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The NADH-Specific ß-Ketoacyl (Acyl Carrier Protein) Reductase from the Plastids of Avocado (*Persea americana*) Fruit Mesocarp

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ABSTRACT. *The enzyme NADH-dependent ß-ketoacyl (ACP) reductase, a putative component of the fatty acid synthetase system, has been purified from avocado fruit mesocarp. Both SDS-PAGE and gel filtration analyses have shown this enzyme to have a molecular mass of 32 kDa suggesting it to be a monomer. An antiserum raised against the enzyme was demonstrated to inhibit enzyme activity. By electron microscopy using the immunogold technique this antiserum was used to show that the enzyme was localized solely in the chloroplasts and plastids and was absent from the mitochondria of avocado and sunflower tissues. Immunoprecipitation of the products of in vitro avocado mesocarp polysomal protein revealed on SDS-PAGE analysis a single labelled product of molecular mass 37 kDa, indicating the presence of a leader sequence of 5 kDa. Two partial amino acid sequences obtained from the peptides derived from Cleveland mapping exhibited homology with a number of dehydrogenases.*

INTRODUCTION

Fatty acid synthesis in plants has been shown to occur in the chloroplasts of green leaves (Stumpf and James, 1963) and in the plastids of non photosynthetic tissues (Zilkey and Canvin, 1972; Vick and Beevers, 1978). The fatty acid synthetase system of avocado fruit mesocarp was shown to be present in plastids by Weaire and Kekwick (1975). Caughey and Kekwick (1982) have shown that the avocado plastid and Shimakata and Stumpf (1982a) the chloroplast fatty acid synthetase systems to be of the prokaryotic type II multicomponent type.

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The initial reduction step in *de novo* fatty acid synthesis is catalysed by ß-ketoacyl (ACP) reductase. Caughey and Kekwick (1982) characterised two forms of the enzyme NADH and the NADPH-specific forms. The NADPH-specific enzyme has been purified to homogeneity from spinach (Shimakata and Stumpf, 1982b), avocado mesocarp (Sheldon *et al.*, 1990) and rape (Sheldon *et al.*, 1992) and has also been characterised in safflower seed (Shimakata and Stumpf, 1982a), barley leaf (Hoj and Mikkelesen, 1982) and *Euglena* (Hendren and Bloch, 1980). The NADH-specific avocado enzyme, the subject of this study, has been partially purified from avocado (Caughey and Kekwick, 1982). It has been demonstrated that the NADPH-specific enzyme has a much greater affinity for acetoacetyl-ACP, which is the physiological substrate, than for acetoacetyl-CoA, while the NADH-specific enzyme has similar Vmax/Km values for both substrates (Caughey and Kekwick, 1982). Since this enzyme is NADH-specific and can utilize CoA esters to a similar effect as the corresponding enzymes of ß-oxidation pathway, it is important to establish its intra-cellular location using a cytochemical method, and thereby, to infer its metabolic function as a component of fatty acid synthetase of plastid or of a component of the ß-oxidation system of peroxisomal or mitochondrial origin.

MATERIALS AND METHODS

Chemicals

Acetoacetyl-CoA, Avidin Peroxidase and Cyclohexylamino-1-propanesulphonic (CAPS) from Sigma Ltd (Poole, Dorset UK); Ultrogel AcA-34 from Pharmacia LKB (Milton Keynes, Bucks., U.K) were used. Blue Dextran Sepharose (BDS) was prepared by the method of Ryan and Vestling (1974). Radio chemicals and anti-rabbit IgG (biotinylated, from goat) from Amersham International (Aylesbury, Bucks., U.K). Anti-rabbit IgG-HRP conjugate from the Department of Immunology, University of Birmingham. Endoproteinase Glu-C (sequencing grade) from Boehringer (Lewes, East Sussex., U.K.). Goat anti-rabbit IgG-colloidal gold from Janssen (Wantage, Oxfordshire, U.K). Reagents for electron microscopy from Agar Scientific (Stansted, Essex., U.K.). Other reagents were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Enzyme extraction and assay

Avocado pears (*Persia americana Var.* Fuerte) were obtained from Sainsbury plc. Plastids were prepared from avocado mesocarp by the procedure of Weaire and Kekwick (1975). Enzyme assay was a modification of that adopted by Caughey and Kekwick (1982).

FPLC

Superose-12 gel filtration column was used on a Pharmacia FPLC system.

Analytical procedures

Protein estimations were carried out either by the dye-binding method (Bradford, 1976) or by measurement of A_{280}. SDS-PAGE was carried out using the discontinuous system of Laemmli (1970). Proteins were revealed by stalning with Coomassie Blue G250 or silver staining (Pharmacia). Edman degradation for protein sequencing was carried out on an Applied Biosystems gas-phase sequencer, model 470.

