2 Optimisation of annealing temperature for a set of primers

An initial experiment to determine the optimal annealing temperature was initially performed on a new set of PCR primers. Thirteen standard PCR reactions (See above) were set up containing 10 pmoles of each of the two primers, along with the relevant template to twelve of the reactions and an equivalent volume of water to the final tube. The amplification was performed in a Stratagene Robocycler, the PCR mixture was identical to that used for the standard PCR protocol with the exception that a range of different temperatures (48-58 °C) were used for the annealing step. After amplification was complete 5 μL of the final PCR reaction was loaded onto a 0.8 % agarose gel to determine the optimum annealing temperature.

2.2.7 cDNA library methods

2.2.7.1 Preparation of plating cells

Phage competent *E. coli* were prepared by diluting an overnight culture of XL1Blue *E. coli* grown under tetracycline selection, 1/100 into fresh 50 mL of LB containing 0.2 % maltose and 10 mM $MgCl_2$. The cells were grown until they reached an OD_{600} 1.0. The cells were harvested by centrifugation, and resuspended in 0.1 volumes of ice cold 10 mM $MgCl_2$. The cells were stored at 4 °C for 1 week, but highest plating efficiencies were achieved within 0 - 2 days.

2.2.7.2 Infection of plating cells for primary cDNA library screen

150, 000 pfu of a developing *Brassica napus* embryo cDNA library was added to 400 L of phage competent XL1Blue *E. coli* and infection allowed to occur by incubating the cells at 37 °C for 15 min. The infected cells were mixed with 40 mL of pre-warmed top agarose (LB, solidified with 0.7 % agarose), and plated onto two LB plates (Nunc, Bioassay plates). The plates were incubated overnight at 37 °C then cooled to 4 °C. The infected cells were transferred to hybond C, and the cellular material removed by placing the membrane on filter that had been soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 min. The filter was then neutralised by placing on filter paper soaked in 1 M Tris.Cl pH 7.4, 1 M NaCl for 2 min. The remaining DNA was fixed to the membrane using a UV Stratalinker (Stratagene). The filters were then transferred to hybridisation tube containing 25 mL of hybridisation solution, and pre-hybridised for 2 h at 65 °C. The hybridisation solution was removed and replaced with a further 25 mL of hybridisation solution containing the castor EST probe. The tubes were incubated overnight at 65 °C, and then the hybridisation solution was removed.

Unbound probe was removed by washing twice in 2 X SSC, 0.1 % SDS (Wash A) at room temperature for 15 min. This was followed by a single wash in 1 X SSC, 0.1 % SDS at 55 °C (Wash B) for 15 min. The filters were removed from the hybridisation tube, and the degree of hybridisation was determined by carefully passing a Geiger counter over the surface of the membranes. A number of isolated radioactive spots could be detected covering the surface of the membrane. To completely remove any remaining unbound probe

the filters were washed again in wash B at 55 °C for 15 min. The filters were then exposed to film overnight and developed.

2.2.7.3 Purifying positive phage

Putative positive plaques were purified by taking through two further rounds of screening. Dilutions of the primary plugs (1 in 1000 and 1 in 10,000) were allowed to infect 100 µL of phage competent XL1Blue *E. coli*. The infected cells were mixed with warm top agarose and plated onto LB in Petri dishes. The dilution, which produced a nice spread of plaques, was lifted and hybridized using identical conditions as used for the primary screen. Positive plaques were identified on all three plates, two putative positives plaques from each plate were transferred to 100 µL of SEM containing 10 µL chloroform using the small bored end of a sterile Pasteur pipette and stored at 4 °C overnight. Two putative positive plaques were identified from each secondary screen plates, and these were taken through a further round of screening to confirm that the plaques were pure. Dilutions of the two putative positive plugs (1 in 100 and 1 in 1000) were allowed to infect 100 µL of phage competent XL1Blue *E. coli*. The infected cells were mixed with warm top agarose and plated onto LB in Petri dishes. Both dilutions were lifted and hybridized using identical conditions as for the two previous rounds of screening. Both plates from all three of the clones were plaque pure. A single plaque was bored from each of the two dilution plates using the small bored end of a sterile Pasteur pipette, and transferred to 100 µL of SEM buffer containing 10 µL of chloroform and stored at 4 °C until required.

2.2.7.4 *In vivo* excision

The ExAssist Interference-resistant helper phage along with SOLR *E. coli* cells (Stratagene) were used for *in vivo* excision of pBluescript from Zap based cDNA libraries (Stratagene). 200 µL of purified phage were incubated with phage competent XL1Blue *E. coli*, along with ExAssist helper phage. Infection was allowed to occur at 37 °C for 20 min. The infected cells were mixed with 3 mL of LB and grown for 2 h at 37 °C. During this period of growth the helper phage encourages the replication, excission, packaging and extrusion of both the helper phage and the pBluescript phagemid. The *E. coli* cells were then killed by heating to 70 °C for 10 min and the dead cells were harvested. 300 µL of the

supernatant allowed to infect phage competent SOLR cells as described for the XL1blue cells. SOLR cells are used, as the helper phage cannot replicate in this strain due to an amber mutation that in the SOLR cells is not suppressed. The infected SOLR cells were plated onto LB containing ampicillin overnight at 37 °C to select for cells containing the released pBluescript phagemid.

2.2.8 Southern hybridisation

2.2.8.1 Production of *Eco*R1 fragment as a probe

The restriction map on the sequence for the clone SD4 shows that the sequence only contains one *Eco*RI recognition site (Figure 1). The SD4 cDNA insert was cloned into the pBluescript phagemid during the initial library construction using *Eco*RI linkers. This leads to two *Eco*RI recognition sequences flanking the cDNA insert and so digestion with *Eco*RI would lead to a 679 bp fragment which contains the majority of the SD4 open reading frame, but does not contain any vector sequence

1µg of pSD4 was incubated with *Eco*RI for 3 h at 37 °C, the whole of the restriction digest was loaded onto a 0.8% agarose gel. The released 679 bp *Eco*RI fragment was excised from the gel using a clean razor blade and purified using the Quiex II gel purification kit (Quiagen). 45 ng of the isolated was labeled with ^{32}P using the redi-prime random prime labeling kit (Amersham). Excess unincorporated nucleotides were removed by applying the labeling reaction to a pre-packed Biospin column (Bio-Rad)

2.2.8.2 Genomic DNA isolation from *Brassica napus*

A number of rape seeds (cv jet neuf) were allowed to germinate, by placing on damp filter paper overnight. The germinating seedlings were placed into moist soil and allowed to grow in a growth cabinet (Sanyo) until the first true leaf was four days old. The tray containing the young rape seedlings was placed into a sealed box for 48 h (until the leaves started to turn yellow) to deplete the carbohydrate content of the leaves. The four-day-old leaves were harvested, weighed and flash frozen in liquid nitrogen. Genomic DNA was extracted using the min-prep method of Dellaporter (Dellaporter *et al.*, 1983). 1 g of the frozen carbohydrate depleted leaf tissue was ground to a fine powder in a pestle and mortar.

The fine powder was then defrosted in 20 mL of extraction buffer (100 mM Tris.HCl pH 8.0, 50 mM EDTA, 500 mM NaCl and 10 mM β-mercaptoethanol at 65 °C. Once defrosted the cells were lysed by the addition of 2.5 mL of 10 % SDS, and were once again incubated at 65 °C. The extracted cells was cooled to 4 °C and 6.25 mL of 5 M potassium acetate added, protein precipitation was allowed to occur by incubating on ice for 20 min. The precipitated proteins were removed by centrifugation 25,000 g for 20 min. The supernatant was passed through two layers of muslin to completely remove any remaining particulate matter. The genomic DNA was isopropanol precipitated, and the DNA recovered by centrifugation. The DNA pellet was washed once in 70 % ethanol, vacuum dried and resuspended in 100 µL of water.

2.2.8.3 Southern Blot

100 ng of genomic DNA was digested with the following restriction endonucleases *Eco*RI, *Bam*HI and *Hind*III (1 unit of enzyme per digestion). The digests were performed in a large reaction volume (200 µL), and incubated at 37 °C overnight to facilitate complete digestion. The digested genomic DNA was ethanol precipitated, and resuspended in 20 µL of DNA loading buffer, and loaded onto a 0.8 % agarose gel. As a positive control a serial dilution of an *Eco*RI digest of SD4 was also loaded onto the same gel. The gel was run at 30 volts overnight (until the dye had run three quarters of the way down the gel). The digested genomic DNA was then depurinated in 0.25 M HCl for 15 min, and then denatured for 15 min, the gel was neutralized in 0.4 M NaOH. The gel was transferred to hybond C (Amersham) by capillary blotting using 0.4 M NaOH as the transfer buffer. The filter was immediately neutalised in 1 M Tris.HCl pH 7.4 and the DNA fixed to the membrane using a UV stratalinker (Stratergene). The blot was hybridized at 65 °C overnight with the radiolabeled *Eco*RI restriction fragment isolated from pSD4 as the probe. Unbound probe was removed by washing the membranes twice in wash A (2 X SSC, 0.1 % SDS) at 65 °C for 15 min. The filters were then washed twice in wash B (1 X SSC, 0.15% SDS) at 65 °C for 15 min. The Southern blot was then exposed to film (Fuji), and stored at -80 °C for 1 week prior to being developed.

2.2.9 Protein Methods

2.2.9.1 PAGE electrophoresis

All PAGE gels were performed in a using Bio-Rad Mini Protean II Electrophoresis cells. Gel dimensions used were 10 cm x 10 cm x 1.5 mm.

2.2.9.1.1 SDS PAGE gel electrophoresis

Discontinous Tris-glycine gel electrophoresis was performed using the method developed by (Laemmli, 1970). Resolving gels contained 15 % acrylamide (Bio-Rad), 0.375 mM Tris.HCl pH 8.8, 0.1 % SDS (w/v). The upper stacking gel contained 4.5 % acrylamide (Bio-Rad), 0.125 mM Tris.HCl pH 6.8, 0.1 % SDS. Polymerisation was catalysed by the addition of 0.1 % ammonium persulphate and 0.01 % TEMED. Protein samples were boiled in SDS PAGE loading buffer (50 mM Tris.HCl pH 6.8, 100 mM DTT, 2 % SDS, 10 % glycerol, 0.1 % bromophenol blue). After boiling the samples were loaded onto the gel and the gels ran at constant voltage (100 V) until the dye front had reached the base of the resolving gel in SDS PAGE running buffer (25 mM Tris base, 250 mM glycine and 0.1 % SDS).

2.2.9.1.2 Native PAGE gel electrophoresis

Native PAGE gels were performed as for by the method developed by Rock and Cronan (Rock and Cronan, 1979). The method is identical to that for SDS PAGE gel electrophoresis with the following exceptions. Resolving gels contained 18 % acrylamide and 0.375 mM Tris.HCl pH 9.0 only. The upper stacking gel contained 4.5 % acrylamide and 0.125 mM Tris.HCl pH 6.8 only. Protein samples were mixed with native gel loading buffer (50 mM Tris.HCl pH 6.8, 10 % glycerol and 0.1 % bromophenol blue) prior to loading onto the gel. Protein samples were run in native PAGE gel electrophoresis buffer (25 mM Tris base, 250 mM glycine) at constant voltage (70 V) until the dye front reached the base of the resolving gel. Only proteins that have a negative charge at pH 9.0 would enter the gel under these conditions.

2.2.9.1.3 Gel electrophoresis of ACP and acyl ACP

Electrophoresis on ACP and its acyl ACP derivatives was performed using the method developed by Post-Bettinmiller (Post-Bettinmiller *et al.*, 1991). The general method is identical to the native gel electrophoresis method described above with the major exception that both the resolving and stacking gel contain urea as a denaturant. The concentration of urea used depended on the ACP or acyl ACP derivative be separated (Table 5).

ACP or derivative	Concentration of urea
holo / apo	0.5 M urea
C2 -C8	0.5 M urea
C8 - C16	2.5 M urea
C16 - C18	5 M urea

Table 5: Urea concentration for the separation of ACP

2.2.9.1.4 Visualisation of electrophoresied proteins

Proteins gels were visualised using Coomassie staining, the stain contains Coomassie Brillialnt Blue R-250. The Coomassie stain binds to aromatic amino acids. Run PAGE gels were stainied in Coomassie stain (0.25 % Coomassie Brilliant Blue R-250 of a methanol : acetic acid (v:v)). for 1 h. The excess stain was removed by incubating the solution in a methanol : acetic acid (v:v) solution.

2.2.9.1.5 Transfer of electrophoresied proteins to nitrocellulose membrane.

Protein blotting involves the transfer of proteins separated by PAGE gel electrophoresis to a nitrocellulose membrane (Towbin *et al.*, 1979, Burnette 1981). A Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell was used to transfer proteins form to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Amersham) in 25 mM Tris base, 250 mM glycine, 0.01 % SDS and 20 % methanol, overnight at 30 V. Blotted proteins were stained in Ponceau S stain, excess stain was removed in TBS: Tween 20.

2.2.10 Overexpression Methods

2.2.10.1 Seed stock induction

100 ng of the overexpression construct was transformed into competent BL21 (DE3) *E. coli* cells. The transformants were plated out onto LB plates containing the relevant antibiotic (ampicillin or kanamaycin) and the cells allowed to grow until the colonies were just visible (~10 h). The small colonies were transferred to 100 μL of LB containing 20 % glycerol and stored at –80 °C until required, these are called seed stocks. A single seed stock is inoculated into LB containing the relevant antibiotic and grown until an OD_{600} of 0.6 had been reached. 100 μL of culture was removed, harvested and resuspended in SDS-PAGE loading buffer (non-induced). 0.5 mM IPTG was added to the remaining culture and the cells allowed to grow for a further 3 h. Again 100 μL of culture was removed, harvested and resuspended in SDS-PAGE loading buffer (induced). Both the non-induced and induced samples were loaded onto a SDS-PAGE gel to confirm that the recombinant protein had been induced.

2.2.10.2 Overexpression using pET vectors

100 ng of the overexpression construct was transformed into competent BL21 (DE3) *E. coli* cells. The transformants were plated out onto LB plates containing the relevant antibiotic (ampicillin or kanamaycin) overnight at 37 °C. Transformants were scraped into LB containing the relevant antibiotic (ampicillin or kanamaycin) and grown to at 37 °C an OD_{600} of approximately 0.6 at 37 °C had been reached. The remaining culture was then treated as described in the seed stock method.

2.2.11 Protein extraction methods

2.2.11.1 Sonication

After harvesting the induced cells, the pellet was resuspended in 0.1 volumes of the relevant extraction buffer, and the cells were again harvested. The cells were then

resuspended in relevant extraction buffer (4 mL of buffer per 100 mL of starting culture). The cells were sonically disrupted by five-15 sec burst using a sonic probe. In between bursts the cells were placed on ice for 15 sec to prevent the cell lysate from warming up. The disrupted cells were then centrifuged at 10,000 g to remove any cell debris and any remaining intact cells. The supernatant containing the soluble protein was carefully transferred to a fresh tube. The pellet was either discarded or resuspended in buffer depending on the relevance to the experiment.

2.2.11.2 High pressure

The induced cells were harvested and washed as previously described in the sonication method. After washing the cells were resuspended in 1:4 (w/v) of the relevant extraction buffer containing 3 mM $MgCl_2$. The cells were disrupted by passing twice through a cooled cell disrupter (Constant systems) at 20 Kpa. After passing through the cells 50 µg of DNAase I was added and the lysate incubated at 4 °C for 30 min. Cell debris was removed by centrifugation at 40,000 g for 30 min. The supernatant was carefully transferred to a fresh tube, the pellet was either resuspended in buffer or discarded depending on the experiment being performed.

2.2.11.3 Freeze thaw extraction

Freeze thaw extraction was performed as described in the method of Johnson and Hecht (Johnson and Hecht, 1994). This method uses repetitive cycles of freezing and thawing to selectively release recombinant proteins from *E. coli* cells. Test experiments were performed on 3 mL of *E. coli*. After the completion of the induction the cells were harvested and submerged in a dry / ice ethanol bath (-78 °C) for 2 min. The cells were then allowed to thaw by placing the cells in to a ice / water (4 °C) bath fo 8 min. The cycle was repeated three times. After the third cycle the cells were resuspended in 50 µL of water and left at 4 °C for 30 min. The *E. coli* cells were harvested and the supernatant transferred to a fresh tube. 8 µL of the supernatant was loaded onto an SDS-PAGE gel to determine if the recombinant protein had been released.

Large scale extractions were accomplished using a similar system as described above with the exception that that the cells were frozen for 10 min, in the dry ice ethanol bath, and

were allowed to defrost in the ice water bath for 30 min. After the third cycle the cells were resuspended in the relevant extraction buffer and incubated at 4 °C prior to the cells being harvested. Approximately 50 % of the recombinant protein is released using this method.

2.12. Protein purification methods

2.2.12.1 Purification of ACP from *E. coli*

2.2.12.1.1 Preparation of an ACP enriched fraction

250 grams of frozen fermenter grown *E. coli* were shattered into tiny fragments by a large steel mallet. The thin slivers were defrosted quickly in 1 L of 10 mM potassium phosphate buffer pH 6.2 containing 0.1 % β-mercaptoethanol (Buffer A). All subsequent procedures were carried out at 4 °C. The defrosted cells were broken by passing twice through a cell disrupter (Constant systems) at 2500 psi. DNA was removed from the bacterial lysate by incubating the broken cells with DNAase I in the presence of 3 mM $MgCl_2$ for 30 min. The cell debris was removed by centrifugation at 40,000 g for 30 min. The cell lysate was carefully decanted into a fresh centrifuge tube and centrifuged at 40,000 g for 15 min. to remove any remaining cellular material; this was repeated until a cleared lysate was obtained. The supernatant was then made 60 % (w/v) with respect to ammonium sulphate and the pH adjusted to 7.0 with dilute NaOH, then incubated for 30 min with continuous stirring. Precipitated proteins were removed by centrifugation at 40,000 g for 15 min, and the supernatant was carefully decanted and acidified to pH 1.0 with concentrated HCl. The acidified mixture was then left for 4 h to allow complete precipitation to occur. The denatured proteins were collected by centrifugation at 40,000 g for 30 min and the supernatant discarded. The pellet was homogenized into a minimum volume of buffer A, containing a few drops of 2 M Tris.HCl pH 8.0 to help raise the pH quickly, and so aid solubilization. Any remaining insoluble material was removed by centrifugation at 40,000 g for 15 min. The supernatant containing the resolubilized protein was dialyzed against 6 L of buffer A using Spectra/Por dialysis tubing with a molecular weight cut off of 3000 Da (Spectrum) to prevent ACP loss.

2.2.12.1.2 Ion Exchange chromatography on enriched ACP extract

A 55 mL high load Q column (Pharmacia) attached to a high load chromatography workstation (Pharmacia) was equilibrated in 2 M NaCl in order to ensure the column was completely charged with sodium ions. The column was then equilibrated in buffer A. The ACP fraction had its conductivity adjusted to <2.0 mS with buffer A and its pH adjusted to 6.2 with dilute HCl, and was then loaded onto the column. The column was washed with buffer A until the optical density (OD) at 280 nm had returned to zero. Subsequent elution was carried out with a linear gradient of 0-100 % buffer B (buffer A containing 0.5 M LiCl) over 500 mL. 200 μL aliquots were removed from each fraction with the remainder of the fraction flash frozen in liquid nitrogen and stored at -80°C until required.

2.2.12.1.3 Storage of ACP

ACP containing fractions were dialysed against 6 L of 50 mM imidazole.HCl pH 7.4, containing 1 mM DTT and 1 mM EDTA (ACP storage buffer) using Spectra/Por tubing with a molecular cut off of 3000 Da (Spectrum), and stored at -80 °C until required.

2.2.12.2 Purification of recombinant ACP

Harvested induced *E. coli* were washed in 10 mM potassium phosphate buffer containing, 10 % β-mercaptoethanol, and the recombinant ACP extracted by freeze thawing. The freeze thaw extract, had its pH and conductivity adjusted to 6.2 and <2.0 mCi respectively, and was then loaded onto a 55 mL mono-Q Sepharose 5/5 column (Pharmacia, attached to a Bio-Rad, Biologic work station). The column had been equilibrated in 10 mM potassium phosphate pH 6.2 containing, 0.1 % β-mercaptoethanol (buffer A) prior to loading of the sample. Unbound protein was removed by washing the column with 5 column volumes of buffer A, until the OD at 280 nm had returned to zero. The subsequent elution was carried out with a linear gradient of 0-100 % buffer B (buffer A containing 0.5 M LiCl) over thirty column volumes. 7 mL fractions were collected from the start of the gradient until the end.

2.2.12.3 Purification of recombinant holo ACP synthase

Induced *E. coli* were washed in 20 mL of 50 mM Tris.HCl pH 8.0 containing 10 mM $MgCl_2$ and 1 mM DTT (Buffer A). They were then harvested and resuspended in 1:4 (w/v) of Buffer A, all subsequent procedures were then carried out at 4 °C. The *E. coli* suspension was passed twice through a cell disrupter (Constant systems). Cation exchange chromatography using a S-Sepharose column was used in the purification of holo ACP synthase from *E. coli*, as well as in purification of the recombinant enzyme (Lambalot and Walsh, 1995). 1 mL of crude holo ACP synthase extract was loaded onto a 1 mL mono-S 5/5 column (Pharmacia, attached to a Pharmacia FPLC workstation), which had been equilibrated in buffer A. The column was then washed in buffer A until the OD at 280 nm had returned to zero. Subsequent elution was achieved with a linear gradient of 0-100 % buffer B (buffer A containing 1 M NaCl) over 30 column volumes. 1 mL fractions were collected from the start of the linear gradient until the end.

2.2.13 Synthesis of acyl ACP

2.2.13.1 Conversion of apo to holo ACP

5 mg of purified recombinant rape and *E. coli* ACP were dialysed against water that was buffered with a few milligrams of ammonium bicarbonate to pH 7.0. The dialysed ACP was then lyophilised by freeze-drying. The freeze dried ACP was resuspended in a small volume of 50 mM potassium phosphate buffer pH 7.3. The 5 mg (333 μM) ACP was added to a reaction cocktail containing 50 mM potassium phosphate buffer pH 7.3, 2 mM EDTA, 10 mM $MgCl_2$, and 666 μM Co-enzyme A (Sigma) the final volume of the reaction mix was made up to 1.5 mL with water. The reaction was initiated by the addition of recombinant holo ACP synthase; and the mixture incubated at 37 °C for 3 h. The reaction was terminated by flash freezing in liquid nitrogen.

2.2.13.2 Synthesis of acyl ACP using recombinant acyl ACP synthetase

E. coli ACP (600 μg) was reduced by incubating with 5 mM DTT for 1 h at 4 °C. All the reduced ACP was immediately added to a reaction cocktail containing 2 mM DTT, 100 mM Tris.HCl pH 8.0, 5 mM ATP, 2.5 mM $MgCl_2$, 400 mM LiCl, 2 % Triton X-100, 60 μM

(2*E*)-tetradecenoic acid and 760 µL acyl ACP synthetase and the reaction made up to 2.5 mL with water. The reaction cocktail was then incubated at 30 °C for 48 h. After 48 h, 16 µL was removed from the reaction and the remainder of the reaction mixture was flash frozen in liquid nitrogen and stored at -20 °C until required.

2.2.13.3 Synthesis of [^{3}H] palimitoyl ACP

[^{3}H] palimitoyl ACP was synthesised using 37.5 µg of reduced *E. coli* ACP, and 60µM [^{3}H] palmitate (specific activity 550 mCi/mol), in a final reaction volume of 250 µL, using the same concentration of reagents as described above. The reaction was incubated at 30 °C for 24 h. The synthesis reaction was terminated by flash freezing in liquid nitrogen and stored at -20°C until required.

2.2.13.4 Purification of acyl ACP

2.2.13.4.1 Q-Sepharose chromatography on acyl ACP

A 1 mL High trap Q column (Pharmacia) was equilibrated in buffer A (20 mM Bis-Tris.HCl pH 6.8). The acyl ACP synthesis reaction was diluted ten fold with buffer A, to reduce the salt concentration sufficiently to allow the ACP to bind to the Q-Sepharose column. Unbound fatty acid was removed by washing the column with 10 column volumes of buffer A. The column was then washed with 20 column volumes of buffer A containing 80 % isopropanol to remove any remaining Triton X-100. The column was then washed with a further 10 column volumes of buffer A to remove any remaining isopropanol. The ACP fraction was step eluted with 10 mL of buffer B (buffer A containing 0.5 M LiCl). 1 mL fractions were collected for the step elution step only, and 20 µL was removed from each fraction and counted for 1 minute.

2.2.13.4.2 Purification of ACP derivatives using octyl Sepharose

A 10 mL octly Sepharose C4-LB (Pharmacia) column was stripped with 20 mM Tris.HCl pH 7.4 containing 80 % isopropanol. The column was then equilibrated in buffer A, and the pooled radiolabeled palmitoyl ACP containing fractions were loaded onto the column. The column was washed with 5 column volumes of buffer A to remove any

unbound material. The column was then equilibrated 5 column volumes of the volatile buffer 20 mM NEMA pH 7.4. The acyl ACP was step eluted with 20 mL of buffer B (Buffer A containing 30 % isopropanol). 1mL fractions were collected during the step elution step, and 20 μL was removed from each fraction and counted for 1 minute.

2.2.13.4.3 Blue-Sepharose purification of acyl ACP derivatives

All of the synthesis reaction was loaded onto a fast flow blue Sepharose column (Pharmacia) which had been equilibrated in 50 mM Tris.HCl pH 8.0 containing 2 % Triton X-100 (Buffer A). The complete run through from the column was collected and the column was then washed with a further 20 mL of buffer A, to ensure complete recovery of the acyl ACP. The sample was then either immediately applied to a Q-Sepharose column or stored at 4 °C until required.

2.2.13.4.4 Concentration of ACP derivatives

ACP derivatives eluted from a octyl Sepharose column in the volatile solvents NEMA and isopropanol were concentrated under vacuum. The acyl ACP containing fractions were placed in a vacuum centrifuge (Jouan), and the buffer was evaporated off until approximately 75 μL was remaining. The acyl ACP was not allowed to completely dry as the least volatile component of the elution buffer is the NEM, and thus reducing the volume to far would produce an alkaline environment which will hydrolyse the thioester bond leading to a reduced yield of acyl ACP.

.2.2.13.4.5 Synthesis of *trans*-2-octenoyl chloride

trans-2-octenoic acid was chosen as the acyl group, as acyl ACPs of six carbons or less have insufficient hydrophobic character to be able to separate the acyl ACP from any unesterified ACP by hydrophobic interaction chromatography. 5 mg of *trans*-2-octenoic acid (Avocado) was dissolved in dry benzene (100 mL). To this a few drops of dimethylformamide was added, as a catalyst. The reaction was initiated by the addition of oxyl chloride ($COCl_2$), the reaction was then allowed to proceed for 2 h at room temperature. The benzene was evaporated off under vacuum at 35 °C, leaving behind a dark brown oily liquid. An infra red spectrogram revealed this was the oily brown liquid is octenoyl chloride,

it also showed there was little if no contamination with the free fatty acid (Data not shown). The oily brown liquid was then washed in dry dichloromethane, and the acid chloride distilled by using bulb to bulb distillation. Here the dissolve octenoyl chloride is evaporated under vacuum in one chamber (the original reaction vessel) and the gaseous *trans*-2-octenoyl chloride is condensed in a second bulb which is cooled to -80 °C with dry ice. A clear liquid was collected in the second bulb while a dark brown residue remained in the original reaction vessel. The clear liquid was then subjected to an NMR spectra which confirmed the clear liquid to be pure *trans*-2-octenoyl chloride (Personal communication Pat Steele). The *trans*-2-octenoyl chloride was stored under argon at room temperature.

2.2.13.4.6 Synthesis thesis of the *trans*-2-octenoic-N-acylimidazole

0.5 g of the *trans*-2-octenoyl chloride was dissolved in 15 mL of 3:2 trihydrofluorine/ethyl diether, both solvents were distilled immediately prior to use to remove any water. The dissolved *trans*-2-octenoyl chloride was placed in an ice water bath and the reaction was initiated by the addition of 413.5 g of imidazole. The reaction was allowed to proceed for exactly 1 min, if the reaction proceeds further then the imidazole can add across the reactive *trans* double bond (Personal communication Dr. Pat Steele). A white precipitate formed immediately upon the addition of the imidazole. The solvents were removed by evaporation under vacuum, leaving behind the white precipitate. The precipitate was then washed with dry ethyl diether, and any undissolved precipitate was removed by filtering through a Buchner flask. The diethyl ether was removed by evaporation under vacuum, leaving behind an oily brown liquid. A yield of 90 mg was obtained giving a conversion of 18 % of the acid chloride to the N-acylimidazole. NMR on the oily brown liquid revealed it to be the *trans*-2-octenoic-N-acylimidazole (Personal communication Pat Steele). The NMR spectrum also showed there were still proton peaks which were derived from the solvents. During the synthesis all glassware was baked to 200 °C and all solvents were distilled, to ensure a completely non-aqueous environment was maintained. This complete removal of water is essential as water can add across the reactive *trans* double bond (Personal communication, Dr. Pat Steele).

2.2.13.4.7 Synthesis of crotonyl ACP

5 mg of ACP was dialyzed into water (containing a few mgs of ammonium bicarbonate to buffer the pH to 7.0), using Spectra/pore dialysis tubing (Spectrum) with a 3000 Da molecular cut off After dialysis the ACP was flash frozen and then freeze dried. The lyophilised ACP was resuspended in 2.5 mL of 500 mM potassium phosphate buffer pH 6.2 and then added to a reactivial and stirred vigorously. A 50 fold molar excess of crotonic anhydride was added directly into the vortex, and the reaction was allowed to proceed with continuous stirring at room temperature for 20 min. The whole of the reaction was then applied to a PD-10 (Pharmacia) desalting column and treated as per the manufactures instructions. The ACP is eluted from the column in a final volume of 3.5 mL of, this was all collected but not fractionated. The presence of crotonyl ACP was then tested by using a enoyl ACP reductase assay (Slabas et al., 1986).

2.2.14 Assay Methods

2.2.14.1 β-hydroxyacyl ACP dehydratase assay

β-hydroxyacyl ACP dehydratse assays were set up containing either 50 μM (1.5 μg) of purified (2*E*)-tetradecenoyl ACP or 100 μM (3 μg) (3*R*)–hydroxymyristoyl ACP, and 1mg/mL crude bacterial extract and the final reaction volume was made up to 4 μL with 25 mM potassium phosphate buffer pH 8.0. The reactions were initiated by the addition of sonicated bacterial crude extract and were incubated at 30 °C for 15 min. The reactions were terminated by the addition of 1 μL native PAGE loading buffer, and the samples immediately loaded onto a 2.5 M urea confirmational gel.

2.2.14.2 β-Hydroxyacyl ACP dehydratase assay using crotonyl ACP

30 μL of 25 mM potassium phosphate buffer pH 8.0, and 20 μL of bacterial crude sonicated extract were placed in to a 100 μL quartz cuvette. The absorbance at 263 nm was taken and the baseline zeroed. To the cuvette 50 μL of crotonyl ACP was added. The mixture was mixed by gently tapping the cuvette on the side and was immediately place back into the spectrophotometer. The decrease in optical density at 263 nm was measured.

2.2.14.3 Enoyl ACP reductase assay

Enoyl ACP reductase assays were performed using the method described by Slabas *et al.*, (Slabas *et al.*, 1986) and recombinant enoyl ACP reductase (kindly provided by Dr. Tony Fawcett). The following was added to a 100 µL quartz cuvette 1.2 mM crotonyl CoA, and 88 µL of 10 mM potassium phosphate buffer pH 6.2. The cuvette was then placed into a spectrophotometer set to measure the absorbance at 340 nm, and the baseline zeroed. To this was added 1.4 mM NADH and the reaction was initiated by the addition of 1 µL of purified recombinant enoyl ACP reductase. The assay mixture was mixed by gently tapping on the side of the cuvette to remove any air bubbles and was then immediately replaced in the spectrophotometer.

2.2.14.4 ACP assay

The assay system was that described by Majerus *et al.* (Majerus *et al.*, 1969). This assay system utilizes the first two enzymatic steps of *E. coli* fatty acid synthetase Fraction A a washed 70 % ammonium sulphate precipitate of *E. coli* crude extract is a rich source of fatty acid biosynthesis enzymes and is essentially ACP free. When ACP is added to the fraction A along with substrates malonyl CoA and hexanoyl CoA fatty acid biosynthesis is stimulated. If the reaction is allowed to reach equilibrium then the carbon dioxide generated by the condensation reaction is incorporated into malonyl ACP. Radiolabeled CO_2 in the form of bicarbonate is also added to the reaction cocktail and the radiolabel is incorporated into malonyl ACP, which is acid precipitable. The amount of CO_2 incorporated into malonyl ACP is proportional to the amount of ACP used to stimulate the reaction). Fraction A was kindly providied by Miss Sian Bithell, and was desalted to remove ammonium sulphate using a PD-10 column (Pharmacia) as per the manufactures instructions. A reaction cocktail containing 2 mM hexanoyl COA. 9mM malonyl CoA, fraction (an amount which fixes 500-1000 cpm, without the addition of ACP), 250 mM $KH[^{14}CO_2]0_3$ (specific activity 0.2 µCi/µmole). The reaction is initiated by the addition of reduced ACP, the reaction cocktail was then incubated at 30 °C for 15 min. The reaction was terminated by the addition of 10 % perchloric acid. The reaction were then left to stand for 30 min, to allow the precipitation of proteinacous material and the complete breakdown of the bicarbonate to carbon dioxide. The

supernatant is removed and the pellet vacuum dried at 70 °C. The pellet was resuspended in 500 μL of water, mixed with scintallant and counted.

2.2.14.5 Acyl ACP synthetase assay

Acyl ACP synthetase assay was performed as described in the [^{3}H] palmitoyl ACP synthesis section. With the following exceptions after the synthesis reaction was complete 100 μL of the synthesis reaction was added to 2 mL of stop solution (5 mM KPO_4 pH 7.2, 0.2 mg/mL BSA, 10 mM $MgCl_2$ and 50 % isopropanol). The unincorporated fatty acids are removed from the aqueous layer by extracting three times with petroleum ether, saturated with 50 % isopropanol, containing 1 mg/mL palmitate to help with the partitioning, then 1 mL of the aqueous phase is then counted.

Chapter 3

Cloning of a putative β-hydroxyacyl ACP dehydratase from *Brassica napus*.

3.1 Introduction

Type II fatty acid synthetases have been well studied in plants and this has led to the isolation of cDNA clones for all of the component enzymes with the exception of β-hydroxyacyl ACP dehydratase (Harwood, 1996). The major aim of the project is to clone a cDNA for β-hydroxyacyl ACP dehydratase from a plant source. The isolation of the cDNA for β-hydroxyacyl ACP dehydratase should allow the overexpression of the recombinant protein in *E. coli*. This would produce a supply of recombinant protein that may be used for reconstitution of the *Brassica napus* type II fatty acid synthetase *in vitro*. The purified recombinant protein can also be used as a source of material for crystallization trials as the 3D structure of β-hydroxyacyl ACP dehydratase has yet to be solved. This is an approach that has been successfully employed to solve the 3D structure of enoyl ACP reductase from *Brassica napus* (Rafferty *et al.*, 1994).

There are a number of potential strategies available to clone a cDNA for β-hydroxyacyl ACP dehydratase. A classical biochemical approach would involve purification of the enzyme to homogeneity followed by N-terminally sequencing the purified protein. This amino acid sequence information could be used to deduce possible nucleotide information that can form the basis of a strategy to clone a cDNA for β-hydroxyacyl ACP dehydratase. Purified β-hydroxyacyl ACP dehydratase protein could also be used as a source of material to raise an antibody, which can be used to screen an expression library.

As well as the biochemical approach there is a potential genetic approach for cloning a cDNA for β-hydroxyacyl ACP dehydratase. Complementation of a bacterial mutant with a plant cDNA library may identify a cDNA clone that can functionally complement for the β-hydroxyacyl ACP dehydratase mutation. An alternative molecular approach would be to use regions of conserved sequence homology present in known β-hydroxyacyl ACP dehydratase as a basis for gene identification. Degenerate PCR primers could be designed to these sequences and used to amplify a region of DNA which could be used to screen a cDNA library for a full-length clone. A second molecular approach would be to exploit the growing sequence data deposited in the

expressed sequence tag (EST) and genomic databases. These databases can be interrogated with a known β-hydroxyacyl ACP dehydratase amino acid sequence to try and identify an unknown plant sequence that shows strong conserved homology to the source sequence. The identified plant sequence can be overexpressed and the recombinant protein tested for β-hydroxyacyl ACP dehydratase biological activity.

Previously β-hydroxyacyl ACP dehydratase has been purified to homogeneity from *Spinacia oleracea* (Spinach) leaves (Shimakata and Stumpf, 1982). The purified protein has a subunit size of 19 kDa and has a α_4 tetrameric solution structure as determined by gel filtration (Shimakata and Stumpf, 1982). The purified protein was not N-terminally sequenced. Spinach leaves is not ideal material to purify fatty acid synthetase enzymes from, as they are not actively synthesizing large quantities of fatty acids. The best source of material would be to choose a tissue which is actively synthesizing fatty acids for incorporation into triacylglycerides (TAG's) e.g. the mesocarp of *Persinia americana* (Avocado) fruits. Tissue actively synthesizing fatty acids act as an enriched source for fatty acid synthetase enzymes. Applying the purification procedure developed by Shimakata and Stumpf (1982) for spinach leaves to avocado mesocarp should yield homogeneous β-hydroxyacyl ACP dehydratase in sufficient quantities to facilitate N-terminal sequencing, or the use of the purified protein to raise an antibody.

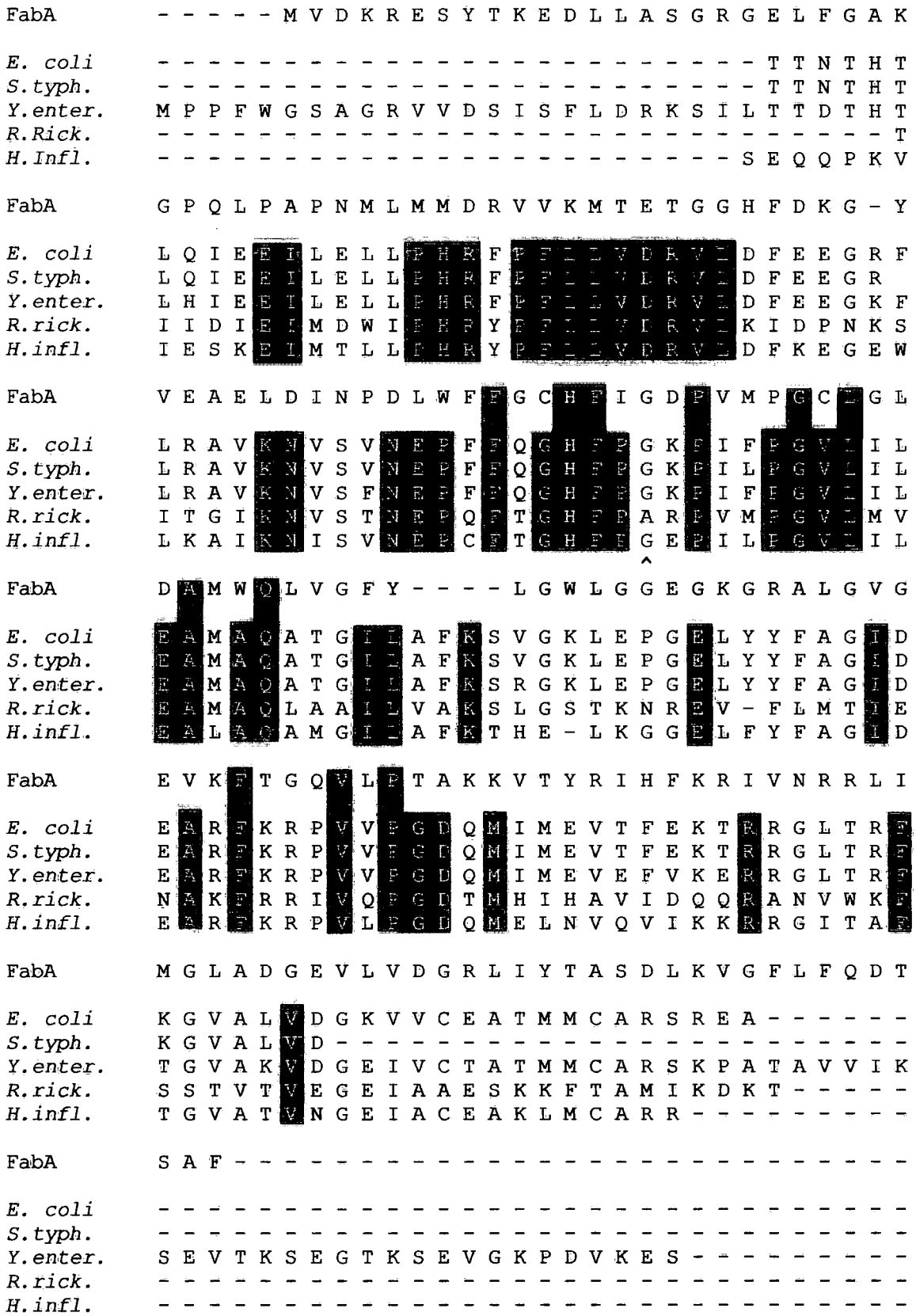
An *E. coli* gene which encodes for β-hydroxyacyl ACP dehydratase (*fabZ*) was identified as a gene that accumulated mutations that can suppress pre-existing mutations in the flanking *lpxA* gene (Mohan *et al.*, 1995). The deduced amino acid sequence from the *fabZ* open reading frame has 26 % identity and 66 % homology to the β-hydroxydecanoyl ACP thioesterase (FabA) gene. The deduced amino acid sequence encoded by *fabZ* contains a conserved histidine residue (His 53) that is present at the base of the active site cleft of FabA (Leesong *et al.*, 1996). It is this conserved histidine that has been shown to be the proton acceptor that initiates the dehydration reaction of β-hydroxydecanoyl ACP thioester dehydratase (Helmkamp *et al.*, 1968; Leesong *et al.*, 1996). The amino acid sequence derived from the *fabZ* sequence shows homology to a number of peptides from enterobacter that have been deposited in the genbank database (Mohan *et al.*, 1995) this sequence alignment can be seen in figure 1. A number of these peptides have been demonstrated to encode for β-hydroxyacyl ACP dehydratase (Servos *et al.*, 1996). The *fabZ* sequence was overexpressed in *E. coli* and the biological activity

of the recombinant enzyme was tested using β-hydroxymyristoyl ACP as the substrate. The recombinant enzyme was capable of converting the substrate (3*S*) hydroxymyristoyl ACP to (3*E*) tetradecenoyl ACP so confirming its identity as the β-hydroxymyristoyl ACP dehydratase. Recombinant FabZ has also been used as a source of material for the reconstitution of the complete *E. coli* fatty acid synthetase *in vitro* (Heath and Rock, 1996). Using this reconstituted system it has been demonstrated that the *fabZ* gene product can participate in rounds of elongation of both saturated and unsaturated fatty acids, therefore proving that *fabZ* does indeed encode for β-hydroxyacyl ACP dehydratase (Heath and Rock, 1996) of fatty acid biosynthesis. Surprisingly this reconstituted system showed that FabA was unable to participate in rounds of elongation of unsaturated fatty acids.

