PARTIAL PURIFICATION OF β-KETOACYL–ACYL CARRIER PROTEIN SYNTHASE FROM A HIGHER PLANT

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1. Introduction

The enzymes of acyl carrier protein (ACP)-dependent fatty acid synthesis in bacteria, particularly in *Escherichia coli,* have been studied extensively [1]. Although ACP-dependent fatty acid synthesis has been demonstrated in higher plants [2], the isolation of the individual enzymes from such organisms has not been reported. So far, chloroplasts from photoautotrophically-grown *Euglena gracilis,* a unicellular phytoflagellate, have been the only source of partially purified enzymes of ACP-dependent fatty acid synthesis from a eukaryote [3].

Here, we describe for the first time the partial purification and characterization of the key enzyme of ACP-dependent fatty acid synthesis, β-ketoacyl-ACP synthase, from a higher plant. We have used cell suspension cultures of parsley (*Petroselinum hortense*) as a convenient source of the enzyme. These cultures offer the advantage of being easily propagated in large amounts and have proved very useful for the isolation and purification of various enzymes, including acetyl-CoA carboxylase, an enzyme closely related to fatty acid synthesis [4], and chalcone synthase, an enzyme catalyzing a condensation reaction of acetate units similar to that of β-ketoacyl-ACP synthase, the 'condensing enzyme' of fatty acid synthesis [5].

2. Materials and methods

2.1. *Enzyme assays*

β-Ketoacyl-ACP synthase, CO_2-exchange assay [6]: The mixture contained 10 μmol imidazole buffer (pH 6.2), 4 μmol 2-mercaptoethanol, 36 nmol malonyl-CoA, ~4 nmol *E. coli* ACP (40 μg, with malonyl-CoA: ACP malonyltransferase activity), 2.5 μmol $NaH^{14}CO_3$ (0.5 Ci/mol) and enzyme solution in 100 μl total vol. The reaction was carried out for 30 min at 30°C. Assay for product identification: 10 μmol potassium phosphate buffer (pH 8.0), 10 nmol acetyl-CoA (or butyryl-CoA), 4 nmol ACP (as above), 1.4 nmol [2-^{14}C]malonyl-CoA (25 Ci/mol) and 8–15 μg partially purified β-ketoacyl-ACP synthase in 100 μl total vol. were incubated for 10 min at 30°C and then treated with 10 μl NaOH (1 mol/l). After 30 min at 30°C, the mixture was acidified with 10 μl HCL (6 mol/l) and extracted with ethyl acetate. The organic phase was used for thin-layer chromatography on cellulose (Merck) with *n*-amyl alcohol/formic acid/water (80:15:11) or on silica gel (Merck) with chloroform/methanol/water (65:25:4). High-pressure liquid chromatography was carried out on an RP-18 column with 0.1 mol/l $NaClO_4$ (pH 4.0), using a gradient from 0–20% methanol. Acetyl-CoA:ACP *S*-acetyltransferase (EC 2.3.1.38) and malonyl-CoA:ACP *S*-malonyltransferase (EC 2.3.1.39) assays were as in [7]. The *E. coli* ACP used for this assay was found to be transferase-free after purification through the second DEAE-cellulose step [6]. For all other purposes, ACP was purified by a single DEAE-cellulose step [6]. [2-^{14}C]Malonyl-CoA (59 Ci/mol; diluted with unlabelled material from Sigma), [1-^{14}C]acetyl-CoA (57 Ci/mol) and $NaH^{14}CO_3$ (59.5 Ci/mol) were from Amersham-Buchler (Braunschweig).

2.2. *Enzyme purification*

Cell suspension cultures of parsley (*Petroselinum hortense*) were propagated in medium I containing 1 mmol/l $CaCl_2$ and were harvested, when the conductivity of the medium was 1.2–1.5 mΩ [8]. The purification procedure was similar to that in [5] for

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chalcone synthase from parsley. All steps were carried out at 4°C. Frozen cells (750 g) were extracted in a mortar with 750 ml 100 mmol/l potassium phosphate (pH 8.0) containing 20 mmol/l 2-mercaptoethanol, and treated with Dowex 1X2 [5]. A protein fraction precipitating between 50–70% NH_4SO_4 was dissolved in 15 ml same buffer (except that this and all of the following buffers contained 10 mmol/l 2-mercaptoethanol), dialyzed and fractionated on a DEAE-cellulose column (2 × 9 cm) using a linear gradient (200 ml) of 50–300 mmol/l NaCl in 50 mmol/l potassium phosphate (pH 8.0) containing 10% glycerol. Fractions with synthase activity, which eluted between 100–200 mmol/l NaCl, were concentrated by ultrafiltration, dialyzed, and chromatographed on a hydroxyapatite column (1.4 × 5 cm) with a linear gradient (60 ml) of 10–50 mmol/l potassium phosphate (pH 7.8) in 20% glycerol. Synthase activity was eluted at 20–40 mmol/l potassium phosphate.