Immunisation of rabbits with ß-ketoacyl (ACP) reductase

This was carried out as described in Howe *et al.*, (1982).

Western blotting

Blotting was carried out according to the method of Towbin *et al.*, (1979).

ELISA

The ELISA was performed as described by Catty *et al.*, (1983).

Cleveland mapping of polypeptides

The method used is a modification adapted for minigels of that described by Cleveland (1983).

Electroblotting of proteins on to PVDF membrane

This was carried out as described in Amersham technical notes. Proteins were visualised by staining with 0.1% Coomassie Blue in 50% methanol for 5 min.

Extraction of polysomes from avocado mesocarp and *In vitro* protein synthesis

The method was modified from the procedure for isolating *Hevea* latex polysomes developed by Tupy (1988). Labelled polypeptides were selectively precipitated with the antiserum as per Howe *et al.*, (1982).

Autoradiography

The procedure used was that adopted by Jen and Thatch (1982).

Immuno electron microscopy

The methods were performed as described in Mackie *et al.*, (1991). Mitochondrial pellet was prepared as per Baker *et al.*, (1968). A 1:10 dilution of antibody was used for the incubation of sections.

RESULTS AND DISCUSSION

Purification of the enzyme

The enzyme was precipitated in 0-55% $(NH_4)_2SO_4$ fraction, which was subjected to gel filtration on Ultrogel AcA-34 column (80x2.5 cm) previously equilbrated in 50 mM phosphate buffer pH 7, containing 0.5mM EDTA and 1mM Dithiothreitol (DTT). A flow rate of 0.5ml/min was maintained (Figure 1).

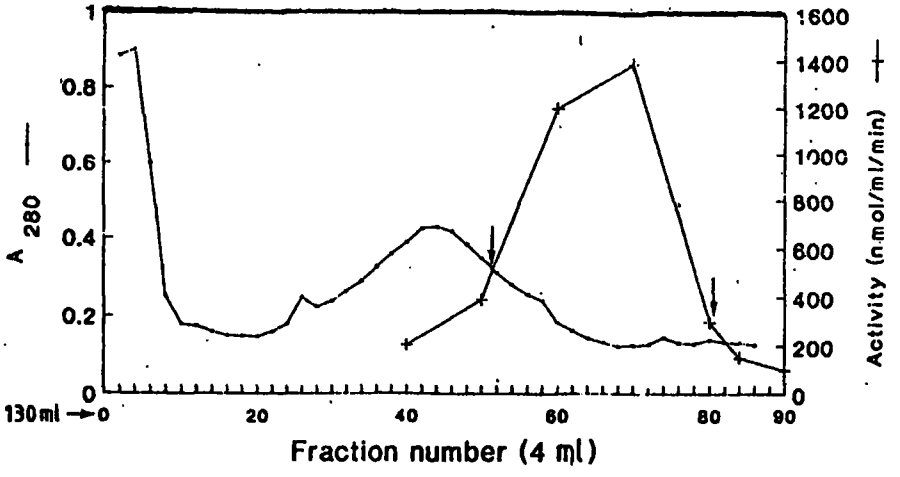

Figure 1. **Gel filtration of 0-55% saturated ammonium sulphate fraction of ß-ketoacyl (ACP) reductase on a 390 ml Ultrogel AcA-34 column. The active fractions of highest specific activity were pooled as indicated with arrows.**

Active fractions of highest specific activity were pooled and were subjected to two successive BDS columns (Figure 2a and 2b) previously equilibrated in 10mM phosphate buffer pH 7 containing 0.5mM EDTA, 1mM DTT. The flow rate was 1.5 ml/min. The bound enzyme was eluted with a linear salt gradient of 0-2M KCl over a volume of 100 ml and at a flow rate of 1 ml/min. The active fractions were pooled and de-salted by dialysis and were then concentrated and fractionated on a Superose-12 column pre-equilibrated in de-gassed and vaccum filtrated 10mm phosphate buffer containing 200mM NaCl, 0.5mM EDTA and 1mM DTT. 200μl of the concentrated BDS(II) fraction was injected, a flow rate of 0.20ml/min was maintained (Figure 3). Table 1 shows a typical purification of ß-ketoacyl (ACP) reductase (NADH-specific). The enzyme was purified approx. 98 fold with a yield of 6%. On SDS-PAGE, the purified preparation showed two polypeptide components migrating at distances corresponding to molecular masses of 29 and 32 kDa (Figure 4, lanes 2-9) SDS-PAGE analysis in which ß-mercaptoethanol has been omitted from the sample buffer gave identical analyses indicating that the peptides were not joined by S-S bonds in the native state (Figure 5).