No null mutants for *fabZ* have been identified in *E. coli* and so for a complementation experiment to be attempted then the mutation would have to be first created. An in frame deletion of the chromosomal copy of *fabZ* could be created by cross hybridisation PCR (Ho *et al.*, 1989; Horton *et al.*, 1989; Link *et al.*, 1997). The in-frame deletion can be used to replace the endogenous *fabZ* gene by homologous recombination so preventing polar disruptions on down stream genes. Recent data suggests that FabA and FabZ have overlapping substrate specificities for saturated fatty acid biosynthesis (Heath and Rock, 1996) and it is not inconceivable that the endogenous FabA may functionally complement any *fabZ* null mutant. If this were the case then under normal growth conditions the *fabZ* null mutant would have no visible phenotype. The *fabZ* mutant would be unable to grow at low growth temperatures because of its inability to synthesize unsaturated fatty acids a phenotype that is similar to the *fabA* mutant.

The *E. coli fabZ* sequence can not be used to screen a plant cDNA library directly as the phagemid is proliferated in an *E. coli* background which would lead to significant cross hybridization with the endogenous *fabZ* sequence producing a high background. Instead the *E. coli FabZ* sequence can be used to interrogate both the EST and genomic database in an attempt to identify a homologous nucleotide sequence. Any identified nucleotide sequence can be used to heterologous screen a plant cDNA library so avoiding this problematic cross hybridization. As the number of sequences deposited in the plant DNA databases is continually increasing a continual database homology search strategy was chosen as the starting point for the potential identification of a DNA sequence encoding β-hydroxyacyl ACP dehydratase from plants.

Figure 1: Comparison of Orf17 (FabZ) against predicted amino acid sequences from several Gram-negative bacteria, and with the *fabA* gene product. Regions of global homology are boxed. The active site histidine (HIS 70) from FabA is marked with ^ (Cronan *et al.*, 1988; Schwab *et al.*, 1986). The *E. coli* FabA and FabZ sequences share 26 % identity and 66 % homology. The sequences of the Gram-negative bacteria were taken from the Swiss-Prot database. ***E. coli*** Accession No. AAC73291 (Coleman and Raetz, 1990), ***Salmonella typhimurium*** Accession No. P21773 (Hirvas *et al.*, 1990); ***Yersinia enterocolitica*** Accession No.P32205; ***Rickettsia ricketsii*** Accession No. AAA26385 (Shaw and Wood, 1994) and ***Heamophilus influenza*** Accession No. P45012 (Servos *et al.*, 1996).


FabA - - - - - M V D K R E S Y T K E D L L A S G R G E L F G A K
E. coli - T T N T H T
S.typh. - T T N T H T
Y.enter. M P P F W G S A G R V V D S I S F L D R K S I L T T D T H T
R.Rick. - T
H.Infl. - S E Q Q P K V
FabA G P Q L P A P N M L M M D R V V K M T E T G G H F D K G - Y
E. coli L Q I E E I L E L L P H R F P F L L V D R V L D F E E G R F
S.typh. L Q I E E I L E L L P H R F P F L L V L R V L D F E E G R
Y.enter. L H I E E I L E L L P H R F P F L L V L R V L D F E E G K F
R.rick. I I D I E I M D W I P H P Y P F L L V I R V L K I D P N K S
H.infl. I E S K E I M T L L P H R Y P F L L V D R V L D F K E G E W
FabA V E A E L D I N P D L W F F G C H F I G D P V M P G C L G L
E. coli L R A V K N V S V N E P F F Q G H F P G K P I F P G V L I L
S.typh. L R A V K N V S V N E P F F Q G H F P G K P I L P G V L I L
Y.enter. L R A V K N V S F N E P F F Q G H F P G K P I F P G V L I L
R.rick. I T G I K N V S T N E P Q F T G H F P A R P V M P G V L M V
H.infl. L K A I K N I S V N E P C F T G H F P G E P I L P G V L I L
FabA D A M W Q L V G F Y - - - - L G W L G G E G K G R A L G V G
E A M A Q A T G I L A F K S V G K L E P G E L Y Y F A G I D
S.typh. E A M A Q A T G I L A F K S V G K L E P G E L Y Y F A G I D
Y.enter. E A M A Q A T G I L A F K S R G K L E P G E L Y Y F A G I D
R.rick. E A M A Q L A A I L V A K S L G S T K N R E V - F L M T I E
H.infl. E A L A Q A M G I L A F K T H E - L K G G E L F Y F A G I D
FabA E V K F T G Q V L P T A K K V T Y R I H F K R I V N R R L I
E. coli E A R F K R P V V P G D Q M I M E V T F E K T R R G L T R F
S.typh. E A R F K R P V V P G D Q M I M E V T F E K T R R G L T R F
Y.enter. E A R F K R P V V P G D Q M I M E V E F V K E R R G L T R F
R.rick. N A K F R R I V Q P G D T M H I H A V I D Q Q R A N V W K F
H.infl. E A R F K R P V L P G D Q M E L N V Q V I K K R R G I T A F
FabA M G L A D G E V L V D G R L I Y T A S D L K V G F L F Q D T
E. coli K G V A L V D G K V V C E A T M M C A R S R E A - - - - - -
S.typh. K G V A L V D -
Y.enter. T G V A K V D G E I V C T A T M M C A R S K P A T A V V I K
R.rick. S S T V T V E G E I A A E S K K F T A M I K D K T - - - - -
H.infl. T G V A T V N G E I A C E A K L M C A R R - - - - - - - - -
FabA S A F -
E. coli -
S.typh. -
Y.enter. S E V T K S E G T K S E V G K P D V K E S - - - - - - - - -
R.rick. -
H.infl. -

3.2 Results

3.2.1 Identification of an EST homologous to *E. coli fabZ*

The *E. coli* FabZ amino acid sequence was used to interrogate the EST databases. A single hit from *Ricinus communis* (castor) was obtained, the identified EST showed 48 % identity and 73.3 % homology to the *E. coli* FabZ sequence (Figure, 2). The EST had been previously assigned as being closely related to a rifampicin resistance protein from the Proteobacteria *Rickettsia ricketsii* (Vandeloo *et al.*, 1995). This rifampicin resistance protein from *Rickettsia ricketsii* has since had its designation changed to a putative β-hydroxyacyl ACP dehydratase based on its homology to the *E. coli fabZ* sequence (Mohan *et al.*, 1995). The identified castor EST sequence is 512 bp in length and contains a number of N's producing indeterminable sequence at the 3' end. The deduced amino acid sequence from one open reading frame from the castor EST contains the same regions of conserved homology previously seen in the β-hydroxyacyl ACP dehydratase sequences from the Gram-negative bacteria (Figure 2).

```
E. coli FabZ      LQIEEILELLPHRFPFLLVDRLDFEE-GRFLRAVKNVSVNEPFFQGHFPG
                  + I +I E+LPHRFPFLLVDR+++   G    A+KNV++N+ FF GHFP+
T15187            MDINQIREILPHRFPFLLVDRVIEYNPGVSAVAIKNVTINDNFFPGHFPE

E. coli FabZ      KPIFPGVLI-EAMAQATGILAFK-SVGKLEPGELYYFAGIDEARFKRPVV
                   PI PGVL+ EAMAQ  G++  +  VG  +    ++FAGID+ RF++PV+
T15187            RPIMPGVLMVEAMAQVGGLV MLQPEVGGSR--DNFFFAGIDKVRFRKPVI

E. coli FabZ      PGDMIMEVTFEKTRRGLTRF
                   GD+++
T15187            XGDTLVIRITVXKLK
```

Figure 2: Identification of an EST from *Ricinus communis* that is homologous to the *E. coli* FabZ sequence. The EST database was interrogated with the *E. coli* FabZ sequence a single hit from *Ricinus communis* was identified. The castor EST showed 48.2 % identity and 73.3 % homology to the *E. coli* FabZ sequence at the amino acid level.

3.2.2 Cloning of the EST sequence from *Ricinus communis*

PCR primers SDRCDEH1 5' AAA AGC AGA AGA AAC TCC TAT TG 3' and SDRCDEH2 5' CCG TAA TTC TAA TGA CTA AGG TA 3' were designed to the castor sequence to encompass the regions of homology seen in the gram negative bacteria and avoiding the areas of undeterminable sequence (Figure, 3).

1 TAAAGACCCA **AAAGCAGAAG AAACTCCTAT TG**AATTAAAA TACCCGGCTT

51 ATCCTACTGT CATGGACATT AATCAGATTC GCGAGATTTT ACCTCACCGG

101 TTTCCATTTC TCCTTGTGGA TAGAGTGATT GAGTACAATC CTGGAGTTTC

151 AGCTGTTGCT ATCAAGAATG TCACTATCAA TGACAATTTC TTTCCTGGAC

201 ATTTCCCTGA AAGACCAATT ATGCCAGGTG TCCTCATGGT TGAGGCAATG

251 GCACAAGTTG GTGGCTTGGT CATGCTGCAA CCAGAAGTTG GAGGTTCTCG

301 TGACAATTTC TTCTTTGCTG GTATTGACAA AGTAAGATTT AGGAAGCCGG

351 TAATTNCAGG TGA**TACCTTA GTCATTAGAA TTACGGT**TNT GAAGCTCAAG

401 AACCGGTTTG GATANAAAAN ATGGAGGGAA ANTTATTTTT GGGGGTNTGT

451 AGTTTTTNNG GGGGGNTTTT TATGCCATGG GNGGTGGGAC CCNCTGGTTA

501 GNGGTGGGAC CCNCTGGTTA

Figure 3: The sequence of the castor EST (T15187). PCR primers SDRCDEH1 (5') and SDRCDEH2 (3') were designed to the sequence encompassing the regions of global homology and avoiding the regions of indeterminable sequence.

A *Ricinus communis* cDNA library was obtained from Pioneer Hybrid (Coughlan *et al.*, 1996). The library was constructed from mRNA isolated from developmental stage V castor endosperm (25 days after flowering; Greenwood and Bewley, 1981). Oil deposition in *Ricinus communis* embryos occurs immediately after stage V (Simcox *et al.*, 1978). The mRNA isolated from these embryos should be enriched for fatty acid synthetase components. A band of the correct predicted size (378 bp) was initially amplified at annealing temperatures below 55 °C, with the optimum annealing temperature being 50 °C (Figure, 4).

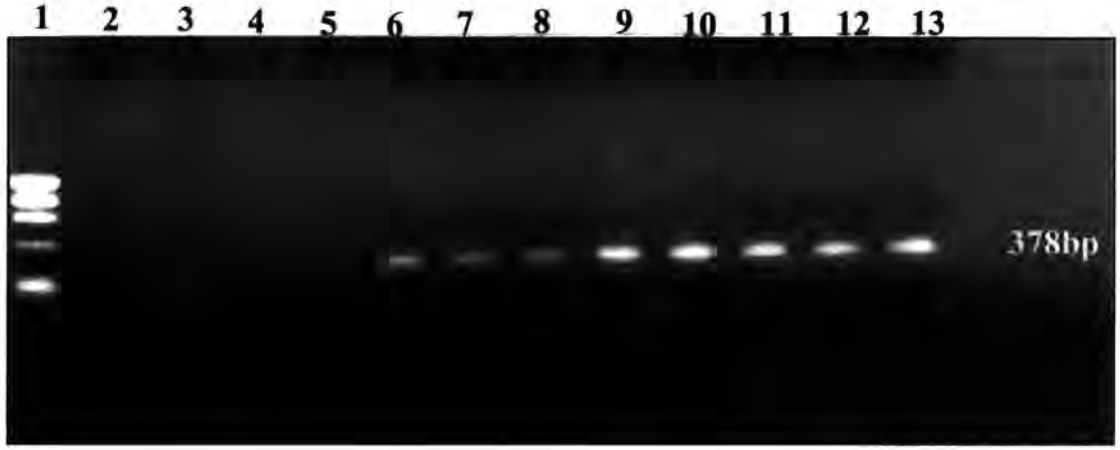


Figure 4: Temperature gradient PCR on *Ricinus communis* cDNA library to amplify the EST sequence. A temperature gradient PCR reaction was performed on the *Ricinus communis* cDNA library using primers SDRCDEH1 and SDRCDEH2. A band of 378 bp was amplified in the PCR reactions performed, with an annealing temperature of below 55 °C. **Lane 1**: ϕX174 / HaeIII markers; **Lane 2**: No Primer control; **Lane 3**: 58 °C; **Lane 4**: 57 °C; **Lane 5**: 56 °C; **Lane 6**: 55 °C; **Lane 7**: 54 °C; **Lane 8**: 53 °C; **Lane 9**: 52 °C; **Lane 10**: 51 °C; **Lane 11**: 50 °C; **Lane 12**: 49 °C; **Lane 13**: 48 °C.

The amplified EST sequence was cloned in order to prepare large quantity of the amplified EST product for use as a probe by amplifying the sequence using 55 °C as an annealing temperature. The purified PCR product was sequenced with 3.2 pmoles of primers SDRCDEH1 and SDRCDEH2. The resulting nucleotide sequence was identical to the castor EST sequence. This plasmid was designated pEST1

3.2.3 Cloning of a *Brassica napus* homolog to the castor EST

The castor PCR product was used to screen a *Brassica napus* cDNA library. 45 ng of the amplified castor EST sequence was radiolabeled using the random prime method, (Redi-prime kit; Amersham). An amplified *Brassica napus* (cv. jet neuf) developing embryo cDNA library, which had been constructed using pooled mRNA isolated from the different stages of embryogenesis and sub-cloned into pBluescript (λZapII; Stratagene) using *Eco*RI linkers and packaged into lambda phage, was obtained from Johan Kroon (Durham). Form the primary screen 37 putative positive clones were identified these were designated (SD1-37). Three of the putative positives (SD1, SD2, and SD4) were taken through to more rounds of screening until they were plaque pure. To determine the size of the cDNA inserts present in each of the purified phage, the

cDNA insert was amplified using the T3 and T7 primers present on the pBluescript phagemid (Figure 5). In all cases a band was amplified, the phage purified from clones SD1 and SD2 gave PCR products of identical size (ca. 1.5 Kb). The amplified product from clone SD4 gave an insert size of 800 bp. Both plaques taken from the two dilutions at the tertiary screen gave the same size amplified product, confirming that the phage were plaque pure. The purified protein from spinach leaves has an apparent molecular weight of 19 kDa (Shimakata and Stumpf, 1982); this would require a minimum open reading frame of 518 bp. All three isolated clones gave cDNA inserts sizes that are theoretically capable of encoding a β-hydroxyacyl ACP dehydratase open reading frame.

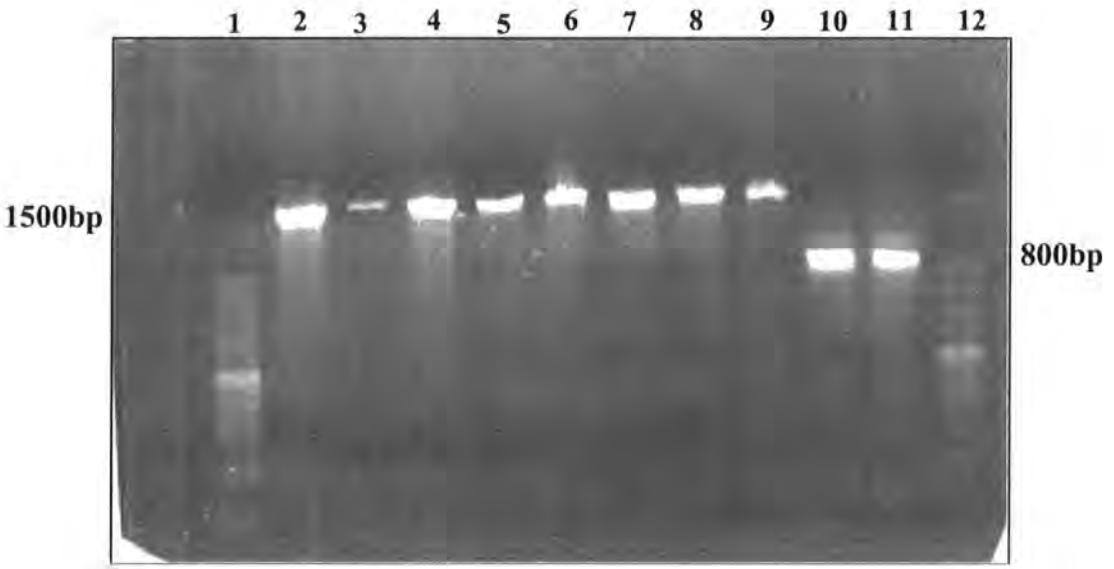


Figure 5: Size of the cDNA inserts from the purified clones SD1, SD2 and SD4 amplified from purified phage. The cDNA inserts from the purified plaques was amplified using the pBluescript phagemid primers T3 and T7. **Lane 1**: 100 bp ladder (GibCo); **Lane 2**: Clone SD1Aa; **Lane 3**: Clone SD1Ab; **Lane 4**: Clone SD1Ba; **Lane 5**: Clone SD1Bb; **Lane 6**: Clone SD2Aa; **Lane 7**: Clone SD2Ab; **Lane 8**: Clone SD2Ba; **Lane 9**: Clone SD2Bb; **Lane 10**: Clone SD4Aa; **Lane 11**: Clone SD4Ab. (A or B, denotes the two plaques picked from the secondary screen; a or b denotes the two pure plaques picked from the tertiary screen plate).

3.2.3.1 Sequencing of Clones SD1, SD2 and SD4

A single purified plaque from SD1, SD2 and SD4 was *in vivo* excised, and plasmid was prepared from the SOLR cells containing the excised phagemid using a mini-prep kit (Hybaid). The cDNA inserts of the isolated plasmids were sequenced using

the forward and reverse primers of the pBluescript phagemid. The sequence data obtained was used to interrogate the genebank database. Both the 5' and 3' sequences from clones SD1 and SD2 were identical, with the 5' sequence identifying a napin cDNA from the database. The 3' sequence showed no significant homology to the napin cDNA or anything else deposited in the database. Upon further analysis this unknown 3' region had an open reading frame which gave a deduced amino acid sequence which was homologous to the castor EST used as a probe (Data not shown). Both the 5' and 3' sequence obtained with clone SD4 was homologous to the castor EST at the nucleotide level and showed significant homology at the amino acid level from one of the predicted open reading frames. Clone SD4 is a good candidate for encoding a *Brassica napus* homolog of the castor EST sequence.

The insert size of clone SD4 is 796 bp, so the sequence strategy chosen was to sequence into the clone using the forward and reverse primers of pBluescript over a number of runs (Figure, 6). This strategy did not allow the complete sequence of both strands to be determined, and a further two sequencing primers SDSEQ1 $^{5'}$GAA CCC TGC TTT GCG TGT C$^{3'}$ and SDSEQ2 $^{5'}$AGC AAC TTC TTT GCT GG $^{3'}$ were designed to complete the sequence.

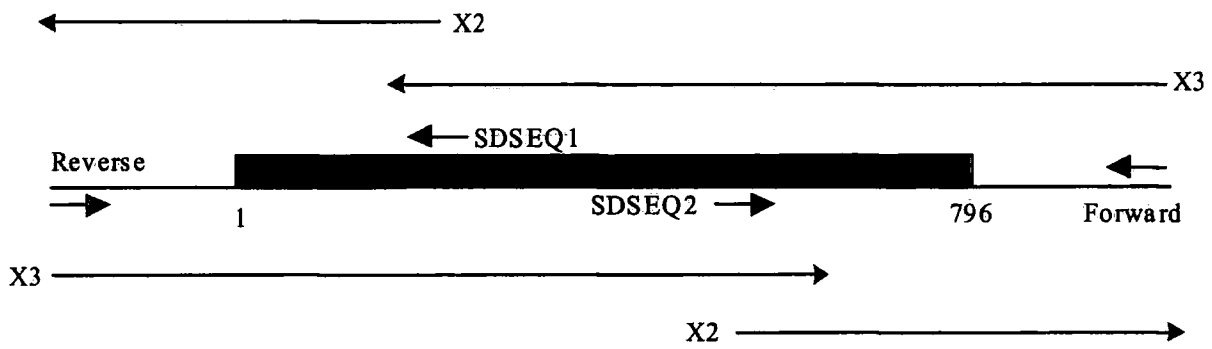


Figure 6: Sequence strategy on clone SD4. The nucleotide sequence of clone SD4 was determined by sequencing into the clone using the forward and reverse primers of pBluescript. The sequence of both the strands was completed using the internal primers SDSEQ1 and SDSEQ 2.

Figure 7: Complete sequence of the clone SD4. The complete nucleotide sequence of clone SD4, also showing the complete deduced amino acid sequence of the largest open reading frame which is shown in blue.

```
1                                                                   60
TTC GAA GTC TCC GTC CCC TTC TCT CGA GAT CAA TCG ACA ATG GCT GCC TCT AAC TCC ATT
F   E   V   S   V   P   F   S   R   D   Q   S   T   M   A   A   S   N   S   I

61                                                                  120
TTC ACC ATC TCT CCG TCG AGA AAT GTT GCA CGT ATC TCT CTT AAC CAC TCC TTA TCG CCG
F   T   I   S   P   S   R   N   V   A   R   I   S   L   N   H   S   L   S   P

121                                                                 180
CCG TTG AGT CTT CCA CTC AAC AGA TCA AGC TCT GTC GCG TTT CGT CCC AAG CCA CGA TCC
P   L   S   L   P   L   N   R   S   S   S   V   A   F   R   P   K   P   R   S

181                                                                 240
AGC TCG CTA GTC TTA TGC TCC ACC GAT GAA TCA AAG ATC ACC GCG GAG AAA GAG ATC CCA
S   S   L   V   L   C   S   T   D   E   S   K   I   T   A   E   K   E   I   P

241                                                                 300
ATA GAG CTC AGG TAC GAG GCT TTT CCG ACA GTG ATG GAC ATT AAC CAG ATA CGT GAA ATT
I   E   L   R   Y   E   A   F   P   T   V   M   D   I   N   Q   I   R   E   I

301                                                                 360
TTA CCT CAC AGG TTC CCG TTT CTG TTA GTG GAT AGA GTG ATA GAG TAC ACA CCT GGT GTA
L   P   H   R   F   P   F   L   L   V   D   R   V   I   E   Y   T   P   G   V

361                                                                 420
TGT GCT GTA GCT ATC AAA AAC GTT ACC ATT AAT GAT AAT TTC TTT CCT GGG CAT TTT CCT
C   A   V   A   I   K   N   V   T   I   N   D   N   F   F   P   G   H   F   P

421                                                                 480
GAG AGG CCC ATT ATG CCT GGA GTC CTC ATG GTT GAG GCC ATG GCT CAG GTG GGA GGT ATA
E   R   P   I   M   P   G   V   L   M   V   E   A   M   A   Q   V   G   G   I

481                                                                 540
GTG ATG CTA CAA CCA GAA GTG GGC GGA TCT AAA AGC AAC TTC TTC TTT GCT GGA ATC GAC
V   M   L   Q   P   E   V   G   G   S   K   S   N   F   F   F   A   G   I   D

541                                                                 600
AAA GTC AGA TTC AGA AAG CCT GTG ACT GCA GGT GAT ACT TTG GTG ATG AAG ATG ACG CTT
K   V   R   F   R   K   P   V   T   A   G   D   T   L   V   M   K   M   T   L

601                                                                 660
GTG AAG ATG CAG AAG AGG TTT GGG ATA GCG AAA ATG GAA GGG AAA GCA TAC GTA GGG AAC
V   K   M   Q   K   R   F   G   I   A   K   M   E   G   K   A   Y   V   G   N

661                                                                 720
ACT GTG GTA TGC GAA GGA GAA TTC TTG ATG GCT ATG GGA AAA GAA GAG TAG TGA TTA TGT
T   V   V   C   E   G   E   F   L   M   A   M   G   K   E   E   Z   Z   L   C

721                                                                 780
TCT CGT GCC TTT TGC TTT TTT TTT TTT TTT TCT TAC CCC TTG TGT TCT ACG AGT TAT CAA
S   R   A   F   C   F   F   F   F   F   S   Y   P   L   C   S   T   S   Y   Q

781                 796
ACA TTT GAT GTT TCC A
T   F   D   V   S
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```
Castor EST          ------------------------------------------KDPK--------------
Brassica napus SD4 MAASNSIFTISPSRNVARISLNHSLSPPLSLPLNRSSSVAFRPKPRSSSLVLCSTDESKI
E. coli FabZ        ------------------------------------------------------------


Castor EST          --AEETPIELKYPAYPTVMDINQIREILPHRFPFLLVDRVIEYNPGVSAVAIKNVTINDN
Brassica napus SD4 TAEKEIPIELRYEAFPTVMDINQIREILPHRFPFLLVDRVIEYTPGVCAVAIKNVTINDN
E. coli FabZ         ----------MTTNTHTLQIEEILELLPHRFPFLLVDRVLDFEEGRFLRAVKNVSVNEP
                                 : . .:::*::* *:***************:::  *    *:***::*:

Castor EST          FFPGHFPERPIMPGVLMVEAMAQVGGLVMLQPEVGGSRDN--FFFAGIDKVRFRKPVIXG
Brassica napus SD4 FFPGHFPERPIMPGVLMVEAMAQVGGIVMLQPEVGGSKSN--FFFAGIDKVRFRKPVTAG
E. coli FabZ         FFQGHFPGKPIFPGVLILEAMAQATGILAFK-SVGKLEPGELYYFAGIDEARFKRPVVPG
                    ** **** :**:****::*****. *:: :: .**  . .  ::*****:.**::**  *

Castor EST          DTLVIRT----------------------------------------
Brassica napus SD4 DTLVMKMTLVKMQKRFGIAKMEGKAYVGNTVVCEGEFLMAMGKEE
E. coli FabZ         DQMIMEVTFEKTRR--GLTRFKGVALVDGKVVCEATMMCARSREA
                    * :::.
```

Figure 8: Amino acid comparison of the *Brassica napus* clone SD4 against the *E. coli* FabZ and castor EST sequences. The deduced amino acid sequence of the putative dehydratase shows 94 % homology to the castor EST used as a probe, and 40.4 % against the *E. coli* FabZ sequence at the amino acid level.

3.2.3.2 SD4 cDNA Analysis

The SD4 cDNA plasmid contains a 796 base pair cDNA which contains an open reading frame initiating at nucleotide 39 and terminating at nucleotide 711, thereby encoding a 224 amino acid polypeptide with a predicted Mr = 24,640 Da (Figure 7). This predicted molecular weight is considerably higher than the molecular weight obtained with the purified spinach enzyme Mr = 19,000 Da (Shimakata and Stumpf, 1982). The open reading frame is 94 % identical at the amino acid level to the deduced castor EST used as the probe. The predicted amino acid sequence is also 40.4 % identical to the *E. coli* FabZ sequence (Figure 8). The predicted protein is hydrophobic. (Figure, 9), but does not contain any predicted membrane spanning domains, and so the protein *in vivo* is predicted to be a soluble protein.

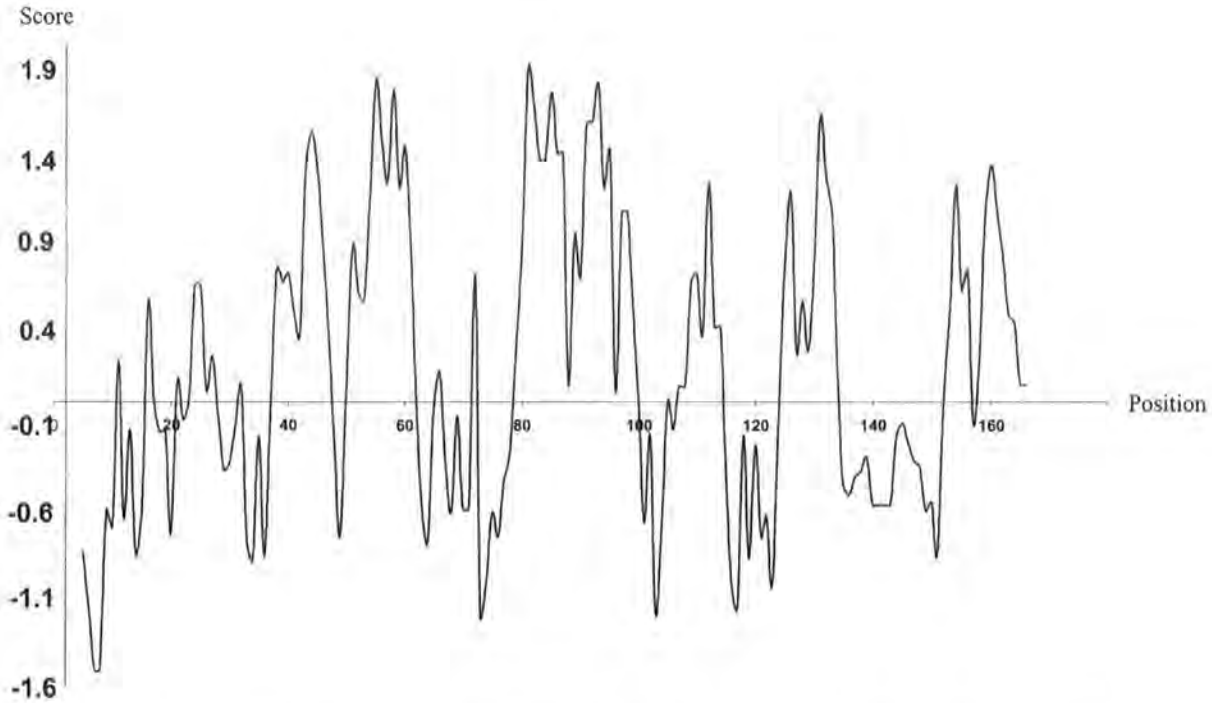


Figure 9: Hydrophobicity plot of the predicted amino acid sequence from clone SD4. A Kyte Doolittle hydrophobicity plot reveals that the protein is a hydrophobic protein. The plot also reveals that there are no predicted membrane spanning domains and so the protein is expected to be a soluble protein.

3.2.3.3 Identification of a putative chloroplast target sequence

The type II fatty acid synthetase of plants is located in the plastid, and an N-terminal extension necessary for directing the mature peptide into the chloroplast would be expected to be present. Chloroplast target sequences have been demonstrated for a number of nuclear encoded proteins, whose intracellular location has been determined to be in the plastid e.g. ACP (Safford *et al.*, 1988) and cyclophilin (Lippuner *et al.*, 1994). Chloroplast target sequences (CTS) do not conform to a conserved sequence motif, instead it is the secondary structure of the N-terminal extension that is important for directing the enzyme into the chloroplast. A series of principals have been defined by von Heijne (von Heijne, 1989) for predicting the presence of a chloroplast target sequence at the N-terminus of a translated protein. These principals are a series of guidelines for the identification of putative CTS, but not every CTS conforms completely to all of the guidelines. Using principals the predicted processing site was identified as being located between the residues cysteine (52) and serine (53). This predicted transit peptide conforms to von Heijnes guidelines in that the region is serine/threonine rich (15 out of 52 residues), and the sequence is lacking in acidic amino acids. The predicted processing site also has an arginine (-8) residue within -10 amino acids of the putative

first residue of the mature protein. The sequence surrounding the putative processing site is a slight variation on the cut site motif V/I-X-A-C/X^A (V-X-X-C^S).

(A)

Sequence of predicted chloroplast target sequence

```
M A A S N S I F T I S P S R N V A R I S L N H S L S P P L S L P L

                        -8          ↓
N R S S S V A F R P K P R S S S L V L C S T D E S K I T A E K E I P I E L R Y E

A F P T V M D
```

(B)

Acid base plot on predicted SD4 protein

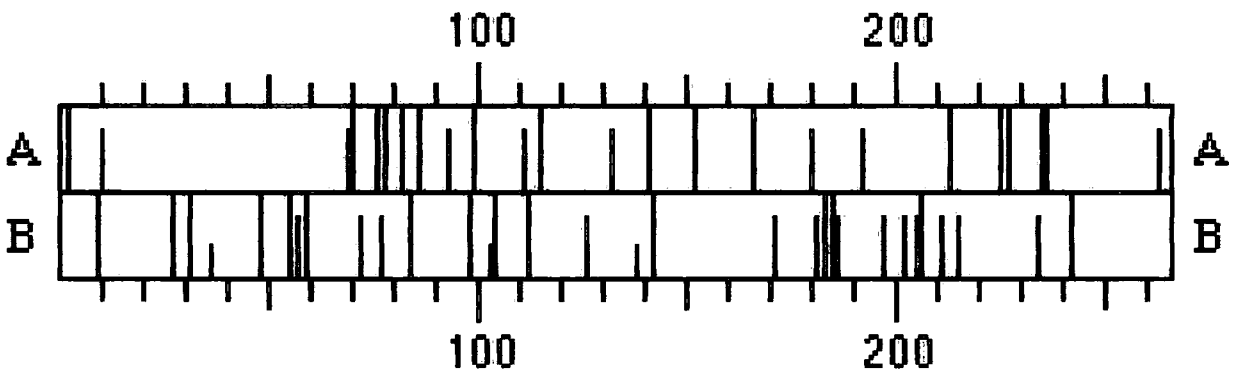


Figure 10: Identification of a putative chloroplast target sequence at the N-terminus of SD4. (A) The N-terminal region of the clone SD4 conforms to a number of principals defined by von Heinje (von Heinje, 1989) for the identification of chloroplast target sequences. The predicted region is serine/threonine rich (Shown in pink). There is also an arginine residue within 10 residues upstream of the putative processing site (shown in magenta). The predicted processing site is marked with a ↓. **(B)** von Heinjes principals also predict that the chloroplast target sequence lacks any acidic amino residues. An acid base plot of the clone SD4 shows a lack of acidic residues present in the first 70 residues of the predicted SD4 protein.

3.2.4 Southern blot on *Brassica napus* genomic DNA

Genomic DNA was extracted from carbohydrate depleted rape leaves by the method of Dellaporter (Dellaporter *et al.*, 1983). From the Southern blot (Figure 11) we

can see that both the *Bam*HI and *Eco*RI digested genomic DNA contain multiple hybridizing bands. There are five strongly hybridizing bands present in the *Eco*RI digested DNA. While there are three strongly hybridizing bands present in the *Bam*HI digest. Two high molecular weight hybridizing bands can be seen in the *Hind*III genomic DNA tract. As the complete sequence of the SD4 genomic clone is unknown from *Brassica napus* then direct copy number from the Southern cannot be determined. But the size of the hybridizing bands present in all three restriction digestions (all greater than 3 Kb) suggests that there are multiple copies of SD4 present in the *Brassca napus* (cv. Jet neuf) genome

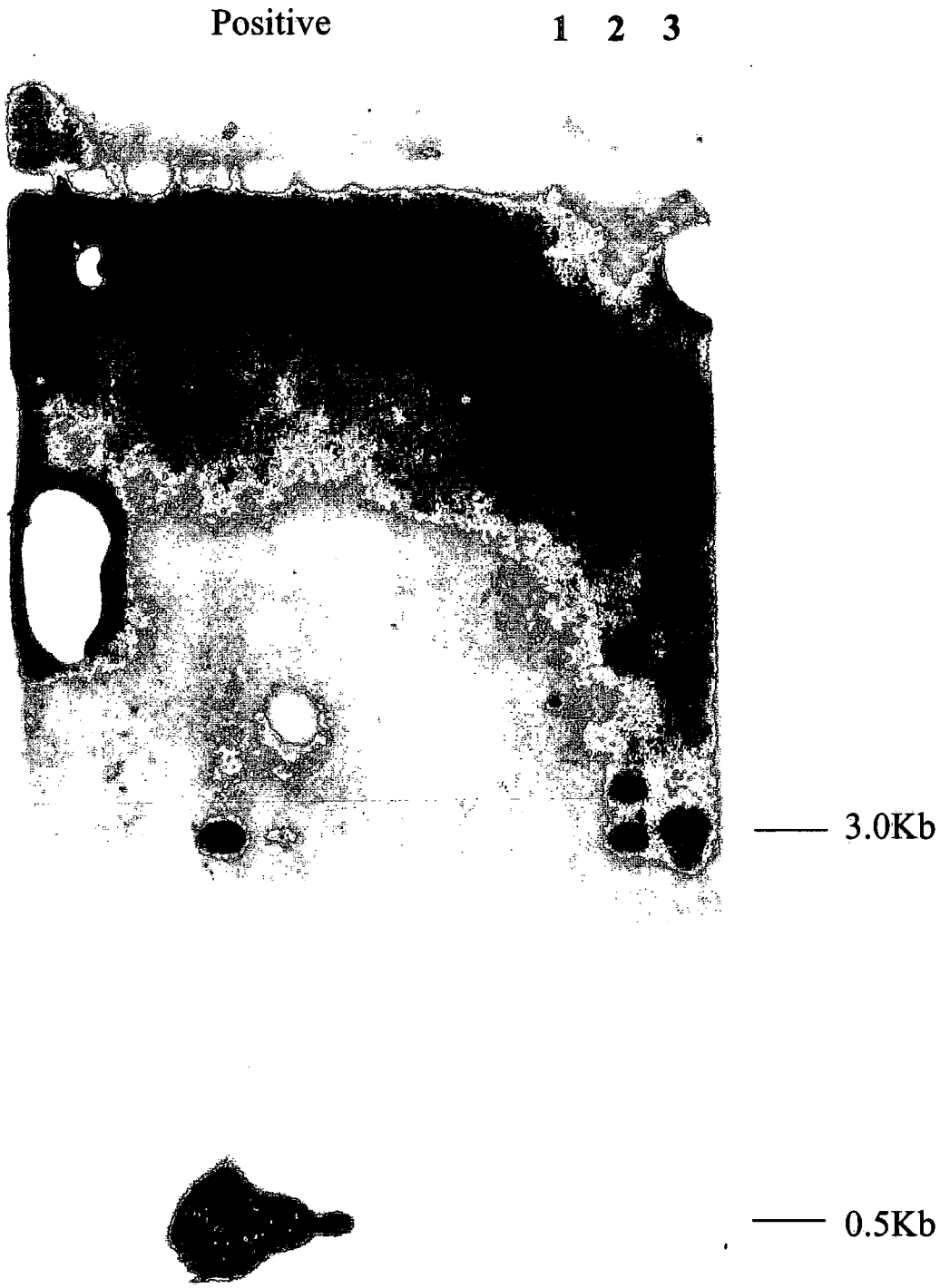


Figure 11: Southern blot on *Brassica napus* (cv. jet neuf) genomic DNA. Genomic DNA from *Brassica napus* was digested with *Hind* III, *Eco*RI and *Bam*HI, The DNA was probed with an *Eco*RI digest of SD4. A serial dilution of the *Eco*RI digest was also loaded onto the gel as a positive control. **Positive:** Serial dilution of an *Eco*RI digest of pSD4; **(1)** *Hind*III; **(2)** *Bam*HI; **(3)** *Eco*RI.

3.2.5 Cloning of paralogs to SD4

The *Brassica napus* rape embryo cDNA library contains a large number of clones which are incomplete and also two or more cDNAs fused together (Personal communication, Johan Kroon), and a number of fused clones were isolated in the initial screen. To narrow down the number of plaques to that have to be purified, clones containing the coding sequence for the mature SD4 protein were identified by PCR.

3.2.5.1 Identification of clones containing the mature SD4 sequence by PCR

PCR primers SDBN1 $^{5'}$GCG CCA TAT GTC CAC CGA TGA ATC AAA AGA TC$^{3'}$ and SDBN2 $^{5'}$GCG CGT CGA CGG CAC GAG AAC ATA ATC ACT A$^{3'}$ were designed to the coding sequence of the predicted mature SD4 protein (Figure 7). Clones SD6, SD9, SD10, SD13, SD14, SD16, SD18, SD20, SD28, SD30, SD32, SD34, SD35, SD36 and SD37 all amplified a product which is consistent as coding for the mature SD4 protein (Figure 12).