3. Results and discussion

The spectrophotometric assay used in [1,3] was too insensitive to detect enzyme activity in parsley cell extracts with sufficient accuracy, at least during the early stages of purification. We used therefore the two radioactive assays described above. The $^{14}CO_2$-exchange reaction proved to be the assay of choice, when a rapid procedure was required, e.g., for screening many samples during enzyme purification. For more specialized purposes, e.g., product identification, the assay using [2-^{14}C]malonyl-CoA as labelled substrate was most appropriate.

Table 1 summarizes a procedure resulting in a 74-fold purification of β-ketoacyl-ACP synthase from cultured parsley cells with a recovery of ~20% of the original activity. The sequence of steps was similar to that used in [3] for the partial purification of β-ketoacyl-ACP synthase from *Euglena gracilis*. In contrast to the studies of acetyl-CoA carboxylase [4] and chalcone synthase [5] from parsley, these experiments were conducted with dark-grown cells, since fatty acid synthesis was not stimulated by light in the cultures used here [9].

Throughout the purification procedure, β-ketoacyl-ACP synthase was inactive in the absence of added ACP. Apparently, all preparations contained sufficient acetyl-CoA:ACP *S*-acetyltransferase and malonyl-CoA:ACP *S*-malonyltransferase activities to ensure an efficient formation of acetyl-ACP and malonyl-ACP from the respective CoA esters, even though both transferases decreased to 0.2% or less of their original activities with increasing purity of the synthase (table 1). Butyryl-CoA was at least as efficient a starter molecule in the combined acyltransferase/synthase assay as was acetyl-CoA. However, a quantitative comparison of various possible starter molecules for the condensation reaction has not been made. This question will be the subject of future studies involving the use of synthetic ACP esters to eliminate the possible influence of acetyl- or other acyltransferase(s) which are present in the assay mixture.

Acetoacetate (3-oxobutyrate) and 3-oxohexanoate were identified as reaction products with acetyl-CoA and butyryl-CoA as substrates, respectively, by co-chromatography with authentic material (TLC, HPLC). Moreover, with acetyl-CoA as starter molecule, acetoacetate was indirectly verified as the condensation product by addition of 20 nmol NADH and 3 units 3-hydroxyacyl-CoA dehydrogenase (Sigma) to the synthase assay. In this case, 3-hydroxybutyrate was isolated from the reaction mixture.

The size of β-ketoacyl-ACP synthase from parsley

Table 1
Partial purification of β-ketoacyl-ACP synthase from parsley cells and separation from acetyl- and malonyltransferases

Purification step	β-Ketoacyl-ACP synthase			Acetyltransferase			Malonyltransferase		
	Spec. act (μkat/kg)	Purif. (-fold)	Yield (%)	Spec. act (μkat/kg)	Purif. (-fold)	Yield (%)	Spec. act. (μkat/kg)	Purif. (-fold)	Yield (%)
Dowex 1X2 supernatant	1.8	1	100	7.1	1	100	3,300	1	100
Fractionation with NH_4SO_4 (50–70% satn.)	10	6	97	0.8	0.1	2	14,800	5	80
DEAE-cellulose column	38	21	49	1.4	0.2	0.5	150	<0.1	0.1
Hydroxyapatite column	134	74	20	4.6	0.6	0.2	460	0.1	<0.1

was estimated by gel chromatography on a Sephacryl S-300 column using the partially purified enzyme. The app. M_r was ~70 000. This M_r-value was similar to those estimated for β-ketoacyl-ACP synthases I (66 000–80 000 [10,11]) and II (76 000–85 000 [10,11]) from *E. coli*, as well as for chalcone synthase from parsley (77 000 [5]). By contrast, a somewhat higher M_r (118 000) was reported for β-ketoacyl-ACP synthase from *Euglena gracilis* [3].

In conclusion, 3 lines of evidence strongly suggest that the partially purified β-ketoacyl-ACP synthase from parsley is a prokaryote-type 'condensing enzyme' of fatty acid synthesis [1], as generally postulated to occur in higher plants [2]: The enzyme catalyzed an ACP-dependent condensation of acetate (or butyrate) with the acetate unit from malonate, was separated to a large extent from acetyl- and malonyltransferase activities, and had a very low M_r, when compared with the multifunctional subunits of animal [12] or yeast [13] fatty acid synthases.

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