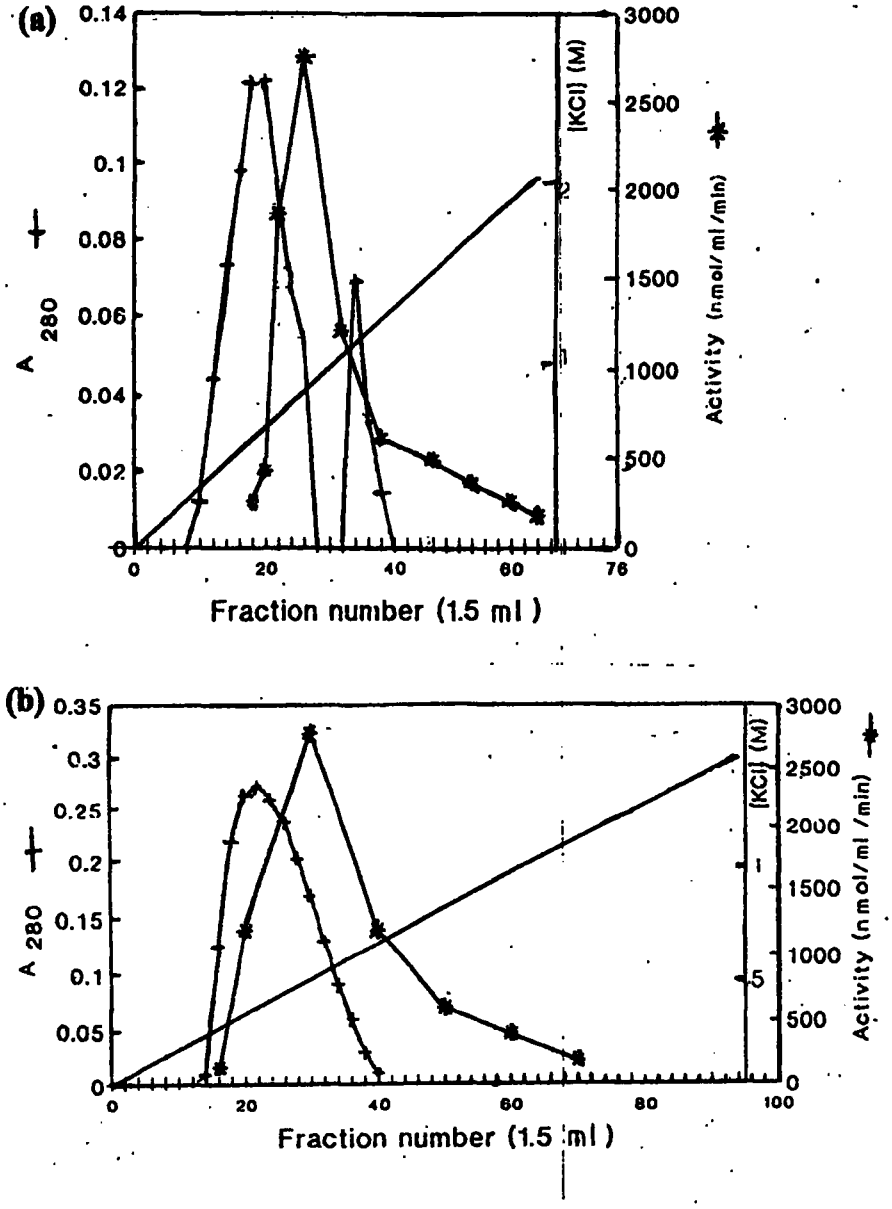

Figure 2. **Blue Dextran Sepharose chromatography steps I and II. (a) The active fractions from the Ultrogel AcA-34 column were applied to a column (6x1.5 cm). (b) Rechromatography of active fractions from BDS column I on a similar column.**

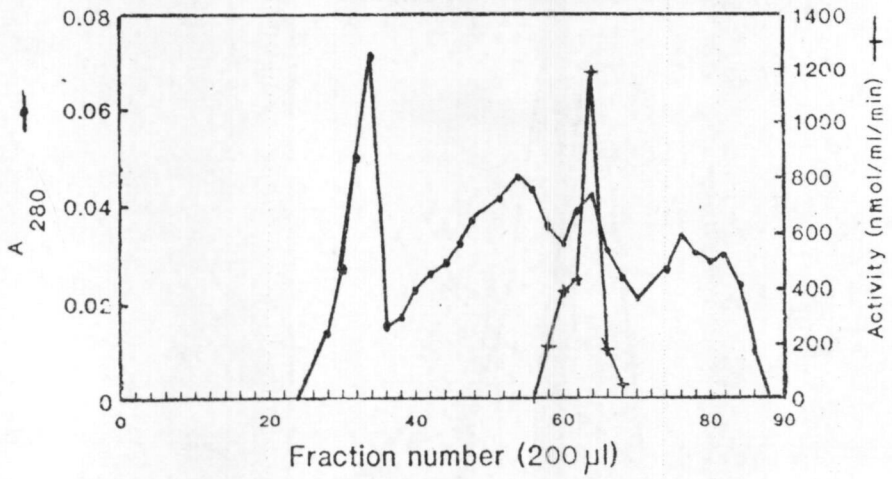

Figure 3. **FPLC gel filtration on a 25 ml Superose-12 column (1x30 cm) of the BDS(II) concentrate.**