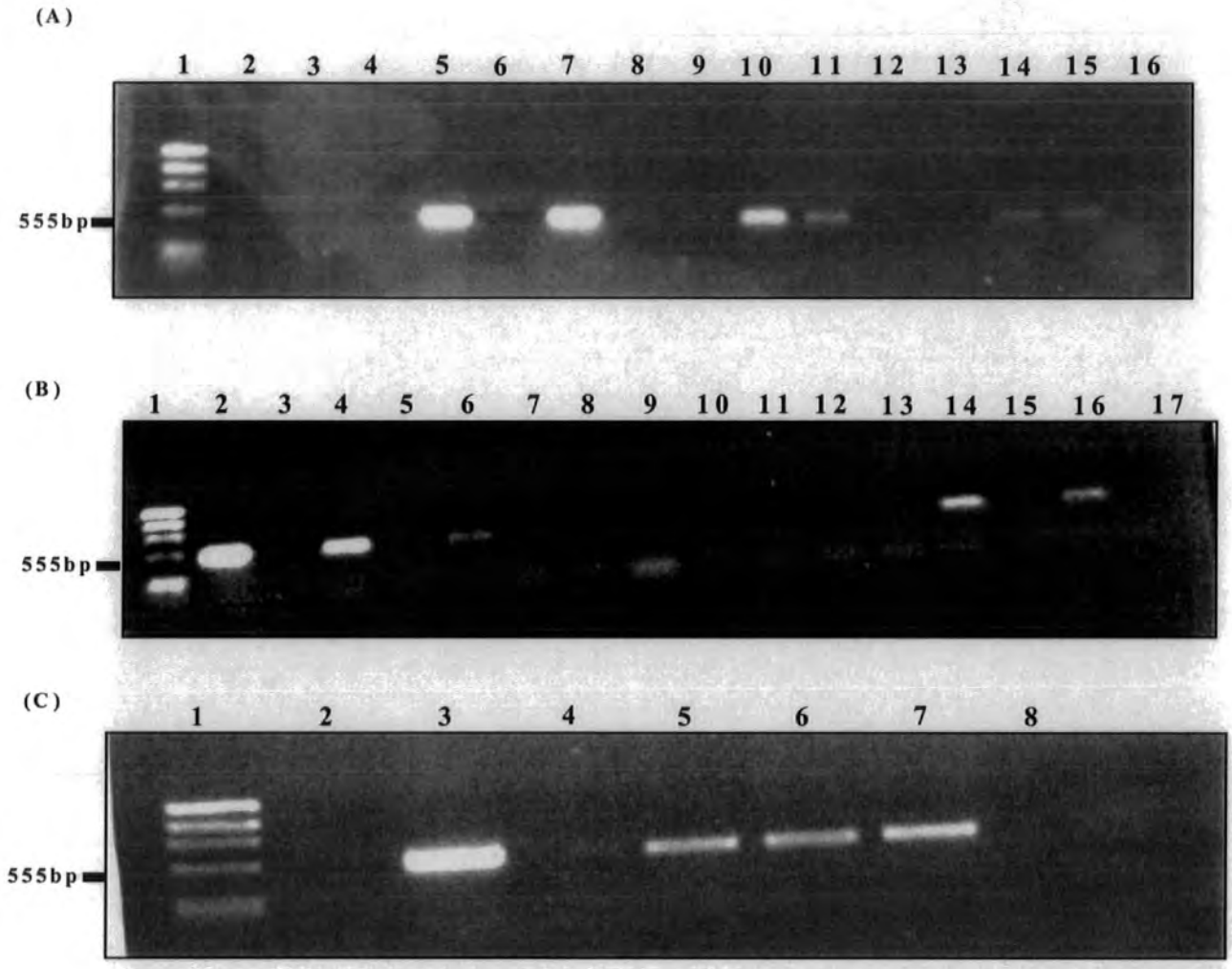


Figure 12: Identification of clones that contain the mature SD4 cDNA coding for the predicted mature protein by PCR. The sequence which encodes the mature SD4 protein can be seen amplified in clones SD4 (Positive control), SD6, SD9, SD10, SD13, SD14, SD16, SD18, SD20, SD28, SD30, SD32, SD34, SD35, SD36 and SD37. **(A) Lane 1** ϕX174 / HaeIII; **Lane 2**: SD1; **Lane 3**: SD2; **Lane 4**: SD3; **Lane 5**: SD4; **Lane 6**: SD5; **Lane 7**: SD6; **Lane 8**: SD7; **Lane 9**: SD8; **Lane 10**: SD9; **Lane 11**: SD10; **Lane 12**: SD11; **Lane 13**: SD12; **Lane 14**: SD13; **Lane 15**: SD14; **Lane 16**: SD15. **(B) Lane 1**: ϕX174/HaeIII; **Lane 2**: SD16; **Lane 3**: SD17; **Lane 4**: SD18; **Lane 5**: SD19; **Lane 6**: SD20; **Lane 7**: SD21; **Lane 8**: SD22; **Lane 9**: SD23; **Lane 10**: SD24; **Lane 11**: SD25; **Lane 12**: SD26; **Lane 13**: SD27; **Lane 14**: SD28; **Lane 15**: SD29; **Lane 16**: SD30; **Lane 17**: SD31. **(C) Lane 1** ϕX174/HaeIII; **Lane 2**: SD32; **Lane 3**: SD33; **Lane 4**: SD34; **Lane 5**: SD35; **Lane 6**: SD36; **Lane 7**: SD37; **Lane 8**: Negative (no phage).

3.2.5.2 Purification of potential paralogs of SD4 from *Brassica napus*

Clones SD6, SD9, SD10, SD12, SD13, SD16, SD18 and SD20, which represent all the putative clones obtained from screening 150,000 pfu, were purified and excised as previously described. Plasmid was prepared from the SOLR cells using a midi-prep kit (Hybaid). The cDNA inserts of the isolated plasmids were sequenced using the forward and reverse primers of pBluescript. The sequence data obtained was compared to the sequence of SD4. Clones SD12 and SD16 gave sequences that had a longer 5' and 3' untranslated regions (UTRs) and also gave similar sequences to SD4 but not identical sequences. The remaining clones were either fused cDNAs (SD10 and SD18; with ribosomal RNA) shorter than SD4 (SD6), or did not give significant sequence differences to SD4, SD12 or SD16 (SD13, SD9, SD20). Clones SD12 and SD16 were identified as good candidates for encoding paralogs of SD4. The sequence strategy chosen was identical to that used for SD4, with the exception that no sequence data was obtained with the primer SDSEQ1 from either clones SD12 or SD16. The sequence of the primer SDSEQ1 contains a difference in the last nucleotide at the 3' end (C in SD4 to A in SD12 and SD16), preventing binding of the primer. A third sequencing primer SDSEQ3 $^{5'}$ GAA ACG GGA ACC TGT GGA GG $^{3'}$ was designed to a region which was homologous in all three clones (Figures 13). The complete sequences of SD12 and SD16 when compared to SD4 shows that the cDNAs fall into two distinctive groups. SD12 and SD16 are closely related and can be assigned to one group (type A), while SD4 is distinct from group A, and form a second group (type B). These differences are more distinct when the amino acid sequences are compared, as changes in the protein sequence are less tolerated than they are in the nucleotide sequence (Figure 14). This is analogous to the two-reductase components of fatty acid synthetase from *Brassica napus*. Both the enoyl ACP reductase and the β-ketoacyl ACP reductase are both coded for by a multigene family, whose members can be assigned into two distinct groups. These two groups arise from the hybrid nature of *Brassica napus*. *Brassica napus* was generated as a cross between *Brassica oleracea* and *Brassica rapa* and each of the families is inherited from one of these two progenitors (Personal communication Dr. Fraser McDonald).

```
Clone SD16    TCGTCCCTTCTTCTTCTTCTTC-TCTCTCTCTCTCTCTCTCTCGATCGGCAATGTCTGCC
Clone SD12    -------TCCTTCGAAATCTCCGTCCCTTCTTCTCTCTCTGTCGATCGGCAATGGCTGCC
Clone SD4     ----------TTCGAAGTCTCC--GTCCCCTTCTCTCGAGATCAATCGACAATGGCTGCC
                        ***    *** *    *    ******    ** **** ***** *****

Clone SD16    CCCAACTCCATTTTCACCACCGCTCCGTCGCGAAATGTCGCACCTCTTTCTCTTCACCAG
Clone SD12    CCCAACTCCATTTTCACCACCGCTCCGTCGAGAAATGTCGCACCTATCTCTCTTCACCAG
Clone SD4     TCTAACTCCATTTTCACCATCTCTCCGTCGAGAAATGTTGCACGTATCTCTCTTAACCAC
               * ***************** * ******** ******* **** * * ****** ****

Clone SD16    TCATTATCTTCACCGTTGAGTCCCCGGATCACTAGATCGCACTCCGTCGCGTTTCGTCCC
Clone SD12    TCATTATCTTCACCGTTGAGTCTCCGGATCACTAAATCGAACTCCGTCGCGTTTCGTCCC
Clone SD4     TCCTTATCGCCGCCGTTGAGTCTTCCACTCAACAGATCAAGCTCTGTCGCGTTTCGTCCC
              ** *****  * **********  *   ***  * ***   *** ***************

Clone SD16    AAACCCCGATCCAGCTCCCTCGTCTTCTGCTCCACCGATGAATCAAACACCGCCGCAGAG
Clone SD12    AAACCCCGATCCAGCTCGCTCGTCTTCTGCTCCACCGATGAATCAAAGATCTCCGCAGAG
Clone SD4     AAGCCACGATCCAGCTCGCTAGTCTTATGCTCCACCGATGAATCAAAGATCACCGCGGAG
              ** ** *********** ** ***** ********************* * * **** ***

Clone SD16    AAAGAGATCCCAATTGAACTCAGGTACGAGGCTTATCCGACAGTGATGGACATTAACCAG
Clone SD12    AAAGAGATCCCAATTGAACTTAGGTACGAGGCTTATCCGACAGTGATGGACATTAACCAG
Clone SD4     AAAGAGATCCCAATAGAGCTCAGGTACGAGGCTTTTCCGACAGTGATGGACATTAACCAG
              ************** ** ** ************ *************************

Clone SD16    ATACGAGAGATTTTGCCTCACAGGTTCCCGTTTCTGCTAGTGGATAGAGTGATAGAGTAC
Clone SD12    ATACGAGAGATTTTGCCTCACAGGTTCCCGTTTCTGTTAGTGGATAGAGTGATAGAGTAC
Clone SD4     ATACGTGAAATTTTACCTCACAGGTTCCCGTTTCTGTTAGTGGATAGAGTGATAGAGTAC
              ***** ** ***** ********************** ***********************

Clone SD16    ACAGCTGGTGAATCTGCTGTAGCTATCAAGAACGTGACCATTAATGACAATTTCTTTCCT
Clone SD12    ACAGCTGGTGAATCTGCGGTAGCTATCAAGAACGTTACCATTAATGACAATTTCTTTCCT
Clone SD4     ACACCTGGTGTATGTGCTGTAGCTATCAAAAACGTTACCATTAATGATAATTTCTTTCCT
              *** ****** ** *** *********** ***** *********** ************

Clone SD16    GGGCATTTCCCTGAGAGACCTATCATGCCTGGTGTCCTCATGGTTGAGGCCATGGCTCAG
Clone SD12    GGGCATTTCCCTGAGAGGCCCATTATGCCTGGTGTCCTCATGGTTGAGGCCATGGCTCAG
Clone SD4     GGGCATTTTCCTGAGAGGCCCATTATGCCTGGAGTCCTCATGGTTGAGGCCATGGCTCAG
              ******** ******** ** ** ******** ****************************

Clone SD16    GTGGGAGGTATAGTGATGCTCAATCCAGAAGTGGGCGGATCTAAAAGCAACTTCTTCTTT
Clone SD12    GTGGGAGGTATAGTGATGCTAAATCCAGAAGTGGGCGGATCTAAAAGCAACTTCTTCTTT
Clone SD4     GTGGGAGGTATAGTGATGCTACAACCAGAAGTGGGCGGATCTAAAAGCAACTTCTTCTTT
              ********************  * ************************************

Clone SD16    GCTGGAGTCGACAAAGTGAGATTCAGAAAGCCTGTGATTGCAGGTGACACTCTGGTGATG
Clone SD12    GCTGGAGTCGACAAAGTGAGATTCAGAAAGCCTGTGATTGCAGGTGACACTCTGGTGATG
Clone SD4     GCTGGAATCGACAAAGTCAGATTCAGAAAGCCTGTGACTGCAGGTGATACTTTGGTGATG
              ****** ********** ******************** ********* *** ********

Clone SD16    AGGATGACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAGGCA
Clone SD12    AGGATGACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCA
Clone SD4     AAGATGACGCTTGTGAAGATGCAGAAGAGGTTTGGGATAGCGAAAATGGAAGGGAAAGCA
              * ***************** ******** ************* ************** ***

Clone SD16    TACGTAGGGAACACTTTGGTATGCGAAGGAGAGTTCTTGATGGCTATGGGAAAAGAAGAT
Clone SD12    TACGTAGGGAACACTTTGGTATGCGAAGGAGAGTTCTTGATGGCTATGGGGAAAGAAGAG
Clone SD4     TACGTAGGGAACACTGTGGTATGCGAAGGAGAATTCTTGATGGCTATGGGAAAAGAAGAG
              *************** ***************** ****************** ********

Clone SD16    GAGTGATCATCTTCTCATGCCTTTTGCTTCTTTTTCTTCT-TTCTTACCC-TTGTGGGTA
Clone SD12    GAGTGATCATGTTCTCATGCCTTTTGCTTTTTTTT------TTCTTACCC-TTGTGGGTA
Clone SD4     TAGTGATTATGTTCTCGTGCCTTTTGCTTTTTTTTTTTTTTTCTTACCCCTTGTGTTCT
               ****** ** ***** ************ *****      ********* *****

Clone SD16    CGAGTTTATCACACATTGAGTCTCATTTCTATCTCTAGTTTCTGTTGTTTGTTCCAAATG
Clone SD12    CGAGTTTATCACACATTGAGTCTCATTTCTATCTCTAGTTTCTGTTGTTTGTTCCAAATG
```

```
Clone SD4     ACGAGTTATCAAACATTTGAT---GTTTCCA----------------------------
              ****** *****   *    **** *

Clone SD16    TTGTTGACTATTTACAAGAAAATAATGAATTGGAGTTATCGCACTTTAAGTGATCAAAAA
Clone SD12    TTGTTGACTATTTACAAGAAAATAATGAATTGGAGTTATCACACAAAAAAAAAAAAAAAA
Clone SD4     ------------------------------------------------------------

Clone SD16    AAAAAAAAAAAAAAAAAA------------------------------------------
Clone SD12    AAAAA------------------------------------------------------
Clone SD4     ------------------------------------------------------------
```

Figure 13: Sequence comparison of the three isolated paralogs of SD4 from *Brassica napus*. Clones SD12 and SD16 encode paralogs of SD4. The sequences fall into two groups SD12 and SD16 into group 1 (Type A) and SD4 into group 2 (Type B). The * indicates nucleotides which are conserved in all three isolated clones. Residues colored in red indicate residues which are conserved in two of the sequences, while those nucleotides highlighted in cyan are different in all three sequences.

```
Clone 12      MAAPNSIFTTAPSRNLAPISLHQSLSSPLSLRITKSNSVAFRPKPRSSSLVFC
Clone 16      MSAPNSIFTTAPSRNLAPLSLHQSLSSPLSPRITRSHSVAFRPKPRSSSLVFC
Clone 4       MAASNSIFTISPSRNVARISLNHSLSPPLSLPLNRSSSVAFRPKPRSSSLVLC
              *.* ***** .****.* .**..*** ***  .. ********************
              ↓
Clone 12      STDESKISAEKEIPIELRYEAYPTVMDINQIREILPHRFPFLLVDRVIEYTA
Clone 16      STDESNTAAEKEIPIELRYEAYPTVMDINQIREILPHRFPFLLVDRVIEYTA
Clone 4       STDESKITAEKEIPIELRYEAFPTVMDINQIREILPHRFPFLLVDRVIEYTP
              *****  .*************.*****************************

Clone 12      GESAVAIKNVTINDNFFPGHFPERPIMPGVLMVEAMAQVGGIV MLNPEVGGS
Clone 16      GESAVAIKNVTINDNFFPGHFPERPIMPGVLMVEAMAQVGGIV MLNPEVGGS
Clone 4       GVCAVAIKNVTINDNFFPGHFPERPIMPGVLMVEAMAQVGGIV MLQPEVGGS
              * .****************************************.******

Clone 12      KSNFFFAGVDKVRFRKPVIAGDTLVMRMTLVKLQKRFGIAKMEGKAYVGNTL
Clone 16      KSNFFFAGVDKVRFRKPVIAGDTLVMRMTLVKLQKRFGIAKMEGKAYVGNTL
Clone 4       KSNFFFAGIDKVRFRKPVIAGDTLVMKMTLVKMQKRFGIAKMEGKAYVGNTV
              ********.*********.*******.*****.******************.

Clone 12      VCEGEFLMAMGKEEEZ
Clone 16      VCEGEFLMAMGKEDEZ
Clone 4       VCEGEFLMAMGKEEZZ
              *************. *
```

Figure 14: Amino acid of the three identified paralogs of SD4 from *Brassica napus*. The amino acid sequences confirm that the sequences fall into two distinctive groups type A (SD12 and SD16) and type B (SD4). Residues which are conserved in all three clones are marked with a *. Residues that are conserved in two of the clones are colored red, residues which are different in all three sequences are colored in cyan.

3.2.5.3 Identification of potential paralogs to SD4 from *Brassica napus*

Both β-ketoacyl ACP reductase and enoyl ACP reductase from *Brassica napus* (Personal communication Dr. Fraser McDonal) are encoded for by multigene families.

So far from the original library screen we have identified three clones that encode for the SD4 protein. All of the isolated phage has not been purified and some of the remaining phage may contain further paralogs of SD4. The phage SD28, SD30, SD32, SD34, SD36 and SD37 have been shown to contain the nucleotide sequence for the mature SD4 protein by PCR. Some of the phage may contain further paralogs of SD4. The 3' untranslated regions of the three parlalogs so far identified show the greatest sequence variability, and so the 3' UTR of the clones SD28, SD30, SD32, SD34, SD36 and SD37 should allow us to assign each of the clones to either type A or B.

The 3' UTR regions of clones SD28, SD30, SD32, SD34, SD36 and SD37 were amplified from the primary phage plugs. The primer SDSEQ2 that had for sequencing on the other isolated clones is present within the SD4 coding sequences but faces into the 3' untranslated region. The SDSEQ2 primer was used in conjunction with either T3 or T7 primes present in the pBluescript phagemid as the original cDNA inserts were not directionality cloned (Figure 15). The purified product was sequenced using the forward and reverse primers of pBluescript, the resulting sequence was compared to a type A and type B cDNA, with the exception of clone SD37, as no sequence data was obtained. The sequences prior to the translation termination codon, allows the assignment of clones SD28, SD30, SD34, SD36 and SD37 to type A, while clone SD32 belongs to type B (Figure 16). The sequence of the 3'UTR was insufficient for the assignment of SD32 as a second member of type B, or the identification of SD32 as a longer version of SD4.

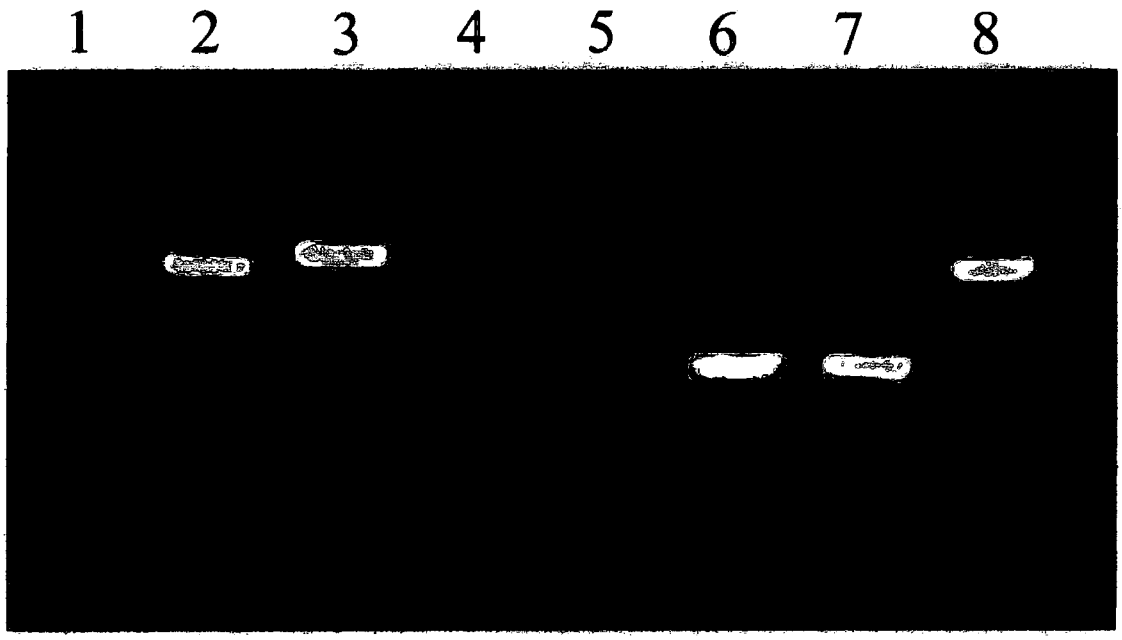


Figure 15: PCR on the 3' untranslated regions of putative SD4 paralogs. The 3' UTR of the clones SD28, SD30, SD32, SD33, SD35, SD36 and SD37 were amplified using primers SDSEQ2 and either the T3 and T7 primers from the pBluescript phagemid. **Lane 1**: ϕX174 / HaeIII markers; **Lane 2**: SD28/T7; **Lane 3**: SD30/T7; **Lane 4**: SD32/T3; **Lane 5**: SD33/T3; **Lane 6**: SD35/T3; **Lane 7**: SD36/T7; **Lane 8**: SD37/T7.

```
Clone SD16      TCGACAAAGTGAGATTCAGAAAGCCTGTG-ATTGCAGGTGACACTCTGGTGATGAGGATG
Clone SD4       TCGACAAAGTCAGATTCAGAAAGCCTGTG-ACTGCAGGTGATACTTTGGTGATGAAGATG

Clone SD28      TCGACAAAGTGAGATTCAGAAAGCCTGTG-ATTGCAGGTGACACTCTGGTGATGAGGATG
Clone SD30      TCGACAAAGTGAGAT-CAGAAAGCCTGTG-ATTGCAGGTGACACTCTGGTGATGAGGATG
Clone SD32      TCGACAAAGTCAGATTCAGAAAGCCTGTG-ACTGCAGGTGATACTTTGGTGATGAAGATG
Clone SD33      TCGACAAAGTGAGATTCAGAAAGCCTGTG-ATTGCAGGTGACACTCTGGTGATGAGGATG
Clone SD35      TCGACAAAGTGAGATTCAGAAAGCCTGTG-ATTGCAGGTGACACTCTGGTGATGAGGATG
Clone SD36      TCGACAAAGTGAGATTCAGAAAGCCTGTGGATTGCAGGTGACACTCTGGTGATGAGGATG
                ********** **** ************ * ********* *** ********* ****

Clone SD12      ACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCATACGTA
Clone SD4       ACGCTTGTGAAGATGCAGAAGAGGTTTGGGATAGCGAAAATGGAAGGGAAAGCATACGTA

Clone SD28      ACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCATACGT-
Clone SD30      ACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCATACGT-
Clone SD32      ACGCTTGTGAAGATGCAGAAGAGGTTTGGGATAGCGAAAATGGAAGGGAAAGCATACGT-
Clone SD33      ACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCATACGT-
Clone SD35      ACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCATACGT-
Clone 36        ACGCTTGTGAAGCTGCAGAAGCGGTTTGGGATAGCCAAAATGGAAGGGAAAGCATACGT-
                ************ ******** ************ ***********************

Clone SD12      GGGAACACTTTGGTATGCGAAGGAGAGTTCTTGATGGCTATGGGGAAAGAAGAGGAGTGA
Clone SD4       GGGAACACTGTGGTATGCGAAGGAGAATTCTTGATGGCTATGGGAAAAGAAGAGTAGTGA

Clone SD28      GGGAACACTTTGGTATGCGAAGGAAA-TTCTTGATGGCTATGGGGAAAGAAGAG-AGTGA
Clone SD30      GGGAACACTTTGGTATGCGAAGGAAAGTTCTTGATGGCTATGGGGAAAGAAGAGGAGTGA
Clone SD32      GGGAACACTGTGGTATGCGAAGGAAAATTCTTGATGGCTATGGGAAAAGAAATA--GTGA
Clone SD33      GGGAACACTTTGGTATGCGAAGGAA--TTCTTGATGGCTATGGGGAAAGAAGAG-AGTGA
Clone SD35      GGGAACACTTTGGTATGCGAAGGAA--TTCTTGATGGCTATGGGGAAAGAAGAG-AGTGA
Clone SD36      GGGAACACTTTGGTATGCGAAGGAAAATTCTTGATGGCTATGGGGAAAGAAAAG-AGTGA
                ********* **************   ***************** ******     ****

Clone SD12      TCATGTTCTCATGCCTTTTGCTTTTTTTTTTCTTACCCTTGTGGGTACGAGTTTATCACA
Clone SD4       TTATGTTCTCGTGCCTTTTGCTTTTTTTTTTTTTTTCTTACCCCTTGTGTTCTACGAGT

Clone SD28      TCATGTTCTCATGCCTTTTGCTTTTTTTTTCCTTACCCTTGTGGGTCAATTTTATC-C-C
Clone SD30      TCATGTTCTCATGCCTTTTGCTTTTTTTTTCCTTACCCTTGTGGGTCAATTTTATC-C-C
Clone SD32      TTATGTTCTCGTGCCTTTTGCTTTTTTTTTTATTTTTTTTTTTT--TTAATTTTTTT----
Clone SD33      TCATGTTCTCATGCCTTTTGCTTTTTTTTTCCTTACCCTTGTGGGTCAATTTTATC-CAC
Clone SD35      TCATGTTCTCATGCCTTTTGCTTTTTTTTTCCTTACCCTTGTGGGTCAATTTTATC-CAC
Clone SD36      TCATGTTCTCATGCCTTTTGCTTTTTTTTTC-TTACCCTTGTGGGTCAATTTTATCACAC
                * ******** ******************  **    **     *     *

Clone SD12      CATTGAGTCTCATTTCTATCTCTAGTTTCTGTTGTTTGTTCCAAATGTTGTTGACTATTT
Clone SD4       TATCAAACAT--TTGATGTTTCCA-----------------------------------

Clone SD28      ATTGATTCCCATTCTACCTATT-CTGTTGTTTGT-CCAAATTTTT--ACT-TTTACAAAA
Clone SD30      ATTGATTCCCATTCTACCTATT-CTGTTGTTTGT-CCAAATTTTT--ACT-TTTACAAAA
Clone SD32      --TTTTTTTTTCCCACCCAAAAGGGGGCCCTCGGGCGGGCCCCCC------CCCCCTAAA
Clone SD33      ATTGATTCCCATTCTACCTATT-CTGTTGTTTGT-CCAAATTTTT--ACTATTTACAAAA
Clone SD35      ATTGATTCCCATTCTACCTATT-CTGTTGTTTGT-CCAAATTTTT--ACTATTTACAAAA
Clone SD36      ATTGATCCATTTCTATCCTATTTCTGTTGTTTGTTCCAAATTTGTTGACTATTTACAAAA
                  *

Clone SD12      ACAAGAAAATAATGAATTGGAGTTATCACACAAAAAAAAAAAAAAAAAAAAA--------
Clone SD4       ------------------------------------------------------------

Clone SD28      AAATAATGAATTGGATTTCCCCTTTAATTACCTTA-------------------------
Clone SD30      AAATAATGAATTGGATTTCCCCCTTTAATTATCCAAAAAAAAAAAAAAAAAAA-------
Clone SD32      A-----------------------------------------------------------
Clone SD33      AAATAATGAATTGGATTTTCCCAAAAAAAAAAAAAAAA----------------------
```

```
Clone SD35      AAATAATGAATTGGATTTTCCCAAAAAAAAAAAAAAAA----------
Clone SD36      AAATAATGAATTGGATTTCCCTTTAATATCATAAAAAAAAAA----------
```

Figure 16: Assignment of remainding clones to type A or B. Clones SD28, SD30, SD32, SD33, SD35, SD36 are type A cDNA's, while SD32 is a type B cDNA. The differences from the type A and B clones are highlighted in red.

3.3 Discussion

β-hydroxyacyl ACP dehydratase is a fundamental enzyme involved in *de novo* fatty acid biosynthesis. No cDNA encoding this enzyme has been cloned from any plant source. In this chapter the cloning of a putative β-hydroxyacyl ACP dehydratase from *Brassica napus* is described. The putative clone was isolated by screening a *Brassica napus* developing embryo cDNA library with a PCR product from *Ricinus communis* which shows strong homology to the *E. coli* FabZ sequence at the amino acid level (Vandeoo *et al.*, 1995). The *E. coli fabZ* locus has been shown to encode for an enzyme that is capable of dehydrating β-hydroxymyristoyl ACP (Mohan *et al.*, 1995). The *E. coli* FabZ can also be functionally reconstituted into an active isolated fatty acid synthetase *in vitro* (Heath and Rock, 1996). Using this reconstituted system it has been shown that the FabZ can participate in rounds of elongation of both saturated and unsaturated fatty acids. Identification of a number of homologs to FabZ present in other enterobacterial genomes identified a number of highly conserved regions present in the FabZ protein. These same regions are also conserved in both the EST from *Ricinus communis* as well as the isolated homolog from *Brassica napus*. This sequence shows that the isolated *Brassica napus* sequence (SD4) is homologous to β-hydroxyacyl ACP dehydratase from *E. coli*.

Analyzing the deduced amino acid sequence from the isolated clone provides further confirmatory evidence that this clone potentially encodes for the first β-hydroxyacyl ACP dehydratase to cloned from plants. The sequence of the deduced protein contains an N-terminal sequence which is consistent with a chloroplast target sequence (von Heinje, 1989). This suggests that the intracellular location of the mature protein is in the chloroplast, which is the intracellular location of other fatty acid synthetase enzymes (Sheldon *et al.*, 1990; Safford *et al.*, 1988). The mature protein has a predicted molecular mass of 19 kDa, which is similar in size to β-hydroxyacyl ACP dehydratase purified from *Spinacea oleracea* leaves (Shimakata and Stumpf, 1982).

During studies on the purification of acyl ACP thioesterase from *Brassica napus* embryo's, it was found that two proteins co-purified with the acyl ACP thioesterase under native conditions (Hellyer *et al.*, 1992). Both of these unknown proteins were N-terminally sequenced one of the proteins was identified as being enoyl ACP reductase. The second unknown protein showed no homology to any known protein in the database. The second unknown protein was 20kDa in size and its N-terminal sequence is identical to the predicted SD4 mature protein immediately downstream of the predicted chloroplast-processing site (Figure 17).

(A) S T D E S K I T A - K E I P

(B) S T D E S K I T A E K E I P

Figure 17: Comparison of the N-terminal amino acid sequence from previously sequenced unknown protein and the predicted N-terminus of the SD4 protein. (A) The N-terminal sequence of a unknown 20kDa protein which co-purifies with acyl ACP thioesterase and enoyl ACP reductase under native conditions. **(B)** The predicted N-terminal of the mature SD4 protein once the predicted chloroplast target sequence has been removed.

The predicted N-terminus of the mature SD4 protein is identical to the N-terminal sequence of the unknown protein that co-purifies with both enoyl ACP reductase and acyl ACP thioesterase. This suggests that the SD4 gene product closely associated with two known components of fatty acid synthetase in *Brassica napus* embryos.

Analysis of the amino acid sequence of SD4 and the castor EST sequences demonstrate that compared to the *E. coli fabZ* sequence there are a number of conserved residues. A His residue is conserved in both the isolated *Brassica napus* sequence and in both FabA and FabZ, this histidine has been shown to be the proton acceptor that initiates the reaction in FabA (Leesong *et al.*, 1996). Other residues which are conserved are

amino acids (R171, F172, P175, R192) which when mutated form mutations which are capable of suppressing mutations in lipid A biosynthesis (Mohan *et al.*, 1995; Kloser *et al.*, 1998). All this data predicts that the gene product of SD4 is a β-hydroxyacyl ACP dehydratase homolog from *Brassica napus*. Whilst the homology is strong, definitive proof would have to come from demonstrating the biological activity of the gene product.

The isolated clone SD4 is a member of a multigene family that contains at least two other members SD12 and SD16 and possibly a fourth (SD32). These isolated clones are paralogs of SD4 and can be assigned into two distinct groups. Type A that contains SD12 and SD16 and type B that contains SD4. This pattern has been previously seen with other fatty acid synthetase enzymes in *Brassica napus*, notably with the two reductases (Personal communication Dr. Fraser Mcdonald). This pattern is due to the hybrid nature of oil seed rape, *Brassica napus* was isolated as a cross between *Brassica rapa* and *Brassica oleracea*. With the each family group being inherited from one of the progenitors.

Chapter 4

Overexpression of the mature SD4 protein in *Escherichia coli*

4.1 Introduction

In the previous chapter the cloning of a cDNA encoding a putative β-hydroxyacyl ACP dehydratase from *Brassica napus* has been described. Although the evidence presented is circumstantial, definitive proof would come from demonstrating that the SD4 gene product has β-hydroxyacyl ACP dehydratase activity. A number of methods have been developed for the expression of recombinant proteins in a number of different host organisms, with the most well developed being expression in the gram negative bacterium *Escherichia coli*. Recombinant proteins can be expressed in *E. coli* using a wide variety of vectors that have been specifically designed to produce efficient overexpression, as well as providing systems for the rapid purification of the recombinant protein. To be able to assay for the SD4 protein activity then the expressed SD4 protein must retain as native structure as possible, and thus be biologically active.

The pET (Novagen) vectors have been specifically designed for the overexpression of native recombinant proteins in *E. coli* (Studier and Moffatt, 1986; Rosenberg *et al.*, 1987; Studier *et al.*, 1990). Using this system target genes are expressed under the control of strong bacteriophage T7 transcription. Expression of the recombinant protein is achieved by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is very specific for the T7 promoter and is also very active and so during induction the majority of the *E. coli* cell resources are diverted to the expression of the target gene. This can lead to the recombinant protein constituting greater than 50% of the total cellular proteins after only a few hours of induction. The nucleotide sequence encoding the desired recombinant protein is cloned into the pET vector, and the vector is transformed into a host *E. coli* strain bearing a chromosomal copy of T7 RNA polymerase. These *E. coli* hosts are lysogens of the bacteriophage DE3. DE3 is a phage lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lac* repressor (LacI). The DE3 lysogen also contains T7 RNA polymerase under the control of the *lacUV5* promoter (Studier and Moffatt, 1986). The fragment is inserted into the *int* gene that prevents integration or excision of the DE3 phage without the help of a helper phage. The *lacUV5* promoter is inducible by IPTG, and so addition of IPTG to the culture medium induces T7 polymerase that in turn

transcribes the target DNA in the pET plasmid. The recombinant protein can then be purified by a series of protein purification steps, this is easier than purifying the native protein direct from plant tissue as the recombinant protein can constitute greater than 50% of the proteins in a crude cell lysate made from the induced bacteria.

Once the recombinant SD4 protein has been overexpressed and purified then biological activity of the recombinant SD4 protein can be assayed. The recombinant SD4 protein can also be used in structural studies as the 3D structure of β-hydroxyacyl ACP dehydratase has yet to be solved. The crystal structure of the related *E. coli* protein FabA has been solved (Leesong *et al.*, 1996), and as the structure of FabA is known the structure of any crystals formed by the recombinant SD4 protein could be solved by mapping the derived crystal structure over the crystal structure of FabA. As it is unknown if the recombinant SD4 protein will crystallize then it would be prudent to overexpress and purify the *E. coli* FabZ protein as this may form crystals. A source of recombinant *E. coli* FabZ would also be a useful tool in the development of an assay for β-hydroxyacyl ACP dehydratase.

4.2 Results

4.2.1 Cloning of the *E. coli* FabZ sequence into pET11d

The *E. coli* β-hydroxyacyl ACP dehydratase has been shown to be encoded by the *E. coli* gene *fabZ* (Mohan *et al.*, 1995). PCR primers SDECDEH1 $^{5'}$GCG CCC AAT GGG GAC TAC TAA CAC TCA TAC TCT G $^{3'}$ and SDECDEH2 $^{5'}$GCG CGG ATC CAC AAA GGC GGA TTT ATC AAT C$^{3'}$ were designed to the *E. coli fabZ* sequence present in the genebank database (Accession number D83536). The 5' primer was designed to contain a *Nco*I site, as there is an internal *Nde*I site present within the *fabZ* open reading frame. The *fabZ* gene does not initiate from a methionine (ATG) but instead initiates from a leucine (TTG). The *fabZ* sequence was sub-cloned into the pET11d vector via *Nco*I (CCATGG) and, so to maintain the correct frame an additional glycine residue (GGG) was also inserted immediately adjacent to the *NcoI* site (Figure 1). The 3' primer was designed to contain a *Bam*HI site.

The optimumal annealing temperature of the primers were determined using a Stratagene robocycler using XL1Blue *E. coli* as a template. A band of the correct predicted size (487 bp) was visible at annealing temperatures below 57 °C, with an

optimum annealing temperature of 54 °C (Data not shown). The PCR was repeated in a Perkin-Elmer thermocycler using standard PCR conditions with an annealing temperature of 55 °C a single band of 487 bp was amplified (Data not shown). The amplified product was cloned into the PCR cloning vector pGEM-T (Promega), and the ligated vector transformed into XL1Blue *E. coli*. A number of white transformants white were tested using the quick screen plasmid isolation method, all six yielded a band of the correct size (478 bp) upon digestion with *Nco*I and *Bam*HI. A Glycerol stock was made and designated pSD101. The amplified *fabZ* open reading frame was excised form the vector using the enzymes incorporated into the amplification primers, and sub-cloned into pET11d. The presence of the insert was confirmed by restriction digest and by DNA sequencing. The insert was sequenced using the pET T7 promoter and T7 terminator primers. This sequence strategy allows both strands to be completely sequenced. The obtained sequence was identical to the nucleotide sequence deposited in the database. This plasmid was designated pFabZ.

```
1                                                                   48
atg ggg act act aac act cat act ctg cag att gaa gag att tta gaa
M   G   T   T   N   T   H   T   L   Q   I   E   E   I   L   E

49                                                                  96
ctt ctg ccg cac cgt ttc ccg ttc tta ctg gtg gat cgc gtg ctg gat
L   L   P   H   R   F   P   F   L   L   V   D   R   V   L   D

97                                                                  144
ttt gaa gaa ggt cgt ttt ctg cgc gca gta aaa aat gtc tct gtc aat
F   E   E   G   R   F   L   R   A   V   K   N   V   S   V   N

145                                                                 192
gag cca ttc ttc cag ggc cat ttc cct gga aaa ccg att ttc ccg ggt
E   P   F   F   Q   G   H   F   P   G   K   P   I   F   P   G

193                                                                 240
gtg ctg att ctg gaa gca atg gca cag gca aca ggt att ctg gcg ttt
V   L   I   L   E   A   M   A   Q   A   T   G   I   L   A   F

241                                                                 288
aaa agc gta gga aaa ctg gaa ccg ggt gag ctg tac tac ttc gct ggt
K   S   V   G   K   L   E   P   G   E   L   Y   Y   F   A   G

289                                                                 336
att gac gaa gcg cgc ttc aag cgc ccg gtc gtg cct ggc gat caa atg
I   D   E   A   R   F   K   R   P   V   V   P   G   D   Q   M

337                                                                 384
atc atg gaa gtc act ttc gaa aaa acg cgc cgc ggc ctg acc cgt ttt
I   M   E   V   T   F   E   K   T   R   R   G   L   T   R   F

385                                                                 432
aaa ggg gtt gct ctg gtc gat ggt aaa gta gtt tgc gaa gca acg atg
K   G   V   A   L   V   D   G   K   V   V   C   E   A   T   M

433                                 468
atg tgt gct cgt agc cgg gag gcc tga
M   C   A   R   S   R   E   A   Z
```

Figure 1: Sequence of the amplified *E. coli fabZ* gene. The *E. coli fabZ* gene was amplified by PCR and cloned in to the *E. coli* overexpression vector pET11d. To allow sub-cloning of the PCR product into pET11d the initiation codon was changed from TTG to ATG, and to maintain the correct frame then an extra glycine residue was added immediately after the initiation codon. Both the changes are highlighted in red.

4.2.2 Test induction on pFabZ

BL21 (DE3) *E. coli* carrying the pFabZ plasmid was induced with 0.5 mM IPTG. A band corresponding to the predicted molecular weight of the *E. coli* FabZ (17 kDa) can be seen in the induced cells, but is absent from the same cells prior to induction. The cells were broken by sonication and by freeze thaw extraction. Both the soluble

(supernatant) and the pellet (insoluble) fractions from both extraction methods were loaded onto a 15 % SDS-PAGE gel. The majority of the recombinant FabZ protein can be found located in the insoluble fraction (Figure 2). The FabZ protein purified previously purified from *E. coli* (Birge and Vagelos, 1972) is a soluble protein. Thus, it is not the physical nature of the protein that is causing the recombinant protein to associate with the insoluble fraction but the manner in which the protein has been overexpressed.

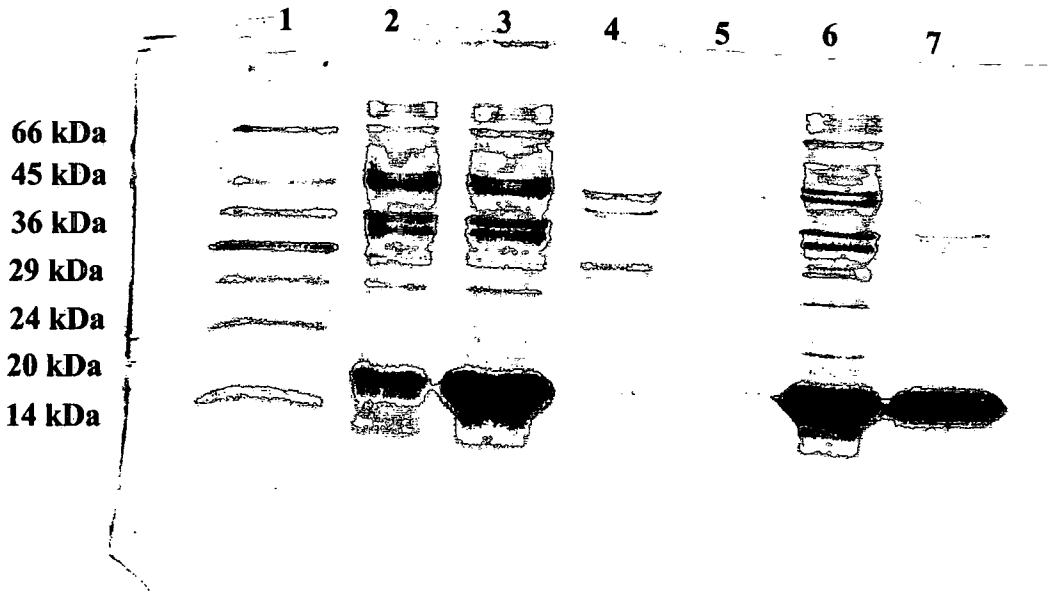


Figure 2: Induction and extraction of recombinant *E. coli* FabZ. BL21 (DE3) *E. coli* carrying the pFabZ plasmid were induced with 0.5 mM IPTG. A band corresponding to the predicted molecular weight of the *E. coli* FabZ (17 kDa) can be seen in the induced cells, but is absent prior to induction. The cells were broken by sonication and the cell lysate separated into the soluble and insoluble fractions by centrifugation. The recombinant FabZ is exclusively located in the insoluble fraction. **Lane 1**: SDS 7 markers; **Lane 2**: non-induced pFabZ; **Lane 3**: induced pFabZ; **Lane 4**: Freeze thaw supernatant (soluble); **Lane 5**: Sonicated supernatant (soluble) **Lane 6**: Sonicated pellet (insoluble); **Lane 7**: freeze thaw pellet (insoluble).

4.2.3 Sub-cloning of the nucleotide sequence encoding the mature SD4 protein into pET24a

To produce a supply of the recombinant SD4 protein the nucleotide sequence for the mature SD4 protein was sub-cloned by PCR into the *E. coli* overexpression vector pET24a. PCR primers SDBN1 $^{5'}$ GCG CCA TAT GTC CAC CGA TGA ATC AAA AGA TC $^{3'}$ and SDBN2 $^{5'}$GCG CGT CGA CGG CAC GAG AAC ATA ATC ACTA$^{3'}$ were designed to the mature SD4 nucleotide sequence (Figure 3). The 5' primer was designed to contain an *Nde*I site, while the 3' primer was designed to contain a *Sal*I site, these are required for cloning of the PCR product into the multiple cloning site of pET24a. As the translation of the SD4 protein initiates from an ATG found at the beginning of the predicted chloroplast target sequence which is subsequently remove. An ATG was also added to the 5' end of the sequence of the predicted mature protein during the sub-cloning to act as an initiation coding (Figure 3). The optimal annealing temperature of the primer was determined to be 55 °C using a Statagene robocycler. Using this optimal annealing temperature the nucleotide sequence that encodes for the mature SD protein was amplified using the excised SD4 phagemid as a template. A single band of the corrected predicted (574 bp) size was amplified. The amplified product was cloned into the PCR cloning vector pGEM-T. The presence of the insert was confirmed by restriction digest and the subsequent plasmid was designated pSD102. The nucleotide sequence form the mature SD4 protein was removed using the restriction sites engineered into the amplification primers and sub-cloned into pET24a. Restriction and digest confirmed the presence of the insert. The insert was sequenced using the T7 promotor and T7 terminator present on the pET24a vector. This strategy allowed both strands of the insert to be sequenced in both directions. The obtained sequence was identical to the original sequence obtained for pSD4. This plasmid was designated pRFabZ.

```
1                                                                   60
ATG TGC TCC ACC GAT GAA TCA AAG ATC ACC GCG GAG AAA GAG ATC CCA ATA GAG CTC AGG
M   C   S   T   D   E   S   K   I   T   A   E   K   E   I   P   I   E   L   R

61                                                                  121
TAC GAG GCT TTT CCG ACA GTG ATG GAC ATT AAC CAG ATA CGT GAA ATT TTA CCT CAC AGG
Y   E   A   F   P   T   V   M   D   I   N   Q   I   R   E   I   L   P   H   R

122                                                                 182
TTC CCG TTT CTG TTA GTG GAT AGA GTG ATA GAG TAC ACA CCT GGT GTA TGT GCT GTA GCT
F   P   F   L   L   V   D   R   V   I   E   Y   T   P   G   V   C   A   V   A

183                                                                 243
ATC AAA AAC GTT ACC ATT AAT GAT AAT TTC TTT CCT GGG CAT TTT CCT GAG AGG CCC ATT
I   K   N   V   T   I   N   D   N   F   F   P   G   H   F   P   E   R   P   I

244                                                                 304
ATG CCT GGA GTC CTC ATG GTT GAG GCC ATG GCT CAG GTG GGA GGT ATA GTG ATG CTA CAA
M   P   G   V   L   M   V   E   A   M   A   Q   V   G   G   I   V   M   L   Q

305                                                                 365
CCA GAA GTG GGC GGA TCT AAA AGC AAC TTC TTC TTT GCT GGA ATC GAC AAA GTC AGA TTC
P   E   V   G   G   S   K   S   N   F   F   F   A   G   I   D   K   V   R   F

366                                                                 426
AGA AAG CCT GTG ACT GCA GGT GAT ACT TTG GTG ATG AAG ATG ACG CTT GTG AAG ATG CAG
R   K   P   V   T   A   G   D   T   L   V   M   K   M   T   L   V   K   M   Q

427                                                                 487
AAG AGG TTT GGG ATA GCG AAA ATG GAA GGG AAA GCA TAC GTA GGG AAC ACT GTG GTA TGC
K   R   F   G   I   A   K   M   E   G   K   A   Y   V   G   N   T   V   V   C

488                                                                 548
ACT GTG GTA TGC GAA GGA GAA TTC TTG ATG GCT ATG GGA AAA GAA GAG TAG TGA TTA TGT
T   V   V   C   E   G   E   F   L   M   A   M   G   K   E   E   *   *   -   -

549     557
TCT CGT GCC
-   -   -
```

Figure 3: The predicted sequence of the mature SD4 protein. PCR primers SDBN1 and SDBN2 were designed to sub-clone the nucleotide sequence that encodes for the predicted mature SD4 protein into pET24a. The two primer regions are highlighted in red, while the amino acid sequence of the mature SD4 protein is highlighted in blue. During the sub-cloning a initiation coding ATG was added to the 5' end of the predicted mature protein.

4.2.4 Test induction on pRFabZ

BL21 (DE3) *E. coli* carrying the pRfabZ plasmid were induced with 0.5 mM IPTG. A band corresponding to the predicted molecular weight of the mature SD4 protein (19 kDa) can be seen in the induced cells, but is absent from the same cells prior to induction. The cells were broken by sonication and the soluble (supernatant) and pellet (insoluble) fractions were loaded onto a 15 % SDS-PAGE gel. The majority of the recombinant mature SD4 protein can be found located in the insoluble fraction, whilst a

small percentage remains in the supernatant. (Figure 2). Previously the SD4 homolog identified form *Brassica napus* (Hellyer I., 1992) is a soluble protein. Previously purified β-hydroxyacyl ACP dehydratase form spinach leaves has also been showed to be a soluble protein (Shimakata and Stumpf, 1982). The hydrophobicity plots of the SD4 protein predicted that the mature SD4 gene product would be a soluble protein, as the recombinant protein associates with the insoluble fraction is probably due to the nature of the overexpression system.

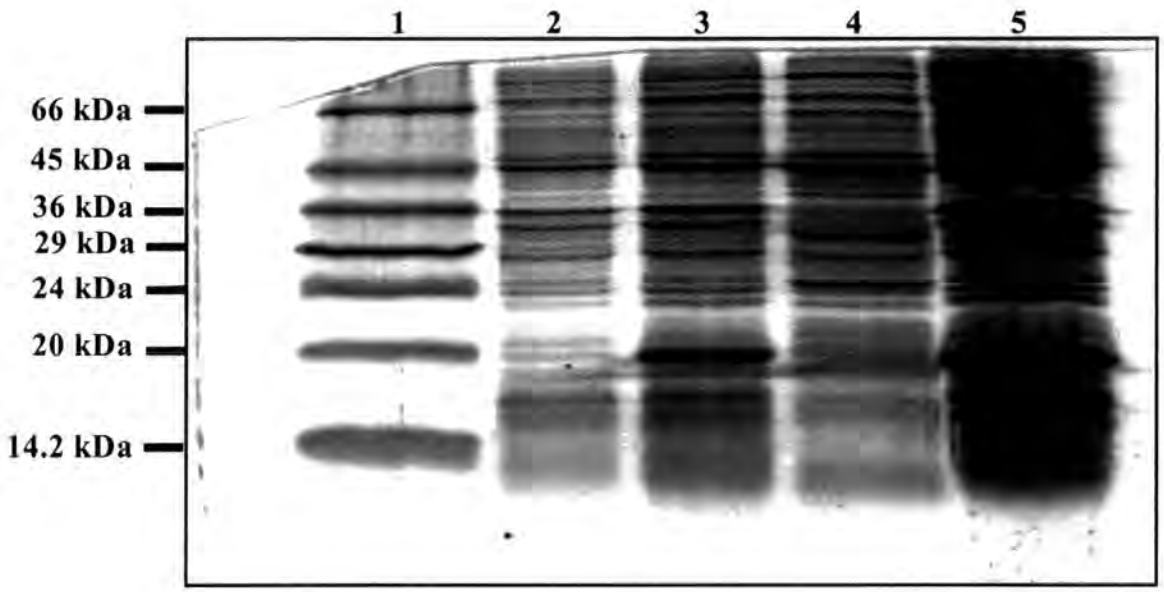


Figure 4: Induction and extraction of recombinant SD4. BL21 (DE3) *E. coli* carrying the pRFabZ plasmid was induced with 0.5 mM IPTG. A band corresponding to the predicted molecular weight of the predicted mature SD4 protein (19 kDa) can be seen in the induced cells, but is absent from the same cells prior to induction. The cells were broken by sonication and the soluble (supernatant) and pellet (insoluble) fractions were loaded onto a 15 % SDS-PAGE gel. The majority of the recombinant mature SD4 protein can be found located in the insoluble fraction whilst a small percentage can be seen in the supernatant. **Lane 1**: SDS 7 markers; **Lane 2**: non-induced pRFabZ; **Lane 3**: induced pRFabZ; **Lane 4**: supernatant (soluble); **Lane 5**: pellet (insoluble).

4.2.5 Effect of temperature on solubility on both the recombinant *E. coli fabZ* and SD4

For some proteins induction and growth at 37 °C can cause some proteins to accumulate as inclusion bodies. Performing the induction at 30 °C can lead to the recombinant protein remaining in the soluble phase (Schein and Noteborn, 1989). To test if performing the induction at 30 °C would improve the solubility of both recombinant SD4 and *E. coli* FabZ. BL21 (DE3) *E. coli* carrying both the pFABZ and pRfabZ plasmid were induced with 0.5 mM IPTG. Immediately after the induced cells were transferred to 30 °C for the remainder of the induction protocol.

At the completion of the induction protocol the cells were broken by sonication and the soluble and insoluble fractions were separated by centrifugation. The *E .coli* FabZ is exclusively found in the insoluble fraction, and no corresponding band can not be seen in the soluble fraction (Figure 5). While the majority of the recombinant SD4 associates with the insoluble fraction, a band of similar molecular weight can be seen in the soluble fraction (Figure 6).

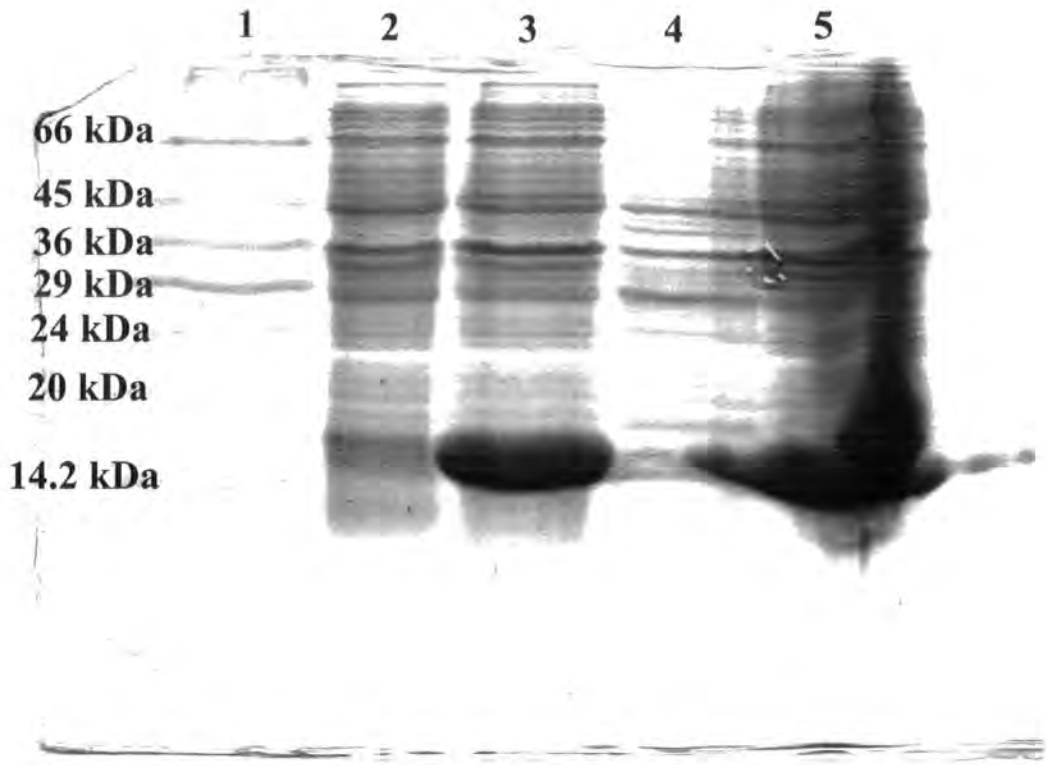


Figure 4: Effect on temperature of induction on solubility of *E. coli* FabZ. pFabZ was transformed into BL21(DE3) *E. coli*, and a number of colonies were scraped into LB/amp and grown at 37 °C until an OD_{600} of 0.6 had been reached. 0.5 mM IPTG was then added to the remaining culture and then the culture shifted to 30 °C and grown for a further 3 h. The cells were harvested and broken by sonication. The cell lysate was separated into the soluble and insoluble fractions by centrifugation at 10,000 g. All of the *E. coli* FabZ associate with the insoluble fraction. **(A) Lane 1**: SDS7 markers; **Lane 2**: non-induced pFabZ**; Lane 3**: induced pFabZ; **Lane 4**: pFabZ supernatant (soluble); **Lane 5**: pFabZ pellet (insoluble).

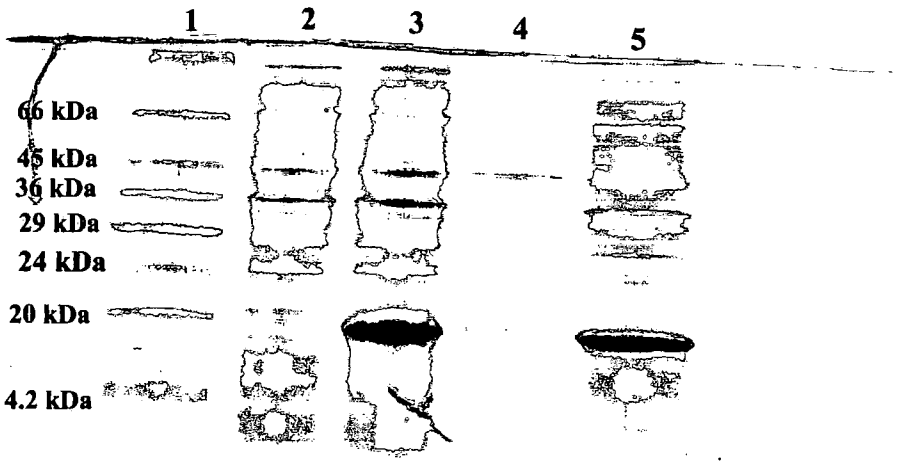


Figure 5: Effect on temperature of induction on solubility on recombinant SD4.. pRFabZ containing BL21 (DE3) were grown at 37 °C until an OD_{600} had been reached. The cells were induced with 0.5 mM IPTG and the culture immediately shifted to 30 °C and added grown for a further 3 h. The cells were harvested and broken by sonication. The soluble and insoluble fractions were separated by centrifugation at 10,000 g. All of the recombinant SD4 associates with the insoluble fraction. **Lane 1**: SDS7 markers; **Lane 2**: non-induced SD4; **Lane 3**: induced SD4; **Lane 4**: SD4 supernatant (soluble); **Lane 5**: SD4 pellet (insoluble).

4.2.6 Confirmation that some recombinant SD4 remains in the soluble fraction

Lowering of the growth temperature during induction failed to improve the solubility of the recombinant proteins. When recombinant proteins form inclusion bodies a significant proportion of the target protein can remain soluble even when most of the recombinant proteins aggregates. If a significant proportion of the recombinant SD4 protein is soluble then this can be used to determine the biological activity of the recombinant SD4 protein. To test if any of the recombinant SD4 protein associates with the soluble phase, both pRFabZ and an empty vector control (pET24a) were induced and the cells broken by sonication. After sonication the disrupted cells were centrifuged at 40,000 g in a bench top centrifuge, to completely ensure that all of the insoluble protein was removed both supernatants were carefully decanted into a fifteen-mL falcon tube. The soluble fractions from both the induced recombinant SD4 and the empty vector

control were loaded onto a 15 % SDS-PAGE gel (Figure 6). A band can of 19 kDa is present in the induced SD4 soluble fraction that is absent from the soluble fraction produced from the empty vector control. This indicates that a small but significant proportion of the recombinant SD4 protein remains in the soluble fraction. This soluble recombinant SD4 is not necessarily active or has been folded correctly, but if a significant proportion of the soluble protein is active then this level of soluble protein should be sufficient to assay for biological activity. A induction is carried out on a sufficiently large enough culture the correctly folded active SD4 protein could be purified.

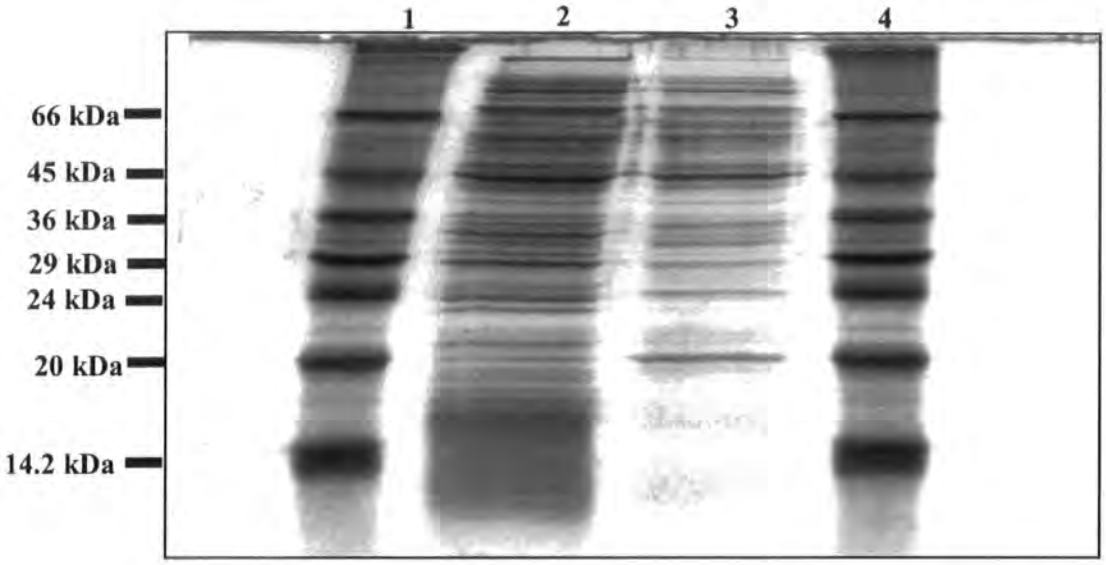


Figure 6: Comparison of soluble sonicated extracts of induced pET24a and pRFabZ. Induced *E. coli* containing either pET24a or pRfabZ were broken by sonication and the cell debris removed by centrifugation at 40,000 g for 30 min. The supernatant was carefully decanted and the protein concentrated by Bradford assay. The protein concentration of both crude extracts was adjusted to 1mg/mL with 50 mM Tris.HCl pH 8.0 containing 1mM DTT and 8 µL loaded onto a 15 % SDS-PAGE gel. **Lane 1**: SDS 7 markers; **Lane 2**: pET24 high speed supernatant; **Lane 3**: pRFabZ high speed supernatant; **Lane 4:** SDS 7 markers.

4.2.7 Solubilization of the recombinant SD4 protein present in the insoluble fraction

Only a small percentage of the recombinant SD4 remains in the soluble phase after the induced cells have been broken and the soluble and insoluble fraction separated by high-speed centrifugation. This soluble SD4 protein can be purified to homogeneity by scaling up the induction and using a biological assay to follow the purification of the recombinant SD4 protein. However the soluble SD4 is not necessarily active and folded correctly. A second potential source of biologically active SD4 protein may be from the aggregated insoluble recombinant SD4 protein. Recently methods have become established for the refolding of insoluble proteins (Rudolph and Lille, 1996; Muckopadhyay, 1997). To determine if the insoluble recombinant SD4 protein can be refolded pRFabZ was induced and the cells broken by sonication the cell debris was removed by centrifuging at 40,000 g for 15 min. The supernatant was carefully removed and flash frozen in liquid nitrogen and stored at –80 °C. The pellet was resuspended in 50 mM Tris.HCl pH 8.0 containing 1 mM DTT and 8 M urea. The pellet was vortexed vigorously for 10 min this did not resolubilize all the proteins in the pellet, as the solution remained cloudy. The resolubilization was left overnight at 4 °C with continuous shaking, this was still not sufficient to completely clear the solution, and the remaining insoluble material was removed by centrifugation at 13,000 g in a bench top centrifuge. 8 μL of the supernatant was loaded onto a 15 % SDS-PAGE gel (Figure 7). The recombinant SD4 protein is now denatured and is now soluble in the 8 M urea solution. To allow the protein to refold the urea was removed by stepwise dialysis by dialyzing 2 mL of the resolubilized pellet against 2 L of 50 mM Tris.HCl pH 8.0 containing 0.1 mM DTT and 6 M urea, followed by 2 h against 2 L 50 mM Tris.HCl pH 8.0 containing 0.1 mM DTT and 4 M urea. This was followed by a further 2 h dialysis against 2 L of both 50 mM Tris.HCl pH 8.0 containing 0.1mM DTT and 2 M urea and finally 50 mM Tris.HCl pH 8.0 containing 0.1 mM DTT.

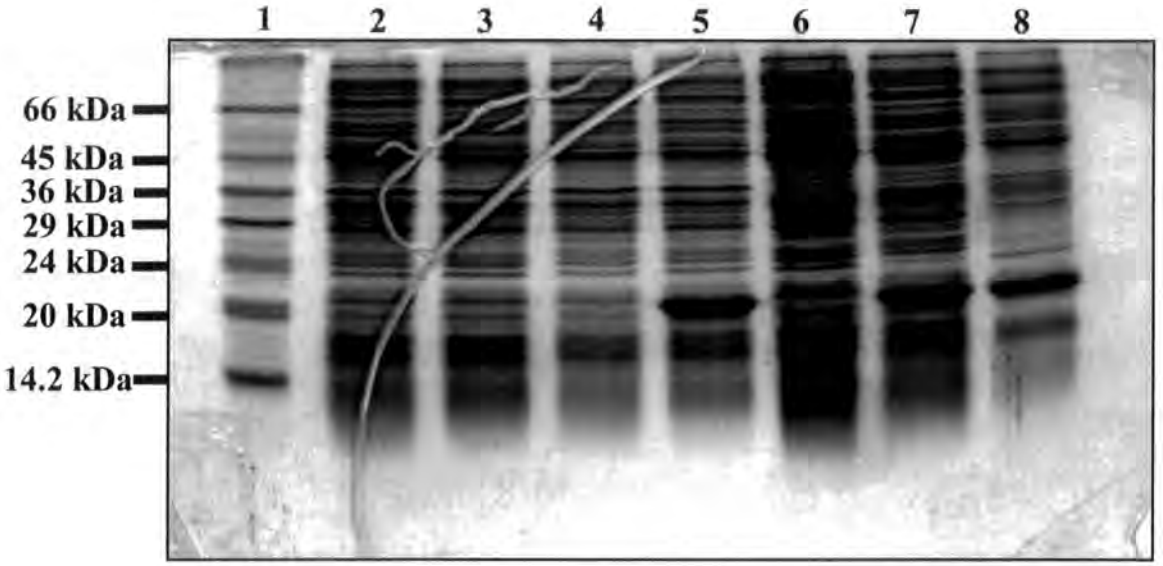


Figure 7: Preparation of induced crude *E. coli* extracts. Both BL21(DE3) *E. coli* bearing either pRFabZ (SD4/pET24a) or pET24a were induced with 0.5 mM IPTG for 3 h. The cells were broken by sonication and cells debris removed by centrifugation at 40,000 g. 8μL from each of the crude extracts were then loaded onto a 15 % SDS-PAGE gel. The recombinant mature SD4 protein can only be seen in the induced pRFabZ crude extract, but not in the induced pET24a extract. The majority of the recombinant SD4 protein located in the pellet was resolubilized with 8 M urea that was removed by stepwise dialysis. Lane 1: SDS 7 markers; Lane 2: non-induced pET24a; Lane 3: induced pET24a; Lane 4: non-induced pRFabZ; Lane 5: induced pRFabZ; Lane 6: pET24a crude extract; Lane 7: pRFabZ crude extract; Lane 8: urea resolubilized pRFabZ pellet.

4.3 Discussion

To produce recombinant SD4 protein the nucleotide sequence which encodes for the predicted mature SD4 protein was sub-cloned by PCR into the *E. coli* overexpression vector pET24a. Once the overexpression was transformed into BL21(DE3) *E. coli* were grown in medium supplemented with IPTG, a protein of 19 kDa was induced. This is the same predicted molecular weight of the mature SD4 protein. When the induced cells were broken and the cell lysate separated into the soluble and insoluble fractions by centrifugation the majority of the recombinant SD4 protein associates with the insoluble fraction. A hydrophobicity plot on the mature SD4 protein revealed that the protein is

predicted to contain regions with hydrophobic nature, but did not show the presence of any trans-membrane spanning domains, thus the SD4 protein was predicted to be soluble. Therefore it is the overexpression of the protein in *E. coli* that is causing the protein to associate with the insoluble fraction.

E. coli proteins are folded into their correct native structure by the action the chaperone GroEL and it's cofactor GroES (Hartl, 1996). Proteins are translated by the polysome and then from a "molten globule" which contains defined secondary structures but lacks the correct tertiary structure of the complete native protein. The "molten globule" contains hydrophobic residues that are exposed to the solvent phase, which are normally buried deep within the center of the protein. The GroEL and GroES form a cage around the translated protein once it is released from the polysome preventing these exposed hydrophobic residues interacting an aggregating. During induction of proteins using the pET system the whole cell machinery is seconded into synthesizing the recombinant protein, leading to the recombinant protein being greater than 50% of the total cell proteins (Studier *et al.*, 1990). This leads to the recombinant being synthesized at a sufficient rate that the exposed hydrophobic cores can interact while the recombinant protein is still bound to the polysome. This causes the recombinant proteins to aggregate forming a complex that includes the incorrectly folded proteins along with the polysomes. This complex associates with the insoluble fraction once the cells are broken. The solubility of the protein could be increased if the rate of translation is decreased, this can be achieved by a number of different ways most notably by lowering the growth temperature at which the induction are performed. Performing induction on the pRfabZ at 30 °C had no effect on the amount of recombinant SD4 protein found in the soluble fraction.

High speed centrifugation comparisons of induced BL21 (DE3) and an empty vector control showed that there is a small but significant amount of recombinant SD4 which remains in the soluble fraction after high speed centrifugation at 40,000 g. This soluble protein is not necessarily folded correctly and thus biologically active. The majority of the recombinant SD4 protein found in the insoluble fraction could provide a source of active SD4 protein. A number of methods have been developed for the correct folding of insoluble proteins (Rudolph and Lille, 1996; Muckopadhyay, 1997). These methods rely on solubilizing the recombinant protein in a denaturants such as urea, and slowly removing the denaturing agent. If the protein does not require specific chaperones to form its native structure then potentially the protein can spontaneously fold into its

correct native structure under these conditions. The insoluble recombinant SD4 protein can be solubilized into solution with 8 M urea, and the recombinant SD4 protein remains in solution when the urea has been removed by stepwise dialysis. Both the recombinant SD4 protein located in the soluble fraction and the resolubilized protein are available to be used as a source of enzyme to test for the biological activity of the recombinant SD4 protein.

Chapter 5:

Isolation and characterisation of ACP from native and recombinant sources

5.1 Introduction

β-hydroxyacyl ACP dehydratase purified from both *E .coli* (Birge and Vagelos, 1972) and spinach leaves (Shimakata and Stumpf, 1982) shows an absolute requirement for ACP as its substrate. Thus any proof of function of the SD4 gene product requires a source of ACP.

ACP is a small (~10 kDa) rod shaped protein, which is characterised by a predominance of acidic amino acids and a lack of aromatic and hydrophobic residues. ACP is synthesised in an apo form lacking the 4'-phosphopantetheine prosthetic group. This prosthetic group is fundamental to ACP function as nascent elongating acyl chains are esterified to ACP via thioesterification of a cysteamine thiol present in the 4'-phosphopantetheine prosthetic group, allowing the ACP to participate in a wide variety of biosynthetic processes. These include the *de novo* synthesis of fatty acids (Magnusson *et al.*, 1993; Harwood, 1996), peptides (Baldwin *et al.*, 1991), polyamino antibiotics (Lipman, 1980), polyketides (Hopwood *et al.*, 1990), the post-translational acylation of proteins (Lawson *et al.*, 1994), and the synthesis of lipopolysaccharides (Shearman *et al.*, 1986). ACP is translated in an inactive form (apo ACP), and is post-translationally modified by the addition of 4'-phosphopantetheine derived from coenzyme A, to form the active holo ACP (Brown, 1959). The transfer of the prosthetic group is catalysed by the enzyme holo acyl carrier protein synthase (Lambalot and Walsh, 1995). Holo ACP synthase transfers the 4'-phosphopantetheine moiety from CoA to a specific serine residue (SER-36 of *E .coli* ACP*)*. All known ACPs have homologous amino acid sequences surrounding the 4'-phosphopantetheine attachment site (Figure, 1).

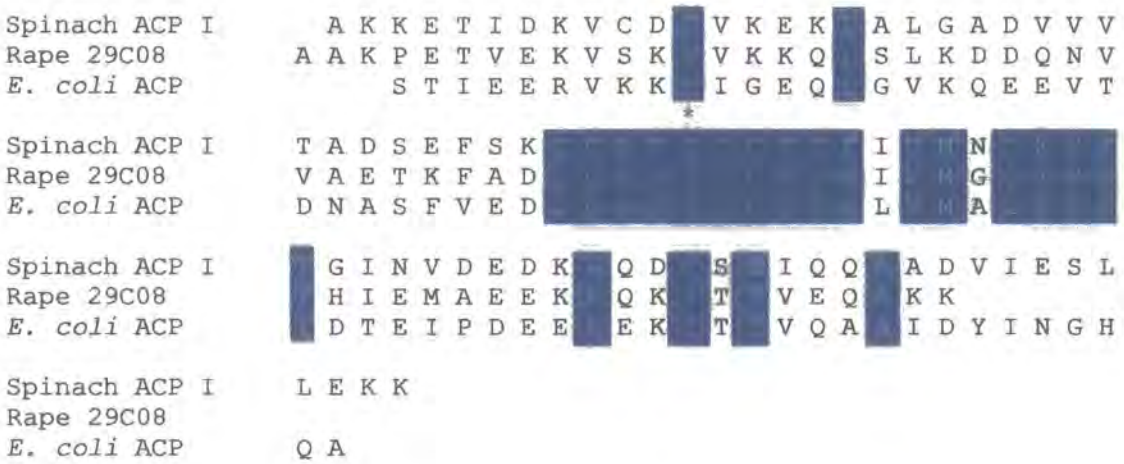


Figure 1: **Amino acid comparison of ACP from different sources.** Amino acid sequence comparison of Spinach ACP I (leaf specific), a rape ACP II (29C08, seed specific) and *E. coli* ACP. The (*) marks the attachment site for the 4'-phosphopantetheine group.

Holo ACP synthetase activity has been purified to homogeneity from *E. coli* (Lambalot and Walsh, 1995). N-terminal acid sequence data from the purified protein revealed that the *dpj* gene, an essential gene that had no previous designated function, encodes the holo ACP synthase in *E. coli.* (Takiff *et al.*, 1992). Holo ACP synthase has not been purified or a cDNA isolated from any plant source, but the activity has been described in chloroplasts (Fernandez and Lamppa, 1990).

Assays measuring β-hydroxyacyl ACP dehydratase activity in *E. coli* (Birge and Vagelos, 1972), safflower and spinach leaves (Shimakata and Stumpf, 1982) have all used ACP purified from *E .coli* (Majerus *et al.*, 1969; Rock and Cronan, 1980). For proof of function of the SD4 gene product, it would be highly desirable to have available rape ACP as a substrate, since specific interactions between the plant enzyme and ACP may be important for the enzyme mechanism and such interactions might be overlooked if a bacterial ACP is used. In the past kinetic analysis using plant ACP has been limited due to the relative difficulty in obtaining large quantities of purified plant ACP (Simoni *et al.*, 1967; Guerra *et al.*, 1986). This problem could be overcome if rape holo ACP could be produced by recombinant means.

The first plant ACP to be expressed in *E. coli* was the spinach ACP-I isoform (Kuo and Ohlrogge, 1984; Beremand *et al.*, 1987), in that case a synthetic ACP gene was constructed using codons biased towards those present in the plant genome. A second synthetic spinach ACP-I gene has also been overexpressed in *E. coli* using codons biased towards high expression in *E. coli* (Broadwater and Fox, 1999). The overexpression of

recombinant rape ACP should allow the advancement of structural studies on ACP. The structure of *E. coli* ACP has been determined by NMR studies (Kim and Prestegard, 1991) but no 3D structure has been determined for ACP using X-ray crystallography. NMR and X-ray crystallography use different principals for structural elucidation. NMR structures provide a "solution structure" whilst crystal derived structures refer to a specific crystallized form. The two techniques are complimentary but the structural solutions are not identical. The use of a recombinant ACP should allow for easy protein purification and provide a system where alterations in the amino acid sequence are possible. These amino acid alterations can be useful for the identification of essential residues as well as the introduction of heavy metal binding sites into the recombinant protein. Furthermore the use of a recombinant protein allows the possibility of producing selenomethionine derivatives, by overexpressing the ACP in a strain of *E. coli* that is auxotrophic for methionine in a culture medium containing selenomethione. In this case selenomethionine will be incorporated into the recombinant protein as opposed to methionine. This technique is becoming increasingly important in solving the structure of proteins using X-ray crystallography (Lee *et al.*, 1997).

5.2 Results

5.2.1. Purification of wild type ACP from K-12 *E. coli*

E. coli ACP was purified from fermenter grown K-12 *E. coli* (kindly provided by Professor Peter Dunhill, University College London). The fermenter grown *E. coli* had been frozen and stored at –80 °C prior to use. Purification of ACP was carried out by a method based substantially on that of Majerus *et al.* (Majerus *et al.*, 1969). This method exploits physical attributes inherent in the ACP i.e. its stability at low pH and high solubility in ammonium sulphate, to produce an extract that is enriched in ACP (Majerus *et al.*, 1969). The ACP was purified from the enriched fraction by anion exchange chromatography. The ACP was bound to a 55 mL Q–Sepharose Fast Flow column (Pharmacia), and eluted with a 10-column volume linear gradient of LiCl (Figure, 2). 2 µL was removed from every eighth fraction and pooled to give a total volume of 16 µL. The pooled fractions were then assayed using the [^{14}C] malonyl CoA exchange reaction (Majerus *et al.*, 1969). Purified *E. coli* ACP was used as a positive control (kindly provided by Bill Simon). The fractions 41-56 stimulated the fixation of CO_2 into acid precipitable counts confirming these fractions contain ACP (Figure 3).

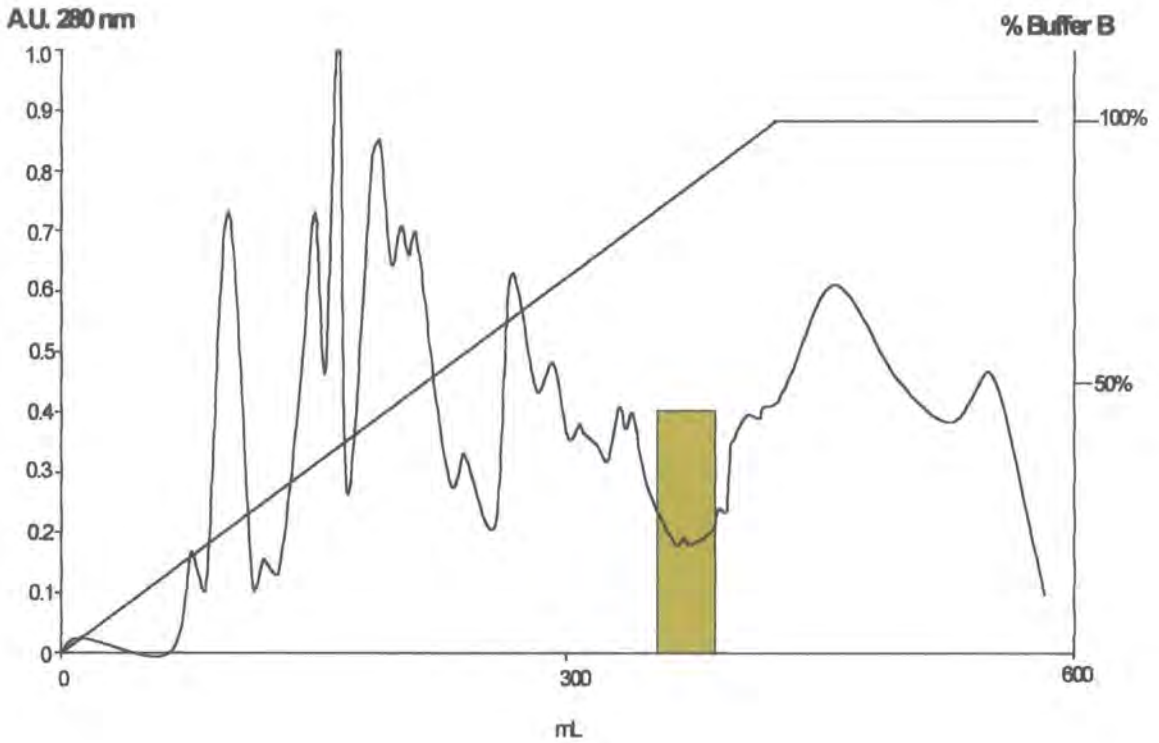


Figure 2: Ion Exchange chromatography on enriched ACP fraction. An enriched ACP fraction was loaded onto a 55 mL high load Q column equilibrated in 10 mM potassium phosphate pH 6.2 containing 0.1 % β-mercaptoethanol. The subsequent elution was carried out with a linear gradient of buffer A containing 0.5 M LiCl. The elution position of the ACP is highlighted by the yellow area as determined by an ACP assay.

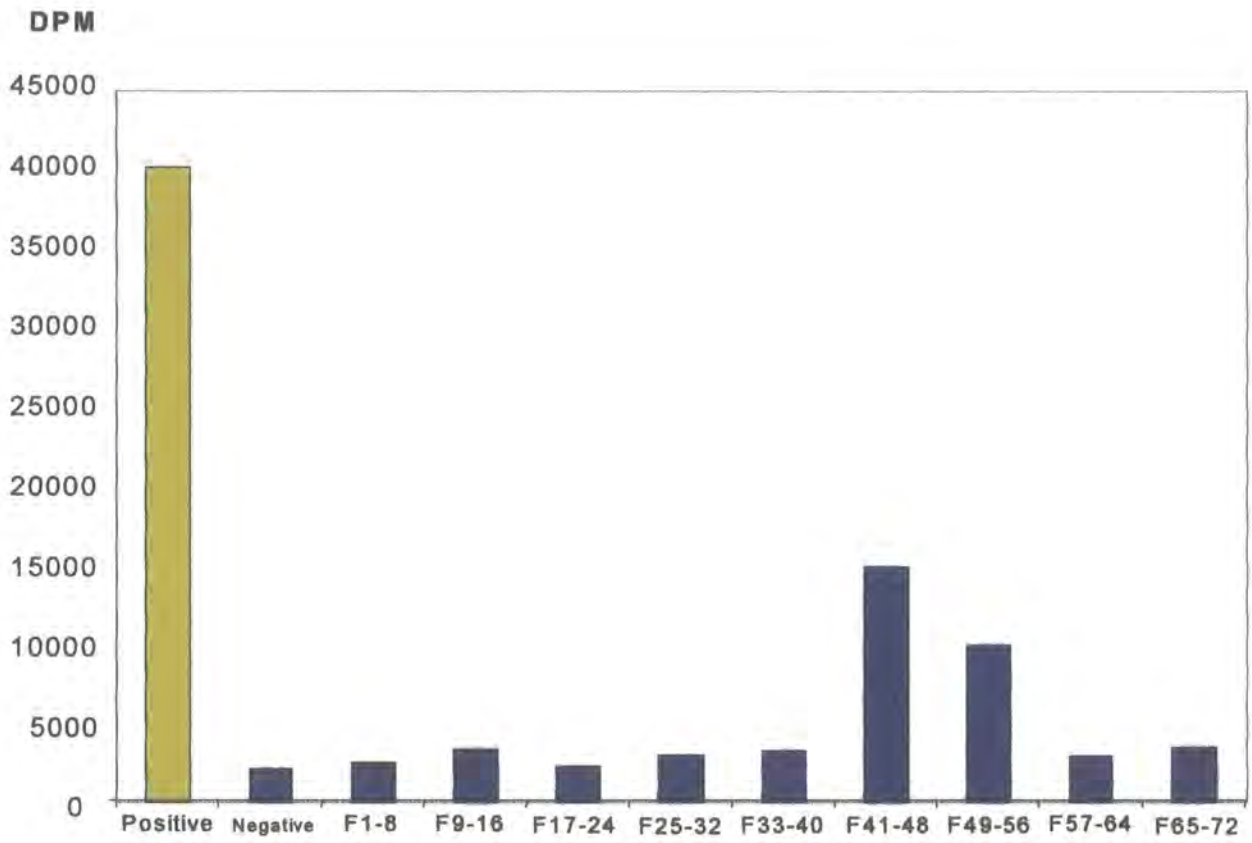


Figure 3: Assay on Q-Sepharose fractions, using the malonyl CoA exchange reaction. 2 µl from every eighth reaction was pooled and assayed using the malonyl CoA exchange reaction (Majerus *et al.*, 1969). Fractions 41-56 contain ACP activity..