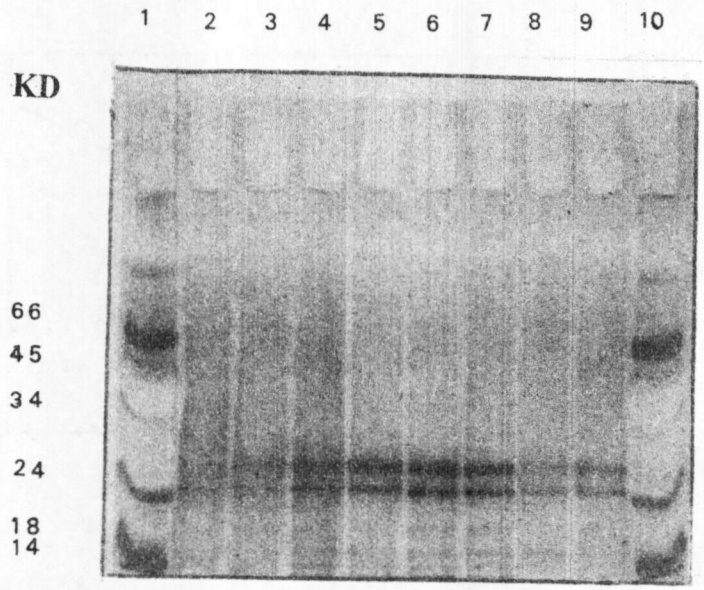

Figure 4. **SDS-PAGE analysis of Superose-12 fractions of ß-ketoacyl (ACP) reductase. A 10% mini gel was silver stained. Lanes 1-8, fractions 59-66.**

Table 1. Purification of b-Ketoacyl (ACP) Reductase (NADH Specific).

Step	Volume (ml)	Activity (nmol/ml/min.)	Total Activity (nmol/min.)	Protein Concentration (mg/ml)	Total Protein (mg)	Specific Activity (nmol/min/mg)	Fold Purific.	Yield %
77,000g Sup.	46	5934	273,000	4.1	188.0	1,452	1.0	100
0.55% sat-$(NH_4)_2So_4$	7	22,903	160,321	8.0	56.0	2,862	1.97	58
Ultrogel AcA-34	170	99	168,640	2.5	42.5	3,968	2.73	61
BDS I	52	2182	113,464	0.48	28.46	4,545	3.13	41
BDS II	50	397	19,840	0.05	2.5	7,936	5.46	7
Superose 12	1	17,097	17,097	0.12	0.12	142,475	98.0	6

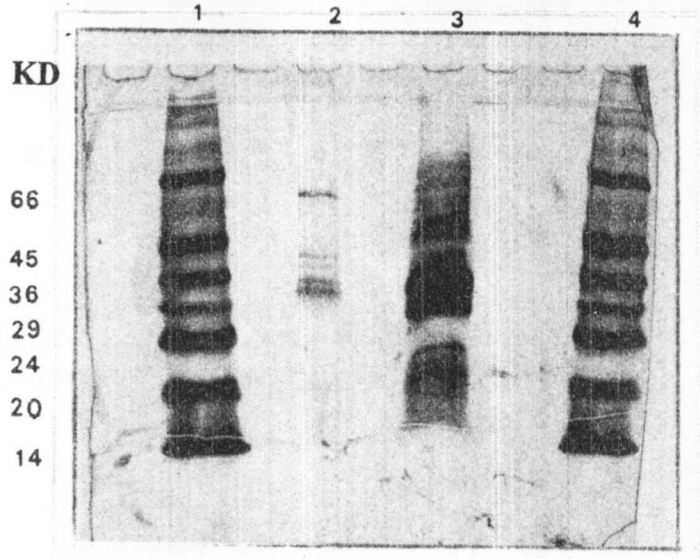

Figure 5. **Analysis of fractions from the Superose-12 FPLC column containing ß-ketoacyl (ACP) reductase by SDS-PAGE. A 10% mini gel was silver stained. Lane 1, ß-ketoacyl (ACP) reductase in the absence of mercaptoethanol; lane 2, ß-ketoacyl (ACP) reductase in the presence of mercaptoethanol; lane 3, molecular weight standards.**

On FPLC gel filtration using a suitably callibrated Superose-12 column, the enzyme activity was eluted as a single peak at a position corresponding to a molecular mass of approx. 32 kDa (Figure 3).