16 µL of each of the ACP containing fractions was loaded onto a 15 % SDS-PAGE gel (Figure, 4) to determine the level of purity. The distinctive ACP band could be seen located in fractions 42-52, with the majority located in the fractions 45-47. Fractions 45-47 were pooled and were estimated to contain a total of 18 mg of ACP by Bradford assay. ACP could also be seen in fractions 48-52, these fractions were pooled separately from the other ACP containing fractions and dialyzed against buffer A. The ACP was concentrated by binding the ACP to a high trap Q-Sepharose column (Pharmacia) and eluted in buffer B, a further 3.6 mg of ACP were isolated from these fractions. Thus a total yield of 21.2 mg of ACP was obtained from 250 grams (dry weight) of *E. coli* using this method. This is equivalent to a yield of 84.4 mg per Kg. Which compares favorably with the yield seen when this method has been used previously (60-80 mg per Kg , Rock and Cronan, 1980).

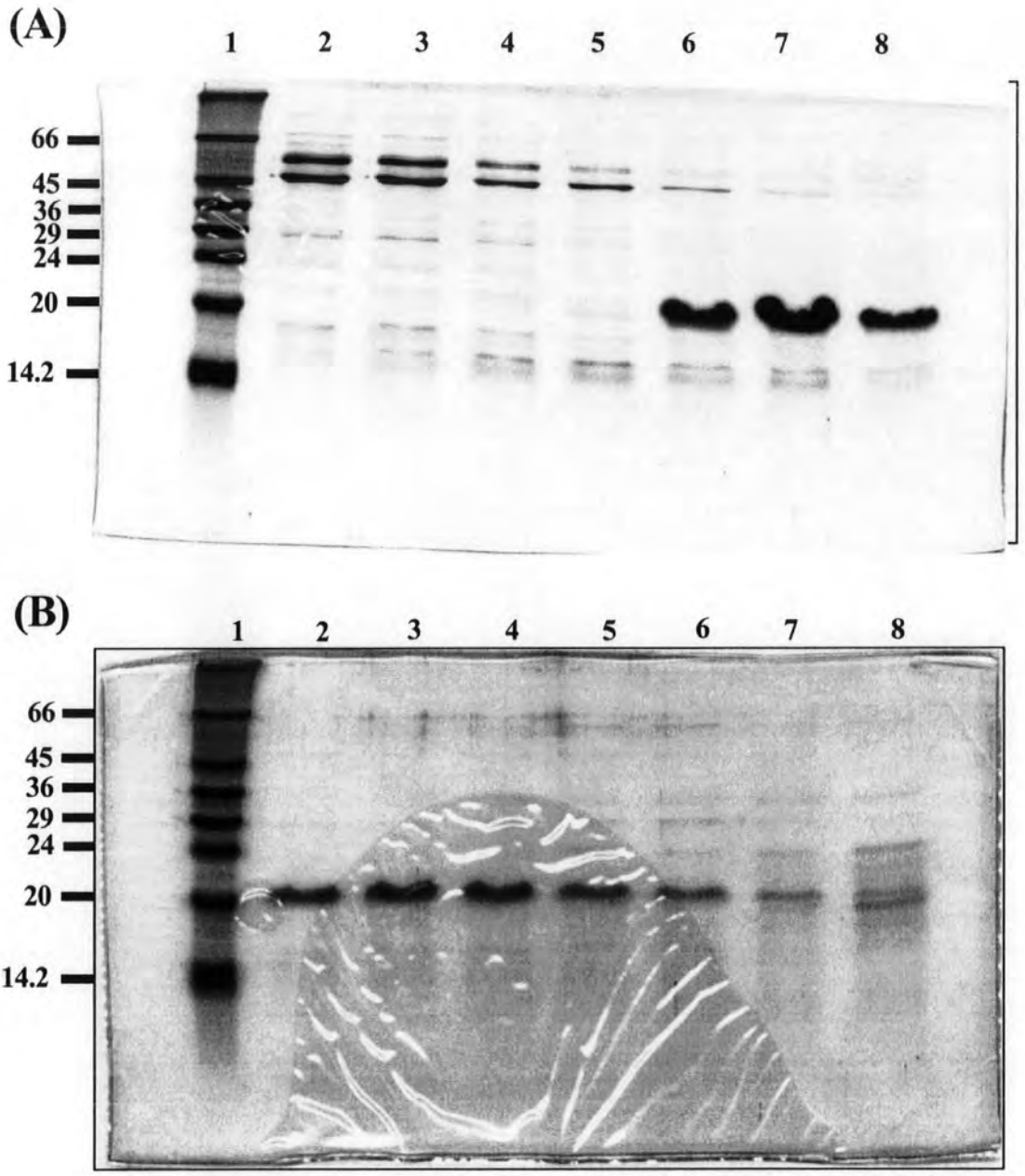


Figure 4: Purification of ACP from K-12 *E. coli*. The distinctive ACP band can be in fractions 45-52, with the majority located in fractions 45, 46 and 47. **(A) Lane 1**: SDS 7 markers; **Lane 2**: Fraction 41; **Lane 3**: Fraction 42; **Lane 4**: Fraction 43; **Lane 5**: Fraction 44; **Lane 6**: Fraction 45; **Lane 7**: Fraction 46; **Lane 8**: Fraction 47; **(B) Lane 1**: SDS markers; **Lane 2**: Fraction 48; **Lane 3**: Fraction 49; **Lane 4**: Fraction 50; **Lane 5**: Fraction 51; **Lane 6**: Fraction 52; **Lane 7**: Fraction 54; **Lane 8**: Fraction 55.

5.2.2 Overexpression of rape ACP

An *E. coli* overexpression vector (pET11d), containing the sequence for the seed specific rape ACP (29CO8, Safford *et al.*, 1988) minus the chloroplast targeting sequence was obtained from Dr Tony Fawcett (Figure 5). This plasmid was designated pRACP1.

```
M A K P E T V E K V S K I V K K Q L S L K D D Q N V
V A E T K F A D L G A D S L D T V E I V M G L E E E
F H I E M A E E K A Q K I T T V E Q A K K
```

Figure 5: Mature peptide sequence of the seed specific rape ACP 29CO8. The nucleotide sequence required for coding the mature 29CO8 ACP sequence was cloned into the overexpression vector pET11d. During the cloning the first alanine residue was excluded. (Work carried out by Dr Tony Fawcett).

5.2.2.1 Induction of rape ACP

BL21 (DE3) *E. coli* carrying the pRACP1 overexpression construct were induced from seed stocks (Figure, 6). 3 mL of the remaining induced culture was freeze thaw extracted, and 8μL of the freeze thaw supernatant containing the released recombinant protein was loaded onto a 15 % SDS-PAGE gel (Figure, 6). A band with the distinctive ACP appearance on SDS-PAGE gels can be seen in the induced cells, the same band is also present in the cells prior to induction. Rape ACP has a predicted Mr. of 9200, the induced band runs just below the 20 kDa marker (ca. 16 kDA). This anomalous mobility of ACP on SDS-PAGE is a phenomenon that has been reported by other workers and is due to the acidic nature and lack of SDS binding sites on the ACP molecule. The induced and extracted ACP does not correspond to endogenous *E. coli* ACP as the two ACPs have different mobility on SDS-PAGE (Figure 6).

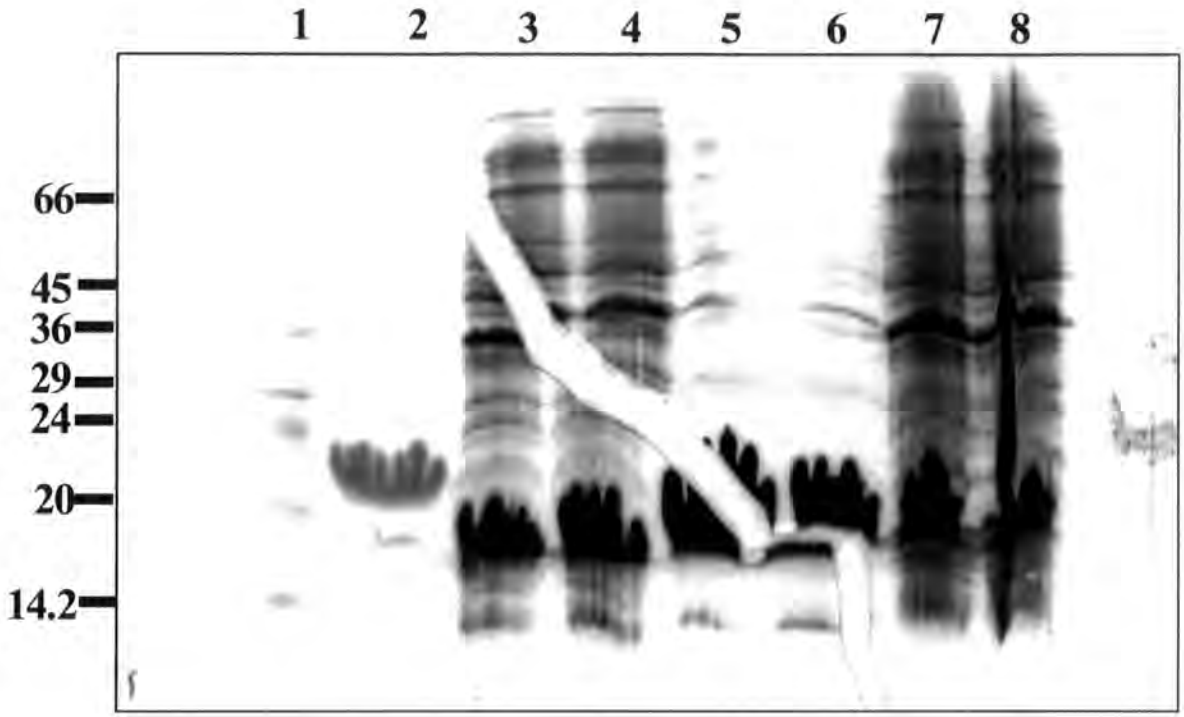


Figure 6: Overexpression and extraction of recombinant rape ACP. Both the non-induced (Lane 3) and induced (Lane 4) *E. coli* contain a band that bears the distinct appearance of ACP when run on SDS-PAGE. The induced ACP does not correspond to endogenous *E. coli* ACP, as both the induced and released ACP has a different mobility than *E. coli* ACP (**Lane 2**). The recombinant ACP is released upon repetitive rounds of freeze thaw extraction (**Lanes 5 and 6**). **Lane 1**: SDS 7 markers; **Lane 2:** *E. coli* ACP; **Lane 3:** Non-induced pRACP; **Lane 4:** Induced pRACP; **Lane 5:** Freeze thaw extract 1; **Lane 6:** Freeze thaw extract 2; **Lane 7:** Remaining cells 1; **Lane 8:** Remaining cells 2.

5.2.2.2 Confirmation that the overexpressed protein is rape ACP

16 μL of the freeze thaw extract was loaded onto a 15 % SDS-PAGE sequencing gel. The gel was transferred onto a PDVF membrane. The recombinant ACP band was excised with a clean razor blade and applied to a 477A pulsed gas phase sequencer (Applied Biosystems, sequencing carried out by John Gilroy). The first five amino acids were sequenced and this gave rise to the following N-terminal sequence AKPET. This corresponds to the deduced amino sequence from the 29CO8 rape ACP cDNA AAKPET (Figure 5; Safford *et al.*, 1988), with the exception that the first alanine is not present, as this had been removed during the sub-cloning (personnal communication Dr Tony Fawcett). The initiation methionine had also been removed in the majority of the recombinant ACP as the second amino acid is an alanine residue, that in prokaryotes is

non-protecting and so allows the cleavage of the initiating methionine residue (Flinta *et al.*, 1986). A small percentage of the recombinant ACP still contains the initiation methionine. The amino acid sequence of the overexpressed protein confirms that the protein is indeed recombinant rape ACP.

5.2.2.3 Purification of recombinant rape ACP

Recombinant rape ACP induction was carried out as above on an 800 mL scale, and then freeze thaw extracted. The freeze thaw extract was applied to a 55 mL mono-Q Sepharose 5/5 column (Pharmacia). The subsequent elution was carried out with a linear gradient of 0-100 % buffer B (buffer A containing 0.5 M LiCl) over thirty column volumes (Figure, 7). 7 mL fractions were collected from the start of the gradient until the end 4 μL from every fourth fraction was removed and pooled, the pooled fractions were loaded onto a 15 % SDS-PAGE gel (Figure, 8). The recombinant rape ACP was located in fractions 22-27. 16 μL from each of these individual fractions was loaded onto a 15 % SDS-PAGE gel (Figure 9). The distinctive ACP band could be seen in fraction 23 and 24. ACP was also present in fraction 25, but here the distinctive ACP band had split into a doublet and so this fraction was discarded. The recombinant rape ACP elutes off at 250 mM LiCl. This differs to the elution position of *E. coli* ACP that elutes at 400 mM LiCl, and so the recombinant rape ACP should not be contaminated with any *E. coli* ACP that is released from the cells by the freeze thaw extraction step.

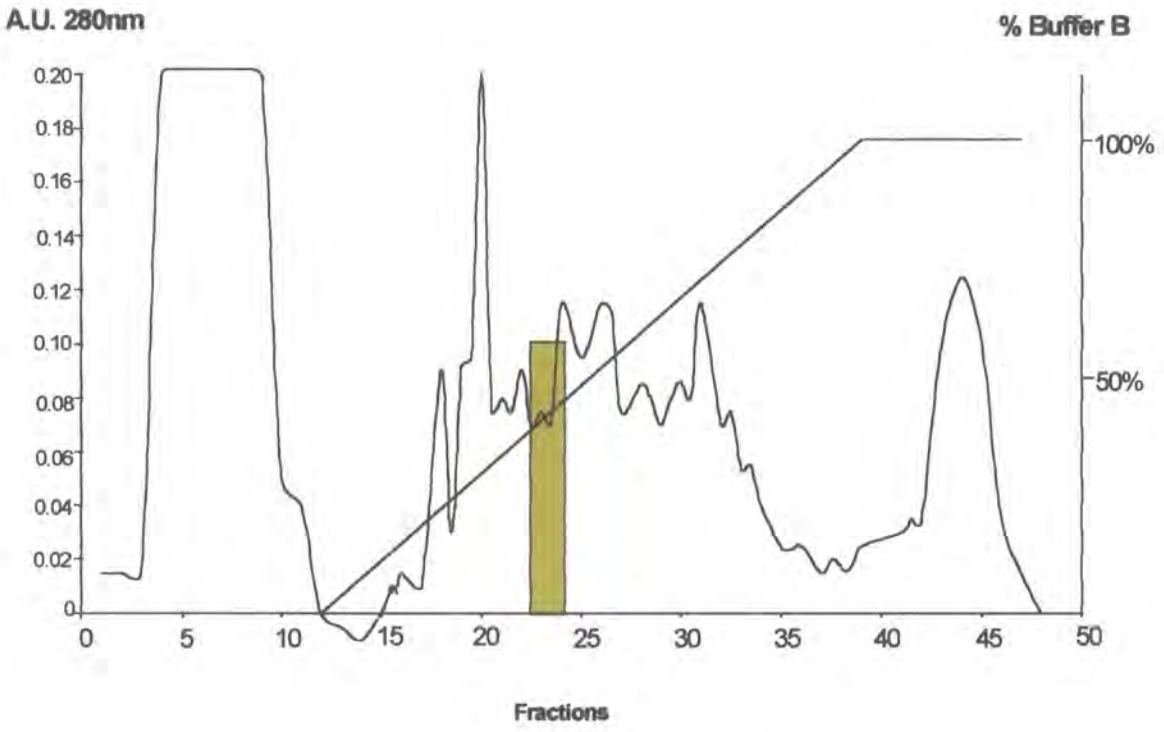


Figure 7: Purification of recombinant rape ACP. A freeze thaw extract of induced recombinant ACP was loaded onto a mono Q column, equilibrated in Buffer A (10 mM potassium phosphate pH 6.2 containing 0.1 % β-mercaptoethanol). The protein mixture was separated by elution with a linear gradient of 0-100 % (Buffer A containing 0.5 M LiCl) over 500 mL. 7 mL fractions were collected, the yellow area shows the elution position of the recombinant rape ACP as determined by SDS-PAGE.

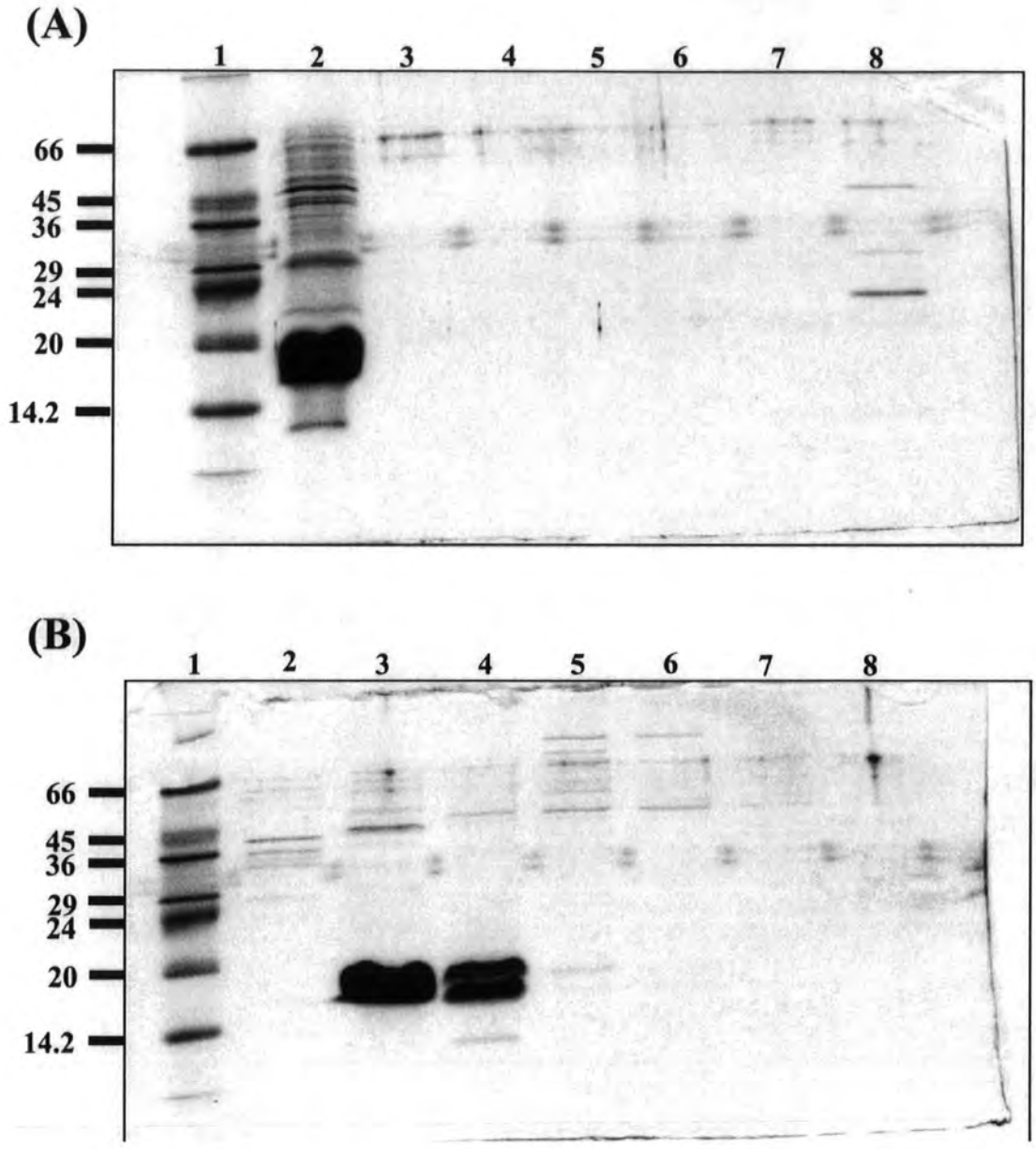


Figure 8: Large scale purification of recombinant rape ACP. The fractions from the large-scale purification of recombinant rape ACP were loaded onto a 15 % SDS-PAGE gel. The distinctive ACP band could be seen located in fractions 22-27. **(A) Lane 1**: SDS 7 markers; **Lane 2**: Freeze thaw extract (8 μL); **Lane 3**: Fractions 1-3; **Lane 4**: Fractions 4-6; **Lane 5**: Fractions 7-9; **Lane 6**: Fractions 10-12; **Lane 7**: Fractions 13-15; **Lane 8**: Fractions 16-18. (B) **Lane 1**: SDS 7 Markers; **Lane 2**: Fraction 19-21; **Lane 3**: Fractions 22-24; **Lane 4**: Fractions 25-27; **Lane 5**: Fractions 28-30; **Lane 6**: Fractions 31-33; **Lane 7**: Fractions 34-36; **Lane 8**: Fractions 37-39.

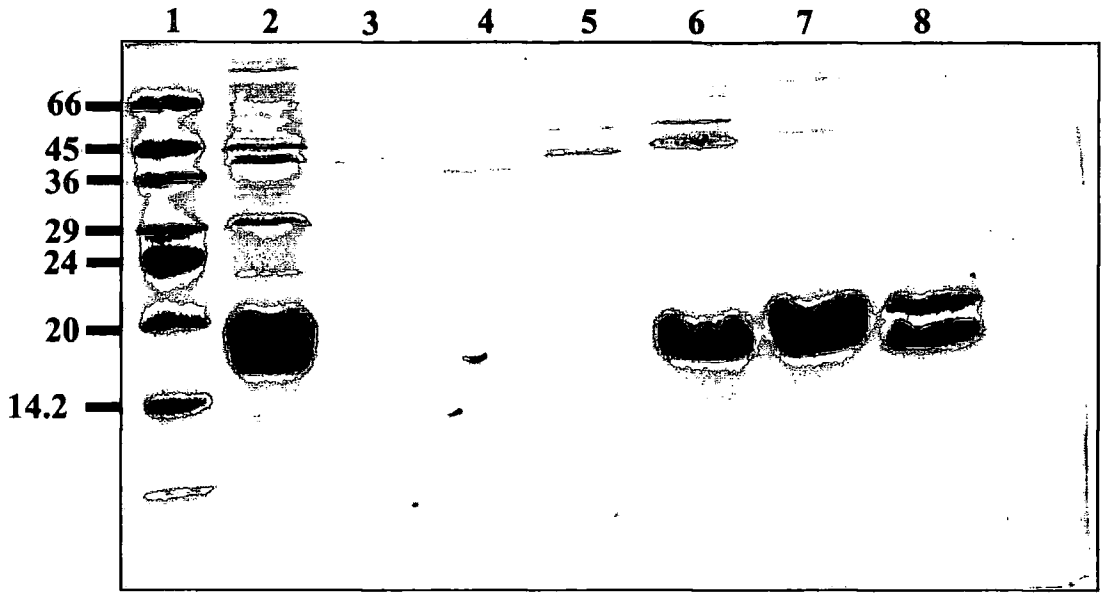


Figure 9: Large scale purification of recombinant rape ACP. The fractions containing the recombinant rape ACP were loaded individually onto 15 % SDS-PAGE gel The ACP could be seen located in fractions 23, 24 and 25. The distinctive ACP band in fraction 25 separates into a doublet. **Lane 1**: SDS 7 markers; **Lane 2**: Freeze thaw extract (6 μL); **Lane 3**: Fraction 20; **Lane 4**: Fraction 21; **Lane 5**: Fraction 22; **Lane 6**: Fraction 23; **Lane 7**: Fraction 24; **Lane 8**: Fraction 25.

5.2.2.4 Overexpressed rape ACP is not correctly post translationally modified

A malonyl CoA exchange reaction (Majerus *et al.*, 1969) was performed on the overexpressed rape ACP, using purified *E. coli* ACP as a positive control (Figure, 10) The recombinant rape ACP showed little stimulation of CO_2 fixation compared to the *E. coli* ACP. The purified *E. coli* ACP was active confirming that the fraction A was active. The result of this assay suggest that the recombinant rape ACP is either unstable or is not post-translationally modified with the 4'-phosphopantetheine prosthetic group required for esterifiication of the radiolabeled malonyl CoA.

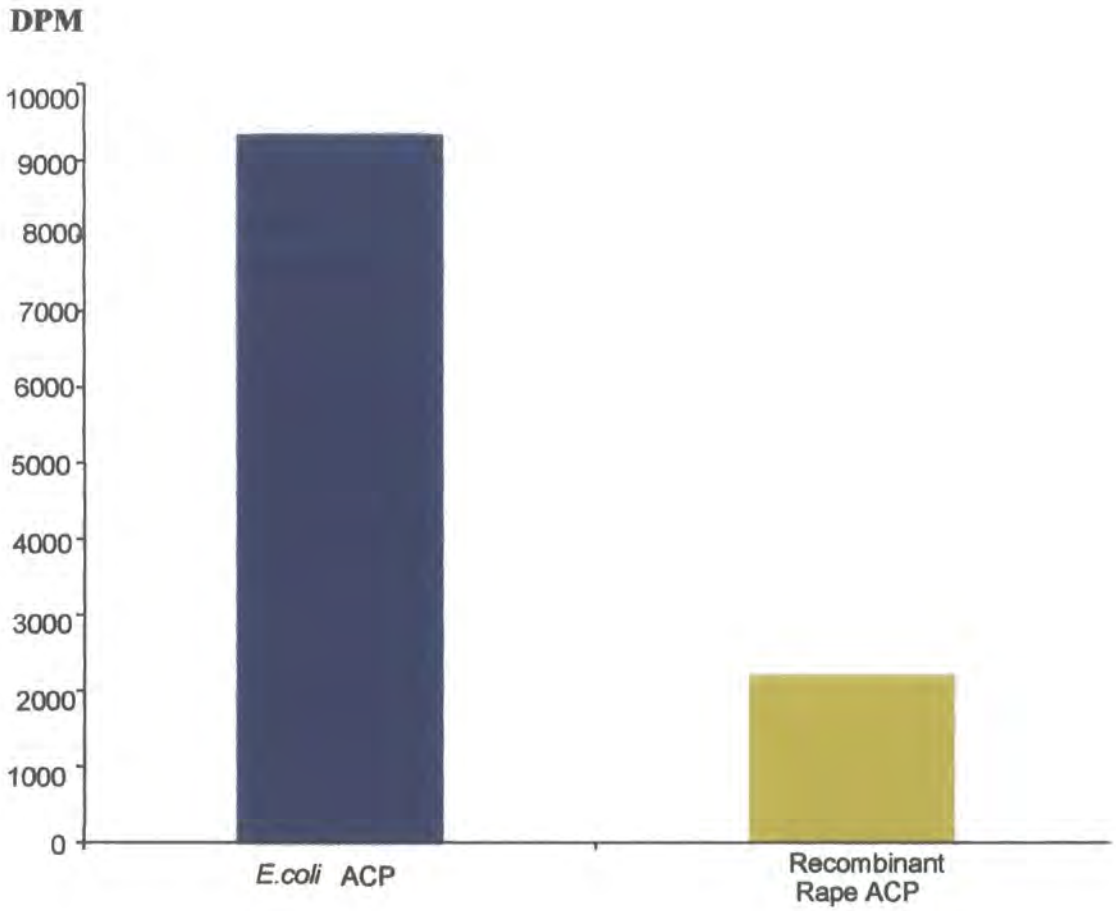


Figure 10: Malonyl CoA exchange assay on recombinant rape on ACP. 1 μg of both recombinant rape ACP, and purified *E. coli* ACP was incubated with 5 mM DTT for 30 min, prior to addition to a malonyl CoA exchange reaction mixture (Majerus *et al.*, 1969). The *E. coli* ACP exchange CO_2 into acid precipitable counts (blue), while the overexpressed rape ACP (yellow) showed little activity.

5.2.2.5 Confirmation recombinant rape ACP is not correctly post-translationally modified

1.5 mg of rape ACP was buffer exchanged into water, using a PD-10 desalting column (Pharmacia). This desalted ACP was applied to an electro-spray mass spectrometer (Professor Jim Staunton's lab, Cambridge). Only one major mass ion was visible in the correct molecular weight region (Figure 11), this corresponds to the predicted molecular weight for apo ACP (9117.8 Da). This result confirms the recombinant rape ACP is not post-translationally modified when overexpressed in *E. coli*. There are three possible hypotheses to explain why this hasn't occurred. Normally

the ratio of CoA to apo ACP inside the *E. coli* cell is 8:1 (Jackowski and Rock, 1981). When the rape ACP is overexpressed in *E. coli* the balance of this equilibrium is disturbed, and as CoA is an important metabolite, this may lead to CoA levels becoming limiting. A second hypothesis is that the pantethylation of ACP progresses through exchange of the prosthetic group from CoA to ACP, catalyzed by the enzyme holo ACP synthase (Brown, 1959; Lambalot and Walsh, 1995). The holo ACP synthase maybe specific for *E. coli* ACP and be inactive with rape ACP. Thirdly there may not be sufficient active holo ACP synthase to cope with the demand from this increased pool of apo ACP. Holo ACP could be synthesized from apo ACP using coenzyme A and holo ACP synthetase *in vitro*.

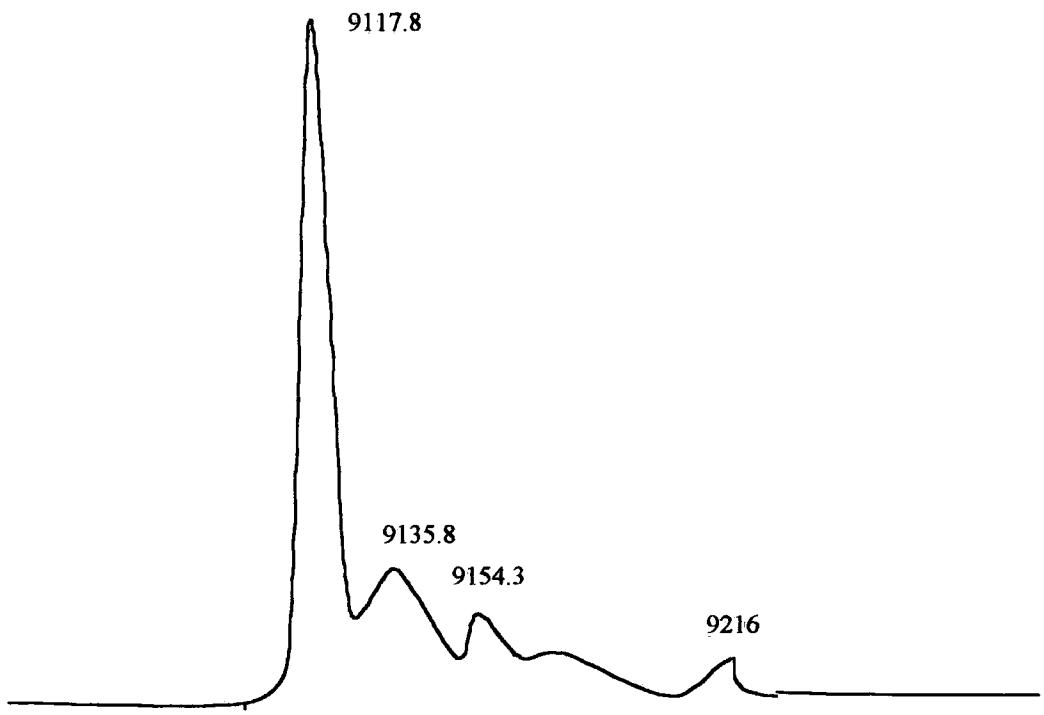


Figure 11: Electro-spray mass spectrogram of purified recombinant rape ACP. Purified recombinant rape ACP was desalted into water using a PD-10 desalting column. This desalted ACP was applied to an electron spray mass spectrometer (work carried out by Philip Sheldon, Cambridge). Only one major mass ion was visible in the correct molecular weight range, this corresponds to the correct molecular weight for apo ACP (9117.8 Da).

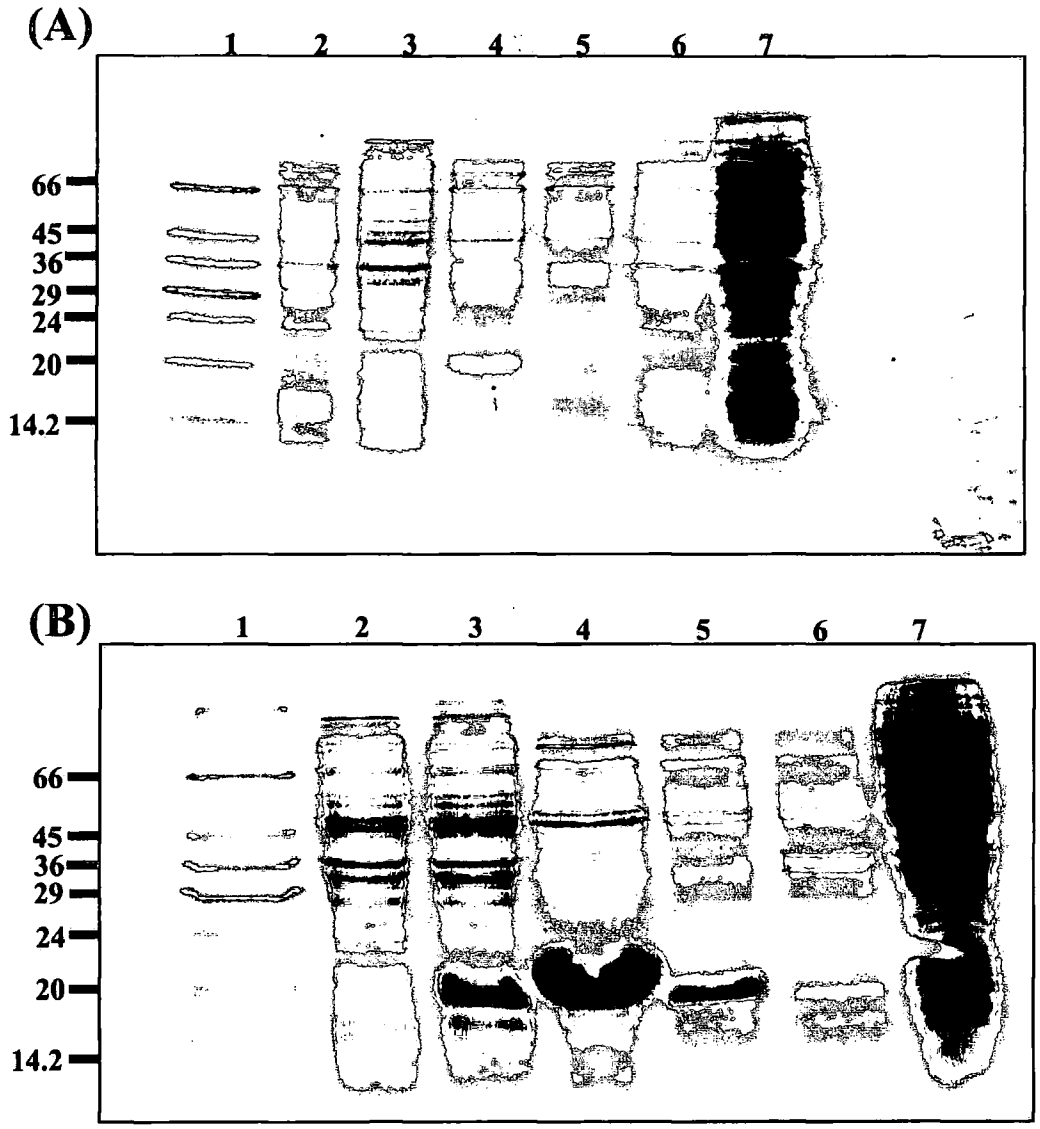


Figure 12: Overexpression of recombinant ACP. The overexpression vectors pRACP and pEACP were induced by the method developed by Dr A.R. Stutjie (Frei University of Amsterdam). (A) **Lane 1**: SDS 7 markers; **Lane 2**: pEACP non-induced; **Lane 3**: pEACP induced; **Lane 4**: pEACP freeze thaw extract (supernatant); **Lane 5**: pEACP Sonicated extract (supernatant); **Lane 6**: pEACP Sonicated pellet; **Lane 7**: pEACP freeze thaw pellet. (B) **Lane 1**:SDS 7 markers; **Lane 2**: pRACP non-induced; **Lane 3**: pRACP induced; **Lane 4**: pRACP freeze thaw extract (supernatant); **Lane 5**: pRACP sonicated extract (supernatant); **Lane 6**: pRACP sonicated Pellet; **Lane 7**: Freeze thaw

5.2.3.2 Confirmation that the induced band is recombinant *E. coli* ACP

The freeze thaw extract from the induced pEACP cells was loaded onto a SDS-PAGE sequencing gel. The gel was transferred to a PDVF membrane, and Ponceau S

stained, the excess Ponceau S stain was removed by washing the membrane with copious quantities of TBS:Tween 20. The induced band was excised with a razor blade and applied to a 477A pulsed gas phase sequencer (Applied Biosystems, sequencing carried out by John Gilroy). The N-terminal amino acid sequence of the induced band gave the sequence STIEERVKK. This corresponds to the deduced amino sequence from the *acp*P gene (Rawlings and Cronan, 1992), with the exception that the initiation methionine had been removed in the majority of the recombinant ACP. As the second amino acid is a serine which in prokaryotes is non-protecting and so allows the removal of the initiating methionine (Flinta *et al.*, 1986).

5.2.3.3 Purification of recombinant *E. coli* ACP

Recombinant *E. coli* ACP was purified using the method developed for recombinant rape ACP (Figure 13). 4 μL from every fourth fraction was pooled, and the pooled fractions loaded onto a 15 % SDS-PAGE gel (Figure, 14). The distinctive ACP band could be seen in fractions 37-40. The degree of purity was tested by loading 16 μL from each of the four ACP containing fractions onto a 15 % SDS-PAGE gel (Data not shown). The recombinant *E. coli* ACP could be seen in all four fractions. The four were pooled and dialysed into ACP storage buffer and flash frozen in liquid nitrogen and stored at -80 °C until required.

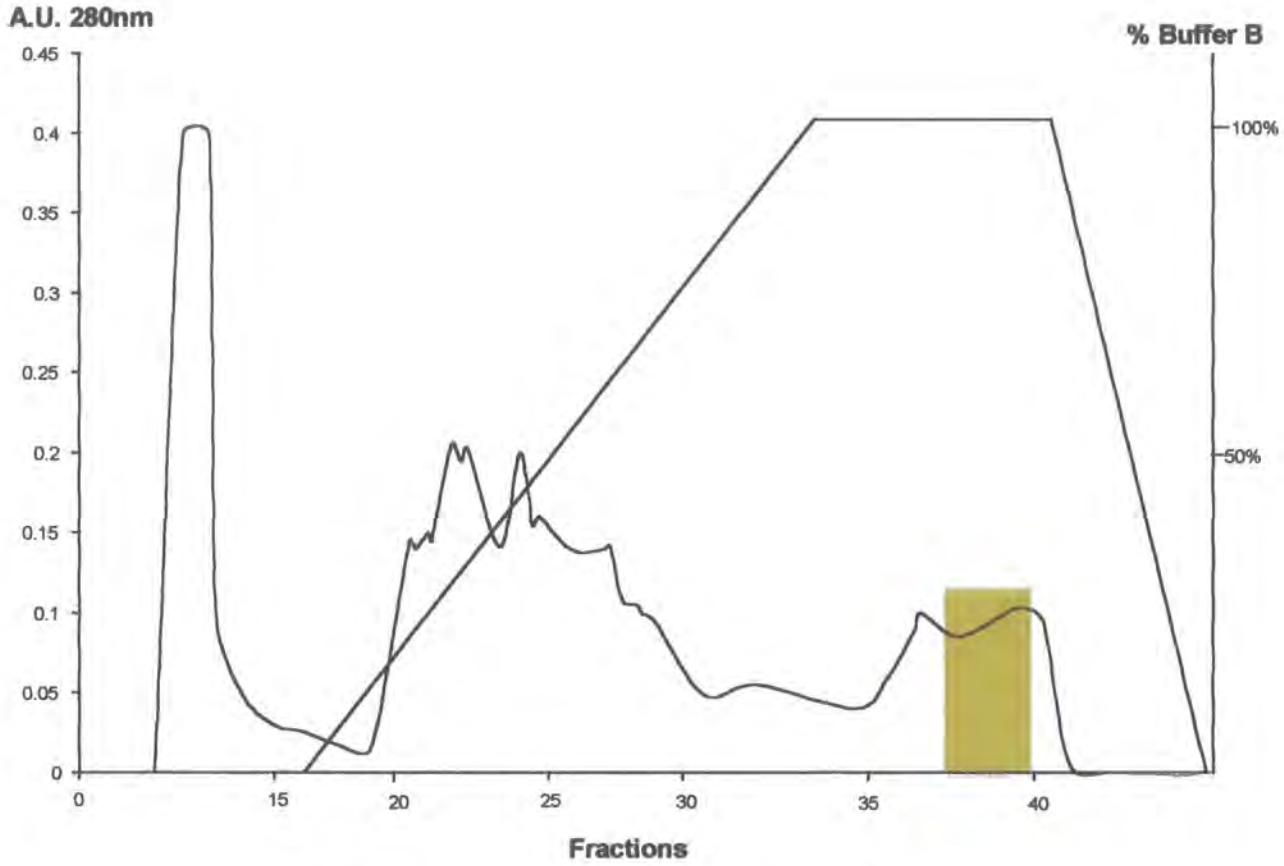


Figure 13: Purification of recombinant *E. coli* ACP. A freeze thaw extract made from overexpressed *E. coli* ACP was loaded onto a 55 mL Q-Sepharose 5/5 (Pharmacia) Column. The column had been equilibrated in 10 mM potassium phosphate buffer pH 6.2, containing 0.1 % β-mercaptoethanol prior to loading of the sample. The subsequent elution was carried out with a linear gradient of 0-100 % buffer B (Buffer A containing 0.5 M LiCl) over thirty column volumes. The yellow area marks the elution point of the recombinant ACP as determined by SDS-PAGE.

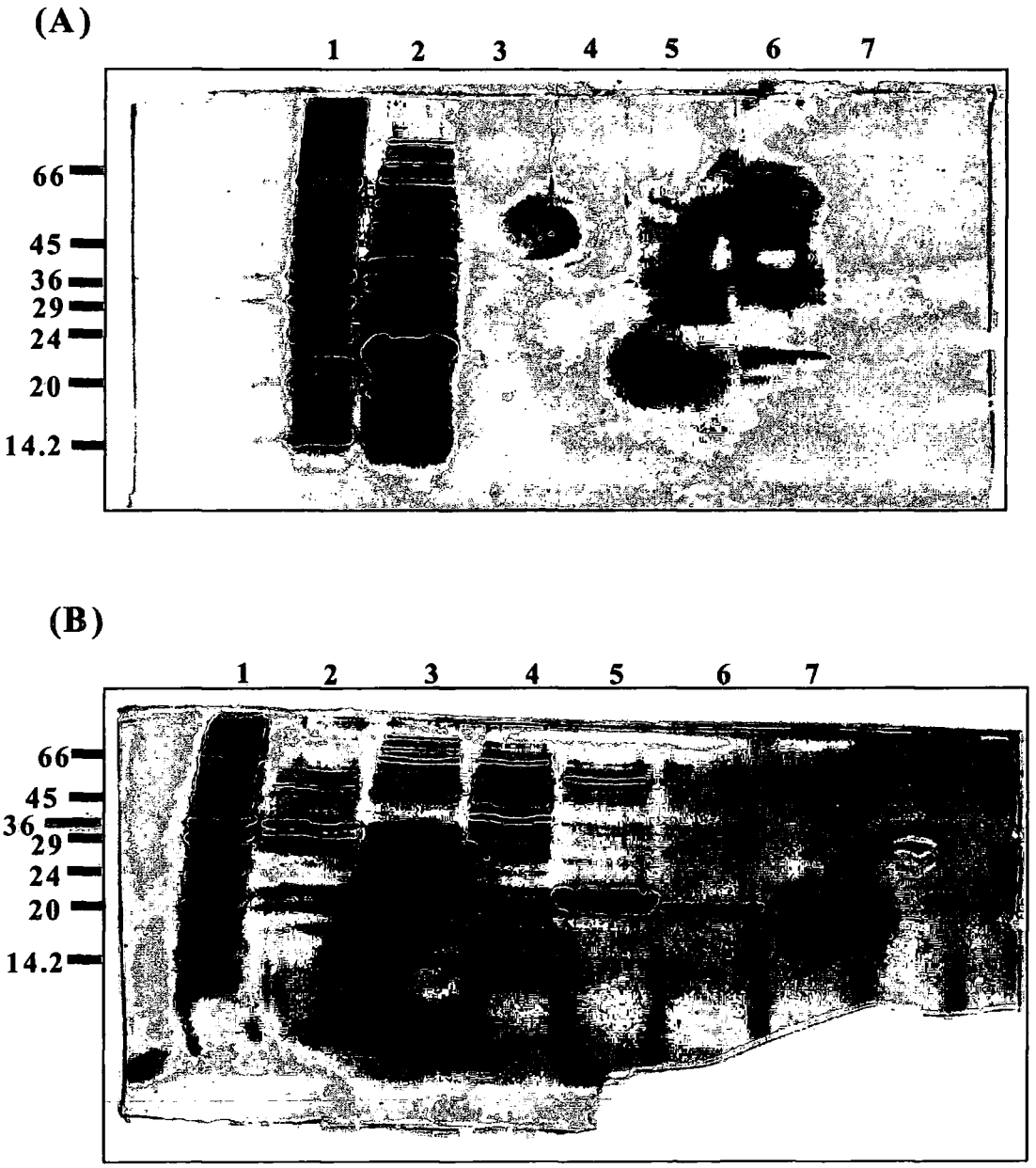


Figure 14: Purification of recombinant *E. coli* ACP. 4 μL was removed from every fourth fraction and then pooled; the pooled fractions were loaded onto a 15 % SDS-PAGE gel. The distinctive ACP band could be seen located in fractions 37-40. **(A) Lane 1**: SDS 7 markers; **Lane 2**: Freeze thaw extract; **Lane 3**: Fraction 1-4; **Lane 4**: Fractions 5-8; **Lane 5**: Fractions 9-12; **Lane 6**: Fractions 13-16; **Lane 7**: Fractions 17-20; **(B) Lane 1:** Fractions 21-24; **Lane 2**: Fractions 25-28; **Lane 3**: Fractions 29-32; **Lane 4**: Fractions 33-36; **Lane 5**: Fractions 37-40; **Lane 6**: Fractions 41-44; **Lane 7**: Fractions 45-48.

5.2.4 Cloning of Holo ACP Synthase (HAS) from *E. coli*

PCR primers SDHAS1 5' GCG CCA TAT GGC AAT ATT AGG TTT AGG C 3' and SDHAS2 5' GCG CGA ATT CCT TCG GGG GTT TTA CTT TTT 3' were

designed to the *dpj* sequence present in the NCBI database (Accession Number AE000343). The 5' primer was designed to contain a *Nde*I site while the 3' primer contained an *Eco*RI site which are required for cloning into the multiple cloning site of pET24a. The ideal annealing temperature for this set of primers was optimized using a Stratagene Robocycler with XL1Blue *E. coli* colonies as template. A band of the predicted size (419 bp) was visible in temperatures below 60 °C, with the optimum annealing temperature being 54 °C (Data not shown). The PCR was repeated in a Perkin-Elmer thermocycler, using standard PCR conditions with an annealing temperature of 54 °C, a single band of 419 bp was amplified (Data not shown). The amplified product was cloned into the PCR cloning vector pGEM-T (Promega), and the ligated vector transformed into XL1Blue *E. coli* and plated out onto LB plates containing ampicillin plus both IPTG and X-gal. Four white colonies had the plasmid prepared and the presence of the cloned PCR product confirmed by an *Eco*R1 digest. Glycerol stocks were made from one of the positive clones and this was designated pHAS1. The *dpj* gene from pHAS1 was removed by digestion with the enzymes present in the PCR primers (*Nde*I and *Eco*R1), and sub-cloned into pET24a. The presence of the insert was confirmed by digestion with both *Nde*I and *Eco*R1 and by sequencing of the insert using the pET T7 promoter and T7 terminator primers. This sequence strategy allowed both strands to be completely sequenced. The obtained nucleotide sequence was identical to the *dpj* sequence present in the database. A glycerol stock was made, and the plasmid designated pEHAS.

5.2.4.1 Induction of recombinant holo ACP synthase

BL21 (DE3) *E. coli* carrying the pEHAS was induced with 0.5 mM IPTG and both the non-induced and induced cells loaded onto a 15 % SDS-PAGE gel. (Figure, 15). A band of the correct size (14 kDa) was induced upon the addition of IPTG; this band was not present in the non-induced cells. The apparent molecular weight of the induced band from the SDS-PAGE gel is the same as the predicted gene product from the *dpj* gene i.e. 14 kDa. 3 mL of the induced culture was then freeze thaw extracted, and the released supernatant loaded onto a 15 % SDS-PAGE gel along with the remaining pellet (Data not shown). In this case approximately 75 % of the induced holo ACP synthase remained inside the cells after the freeze thaw extraction. Thus, freeze thaw extraction

was deemed not to be an appropriate extraction method for the preparation of recombinant holo ACP synthase.

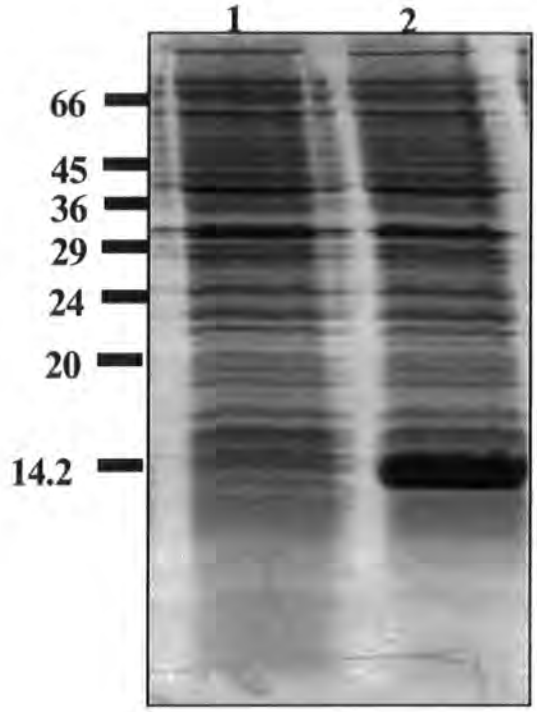


Figure 15: Induction of recombinant *E. coli* holo ACP synthase. *E. coli* cells containing pEHAS induce a band of the predicted molecular weight (14 kDa) upon the addition of IPTG. **Lane 1**: Non-induced cells **Lane 2**: Induced cells.

5.2.4.2 Purification of recombinant *E. coli* holo ACP synthase

The induction of pEHAS was repeated on a 100 mL scale and the cells broken in a cell disrupter (Constant systems). The supernatant was applied to a S-Sepahrose 5/5 column (Pharmacia) and Subsequent elution was achieved with a linear gradient of 0-100 % buffer B (buffer A containing 1 M NaCl) over 30 column volumes (Figure 16). 1 mL fractions were collected from the start of the gradient until the end. Only two peaks were visible on the chromatogram a small peak at the base of a much larger peak. 8 μL was removed from fractions immediately surrounding these two peaks and loaded onto a 15 % SDS-PAGE gel (Figure 17). A band corresponding to holo ACP synthase band (14 kDa) can be seen in the fractions 4, 5 and 6. Fraction 4 corresponds to the small peak

present at the foot of the large peak on the chromatogram (Figure 16). Fraction 5 contains the majority of the recombinant holo ACP synthase, this fraction was dialyzed into buffer A, then flash frozen in liquid nitrogen, in 100 μL aliquots, and stored at -80 °C until required.

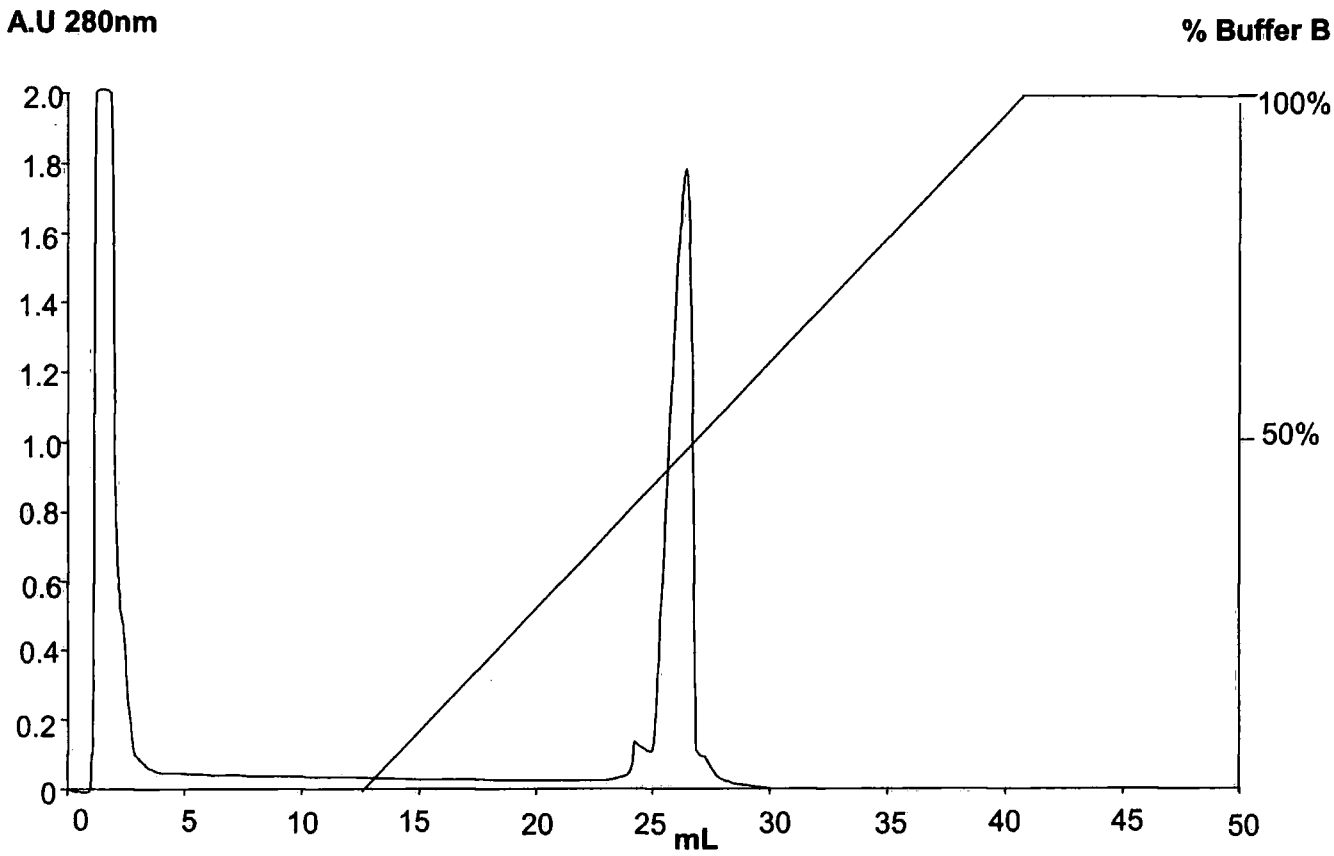


Figure 16: Purification of recombinant holo ACP synthase. 1 mL of crude extract was loaded onto a mono S 5/5 column (Pharmacia), which had been equilibrated in 50 mM Tris.HCl pH 8.0 containing 10 mM $MgCl_2$ and 1 mM DTT (Buffer A). Subsequent elution was carried out with a linear gradient of 0-100 % buffer B (buffer A containing 1 M NaCl) over thirty column volumes. Only two protein peaks were visible during the elution step.

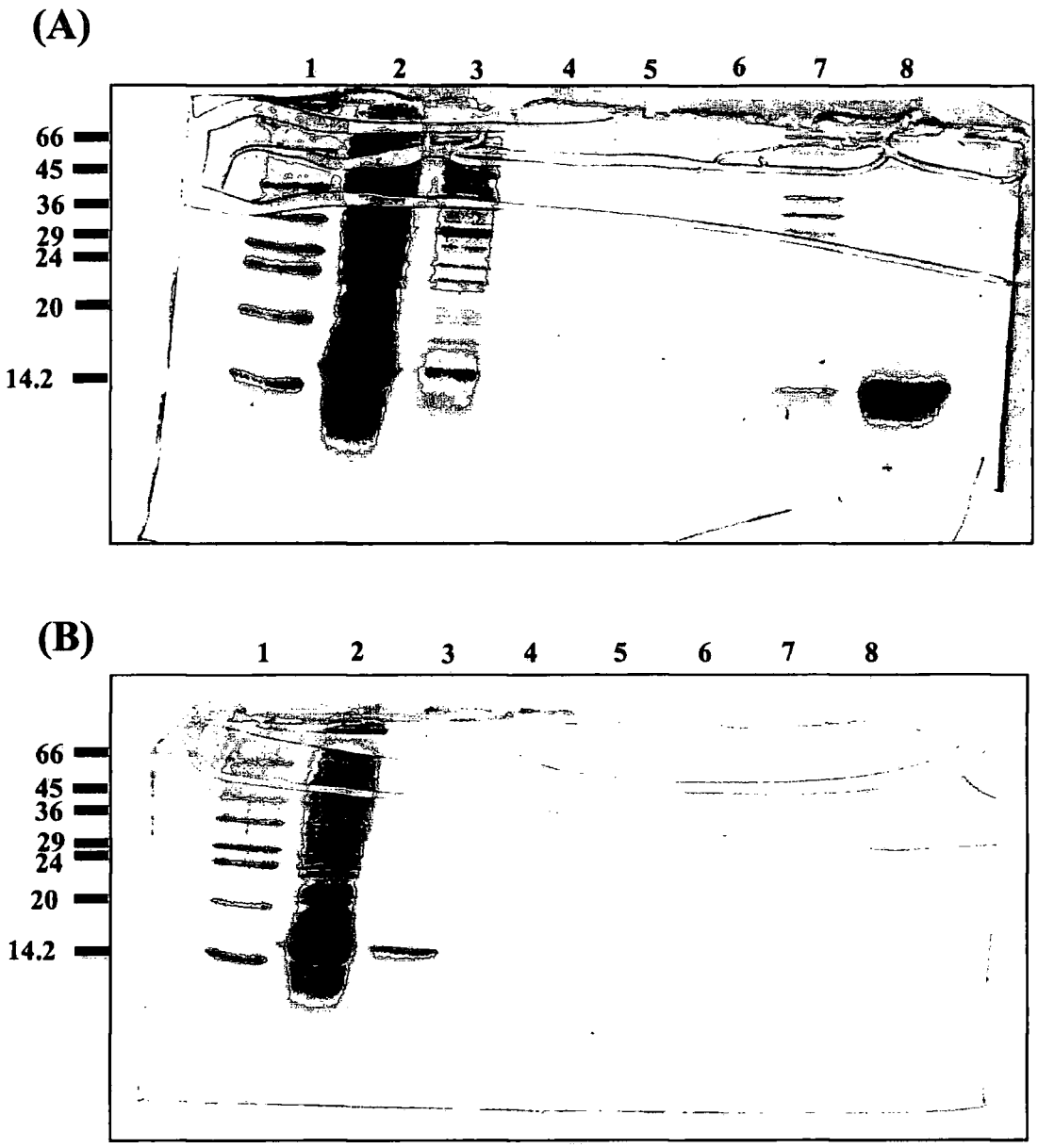


Figure 17: Purification of recombinant holo ACP Synthase. The fractions immediately surrounding the two peaks were loaded onto a 15 % SDS-PAGE gel. The recombinant holo ACP synthase can be seen located in fraction 4, 5 and 6 with the majority found in fraction 5. **(A) Lane 1**: SDS 7 markers; **Lane 2**: holo ACP synthase crude extract; **Lane 3**: Column wash through; **Lane 4**: Fraction 1; **Lane 5**: Fraction 2; **Lane 6**: Fraction 3; **Lane 7**: Fraction 4; **Lane 8**: Fraction 5. **(B) Lane** 1: SDS 7 markers; **Lane 2**: HAS crude extract; **Lane 3**: Fraction 6; **Lane 4**: Fraction 7; **Lane 5**: Fraction 8; **Lane 6**: Fraction 9; **Lane 7**: Fraction 10; **Lane 8**: Fraction 11.

5.2.5 Post-translation modification of recombinant ACPs

Both recombinant rape and *E. coli* ACP have been purified to homogeneity; neither is post-translationally modified with 4'-phosphopantetheine. The pantethylating

enzyme holo ACP synthase has also been overexpressed and purified. Using recombinant holo ACP synthase enzyme and the two recombinant ACPs as substrates, post-translation modification can be attempted *in vitro*. The *in vitro* modification of recombinant ACP was achieved by using the method developed by Dr A.R. Stutjie. 16 μL of the conversion reaction was loaded onto a 0.5 M urea conformational gel, along with an equivalent amount of recombinant apo ACP. (Figure, 18). Both the recombinant apo *E. coli* and rape ACP, run as a single band. After incubation with recombinant ACP in an excess of CoA the position of the single band has shifted to further down the gel, this corresponds to holo ACP. In the case of *E. coli* ACP the same shift of mobility can also be seen, but here a small percentage remains as apo ACP

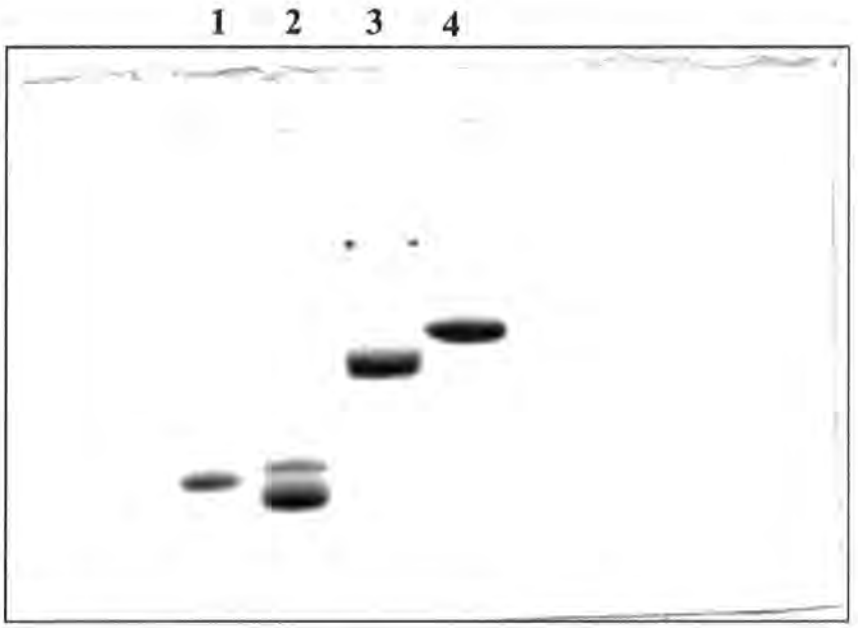


Figure 18: *in vitro* modification of recombinant *E. coli* and rape ACP. 16 μL from both *in vitro* modification reactions were loaded onto a 0.5 M Urea conformational gel, along with recombinant apo rape and *E. coli* ACP. The apo ACP contain only a single band, After incubating with an excess of CoA in the presence of recombinant holo ACP synthase, the mobility position of the band shifts gel to a gel position equivalent to that of holo ACP. **Lane 1**: Recombinant *E. coli* ACP; **Lane 2**: *in vitro* modified *E. coli* ACP; **Lane 3:** *in vitro* modified rape ACP; **Lane 4**: Recombinant rape ACP.

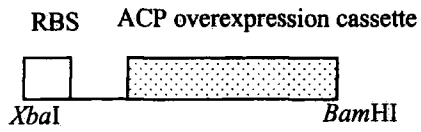
5.2.5.2 Co-expression of holo ACP synthase and recombinant ACP

The *in vitro* modification of apo ACP requires the purification of the substrate ACP as well as recombinant holo ACP synthase, which can be time consuming. An alternative method would be to try and encourage the *E. coli* cell to undertake the post-translation modification. This may be accomplished by co-expression of both the holo ACP synthase and the recombinant ACP in the same cell. This is a method that has been successfully employed to produce synthetic spinach holo ACP-I (Broadwater and Fox, 1999). The *E. coli* holo ACP synthase (kanamycin resistant) and the recombinant rape ACP (ampicillin resistant) have been sub-cloned into overexpression vectors bearing different antibiotic selection, so co-transformation of the two plasmids into one *E. coli* may be a valid method to obtain the desired co-expression. The overexpression plasmid bearing both the *E. coli* and rape ACP open reading frames have shown inherent plasmid instability. This plasmid instability maybe attenuated by the presence of a second overexpression vector present in the same cell. The method chosen was that previously used by Broadwater and Fox (Broadwater and Fox, 1999). These co-workers co-expressed both the holo ACP synthase and the recombinant ACP by placing both open reading frames in an oxerexpression vector.

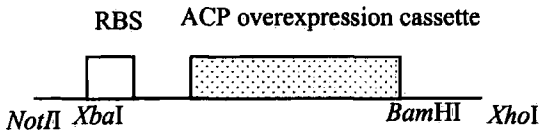
5.2.5.3 Construction of the dual overexpression vector

The ACP cassette (open reading frames plus the ribosome binding site) form both pRACP and pEACP were excised with *Xba*I and *Bam*HI and sub-clone into pBluescript. The presence of the cassettes was confirmed by restriction digest. The sub-clones were designated pRACP2 and pEACP2. The ACP overexpression cassettes were excised form pRACP2 and pEACP2 using *Xho*I and *Not*I and then sub-cloned downstream of the holo ACP synthase expression cassette in pEHAS (Figure 19). The dual overexpression constructs were designated were designated pRACP3 (Rape) and pEACP3 (*E. coli*).

Step 1: The ACP overexpression cassette is excised for pET24a



Step 2: The ACP overexpression cassette is sub-cloned into pBluescript



Step 3: The ACP overexpression cassette is subcloned downstream of holo ACP synthase in pEHAS

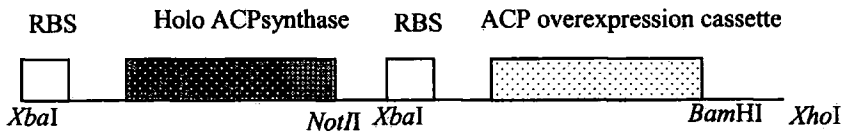


Figure 19: Sub-cloning strategy for creating the dual overexpressing constructs. The ACP overexpression cassettes from pEACP and pRACP are first sub-cloned into pBluescript, and then sub-cloned downstream of holo ACP synthase in pEHAS to form the plasmids pEACP3 and pRACP3.

5.2.5.4 Induction of the dual overexpression vectors pOESD6 and pOESD7

Both pEACP3 and pRACP3 were induced using the pET overexpression system.. The no-induced and induced cells were loaded onto a 15 % SDS-PAGE gel (Data not shown). The distinct ACP band could be seen in both the pEACP and pRACP induced lanes. But a band corresponding to the recombinant holo ACP synthase band was not visible in the induced cells (Data not shown).

To determine if the co-expression had changed the degree of post translation modification of the recombinant ACP, inductions of pRACP, pEACP, pRACP3 and pRACP3 were carried out on a 50 mL scale, then freeze thaw extracted 16 μL of the freeze thaw supernatant was loaded onto a 0.5 M Urea conformational gel (Figure 20). The *E. coli* and rape ACP obtained from pRACP and pEACP are mainly in the apo form. The ACP extracted from pRACP3 and pEACP3 contained both bands corresponding to apo and holo ACP, but the proportion of holo to apo ACP had been increased in both the *E. coli* and rape ACP. The co-expression of holo ACP synthase with either *E. coli* or rape ACP increases the post-translation modification of the recombinant ACP. In both cases not all of the available ACP pool is post-translationally modified and a significant pool of apo ACP remains. The apo ACP would have to be removed from the purified recombinant ACP, as apo ACP may be an inhibitor of β-hydroxyacyl ACP dehydratase by competing for ACP binding sites on the enzyme. The most viable option for producing holo ACP is by *in vitro* modification with holo ACP synthase.

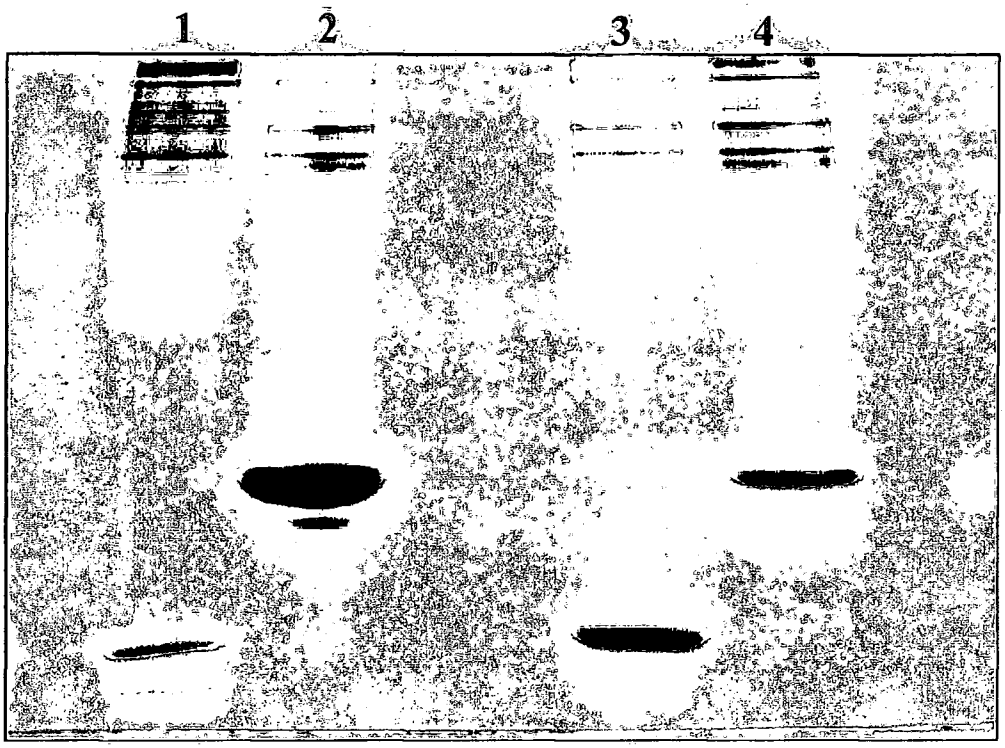


Figure 20: Urea conformational gels on freeze thaw extracts made from dual overexpression constructs. The degree of post-translation modification of ACP extracted from the dual overexpressing cells was tested by loading freeze thaw extracts from induced pRACP (rape ACP), pEACP (*E. coli* ACP), pRACP3 (rape ACP/HAS) and pEACP3 (*E. coli* ACP/HAS) onto a 0.5 M urea conformational gel. **Lane 1**: *E. coli* ACP/HAS; Lane 2: Rape ACP; Lane 3: *E. coli* ACP; Lane 4: Rape ACP/HAS.

5.3 Discussion

In this chapter are described methods for the production of *E. coli* and rape holo ACP a required substrate for the assay of β-hydroxyacyl ACP dehydratase. Previous dehydratase assays (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982; Mohan *et al.*, 1995) have used holo ACP purified from *E. coli* (Majerus *et al.*, 1969; Rock and Cronan, 1980). Holo ACP has been purified from *E. coli* by a modified version of the method of Majerus *et al.* (Majerus *et al.*, 1969). This method exploits a number of physical attributes of the ACP to produce an enriched fraction, consisting of up to 2% of the total protein (Majerus *et al.*, 1969). From this enriched ACP fraction, a single step ion

exchange chromatography column rapidly purifies the ACP. Majerus *et al.*, reported a total yield of ACP (holo and apo) of 116 mg per Kg of *E. coli.* This was later reported as being an exceptionally high yield (Rock and Cronan, 1980), with the usual yield being between 60-80 mg. Using the slightly modified version, a yield of between 75-85 mg per Kg was achieved, this compares favourably with the previously reported yield. The purified ACP was present as 80-90 % holo ACP, as determined by urea confirmation gel analysis. It was observed that the yield of ACP and degree of post-translation modification were dependent on the speed of isolation. In particular the initial defrosting step, if large 250 gram lumps were allowed to defrost overnight in buffer, then the yield of holo ACP was greatly reduced when compared to lumps which had been shattered into tiny pieces and allowed to defrost quickly.

Rape holo ACP cannot be purified directly from *Brassica napus*, as it is unstable and quickly loses its activity (Slabas *et al.*, 1987). An alternative source would be to use recombinant ACP. In this chapter are also described methods for the overexpression, purification and *in vitro* modification of recombinant rape ACP. This is not the first expression of a recombinant ACP in *E. coli.* ACP's have been overexpressed from a variety of sources (Bridges *et al.*, 1991; Morris *et al.*, 1993; Cox *et al.*, 1997; Tropf *et al.*, 1998) these also include the expression of a synthetic ACP-I gene from spinach (Beremend *et al.*, 1987; Broadwater and Fox, 1999). Beremend *et al.* synthesised a gene using a plant codon bias; and achieved a yield of recombinant ACP of 1-6 mg per litre of culture. However this was all present in the holo form. Broadwater and Fox synthesised a similar synthetic ACP-I gene but with codons biased towards high expression in *E. coli* (Broadwater and Fox, 1999). Here a yield of 70 mgs of ACP per litre of culture was achieved, but only 15 % were present in the holo form. This data seems to suggest that there is a correlation between expression levels and degree of post-translation modification. In the case of the recombinant rape ACP the yield is low 10 mg per litre of culture, this doesn't accurately represent the expression level. For ease of purification the extraction method chosen was repetitive rounds of freeze thawing (Johnson and Hecht, 1994). This leads to a significant proportion of the recombinant ACP remaining trapped in the *E. coli* cells, and not available for further purification. The true expression level is high (ca 15 % total protein), and correspondingly the degree of post-translation modification is low so a false picture of the degree of post-translation modification of the recombinant rape ACP may have been obtained during this work. During the purification of the recombinant rape ACP it was observed that the ACP band present in the fractions

corresponding to the rear of the ACP elution peak separated into a doublet. These fractions were discarded and not applied to the electron spray mass spectrometer. Both bands bear the distinctive appearance of ACP when run on SDS-PAGE. It has been previously observed that the mobility of holo and apo ACP differs on SDS-PAGE (Rock and Cronan, 1980). One of the bands present fractions may correspond to the holo form that has been separated from the majority of the apo ACP form by the ion exchange chromatography step. This may explain why no mass ion corresponding to holo ACP was identified by electron spray mass spectroscopy as the holo ACP had been separated from the apo form and discarded and not applied to the mass spectrometer.

The ACP can be modified *in vitro* to produce a supply of holo ACP. The reaction involves incubating the recombinant ACP (apo) with an excess of CoA, in the presence of recombinant holo ACP synthase. This can be used to supply holo ACP for the synthesis of *trans*-2-enoyl rape ACP, the complete substrate for β-hydroxyacyl ACP dehydratase. This correctly post-translation modified rape ACP can also be used as the substrate for a reconstituted type II fatty acid synthetase from *Brassica napus*. The co-expression of recombinant ACP with holo ACP synthase increases the pool of holo ACP. But leaves a significant pool of apo ACP remaining in the cell which would need to be separated from the holo ACP if this is to be used as a source of material for substrate synthesis. A similar result was obtained with the co-expression of the spinach ACP-I isoform with holo ACP synthase (Broadwater and Fox, 1999). In these experiments the co-workers found that co-expression increased the level of post-translation modification from 15% to 40% under normal induction conditions. Performing the induction in a fermenter using controlled oxygen and pH levels, the level of holo ACP was increased to 95%. If these conditions were applied to the recombinant rape and *E. coli* co-expression systems a similar increase in the level of holo ACP may also be obtained

Additionally recombinant ACP can be as a source of material for structural studies. The solution structure of *E. coli* ACP has been determined by NMR studies (Kim and Prestegaard, 1991), but no X-ray structure has yet to be determined. The amino acid sequence of spinach ACP-I can be mapped over the NMR structure of *E. coli* ACP, this confirms that both ACPs have a similar tertiary structure (Kuo and Ohlrogge, 1986). It has been recently determined that a requirement for the formation of ACP crystals, requires both the 4'-phosphopantetheine group to be present plus derivitisation of the thiol group with an acyl moiety (Personal communication Professor Toni Slabas). To solve the 3D structure a suitable defracting heavy metal derivatives of the crystals need

to be formed. The overexpression system for recombinant ACP allows the manipulation of the amino acid composition by mutagenesis to produce a crystals with built in sites for heavy metal derivitisation i.e. the addition of additional cysteine residues. Or the induction can be performed in a methionine deficient strain of *E. coli* with selenomethionine in the growth medium to produce selenomethionine derivatives.

Chapter 6:

Synthesis of substrates for β-hydroxyacyl ACP dehydratase assay

6.1 Introduction

β-hydroxacyl ACP dehydratase catalyses the removal of a molecule of water from β-hydroxyacyl ACP to from *trans*-2-enoyl ACP (Figure 1). β-hydroxyacyl ACP dehydratase is a stereospecific enzyme as β-hydroxyacyl ACP is the only isomer which can act as a substrate and the only product formed by the reaction is *trans*-2-enoyl ACP. At pH 8.0 the equilibrium for the reaction lies 5:1 in favour of the reverse reaction (Shimakata and Stumpf, 1982; Birge and Vagelos, 1972).

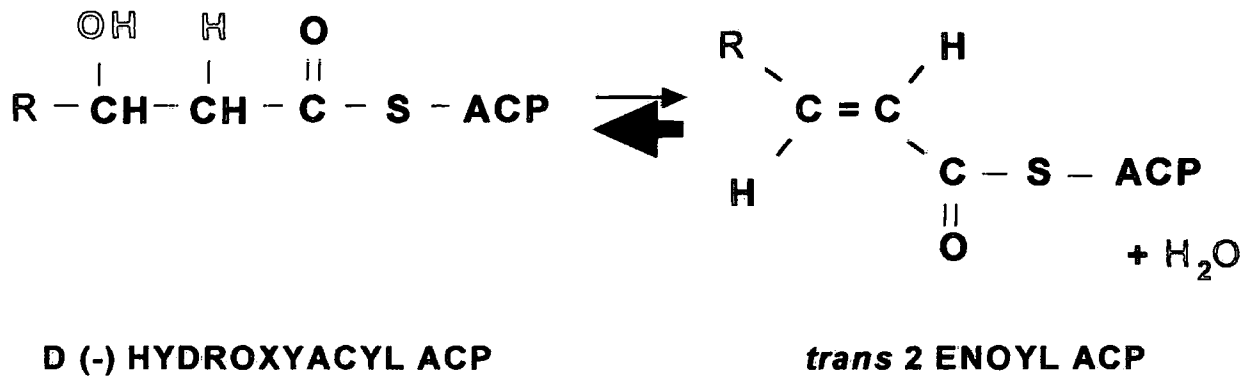


Figure 1: The reaction catalysed by β-hydroxacyl ACP dehydratase. β-hydroxacyl ACP dehydratase catalyses the removal of a molecule of water from β-hydroxyacyl ACP to from *trans*-2-enoyl ACP.

β-hydroxyacyl ACP dehydratase has been purified to homogeneity from spinach leaves (Shimakata and Stumpf, 1982) and the activity has also been partially purified from *E. coli* (Birge and Vagelos, 1972). The β-hydroxyacyl ACP dehydratase purified from spinach leaves was inactive with crotonyl CoA and similarly the partially purified β-hydroyacyl ACP dehydratase from *E. coli* is inactive with both crotonyl CoA and crotonyl-N-acylcysteamine. Thus β-hydroxyacyl ACP dehydratase has an absolute requirement for ACP as a component of its substrate.

6.1.1 Synthesis of *trans*-2-enoyl ACP

trans-2-enoyl ACP can be synthesised by a number of different methods. The crotonyl ACP used to purify β-hydroxyacyl ACP dehydratase from both *E. coli* and spinach leaves was synthesised chemically. Here crotonyl ACP was synthesised by using crotonic anhydride as an acyl donor to S-acylate reduced holo ACP (Weeks and Wakil, 1969). Under these conditions S-acylation is not specific for the 4'-phosphopantetheine group of ACP, and a number of other reactive side chains present in the ACP can also become derivatised (Klages and Cronan, 1981). The reaction does not always proceed to completion, leaving behind a pool of unesterified holo ACP. This remaining pool of ACP can potentially act as an inhibitor of β-hydroxyacyl ACP dehydratase by acting as a competitor for ACP binding sites on the enzyme. The non homogeneous state of the crotonyl ACP precludes its use as a substrate for the accurate measurements of kinetic parameters. The remaining unesterified ACP is difficult to remove as the crotonyl side chain lacks sufficient hydrophobic character to allow purification by hydrophobic interaction chromatography.

A second method for chemical synthesis of acyl ACP has been developed by Klages and Cronan (Klages and Cronan, 1981). This method is based on the observation that specific S-acylation of glutathione can be achieved by using N-acylimidazole as an acyl donor in the presence of imidazole base (Jenks and Carrulo, 1959). Using this system Cronan and Klages (Cronan and Klages, 1981) were able to specifically S-acylate reduced holo ACP with a range of saturated and *cis* unsaturated fatty acids.

An enzymatic synthesis method for the synthesis of acyl ACP has been developed by Rock and Cronan (Rock and Cronan, 1979). In this method the ACP fatty acid thioester linkage is formed by the action of the bacterial enzyme acyl ACP synthetase (Cooper *et al.*, 1989). Acyl ACP synthetase catalyses the ligation of a free fatty acid to ACP, in an ATP dependent reaction. Acyl ACP synthetase from, *E. coli* is encoded for by the *aas* gene. The gene product from the *aas* gene shows 2-acylglycerolphospho-ethanolamine acyltransferase activity (Cooper *et al.*, 1989), the acyl ACP synthetase activity is only a partial activity present only in the presence of high salt (Rock and Cronan, 1979). The acylation of ACP catalyzed by acyl ACP synthetase is specific for S-acylation of 4'-phosphopantetheine and so produces an acyl ACP which only contains a single acyl group. Acyl ACP synthetase shows greatest activity with palmitic (C16:0) and stearic (C18:0) fatty acids as these are the usual products of the *E. coli* fatty acid

synthetase (Rock and Cronan, 1979). Acyl ACP synthetase does have activity with other long chain fatty acids (C10-C14) albeit at a reduced level. Acyl ACP synthetase is inactive with short chain fatty acids (C4-C8), and shows low level activity with unsaturated fatty acids with the level of activity being equivalent to a saturated acyl chain of two carbons less e.g. *trans*-2-tetradecenoic acid (C14:1) shows equivalent activity to that of lauric acid (C12:0) (Rock and Cronan, 1979). The acyl ACP synthesized using acyl ACP synthetase contain long chain fatty acids which have sufficient hydrophobic character to allow purification of the substrate by hydrophobic interaction chromatography.