Partial amino acid sequencing of the protein

N-terminal sequence determination by the Edman degradation technique failed to detect an N-terminal amino acid suggesting that the N-terminus of the protein was blocked. Therefore, internal amino acid sequences were derived by cleavage of the polypepetides, which was achieved by Cleveland mapping, a technique which is a sensitive test for protein identity based upon proteolytic cleavage.

Superose-12 eluted ß-ketoacyl (ACP) reductase was prepared for electrophoresis by dialysis against 20mM N-ethyl morphaline buffer (pH 7) and lyophilisation. SDS-PAGE analysis of the products of partial digestion of the 32 kDa component with Staphylacoccus V8 protease gave a major band in the molecular weight region of 24 kDa and few other bands of lower intensity in the 14-7 kDa molecular weight region (Figure 6). Amino acid sequence analyses of the two bands of molecular weight 14 kDa (polypeptide I) and 7 kDa (Polypeptide II) revealed the following.

Polypeptide I	I Y K V A V L G A
Polypeptide II	L T Y N Y G V D

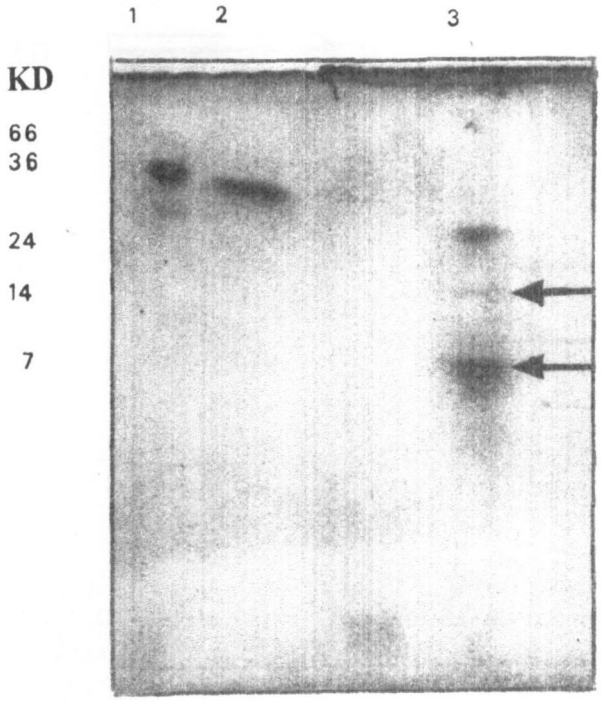

Figure 6. **Cleveland mapping of avocado ß-ketoacyl (ACP) reductase. Lane 1, ß-ketoacyl (ACP) reductase; lane 2, V8 Protease (3.0 mg); lane 3, ß-ketoacyl (ACP) reductase + V8 protease (1.5 mg).**

Reactions of an antiserum to the 32 kDa polypeptide

Western blot with a partially purified avocado mesocarp extract shows that the antiserum specifically reacts with a 32 kDa polypeptide (Figure 7a). A Western blot against a crude sunflower extract gave a very distinct low molecular weight band of about 18 kDa suggesting that there is a certain degree of cross reactivity between the avocado enzyme and a sunflower polypeptide but the molecular weight of the sunflower polypeptide is very much lower (Figure 7b).