6.1.2 β-hydroxyacyl ACP dehydratase assays

The assay used to purify β-hydroxyacyl ACP dehydratase from both *E. coli* and spinach leaves were performed at pH 8.0 using crotonyl ACP as the substrate. The disappearance of the crotonyl ACP is followed spectrophotometrically at 263nm. This is a region of the spectrum where a number of other biological molecules also have absorbance. This high background absorbance makes the measurement of β-hydroxyacyl ACP dehydratase activity in crude extracts difficult to perform using this assay. The crotonyl ACP assay was only performed once the β-hydroxyacyl ACP dehydratase activity had been partially purified from both *E. coli* and spinach leaves (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982).

A second assay which can be used to measure β-hydroxyacyl ACP dehydratase activity in crude extracts by avoiding any spectrophotometric measurements has been developed by Mohan *et al.*, (Mohan *et al.*, 1995). This assay system separates the product of the reaction from the substrate by exploiting the differing mobility of acyl ACP's in native PAGE gels using urea as a denaturant (Post-Bettinemiller *et al.*, 1991). Using this assay Mohan *et al.*, were able to assign β-hydroxymyristoyl ACP dehydratase activity to the recombinant *fabZ* gene product. Mohan *et al.*, used (3*R*)-hydroxymyristoyl ACP synthesized by acyl ACP synthetase as the substrate and were able to separate (2*E*)-tetadecenoyl ACP from (3*R*)-hydroxymyristoyl ACP on urea native PAGE gels. The assay is performed at pH 8.0, and follows the reaction in the forward direction. It has been shown that at pH 8.0 the equilibrium for the reaction lies 5:1 in favor of the reverse reaction (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982), but conversion of

(3*R*)-hydroxymyristoyl ACP to (2*E*)-tetadecenoyl can still be visualized using these assay conditions.

The majority of the recombinant SD4 protein is insoluble with only a small percentage remaining in the soluble fraction. The insoluble SD4 protein has been solubilized using urea but both recombinant proteins are present in crude *E. coli* cell extracts. The Mohan *et al.*, assay system was chosen as the best assay system to use as this can be performed on crude extracts (Mohan *et al.*, 1995) with the exception that the assay would be performed using at pH 8.0 using (2*E*)-tetradecenoyl ACP as the substrate, thus measuring the reaction under kinetically favorable conditions.

6.2 Results

6.2.1 Synthesis of (2*E*)-tetradecenoyl ACP

(2*E*)-tetradecenoic acid is not available commercially, but the free fatty acid was obtained as a lyophilized powder from Dr. Pat Steele (Durham University, Chemistry department). (2*E*)-tetradecenoic acid was dissolved in 10% Triton X-100, and heated to 40 °C to ensure complete solubilization of the fatty acid. (2*E*)-tetradecenoyl ACP was synthesised using partially purified acyl ACP synthetase, obtained from Dr. Ted Shirerer. The acyl ACP synthetase is prepared from microsomes isolated from an *E. coli* strain containing the *aas* gene driven by a heat shock inducible promoter which overexpresses acyl ACP synthetase activity five fold over its normal level (Personnal communication Dr Ted Shirerer). The acyl ACP synthetase was partially purified from isolated microsomes as per the method developed by Rock and Cronan (Rock and Cronan, 1979a). The acyl ACP synthetase purified by this method does not contain any acyl ACP thioesterase activity. This is essential as any residual acyl ACP thioesterase activity present in the acyl ACP synthetase preparation would lead to a reduced yield of acyl ACP, as acyl ACP thioesterase can hydrolyze acyl ACP.

Initially a test synthesis was performed, 16 µL removed from the synthesis and loaded onto a 2.5 M urea conformational gel, along with an equivalent amount of reduced ACP (3.84 µg), plus a 2.5 % dilution of the acyl ACP synthetase (Figure 2). In the reduced ACP lane are two bands which correspond to the two forms of ACP (holo and apo). In the acyl ACP synthetase lane no bands could be seen on the gel at this dilution. The sample removed from the synthesis reaction also contains the two bands that correspond to holo and apo ACP, and also present is a band that has similar mobility

to that of (2*E*)-tetradecenoyl ACP (Mohan *et al.*, 1995). This (2*E*)-tetradecenoyl ACP is a product of the synthesis reaction as the band is not present in either the ACP or the acyl ACP synthetase prior to their addition to the synthesis reaction. The presence of the bands corresponding to holo and apo ACP show the reaction has not proceeded to completion, this is not unexpected as (2*E*)-tetradecenoic acid is not a natural substrate for acyl ACP synthetase (Rock and Cronan, 1979). As unesterified ACP may potentially act as an inhibitor of β-hydroxyacyl ACP dehydratase activity then the synthesized (2*E*)-tetradecenoyl ACP needs to be purified if it is to act as a substrate for β-hydroxyacyl ACP dehydratase.

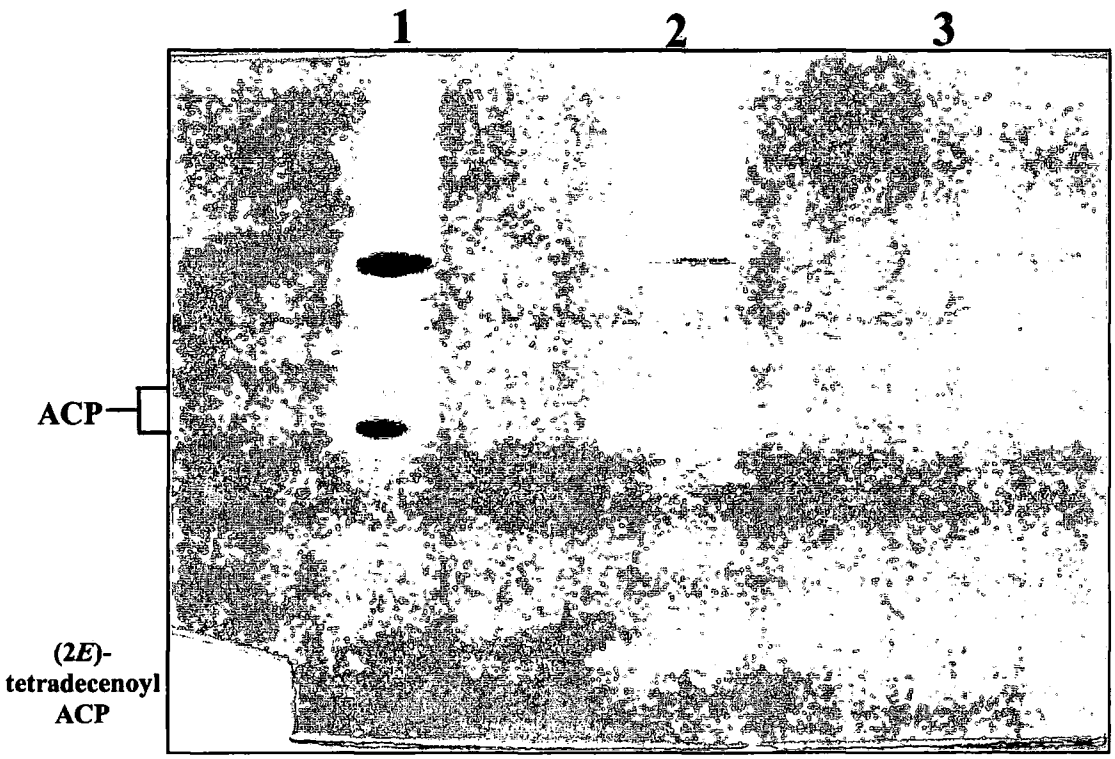


Figure 2: Synthesis of (2*E*)-tetradecenoyl ACP. (2*E*)-tetradecenoyl ACP was synthesised by incubating (2*E*) tetradecenoic acid with reduced ACP in the presence of partially purified acyl ACP synthetase. After 48 hours the reaction contains a band which has similar mobility to that of (2*E*)-tetradecenoyl ACP (Mohan *et al.*, 1995), the reaction also contains two bands which corresponds to holo and apo ACP. The (2*E*)-tetradecenoyl ACP band is a product of the reaction as the band was not present in either the ACP or the acyl ACP synthetase prior to addition to the synthesis reaction. **Lane 1**: *E. coli* ACP (3.84 μg); **Lane 2**: (2*E*)-tetradecenoyl ACP synthesis (3.84 μg); **Lane 3**: Acyl ACP synthetase (2.5 %).

6.2.2 Purification of (2*E*)-tetradecenoyl ACP

The major difference between (2*E*)-tetradecenoyl ACP and the unreacted ACP is the hydrophobic (2*E*)-tetradecenoyl fatty acid group esterified to the ACP. This difference can be exploited to purify (2*E*)-tetradecenoyl ACP by hydrophobic interaction chromatography (Rock and Cronan, 1979; Hellyer *et al.*, 1992). The reaction mixture used to synthesise the (2*E*)-tetradecenoyl ACP contains two hydrophobic compounds which could interfere with any hydrophobic chromatography step i.e. triton X-100 and (2*E*)-tetradecenoic acid. These compounds need to be removed from the (2*E*)-tetradecenoyl ACP before any hydrophobic interaction chromatography can be attempted. Thus the first step in the purification of (2*E*)-tetradecenoyl ACP is to purify the total pool of ACP which includes the (2*E*)-tetradecenoyl ACP from the reaction mixture.

6.2.2.1 Synthesis of [^{3}H] palimitoyl ACP

To be able to follow the purification of (2*E*)-tetradecenoyl ACP a radiolabeled acyl ACP with similar biophysical properties too (2*E*)-tetradecenoyl ACP was synthesized. The efficiency of the synthesis was tested by removing 100 µL from the palmitoyl ACP synthesis and extracting the unreacted fatty acid as for an acyl ACP synthetase assayA total of 556,640 DPM were incorporated into ACP. This represents a total of 93.7 % of the available radiolabeled palmitic acid being esterified to ACP.

6.2.2.2 Purification of the total ACP pool from the synthesis cocktail

The synthesis reaction was diluted 1 in a 100 and 15µg (approximately 100,000 DPM) of radiolabeled palmitoyl ACP was added as a tracer to follow the purification of the (2*E*)-tetradecenoyl ACP, then loaded onto a Q-Sepharose column. 1ml fractions were collected from every stage and then 20 µL was removed from each fraction and counted for 1 min (Figure 3). The radiolabeled unesterified palmitic acid ran straight through the column, this constitutes the majority of the added counts. During washing of the column to remove both the Triton X-100 and isopropanol only a few counts are lost. The radiolabeled palmitoyl ACP elutes from the column upon the addition of 0.5 M LiCl. Of the total counts loaded 48.91 % were recovered during the elution step. As both palmitic acid and palmitoyl ACP have similar biophysical properties to (2*E*)-

tetradecenoic acid and (2*E*)-tetradecenoyl ACP respectively then the elution profile of these compounds are thought to be comparable.

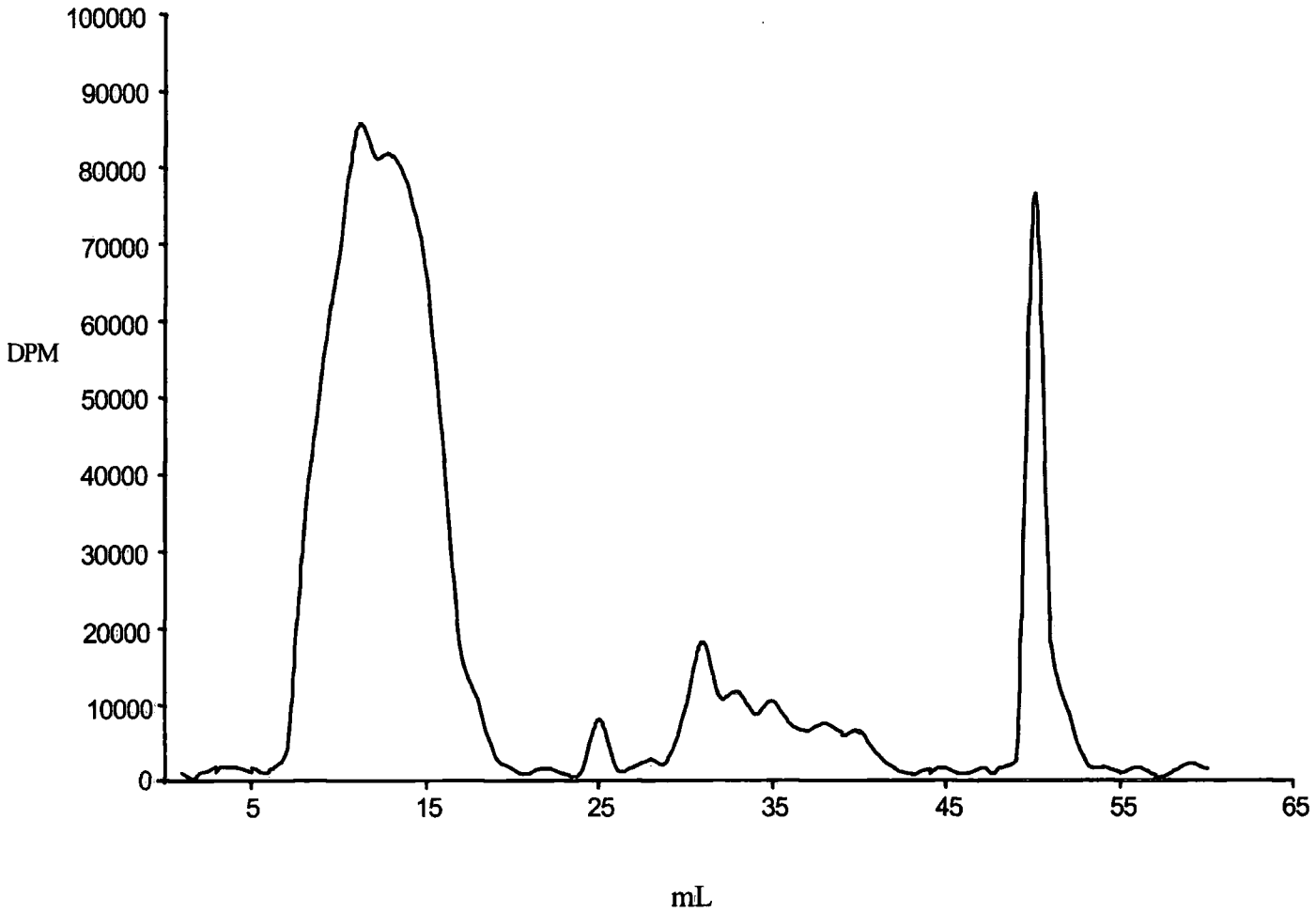


Figure 3: Purification of the total ACP pool from the reaction cocktail. 1 mL of a (2*E*)-tetradecenoyl ACP synthesis reaction was spiked with radiolabeled palmitoyl ACP and applied to a 1mL high trap Q-sepahrose column. The vast majority of the counts pass straight through the column. The remaining Triton X-100 is removed by first washing with buffer A containing 80 % isopropanol and is then washed again with buffer A. The total ACP pool is step eluted with 0.5 M LiCl. A total of 48.91 % of all the total added counts were recovered.

6.2.2.3 Purification of (2*E*)-tetradecenoyl ACP using octyl Sepharose

The eluted fractions form the Q-Sepharose column was loaded onto a 10 mL octyl Sepharose C4LB column. 1mL fractions were collected and 20 μL was removed from each fraction and and counted for 1 minute (Figure 4). No palmitoyl ACP was lost during the buffer exchange into NEMA, and all of the palmitoyl ACP elutes upon the addition of 30% isopropanol with 81.77 % of the added counts being recovered.

To confirm that that the unreacted ACP had been removed and also to confirm that the elution profile of palmitoyl ACP corresponds to that of (2*E*)-tetradecenoyl ACP. 8μl from the pooled palmitoyl ACP containing fractions were loaded onto a 2.5M Urea conformational gel (Figure, 5). The band corresponding to (2*E*)-tetradecenoyl ACP can be seen located in the eluents from both the Q-Sepharose and octyl Sepharose columns, this confirms that the elution profiles of (2*E*)-tetradecenoyl ACP and palmitoyl ACP do co-inside. The two bands corresponding to holo and apo ACP are present in the eluent off the Q-Sepharose column but are not present in the octyl Sepharose eluent. However there is a second band present in the octyl Sepharose eluent, which potentially corresponds to an oligomerization of the (2*E*)-tetradecenoyl ACP

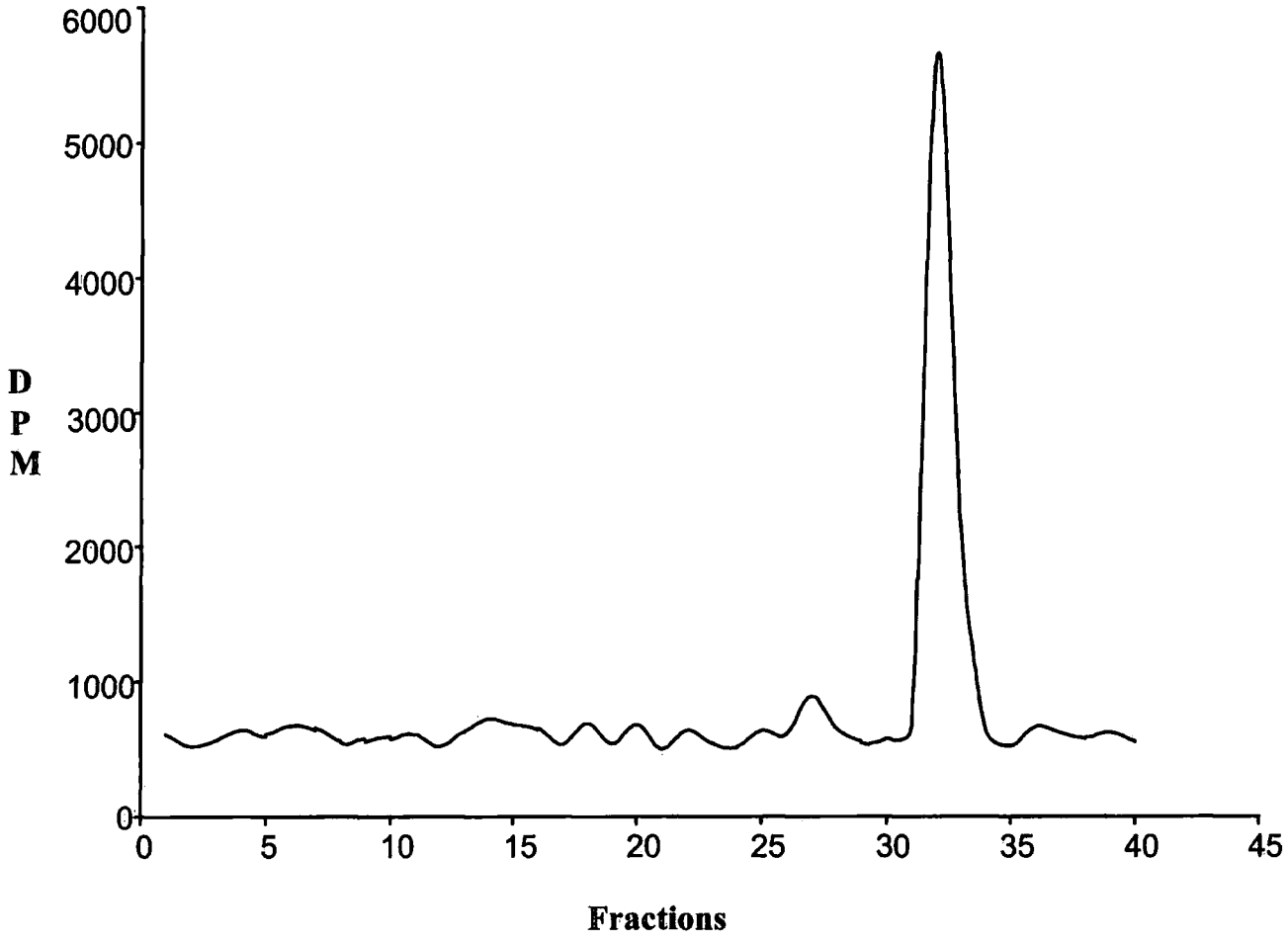


Figure 4: Purification of (2*E*)-tetradecenoyl ACP using octyl Sepharose C4-LB column. The palmitoyl ACP containing fractions form the Q-Sepharose column were pooled and applied to a octly Sepharose C4-LB column. The palmitoyl ACP binds to the column and the column is washed with the volatile buffer 20 mM NEMA pH 7.4 The palmitoyl ACP is eluted with 30 % isopropanol. A total of 81.77 % of the total added counts were recovered.

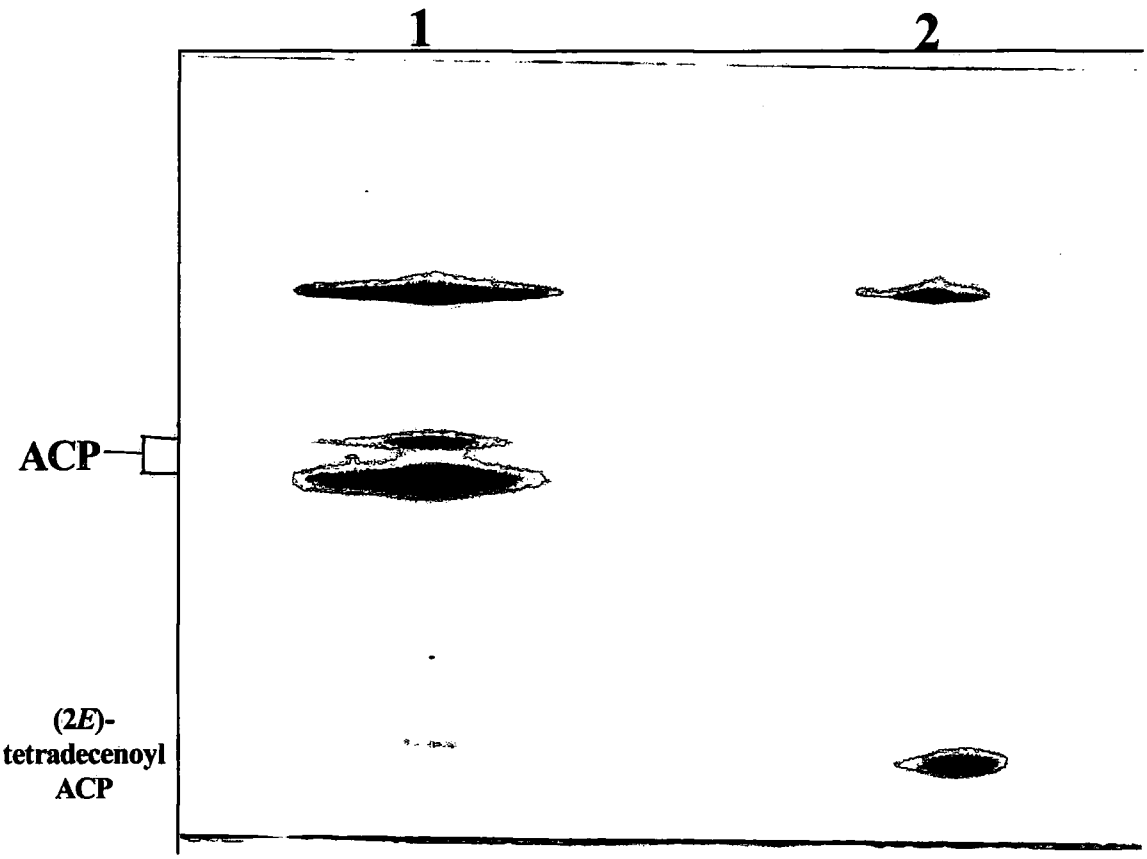


Figure 5: Octyl C4-LB Sepharose purification of (2*E*)-tetradecenoyl ACP. The palmitoyl ACP containing fractions from the Q-Sepharose column were loaded onto an octyl Sepharose C4-LB column. Unbound material was washed through the column with 5 column volumes buffer A. The column was then equilibrated with the volatile buffer 20 mM NEMA pH 7.4, and the palmitoyl ACP step eluted with buffer A containing 30 % isopropanol. **Lane 1:** Pooled radiolabeled palmitoyl ACP containing fractions off the Q-Sepahrose column; **Lane 2**: Pooled radiolabeled palmitoyl ACP containing fractions off octyl Sepharose C4-LB column.

6.2.2.4 Large scale synthesis of (2*E*)-tetradecenoyl ACP

(2*E*)-tetradecenoyl ACP was synthesised using 5 mg of ACP. The acyl ACP synthetase preparation is not homogeneous and still contains a number of other proteins These contaminating proteins potentially may contain enzyme activities which can chemically modify (2*E*)-tetradecenoyl ACP and so affect its viability as a substrate for a β-hydroxyacyl ACP dehydratase assay. The acyl ACP synthetase is prepared from

isolated microsomes using a blue Sepharose column as an affinity purification step. To remove the contaminating proteins the final reaction can be passed through a blue Sepharose fast flow column allowing the previously eluted proteins to re-bind to the blue Sepharose column. This is possible because ACP has no affinity for the blue Sepharose resin and so will flow through the column (Rock and Cronan, 1979). This allows also for the recycling of the acyl ACP synthetase activity as after re-binding to the blue Sepharose resin it can then be re-eluted. The (2*E*)-tetradecenoyl ACP was purified as for the test experiments and the radiolabelled palmitoyl ACP containing fractions were pooled concentrated and then stored at –80 °C until required

6.2.3 Preparation of SD4 extract for assay

Mohan *et al.*, demonstrated that β-hydroxyacyl ACP dehydratase activity could be measured in crude *E. coli* extracts prepared by sonication. BL21 (DE3) *E. coli* carrying the pRfabZ (SD4/pET24a) construcrt and pET24a as a control were induced. cells were broken by sonication . After separating the soluble and insoluble fractions by centrifugation. The pellet from the pRfabZ induced cells was solubilzed as described previously. To confirm the presence of the recombinant SD4 protein the non-induced and induced cells from both pET24a and pRfabZ along with 8 μL of the soluble supernatants plus the urea resolubilized pellet were loaded onto a 15 % PAGE gel (Figure 8). The protein concentration of each of the crude extracts (pET24a, pRfabZ soluble fractions plus the dialyzed solubilized insoluble fraction) were determined by Bradford assay and the concentration adjusted to 1mg/mL with 50 mM Tris.HCl pH 8.0 containing 1 mM DTT. The crude extracts were aliquoted in to 1ml aliquots and flash frozen in liquid nitrogen and stored at –80 °C until required.

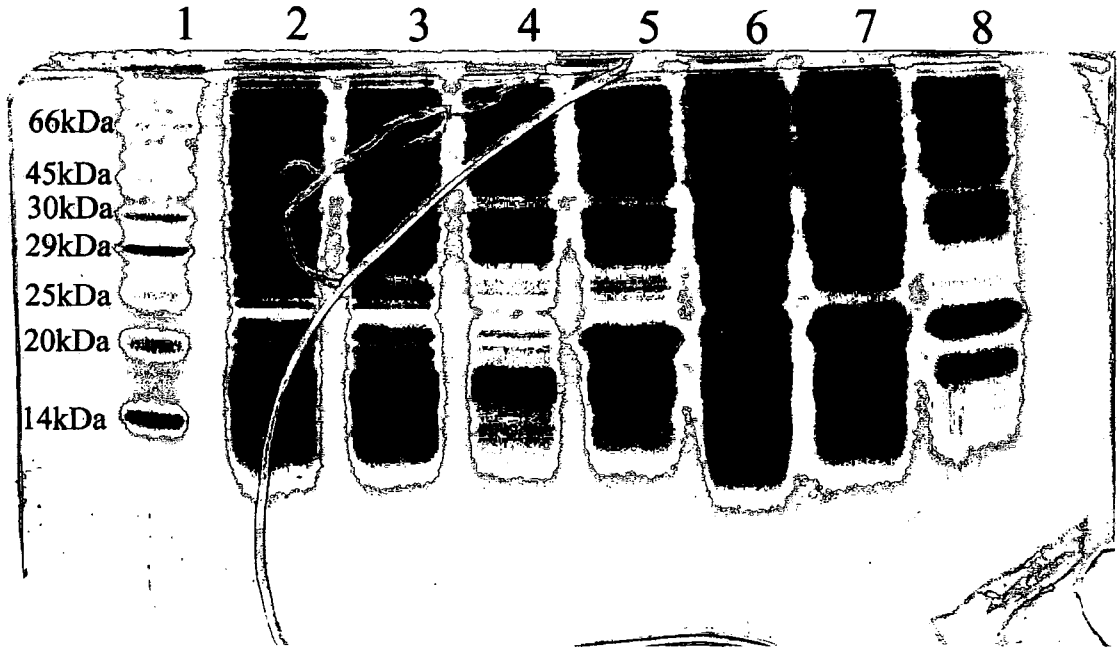


Figure 6: Preparation of induced crude *E. coli* extracts. Both BL21(DE3) *E. coli* bearing either pRfabZ (SD4/pET24a) or pET24a were induced with 0.5 mM IPTG The cells were broken by sonication and cells debris removed by centrifugation at 40,000 g. 8 μL from each of the crude extracts were then loaded onto a 15 % SDS-PAGE gel. The recombinant mature SD4 protein can only be seen in the induced pRfabZ crude extract, but not in the induced pET24a extract. The majority of the recombinant SD4 protein located in the pellet was resolubilized with 8 M urea which was removed by stepwise dialysis. **Lane 1**: SDS 7 markers; **Lane 2**: non-induced pET24a; **Lane 3**: induced pET24a; **Lane 4**: non-induced pRfabZ; **Lane 5**: induced pRfabZ; **Lane 6**: pET24a crude extract; **Lane 7:** pRfabZ crude extract; **Lane 8**: urea resolubilized pRfabZ pellet.

6.2.4 Assay on pET24a extracts with purified (2*E*) tetradecenoyl ACP

To confirm that the (2*E*) tetradecenoyl ACP is an active substrate for β-hydroxyacyl ACP dehydratase, then the (2*E*) tetradecenoyl ACP was used to measure β-hydroxyacyl ACP dehydratase activity *E. coli* crude extracts. The assay conditions used were identical to those described by Mohan *et al.* (Mohan *et al.*, 1995) for use with (3*R*)-

hydroxymyristoyl ACP. Using these conditions the assay is linear for 15 min with a 1mg/mL crude *E. coli* extract.

A series of β-hydroxyacyl ACP dehydratase assays were set up containing varying amounts of a 1mg/mL of induced pET24a crude extract. After the assays were terminated they were immediately loaded onto a 2.5 M urea conformational gel (Figure 9). No shift in mobility of the (2*E*)-tetradecenoyl ACP could be seen for any of the different concentrations of *E. coli* crude extract. This suggests that the synthesized (2*E*)-tetradecenoyl ACP is not acting as a substrate for β-hydroxyacyl ACP dehydratase.

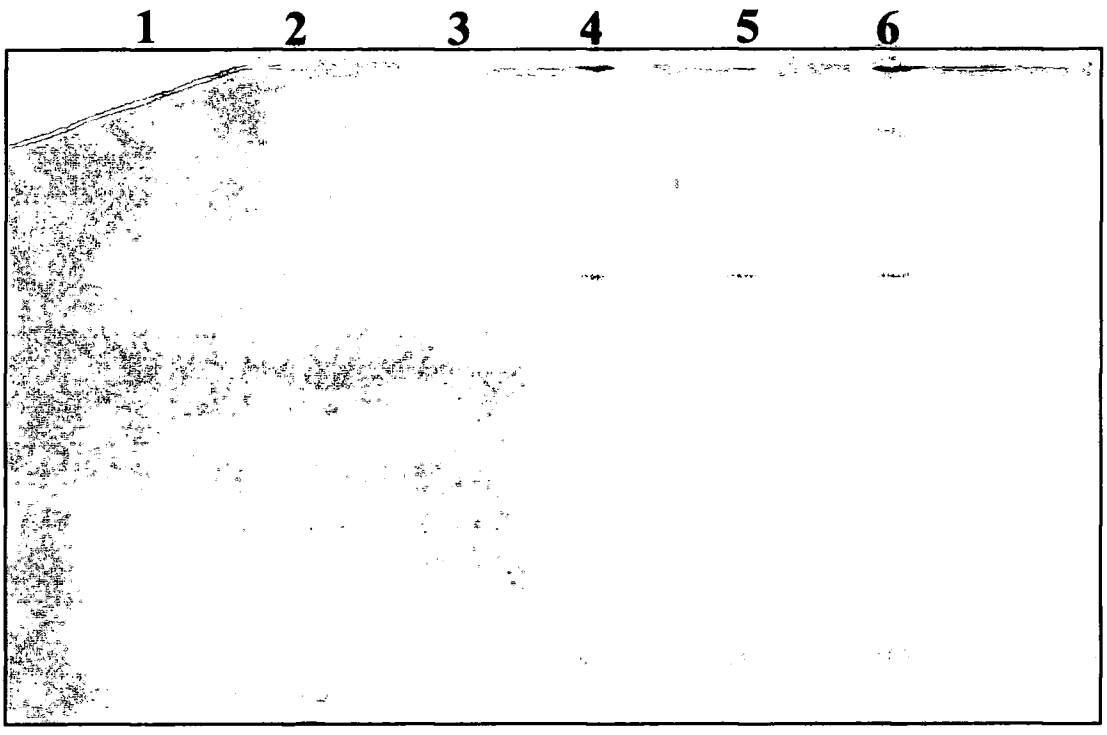


Figure 9: β-hydroxyacyl ACP dehydratase assay on induced pET24a ***E. coli* crude extracts.** A number of assays were set up containing a varying amount of the induced pET24a crude extracts (1mg/mL). Each assay was performed with 2 μL of the (2*E*)-tetradecenoyl ACP at pH 8.0. The assays were incubated at 30 °C for 15 min, and terminated by adding 5X native PAGE loading buffer. Each of the completed assays was then immediately loaded onto a 2.5 M conformational PAGE gel.

6.2.5 Enoyl ACP reductase assay using purified (2*E*) tetradecenoyl ACP

As the purified (2*E*)-tetradecenoyl ACP is inactive with *E. coli* pET24a crude extract when assayed using identical assay conditions as those described by Mohan *et al.* (Mohan *et al.*, 1995) then the integrity of the synthesized substrate is called into question. As *trans*-2-enoyl ACP is a common substrate for both β-hydroxyacyl ACP dehydratase (reverse reaction) and enoyl ACP reductase (forward reaction), then an enoyl ACP reductase assay (Slabas *et al.*, 1986) can be used to check the validity of the purified (2*E*)-tetradecenoyl ACP.

Purified recombinant enoyl ACP reductase was obtained from Dr Tony Fawcett. To confirm that the isolated recombinant enoyl ACP reductase was active a standard enoyl ACP reductase assay was performed (Slabas *et al.*, 1986). An immediate decrease in optical density at 340 nm was observed showing the consumption of NADH and so confirming that the recombinant enoyl ACP reductase is active (Figure 10).

A second reaction was set up containing 20 μL of the purified (2*E*)-tetradecenoyl ACP. The baseline was zeroed and NADH was added followed by 1 μL of the recombinant enoyl ACP reductase. No change in optical density was visualized after 5 minutes. To confirm that no component of the synthesized (2*E*)-tetradecenoyl ACP is inhibitory to enoyl ACP reductase 1.2 mM crotonyl CoA was added to the assay containing the (2*E*)-tetradecenoyl ACP and an immediate drop in optical density at 340 nm was seen. This confirms that the enoyl ACP reductase is still active but is unable to utilize the purified (2*E*)-tetradecenoyl ACP as a substrate. This confirms that the synthesized substrate is not active with either β-hydroxyacyl ACP dehydratase or enoyl ACP reductase. This suggests that the (2*E*)-tetradecenoyl ACP has undergone some modification during the synthesis or purification which is preventing it acting as a substrate for β-hydroxyacyl ACP dehydratase. Mohan *et al.*, used (3*R*)-hydroxymyristoyl ACP as the substrate and not (2*E*)-tetradecenoyl ACP, it was reported that during synthesis the (3*R*)-hydroxyacyl was modified, this problem was overcome by using an acyl ACP synthetase overexpressing strain of *E. coli* as a source of acyl ACP synthetase. As the (2*E*)-tetradecenoyl ACP is more reactive than (3*R*)-hydroxymyristoyl ACP then even though an overexpressing strain was used the substrate is still undergoing chemical modification during the synthesis reaction. If the synthesis and assay was performed using the (3*R*)-hydroxymyristoyl ACP then an active β-hydroxyacyl ACP dehydratase substrate could be purified.

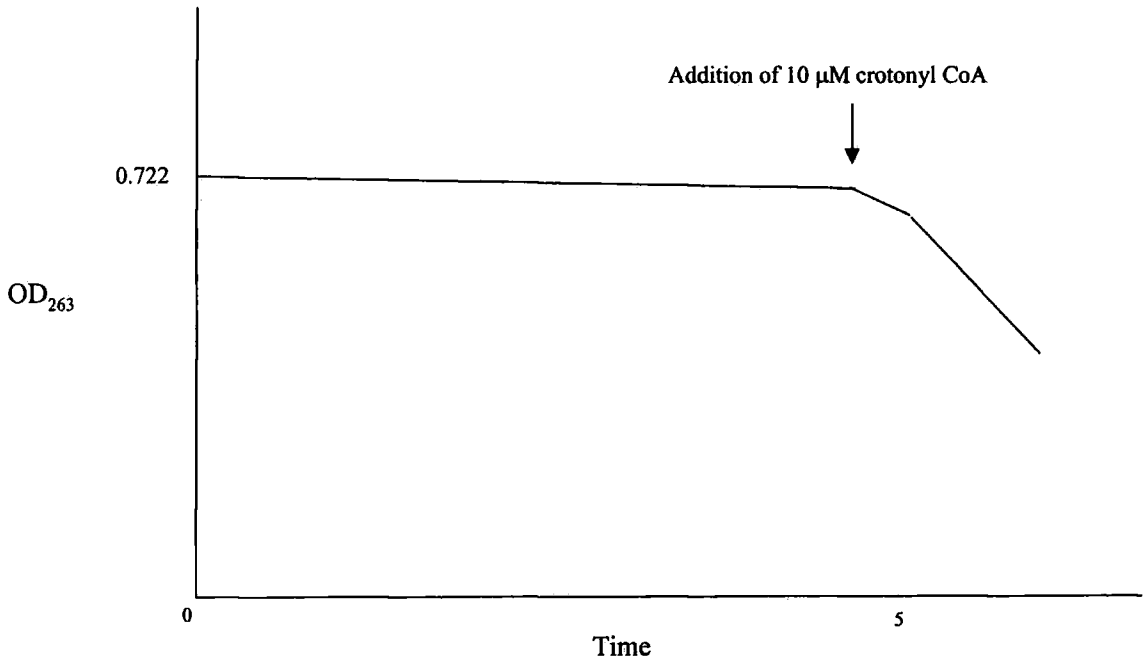


Figure 10: Enoyl ACP reductase assay on synthesised (2*E*)-tetradecenoyl ACP. 1 μL of the synthesised (2*E*)-tetradecenoyl ACP was used in an enoyl ACP reductase assay. There was no decrease in optical density for 5 min after the initiation of the reaction with recombinant enoyl ACP reductase. 10 μM crotonyl ACP was then added and an immediate drop in optical density could be seen confirming that the enoyl ACP reductase was active.

6.2.6 Synthesis of β-hydroxymyristoyl ACP

As for (2*E*)-tetradecenoyl ACP, (3*R*)-hydroxymyristoyl ACP is not available commercially. But, a racemic β-hydroxymyristoic acid was purchased from Larodan Biochem. This compound is racemic and so contains a 50:50 mixture of both the *R* and *S* isomers. It has been shown that the *S* isomer is biologically inactive when used as a substrate for β-hydroxyacyl ACP dehydratase (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982). Thus it was thought the presence of the *S* isomer would not effect the ability of the *R* isomer to act as a substrate for β-hydroxyacyl ACP dehydratase. β-

hydroxymyristoyl ACP was synthesized as the (2*E*)-tetradecenoyl ACP. After the synthesis had been completed 16 μL of the reaction cocktail was loaded on to a 2.5 M urea conformational gel (Figure 11). After a 48 h incubation a band was present in the synthesis reaction that was absent from both the ACP and acyl ACP synthetase prior to the start of the synthesis. This new band has the expected mobility of β-hydroxymyristoyl ACP on a 2.5 M urea conformational gel.