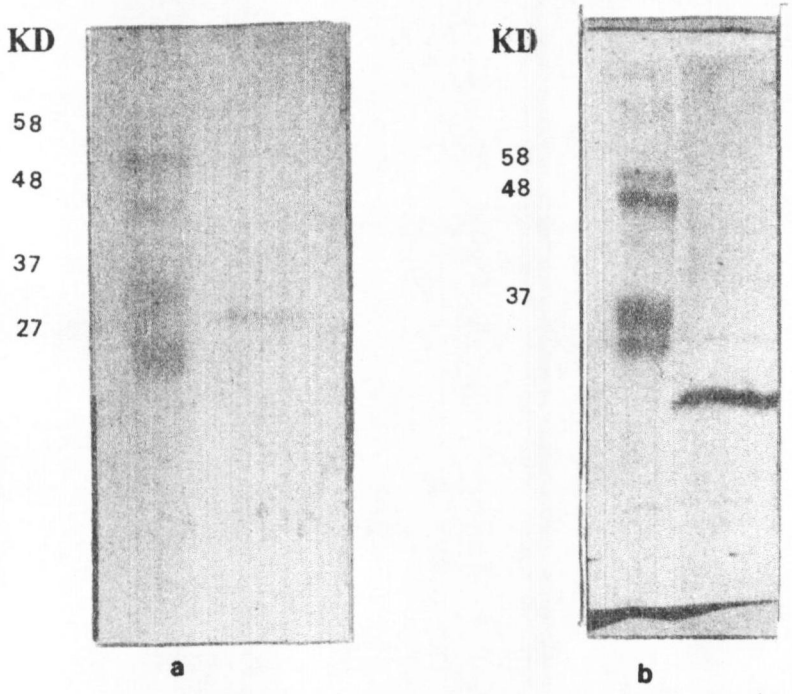

Figure 7. **Western blot analysis of immune serum to avocado mesocarp ß-ketoacyl (ACP) reductase with plant proteins separated by SDS-PAGE on a 10% mini gel. (a) Against a partially purified preparation of avocado mesocarp ß-ketoacyl (ACP) reductase. Lane 1, pre-stained molecular weight standards lane 2, partially purified preparation of avocado mesocarp; (b) Against a sunflower leaf crude extract. Lane 1, pre-stained molecular weight standards lane 2, sunflower leaf 0-55% $(NH_4)_2SO_4$ fraction.**

Effect of antibody on the enzyme

Since studies on inhibition of enzyme activity using the antibody are carried out using the native form of the antigen, it was essential to know the titre of the antibody against the native form of the antigen. On the basis of ELISA the titre of the antibody was found to be 1/10.

Inhibition of enzyme activity with the antibody was carried out using an antibody concentration of 1:10 with reducing amounts of antigen 10-100μg in order to be within the inhibition range. Two controls were used; one with a constant pre-immune serum concentration of 1:10 with reducing amounts of antigen ranging from 10-100 μg and the other with antigen alone ranging from 10-100 μg (Figure 8). It was observed that the total inhibition took place when 10 μg of enzyme was used. The pre-immune serum had no effect on the enzyme activity.

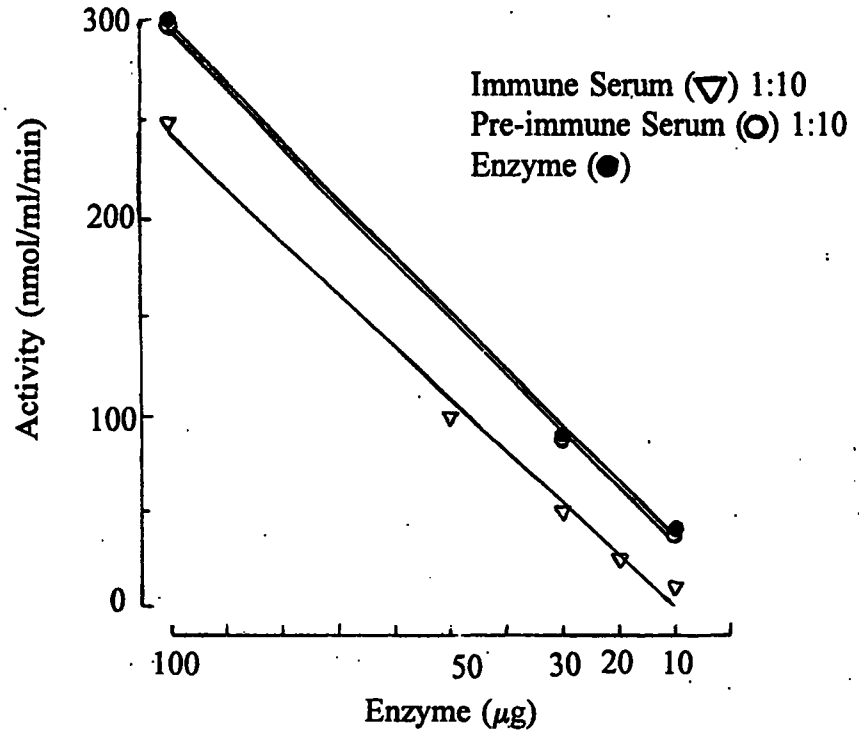

Figure 8. **The effect of pre-immune and immune sera on ß-ketoacyl (ACP) reductase activity.**

Subcellular localisation of ß-ketoacyl (ACP) reductase by immunoelectron microscopy

The subcellular localisation of the enzyme was determined by immunoelectron microscopy of avocado tissue and sunflower leaf tissue. The enzyme was found to be present in chloroplast and non photosynthetic plastids of both avocado and sunflower tissues (Figures 9a, 9b and 9c). In view of the very poor representation of mitochondria in the leaf tissue examined, an avocado mesocarp mitochondrial preparation derived from a total cellular homogenate was used to investigate the location. It is seen in Figure 10c that while the plastid in the field of the EM of the pellet shows the presence of the enzyme it appears to be absent from the mitochondria.