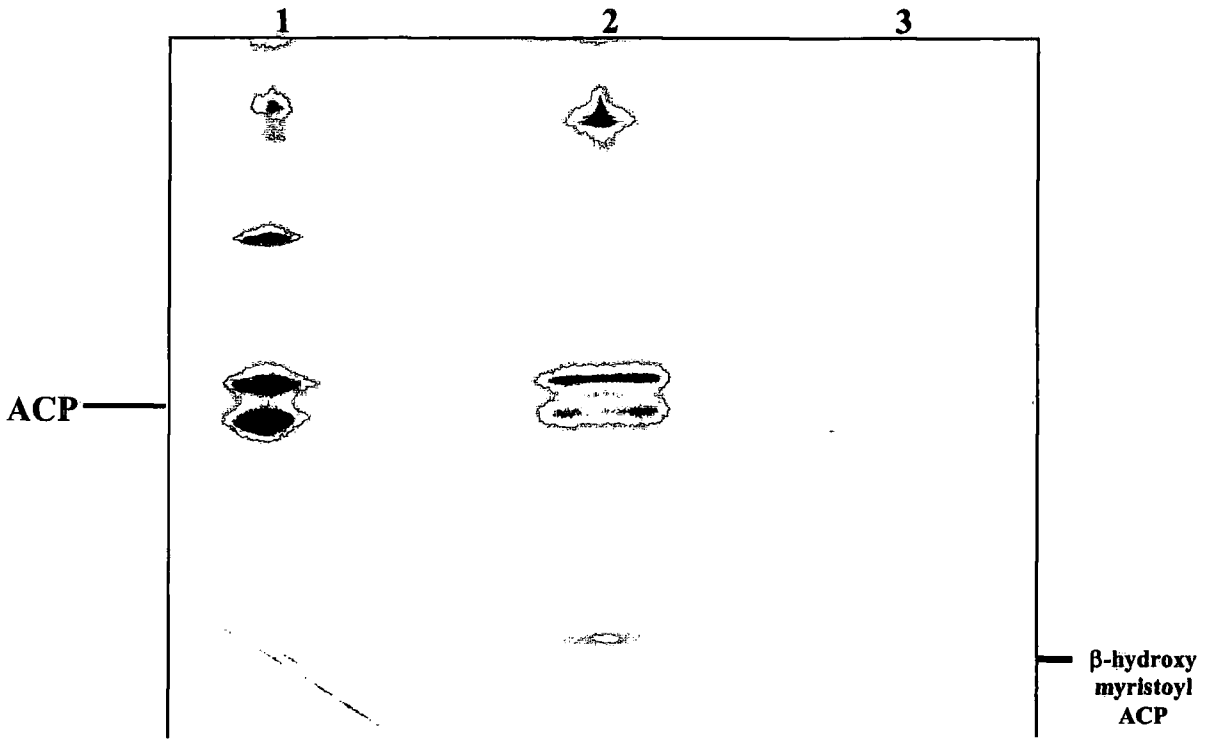


Figure 11: Synthesis of β-hydroxymyristoyl ACP. β-hydroxymyristoyl ACP was synthesized by incubating β-hydroxymyristoic acid with reduced ACP in the presence of partially purified acyl ACP synthetase. After 48 h a band could be seen in the synthesis which is absent from both the ACP and acyl ACP synthetase prior to the start of the synthesis. **Lane 1**: ACP; **Lane 2**: β-hydroxymyristoyl ACP synthesis; **Lane 3**: Acyl ACP synthetase

6.2.7 Purification of β-hydroxymyristoyl ACP

A large scale synthesis and purification of β-hydroxymyristoyl ACP was performed using identical conditions to those used to synthesize and purify (2*E*)-tetradecenoyl ACP. From the octyl-Sepharose column the [H^3] palmitoyl ACP eluted in four fractions (7,8,9 and 10), these fractions were pooled and stored -20 °C until required (Figure 12). The pooled fractions were vacuumed dried to a volume of approximately 75 μL in a vacuum centrifuge (Jouan). 2 μL from the dried sample was loaded onto a 2.5 M urea conformational gel to confirm that the substrate had not become hydrolysed (Data not shown). The vacuum dried β-hydroxymyristoyl ACP was then Bradford assayed and was determined to contain 366.75 μg of ACP. If this is assumed to be all β-

hydroxymyristoyl ACP then as the synthesis was initially performed with 5 mg of ACP a total yield of 7.35 % of β-hydroxymyristoyl ACP was obtained. If the assay conditions of Mohan *et al.*, are followed identically then 50 μM (3*R*)-hydroxymyristoyl ACP is required per assay this equates to 1.5 μg of β-hydroxymyristoyl ACP in a assay volume of 4 μL. But the extent of the activity of acyl ACP synthetase with the *S*-isomer is unknown and so it was assumed that its activity was comparable and so only 50 % of the substrate is (3*R*)-hydroxymyristoyl ACP. Therefore, twice as much substrate is required per assay. The volume of the synthesized substrate was increased to 122.5 μL to give a final concentration of 3 μg/μL. The synthesis and subsequent purification yields sufficient substrate for 61 assays to be performed.

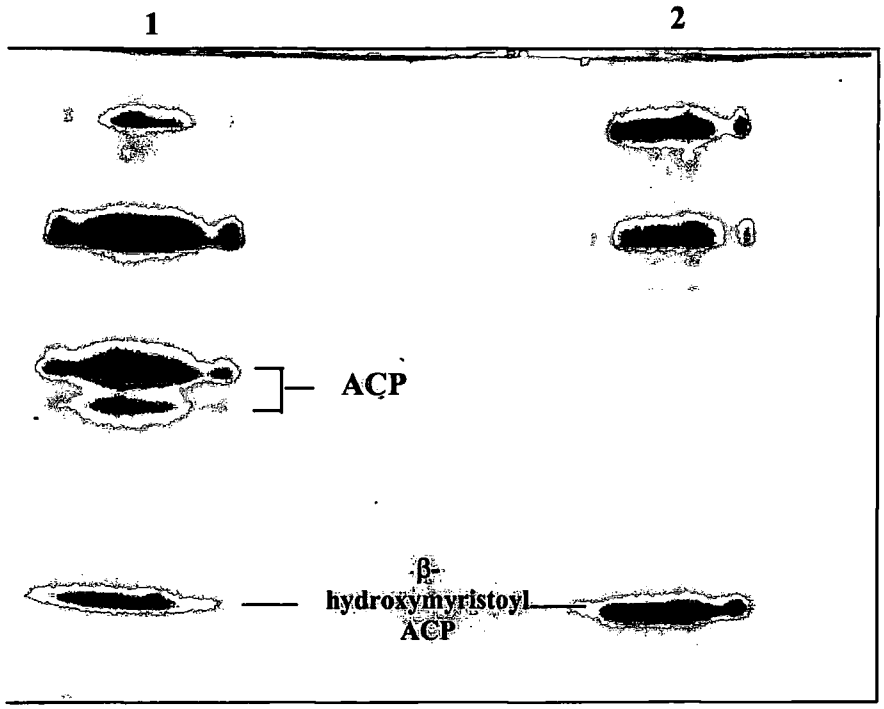


Figure 12: Purification of β-hydroxymyristoyl ACP. The fractions containing palmitoyl ACP from both the Q-Sepharose and octyl Sepharose column were loaded onto a 2.5 M Urea conformational PAGE gel. The β-hydroxymyristoyl ACP eluted from the Q-Sepharose column contains two bands that correspond to holo and apo ACP. These two bands are removed by the hydrophobic interaction chromatography step leaving a band which has the same mobility as (3*R*)-hydroxymyristoyl ACP (Mohan *et al.*, 1995). Present also in the octyl Sepharose eluent is a second band that potentially corresponds to an oligomer of β-hydroxymyristoyl ACP. **Lane 1**: Pooled fractions from the Q-Sepharose column; **Lane 2**: Pooled fractions from octyl Sepharose column.

6.2.8 β-hydroxyacyl ACP dehydratase assay using β-hydroxymyristoyl ACP as a substrate

pET24a and pRfabZ (SD4/pET24a) crude extracts were made as before, and the induction of the recombinant SD4 protein was confirmed by loading the non-induced and induced cells on a 15 % PAGE gel (Data not Shown). The protein concentrations of the crude extracts of both pET24a and pRfabZ were determined by Bradford assay, and the concentration of the crude extracts was adjusted to 1mg/mL with 25 mM potassium phosphate buffer pH 8.0. A β-hydroxyacyl ACP dehydratase assay was performed using 100 μM (3μg) of purified (3*R*)–hydroxymyristoyl ACP, and 1mg/mL sonicated pET24a crude extract. The reactions were incubated at 30 °C for the following time periods 0, 5, 10, 15, 20, 25, and 30 min. The reactions were then terminated by the addition of native PAGE loading buffer and immediately flash frozen in liquid nitrogen. After all the time points were completed the reactions were quickly defrosted and immediately loaded onto a 2.5 M urea conformational gel (Figure 13). No mobility shift for β-hydroxymyristoyl ACP could be seen at any of the time points. This suggests as with the synthesis of (2*E*)-tetradecenoyl ACP that the β-hydroxymyristoyl ACP is not acting as a substrate for β-hydroxyacyl ACP dehydratase. Unlike the (2*E*)-tetradecenoyl ACP there is no assay which can be performed to confirm that the substrate is correct. The β-hydroxymyristoyl ACP is potentially inactive because the substrate has undergone chemical modification during the synthesis reaction and can no longer act as a substrate for β-hydroxyacyl ACP dehydratase. A second possible explanation could be rather than be inactive with the β-hydroxyacyl ACP dehydratase the (3*S*)–hydroxymyristoyl ACP acts as a potent competitive inhibitor by competing for ACP binding sites on the β-hydroxyacyl ACP dehydratase.

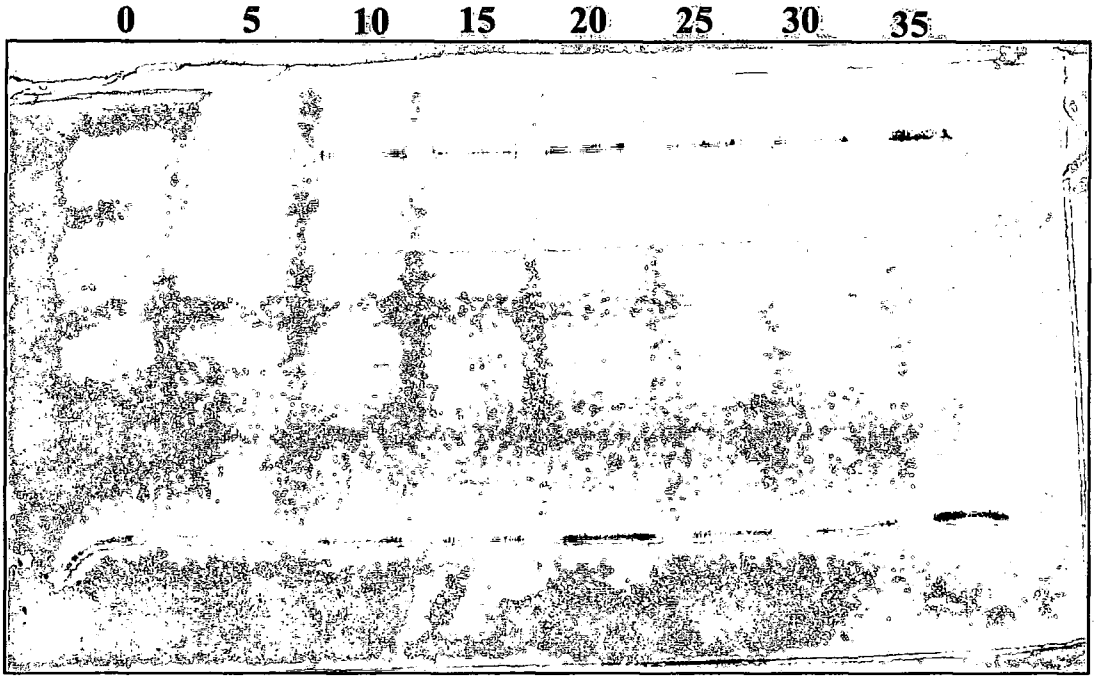


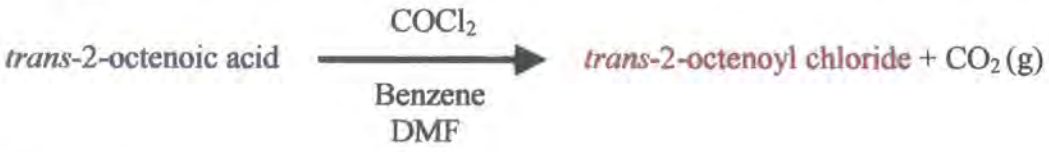
Figure 13: β-hydroxyacyl ACP dehydratase assay on induced pET24a crude *E. coli* extracts using purified β-hydroxymyristoyl ACP as a substrate. Induced *E. coli* pET24a crude extracts (1mg/mL) were incubated with purified β-hydroxymyristoyl ACP over increasing time periods. No change in mobility was observed at any of the time points. **Lane 1**: 0 minutes; **Lane 2**: 5 minutes; **Lane 3**: 10 minutes; **Lane 4**: 15 minutes; **Lane 5**: 20 minutes; **Lane 6**: 25 minutes; **Lane 7**: 30 minutes.

6.2.9 Chemical synthesis of *trans*-2-enoyl ACP

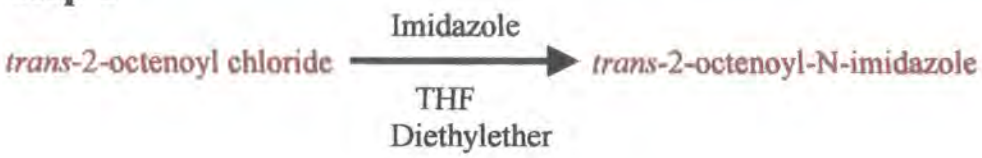
As an enzymatic route to synthesis the substrate for a β-hydroxyacyl ACP dehydratase assay have failed, then a chemical synthesis was attempted. Chemical synthesis using crotonic anhydride as an acyl donor to S-acylate ACP is non-specific and leads to a multi-derivitised ACP molecule. The crotonyl group lacks sufficient hydrophobic character to allow separation of the crotonyl ACP from any unesterified ACP by hydrophobic interaction chromatography. The crotonyl ACP assay cannot be performed on crude cell extracts. A second chemical synthesis method has been developed for the specific S-acylation of the 4'-phosphopantetheine group of ACP. This method could be used to synthesize *trans*-2-enoyl ACPs with sufficient hydrophobic character to allow purification o f the *trans*-2-enoyl ACP from the unreacted ACP. The purified substrate could potentially be used in a Mohan *et al.*, type assay and thus be performed on crude extracts.

The method developed by Klages and Cronan is a multistep method (Figure 14). First the free fatty acid is converted to the more reactive acid chloride, using oxyl chloride in the presence of the catalyst dimethyl formamide (DMF). After the acid chloride has been synthesized and purified this can be used to synthesise the N-acylimidiazole derivative. Once the N-acylimidiazole derivative has been synthesized and purified this can be used to specifically S-acylate ACP in the presence of imidazole base.

Step 1



Step 2



Step 3

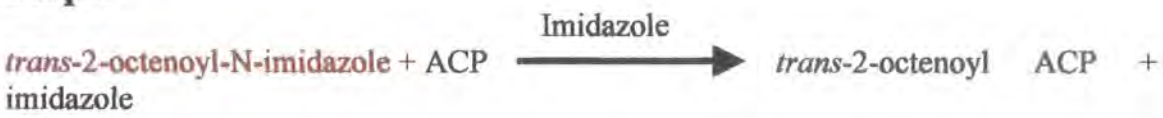


Figure 14: Chemical synthesis of *trans*-2-octenoyl ACP. Step 1: *trans*-2-octenoyl ACP is synthesized from the free fatty acid by first converting *trans*-2-octenoic acid to the more reactive acid chloride (*trans*-2-octenoyl chloride). **Step 2:** The acid chloride is purified and then used to synthesize *trans*-2-octenoyl-N-imidazole. **Step 3:** The *trans*-2-octenoyl-N-imidazole is purified and then be used to S-acylate ACP in the presence of imidazole base (Klages and Cronan, 1981).

6.2.9.3 Synthesis of *trans*-2-octenoyl ACP

The synthesized *trans*-2-octenoyl-N-acylimidazole was used to successfully synthesize *trans*-2-octenoyl CoA, and this was confirmed to be an active substrate for enoyl ACP reductase (Data not shown). Using the same *trans*-2-octenoyl-N-acylimmidazole, *trans*-2-octenoyl ACP could not be synthesized, as the product from this reaction showed no activity with enoyl ACP reductase (work carried out by Dr Wayne Martindale).

6.2.9.4 Synthesis of crotonyl ACP

Crotonyl ACP was synthesised and the presence of crotonyl ACP was then tested by an enoyl ACP reductase assay (Slabas *et al.*, 1986). The enoyl ACP reductase assay was performed 50 μL of the crotonyl ACP, an immediate decrease in absorbance at 340 nm was observed following the addition of the recombinant enzyme, this was followed

by a rapid tailing off. This confirms that crotonyl ACP has been synthesized. (Figure 15).

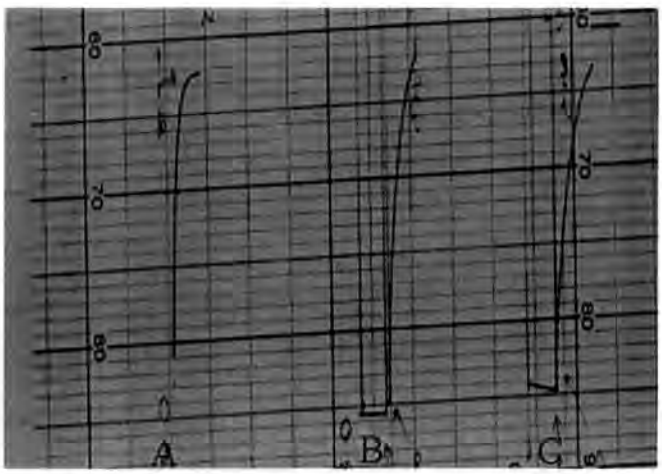


Figure 15: Confirmation that crotonyl ACP active. Crotonyl ACP was confirmed by an enoyl ACP reductase assay. Enoyl ACP reductase assays using **(A)** crotonyl ACP, to confirm the enzyme was active. **(B)** 10 μL of the synthesised crotonyl ACP, (C) 50 μL of the synthesised crotonyl ACP. In all cases a decrease in optical density at 340 nm can be seen.

6.2.9.5 β-hydroxyacyl ACP dehydratase assay using crotonyl ACP

An assay was set up containing 50 μL of crotonyl ACP plus 1mg/mL of the sonicated pRfabZ extract. There was an initial small increase of optical density followed by no further change. The initial increase in optical density is larger than the theoretical decrease due to the addition of the crotonyl ACP ((Figure 16). This masks any potential decrease in absorbance due to the presence of β-hydroxyacyl ACP dehydratase in the cell

lysate. This is a problem which has previously been reported (Birge and Vagelos, 1972). This confirms that β-hydroxyacyl ACP dehydratase activity can not be measured using crotonyl ACP on crude extracts.

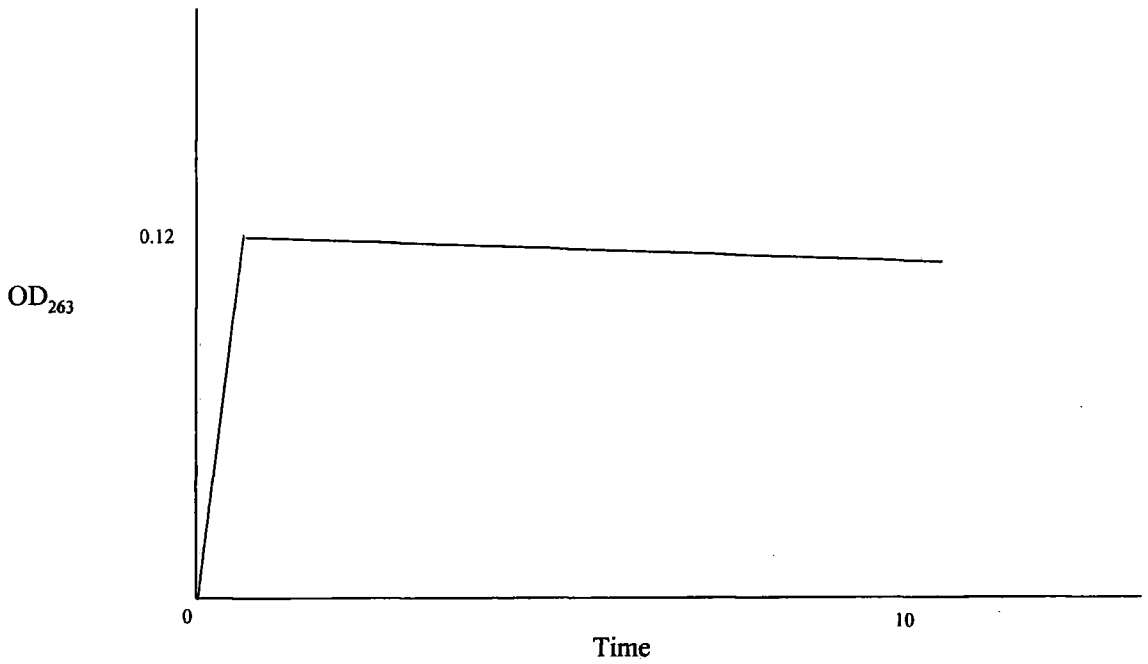


Figure 16: β-hydroxyacyl ACP dehydratase assay using crotonyl ACP. An initial increase in absorbance was seen upon initiation of the reaction with the sonictaed pRfabZ extract. There was no further significant decrease in optical density over the next 10 min.

6.3 Discussion

β-hydroxyacyl ACP dehydratase activity has been purified from spinach leaves (Shimakata and Stumpf, 1982), and also partially purified from *E. coli* (Birge and Vagelos, 1972). The assay used to purify these two enzymes was performed at pH 8.0 using crotonyl ACP as the substrate. This assay follows the disappearance of crotonyl ACP spectrophotometrically at 263 nm. This assay system is not ideal and would not be sufficient for the accurate measurement of kinetic parameters for β-hydroxyacyl ACP

dehydratase. This is due to how the crotonyl ACP is synthesized. Crotonyl ACP is synthesized chemically by using crotonic anhydride as an acyl donor to S-acylate reduced ACP. But, this system is not specific for S-acylation of the 4'-phosphopantetheine group and other reactive side chains become acylated, most notably the single tyrosine residue present in the ACP (Klages and Cronan, 1981). The chemical synthesis of crotonyl ACP also leaves behind a pool of unacylated ACP, this can potentially inhibit the β-hydroxyacyl ACP dehydratase by competing for ACP binding sites on the enzyme. The crotonyl group esterified to ACP lacks sufficient hydrophobic character to allow separation of the ACP from crotonyl ACP by hydrophobic interaction chromatography. A further complication is that the assay is performed at 263 nm a region of the spectrum where a number of other biological molecules have high absorbance. This makes the assay difficult to perform on crude extracts. Both the β-hydroxyacyl ACP dehydratase purified from *E. coli* and spinach leaves were purified using the crotonyl ACP based assay once a number of initial protein purification steps were undertaken to reduce both the protein and nucleic acid concentrations of the crude extract (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982).

A second assay for β-hydroxyacyl ACP dehydratase has been developed by Mohan *et al.*, (Mohan *et al.*, 1995), which eliminates all of the problems associated the crotonyl ACP based assay system. This assay can be performed on crude extracts and the substrate is also homogeneous and so allows the accurate measurements of kinetic parameters. This assay does not use a spectrophotometric system to distinguish between product and substrate, but relies on the observation that the substrate an product can be separated on urea conformation gels (Post-Bettinemiller, 1991), so avoiding the high background produced by other biological molecules present in crude extracts. The substrate for the system is synthesized enzymatically using a acyl ACP synthetase from *E. coli.* Acyl ACP synthetase ligates the free fatty acid direct to ACP, and will utilize both saturated and unsaturated fatty acids down to ten carbons in length (Rock and Cronan, 1979). As the reaction is enzymatic then the S-acylation of the 4'-phosphopantetheine group is specific and a solitary derivatised ACP is formed. As the acyl ACP synthetase only shows activity with medium and long chain fatty acids, these posses sufficient hydrophobic character to allow separation of any unreacted ACP away from the acyl ACP by hydrophobic interaction chromatography (Rock and Cronan,

1979), thus producing a homogeneous substrate. This second assay system was deemed to be the best system to assay the biological activity of the recombinant SD4 protein.

Initially it was decided to use (2*E*)-tetradecenoyl ACP and not (3*R*)-hydroxymyristoyl ACP as used by Mohan *et al.* As at pH 8.0 the equilibrium for the reaction lies 5:1 in favor of the reverse reaction (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982). This would us allow us to perform the assay under kinetically favorable conditions. *trans*-2-enoyl fatty acids are not available to be purchased commercially., but we were able to obtain (2*E*)-tetradecenoic acid from Dr Pat Steele (Durham University Chemistry department). Using recombinant acyl ACP synthetase we were able to synthesize what appeared to be (2*E*)-tetradecenoyl ACP and by hydrophobic interaction chromatography were able to purify the (2*E*)-tetradecenoyl ACP to homogeneity. When this purified substrate was initially used in a β-hydroxyacyl ACP dehydratase assay on *E. coli* crude extracts no activity could be measured. When the substrate was attempted to be verified by an enoyl ACP reductase assay as (2*E*)-tetradecenoyl ACP is also a substrate for this enzyme no activity could be measured. This called into question the validity of the synthesized (2*E*)-tetradecenoyl ACP. Mohan *et al.*, reported that during the synthesis of (3*R*)-hydroxymyristoyl ACP that the acyl ACP synthetase preparation was able to modify the (3*R*)-hydroxymyristoyl ACP during the synthesis reaction This problem was alleviated by using a strain of *E. coli* which had acyl ACP synthetase overexpressed. The synthesis of (2*E*)-tetradecenoyl ACP was accomplished by using acyl ACP synthetase partially purified for *E. coli* which contained the acyl ACP synthetase overexpressed. But in this case we are using the more reactive (2*E*)-tetradecenoyl ACP.

To try and overcome this we attempted to synthesize (3*R*)-hydroxymyristoyl ACP as the substrate. As with (2*E*)-tetradecenoyl ACP, (3*R*)-hydroxymyristoyl ACP is not available to be purchased commercially. We were able to obtain a racemic mixture of both the *R* and *S* isomers from Larodan Biochem. The *S* isomer has been shown to be inactive with β-hydroxyacyl ACP dehydratase purified from both *E. coli* and spinach leaves (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982). The activity of the S-isomer with acyl ACP synthetase is unknown and as a supply of purified (3*S*)-hydroxymyristoic acid was unavailable this was unable to be tested. So it was assumed that the activity would be 50:50 and that half the substrate synthesized would be inactive with the β-hydroxyacyl ACP dehydratase. The racemic β-hydroxymyristoyl ACP was

synthesized using recombinant acyl ACP synthetase and purified using identical conditions to that for (2*E*)-tetradecenoyl ACP. When the purified substrate was used on *E. coli* crude extracts no β-hydroxyacyl ACP dehydratase activity could be measured.. Potentially this could be due to the presence of the *S*-isomer in the substrate. The *S*-isomer has been shown to be inactive with β-hydroxyacyl ACP dehydratase but no competitor studies have been reported, and thus the *S*-isomer may be a potent inhibitor of β-hydroxyacyl ACP dehydratase by competing for the binding site on the active site.

The enzymatic synthesis methods attempted failed to produce an active substrate for a β-hydroxyacyl ACP dehydratase assay, so a chemical synthesis method was attempted. The chemical synthesis method developed by Klages and Cronan (Klages and Cronan, 1981) has been to shown to specifically acylate the 4'-phosphopantetheine group of ACP by using an acyl-N-imidazole as an acyl donor. This method has been used to synthesize both saturated and *cis* unsaturated ACP derivatives, but there was no report of the synthesis of *trans* unsaturated fatty acids. This method potentially can produce a synthesized *trans*-2-enoyl ACP with sufficient hydrophobic character to allow purification of the substrate. *trans*-2-octenoic acid was chosen as the substrate as this fulfilled these criteria. The Klages and Cronan method involves first converting the free fatty acid to the more reactive acid chloride, then converting this to the N-acylimidaole. The *trans*-2-octenoyl-N-acylimidazole was successfully synthesized. This can be then used as an acyl donor for the specific S-acylation of ACP. This specific S-acylation of ACP is dependent on the presence of imidazole base during the reaction. This method was attempted by Dr Wayne Martindale but no active ACP substrate was synthesized. This is potentially due to the presence of the imidazole base. During the synthesis of the N-acylimidazole derivative the acid chloride group is replaced by imidazole if the reaction is allowed to proceed longer then a minute then the imidazole can also add across the reactive *trans* double bond. A situation which may be occurring during the synthesis of *trans*-2-octenoyl ACP.

The final option for a direct measurement of the recombinant SD4 gene product was to use crotonyl ACP as the substrate. Crotonyl ACP was synthesized and used to assay β-hydroxyacyl ACP dehydratase activity in an induced pOESD2 (SD4) *E. coli* crude extract. No activity was seen as the theoretical decrease in absorbance due to the hydration of crotonyl ACP is masked by an initial increase in optical density due to the addition of the crude extract. This is a similar situation to that previously reported by

Birge and Vagelos (Birge and Vagelos, 1972) . Therefore the recombinant SD4 needs to be partially purified before the assay can be attempted. Unfortunately the vast majority of the recombinant SD4 protein is insoluble and only a very small percentage remains in the soluble fraction.

Chapter 7

Discussion

7.1.1 Cloning of β-hydroxyacyl ACP dehydratase from *Brassica napus*

A number strategies to clone β-hydroxyacyl ACP dehydratase from a plant source based on utilising the sequence of the *E. coli fabZ* are potentially available. The *fabZ* gene itself could not be used to directly screen a plant cDNA library since there would be sufficient cross hybridisation between the probe and the *fabZ* present in the *E. coli* used to propagate the lambda phage carrying the cloned cDNAs. The *E. coli* FabZ sequence was used to identify a plant homolog by screening the sequence data present in the EST databases. From the EST database an EST from *Ricinus communis* (Vandeloo, 1995) was identified which showed significant homology to the *E. coli* FabZ sequence at the amino acid level was identified. This EST was not annotated as being a β-hydroxyacyl ACP dehydratase homolog but instead was assigned as being homologous to a rifampicin resistance protein form *Rickettsia ricketsii* as the EST was identified before the assignment of β-hydroxyacyl ACP dehydratase to the *fabZ* open reading frame. The nucleotide sequence of the *Ricinus communis* EST was amplified from a developing castor embryo stage V cDNA library obtained from Pioneer hybrid (Coughlan *et al.*, 1996). The amplified EST was then used to heterologously screen a developing *Brassica napus* embryo cDNA library. From the primary screen 37 putative positive plaques were isolated and a number purified. The cDNA insert from one of the isolated putative clones SD4 contained a single open reading frame which showed homology 40.4 % identity with the and the *E. coli* FabZ amino acid sequence, the sequence also contained the conserved residues seen in both the sequences form the enterobacter as well as the EST used as the probe.

7.1.2 Analysis of SD4 cDNA

The isolated SD4 clone was 796 bp long and contained a single open reading with the predicted gene product having a predicted molecular weight of 24.6 kDa. The β-hydroxyacyl ACP dehydratase activity previously purified from spinach leaves was associated with a 19 kDa protein (Shimakata and Stumpf, 1982). Using von Heijne (von Heijne, 1989) principals for predicting the presence of chloroplast target sequences a putative chloroplast target sequence was identified at the N-terminus of the SD4

translation product. Removing the predicted chloroplast target sequence then a protein with a similar molecular weight (19 kDa) to the spinach β-hydroxyacyl ACP dehydratase remains. A hydrophobicity plot on the predicted SD4 translation product shows that even though the protein contains regions of hydrophobic nature there are no predicted transmembrane spanning domains, and the SD4 protein is predicted to be soluble protein.

7.1.3 SD4 belongs to a multigene family

Further analysis on some of the sequence data obtained from some of the 37 putative positive plaques indicated that SD4 belongs to a multigene family. Sequences were obtained for clones, which have similar translation products as, SD4 but showed significant differences in the 3' untranslated regions of the cDNAs. Complete sequencing of two such clones SD12 and SD16 revealed that not only were their significant differences in the 3'-untranslated regions but, there was also changes in the amino acid sequence of the predicted translation products indicating that these cDNAs were transcription products of paralogs of SD4 and thus SD4 belongs to a multigene family. Comparison of both the nucleotide and amino sequences of SD12 and SD16 Based on this data it was possible to split the multigene family into two distinct groups types A (SD12 and SD 16) and type B (SD4). A similar pattern has been seen with other genes coding for FAS enzymes form *Brassica napus*, for example both β-ketoacyl ACP reductase and enoyl ACP reductase from *Brassica napus* belong to multigene families (Personal communication Dr Fraser McDonald) with each of the cDNA clones being able to be assigned to two subtypes with two members in each subtype.. This possibly arises from the hybrid nature of *Brassica napus,* as *Brassica napus* was originally isolated as a cross between *Brassica oleracea* and *Brassica rapa*. Each of the two subgroups from the multigene family was probably inherited independently from one or other of the progenitors. If the pattern of inheritance of the genes for SD4 mirrors that of β-ketoacyl ACP reductase and enoyl ACP reductase then there is potentially a second type B paralog which was not located in the first batch of primary plaques to be purified. The largest region of variance between type A and B occurs in the 3' untranslated regions of the cDNA clones. To try and identify a potential type B clone amongst the remaining identified primary phage populations the 3' untranslated regions were amplified. and the PCR products sequenced. From this sequence data it was not possible

to assign any of the clones to belonging to type B based on sequence comparison of the untranslated regions. When a more detailed comparison was undertaken by comparing the predicted amino acid sequence of the amplified clones it became clear that SD32 encodes a clone of type B, and should be purified further and sequenced to confirm if this is the missing type B clone.

The data derived from the of SD4 sequence provides a large amount of circumstantial evidence that SD4 codes for a β-hydroxyacyl ACP dehydratase, as well as the predicted intracellular location. Additional evidence for the existence of this protein in *Brassica napus* embryos comes form data obtained during the purification of acyl ACP thioesterase from *Brassica napus* (Hellyer *et al.*, 1992). Hellyer *et al.*, found that acyl ACP thioesterase co-purifies with both a 30 kDa and 20 kDa proteins under native conditions. The N-terminal amino acid sequence of both of these proteins was determined and revealed that 30 kDa protein to be enoyl ACP reductase, while the 20 kDa protein showed no homology to any known protein present in the database. The sequence of the translated SD4 protein from the predicted chloroplast target sequence processing site is identical to that of the unknown 20 kDa protein sequenced by Hellyer *et al*, so indicating that enoyl ACP reductase and acyl ACP thioesterase co-purify with β-hydroxyacyl ACP under native conditions and are possibly associated *in vitro*. This evidence that SD4 codes for b-hydroxyacyl ACP dehydratase is strong it is still circumstantial and direct evidence would be provided by demonstrating that the SD4 gene product has β-hydroxyacyl ACP dehydratase activity.

7.1.4 Overexpression of the recombinant SD4 protein

The nucleotide sequence coding for the predicted mature SD4 protein was sub-cloned into the *E. coli* overexpression vector pET24a. When BL21 (DE3) *E. coli* carrying the overexpression construct was grown in medium supplemented with IPTG a 19 kDa protein was induced. When the induced cells were broken by soniaction the majority of the induced protein associates with the insoluble fraction. The high speed centrifugation supernatants of cells with induced SD4 and an empty vector control were compared this revealed that there was a significant proportion of the recombinant SD4 protein which remained in the soluble phase. This soluble SD4 protein may or may not be folded correctly and thus be biologically active, but the level of this protein should be sufficient to determine the biological activity.

An alternative source of active recombinant SD4 protein may be from the recombinant protein found in the insoluble fraction. A number of methods have been developed for the refolding of proteins from inclusion bodies (Rudolph and Lille. 1996; Muckopadhyay, 1997). The SD4 protein was solubilized with 8 M urea and once the urea was removed by dialysis the recombinant SD4 protein remained in solution. remained in solution once the urea had been dialyzed away. This now soluble SD4 protein may contain active SD4 protein which can also be used to determine the biological activity of the SD4 protein.

7.1.5. β-hydroxyacyl ACP dehydratase assay

β-hydroxyacyl ACP dehydratase purified from both spinach leaves and *E. coli* shows an absolute requirement for ACP derivatives as the substrate over the substrate analogs CoA and N-acylcysteamine (Birge and Vagelos, 1972; Shimakata and Stumpf, 1982). This means that to synthesise a substrate for a β-hydroxyacyl ACP dehydratase assay then a source of ACP is required. I have purified holo ACP directly form K-12 *E..coli* using a modified version of the method developed by Majerus (Majerus *et al.*, 1986). Holo ACP from both rape and *E. coli* has also been synthesized by recombinant means. ACP form both *E. coli* and rape when overexpressed in *E. coli* is not correctly post-translationally modified with the 4'-phosphopantetheine prosthetic group. The enzyme holo ACP synthase which catalyses the attachment of the prosthetic group has been overexpressed in *E. coli* and the recombinant protein purified. Using reaction conditions developed by Dr. A.R Stutjie both *E. coli* and rape ACP were correctly modify *in vitro* using the recombinant holo ACP synthase and an excess of free Coenzyme A. The recombinant ACP from both *E. coli* and rape were co-expressed with holo ACP synthase and this increased the level of post-translation of the extracted ACP, but there still remained a significant pool of apo ACP in both cases. This is a similar result as to the one achieved by Broadwater and Fox with a synthetic spinach ACP-I. Broadwater and Fox found they could increase the level of post-translational modification levels to that of native ACP by performing the induction in a fermener using controlled pH and oxygen levels.

The ACP is only one component of the substrate for β-hydroxyacyl ACP dehydratase and the complete substrate needs to be synthesized. The assay used to purify β-hydroxyacyl ACP dehydratase was performed using crotonyl ACP as the substrate.

But, as the recombinant SD4 protein is not been purified to homogeneity and in is present in an *E. coli* crude extract, then the crotonyl ACP assay cannot be used. The assay chosen was that developed for *E. coli* FabZ by Mohan *et al.*, (Mohan *et al.*, 1995). This assay system can be used to measure β-hydroxyacyl ACP dehydratase activity in crude extracts. Mohan et al., performed their assay using the substrate. (3R)-hydroxymyristoyl ACP at pH 8.0, these are not ideal reaction conditions as the equilibrium of the reaction at pH 8.0 lies 5:1 in favor of the reverse reaction. Instead I choose (2*E*)-tetradecenoyl ACP as the substrate as the substrate so allowing the assay to be performed under kinetically favorable conditions. Using recombinant acyl ACP synthetase we were able to synthesize an acyl ACP which had similar mobility to that of (2*E*)-tetradecenoyl ACP, this acyl ACP was purified by octyl Sepharose chromatography, but the substrate was inactive with recombinant enoyl ACP reductase and an *E. coli* crude extract also showed no β-hydroxyacyl ACP dehydratase activity. Mohan *et al.*, reported that when they synthesized (3*R*)-hydroxymyristoyl ACP using acyl ACP synthetase from K-12 *E. coli* then there was a significant pool of β-hydroxyacyl ACP dehydratase present in the acyl ACP synthetase preparation which converted the substrate to (2*E*)-tetradecenoyl ACP during the purification procedure. Using an acyl ACP synthetase overexpressing strain of *E. coli* as a source of the acyl ACP synthetase alleviated this problem. As (2*E*)-tetradecenoyl ACP is more reactive than (3*R*)-hydroxymyristoyl ACP then the β-hydroxyacyl ACP dehydratase present in the acyl ACP synthetase preparation maybe sufficient to allow modification of the (2*E*)-tetradecenoyl group during purification. Unfortunately no electro-spray mass spectrometer was available to verify the substrate at that time.

The Mohan *et al.*, assay was performed using (3*R*)-hydroxymyristoyl ACP as a substrate, (3*R*)-hydroxymyristoyl ACP is not available commercially but e a racemic mixture of both the *R* and *S* isomers was obtained from Larodan Biochem. Using acyl ACP synthetase a band which showed similar mobility to that of (3*R*)-hydroxymyristoyl ACP was synthesized, this was purified to homogeneity using a similar protocol to that used for (2*E*)-tetradecenoyl ACP. The purified (3*R*)-hydroxymyristoyl ACP was used to assay β-hydroxyacyl ACP dehydratase activity in an *E. coli* crude extract. No mobility shift in the position of the band could be seen using identical conditions to those described by Mohan *et al.* It was initially assumed that the *S*-isomer would be biologically inactive with β-hydroxyacyl ACP dehydratase as the enzyme is

stereospecific. Possibly rather than being inactive the *S*-isomer maybe acting as a powerful inhibitor of β-hydroxyacyl ACP dehydratase activity by competing for the substrate binding site. An alternative explanation could again be that the substrate is modified during the synthesis, but no mass spectrometer was available to confirm this.

7.1.6 Conclusion

Based on a range of circumstantial evidence we had deduced we had cloned the first β-hydroxyacyl ACP dehydratase from a plant source. To provide definitive proof that this was the case we overexpressed the putative β-hydroxyacyl ACP dehydratase in *E. coli*. The majority of the recombinant protein was insoluble but a small percentage remained in the soluble phase. The level of the soluble recombinant protein was deemed sufficient to allow the biological activity to be determined. β-hydroxyacyl ACP dehydratase shows an absolute requirement for ACP as its substrate, and we were able to purify and synthesize holo ACP from both native and recombinant sources. To confirm the biological activity the holo ACP was used to synthesize a potential substrate for a β-hydroxyacyl ACP dehydratase assay, even though a number of different methods were used the correct substrate for a β-hydroxyacyl ACP dehydratase assay that could be used on crude extracts was not successfully synthesized. Thus, definitive proof that the isolated clone SD4 does code for β-hydroxyacyl ACP dehydratase has yet to be shown.

7.2 Future strategies for confirming SD4 activity

During this work we have been unable to demonstrate conclusively that the isolated clone SD4 codes for β-hydroxyacyl ACP dehydratase from *Brassica napus* by using direct assay methods then an alternative approach is required. One of the initial strategies designed for the potential cloning of β-hydroxyacyl ACP dehydratase from a plant source was to complement a *E. coli* FabZ mutant with a rescued plant cDNA library to try and isolate a complementing clone. As a putative β-hydroxyacyl ACP dehydratase complementing clone has been isolated via a different strategy then potential proof may come form using the isolated clone to rescue a FabZ mutant. The only FabZ mutants isolated are point mutations, and these potentially can spontaneously revert back to the wild type sequence, a better method would be to completely remove the FabZ locus and thus any β-hydroxyacyl ACP dehydratase activity completely.

Gene disruption in *E. coli* can be achieved by replacing the gene of interest with the open reading frame disrupted with a antibiotic resistance cassette. *E. coli* co-transcribes related genes on polycistronic mRNAs, the insertion of an antibiotic cassette can cause polar effects on downstream genes. *E. coli* FabZ is surrounded by three genes involved in lipid A biosynthesis (*lpxD*, *lpxA*, *lpxB*), also located downstream are a gene for RNAaseH (*rncH*) and for the α subunit of DNA polymerase III (*dnaE*). Any effect on these genes may also produce a phenotype or could disrupt the transcription of an essential gene. These polar effects can be avoided if the system developed by Link *et al.*, is used for markerless gene replacement (Link *et al.*, 1997). The method developed by Link *et al.*, involves first creating an in-frame deletion by cross hybridization PCR containing the FabZ gene completely removed flanked by two 500 bp sequences required for homologous recombination. The in-frame deletion is cloned into the vector pK03 which is specifically designed for markerless gene replacement and used to replace the FabZ sequence, the resulting mutant can then be complemented..

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