Immunoprecipitation of the products of polysomal protein synthesis with the antiserum

The products of polysomal protein synthesis of molecular weight ranging from 20-80 kDa which were separated on a 10% SDS-PAGE are shown in Figure 10a. It is seen that cycloheximide inhibits the incorporation of L-[^{35}S] methionine to proteins. Immunoprecipitation of the products of polysomal protein synthesis gave a polypeptide in the region of 37 kDa (Figure 10b) which is about 5 kDa more than that of the mature ß-ketoacyl (ACP) reductase.

The purified enzyme preparation was found to contain two polypeptides of molecular weight 32 and 29 kDa, respectively, both in the presence and absence of mercaptoethanol. These may have arisen as a result of the co-purification of two different proteins, or from the dissociation of two subunits of the same protein or from the partial proteolysis of the 32 kDa polypeptide to give the 29 kDa component. Since gel filtration chromatography on Superose-12 indicated that the enzyme had a molecular weight of 32 kDa and the antiserum to the 32 kDa polypeptide, which inhibited the enzyme activity, did not react with the 29 kDa polypeptide in the enzyme preparation was a result of fortuitous co-purification. Since the native enzyme appears to have a molecular weight of 32 kDa on the Superose column and the SDS-PAGE analysis of the preparation showed the presence of a similar peptide, the enzyme appears to be a monomer.

Since this enzyme has a high rate of reduction of CoA thioesters and uses NADH as reductant, it might be argued that this enzyme could be a

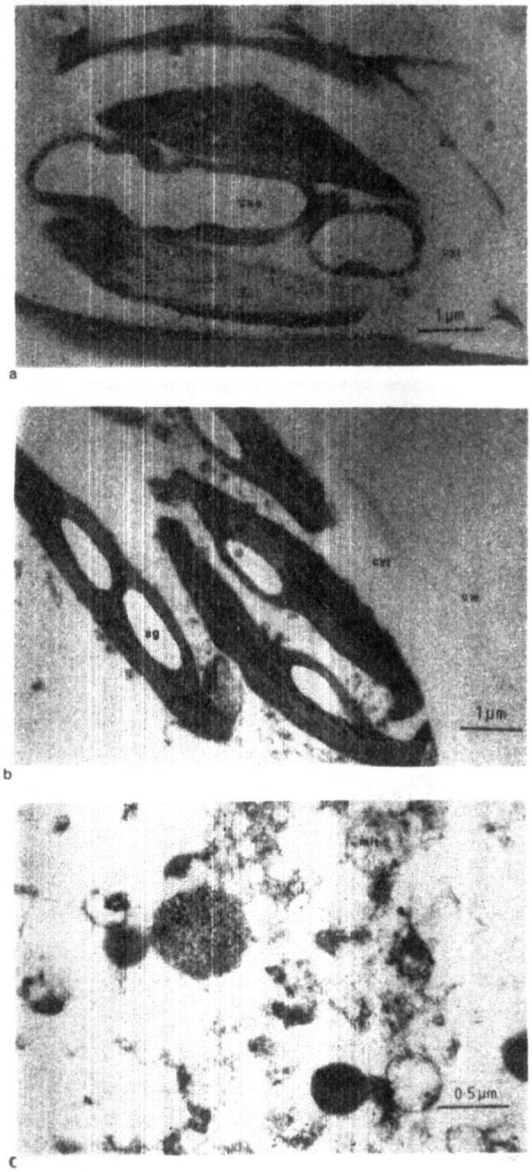

Figure 9. **Immunogold localization of ß-ketoacyl (ACP) reductase in plant tissues. (a) avocado leaf. *ch*, chloroplast; *cw*, cell wall; *vac*, vacuole; *cyt*, cytoplasm. Bar=1 µm;.x 12,200 (b) sunflower leaf. *ch*, chloroplast; *cw*, cell wall; *vac*, vacuole; *cyt*, cytoplasm; *sg*, starch grain. Bar = 1 µm; x10,000 (c) avocado mesocarp mitochondrial pellet. *pl*, plastid; *mit*, mitochondria. Bar = 0.5 µm; x16,500.**

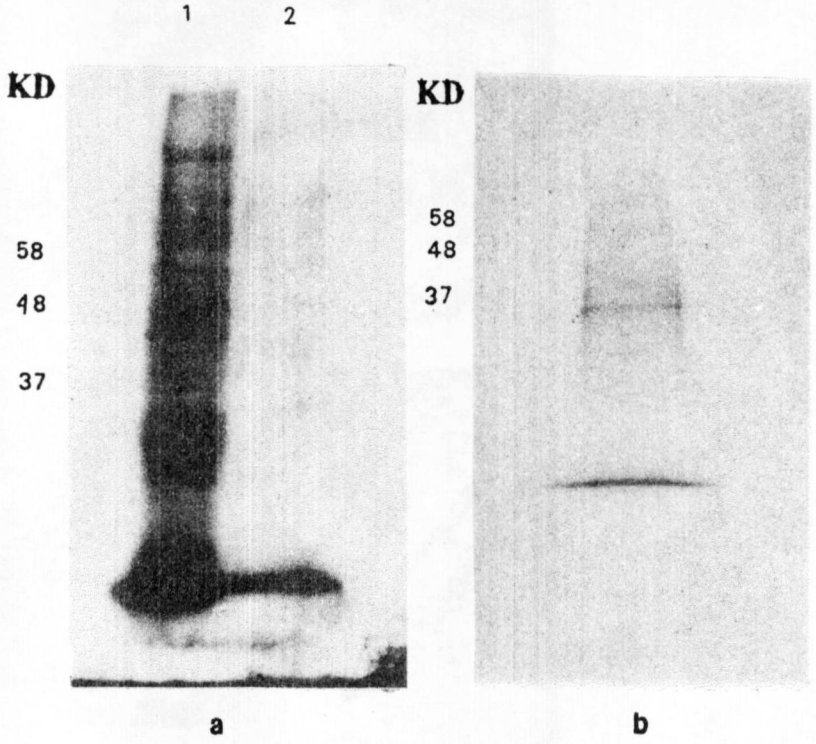

Figure 10. **L-[^{35}S] methionine labelled products of polysomal protein synthesis separated on a 10% SDS-PAGE. (a) Total proteins lane 1, products of total polysomal synthesis; lane 2, products of total polysomal protein synthesis in the presence of 0.2% cycloheximide. (b) Immuno precipitation with anti ß-ketoacyl (ACP) reductase.**

component of the ß-oxidation pathway. If this was the case it should be present in the mitochondria or peroxisomes. In the present study, using immunogold labelling techniques, it has been demonstrated that in both avocado and sunflower tissues, this enzyme is localised within chloroplasts and plastids, but could not be detected in mitochondria. Thus, it is extremely unlikely that this enzyme is a mitochondrial or peroxisomal enzyme and consequently a component of the ß-oxidation pathway.

The higher molecular weight precursor obtained from the immunoprecipitation, with anti-ß-ketoacyl (ACP) reductase of the products of cytoplasmic polysomal protein synthesis, indicates the presence of a leader sequence of molecular weight of about 5 kDa. Sequencing of cDNA clones from *B. napus* seed and *A. thaliana* leaf ß-ketoacyl (ACP) reductase (NADPH-specific) reveals the presence of transit peptides of 59 amino acids (Slabas *et al.*, 1990). These observations together with that of immunogold labelling studies, confirm that these enzymes are nuclear encoded and are targeted to the plastids, being synthesised on cytoplasmic mRNA and possessing a transit peptide.

Comparison of amino acid sequence data of the polypeptides with other known sequences revealed a high percentage of homology with a number of dehydrogenases. Polypeptide I shows 87.5% homology in 8 amino acid overlap with yeast malate dehydrogenase mitochondrial precursor; while polypeptide II shows 66.7% sequence homolgy in 8 amino acid overlap with both subunits of pyruvate dehydrogenase E1 component of *Bacillus subtilis* and glucose 6-phosphate dehydrogenase of the fruit fly (*Drosophila melanogaster*), 100% homolgy is shown in 5 amino acid overlap in the a chain of alcohol dehydrogenase complex of Japonica (*Coturnix cortunix*) and 80% homology in 5 amino acid overlap with type II precursor of pyruvate dehydrogenase E1 component of pig round worm (*Ascaris suum*).

It was found that the antibodies raised against avocado mesocarp ß-ketoacyl (ACP) reductase (NADH-specific) cross-react specifically with sunflower and *Arabidopsis* leaf extracts (results not shown) suggesting that the enzyme may be of general occurrence.

On the evidence presented thus far, the location and properties of NADH-specific ß-ketoacyl (ACP) reductase are consistent with it being a component of the plastid fatty acid synthetase system. The presence of an NADH-specific ß-ketoacyl (ACP) reductase has so far been detected only in avocado mesocarp (Caughey and Kekwick, 1982). A different NADH-specific acetoactyl-CoA reductase has, however, been purified from *Euglena gracilis* (Ernst-Fonberg, 1986) and it has been shown that this enzyme is not directly involved in *de novo* fatty acid biosynthesis nor in the ß-oxidation pathway. It is known that while type-I synthetases show a marked specificity for NADPH (Katiyar and Porter, 1980), type-II systems require both NADH and NADPH for maximal rates (Caughey and Kekwick, 1982; vick and Beevers, 1978).

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