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The glyoxysomal 3-ketoacyl-CoA thiolase precursor from *Brassica napus* has enzymatic activity when synthesized in *Escherichia coli*

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Abstract Glyoxysomal 3-ketoacyl-CoA thiolase is the last enzyme in the β-oxidation of fatty acids in plant glyoxysomes. A full-length cDNA of the glyoxysomal 3-ketoacyl-CoA thiolase from *Brassica napus* and a truncated version, lacking the N-terminal targeting signal were cloned in a T7 promoter-based vector. Both recombinant proteins were expressed in *Escherichia coli* and activity was measured. Full-length and truncated 3-ketoacyl-CoA thiolase have comparable activity in *E. coli*. Moreover, full-length 3-ketoacyl-CoA thiolase was purified from *E. coli* and N-terminal sequencing of the protein confirmed that the precursor form indeed is enzymatically active.

Key words: Expression; Microbody; Presequence

1. Introduction

The 3-ketoacyl-CoA thiolase is a homodimeric enzyme which catalyses the thiolytic cleavage of (C_n)3-ketoacyl-CoA to acetyl-CoA and (C_{n-2})3-ketoacyl-CoA in the presence of CoA [1]. In many eukaryotes 3-ketoacyl-CoA thiolase is located in the mitochondrial matrix, but in plants it is confined to peroxisomes and glyoxysomes [2] and has not been demonstrated in plant mitochondria [3]. Like mitochondrial proteins, peroxisomal and glyoxysomal proteins are synthesized in the cytosol on free polyribosomes and are post-translationally imported into their target organelle. Most mitochondrial proteins are synthesized with an N-terminal targeting signal, which is cleaved off during the translocation process [4]. In contrast, the majority of peroxisomal and glyoxysomal proteins are not proteolytically modified during transport into the organelle, and are further distinguished from mitochondrial proteins in that the targeting signal is located in the extreme C-terminus [5]. This type of targeting signal was first identified in luciferase from firefly and has been referred to as the peroxisomal targeting signal 1 (PTS1). As an exception to this general peroxisomal targeting signal, rat peroxisomal 3-ketoacyl-CoA thiolase is synthesized as a larger precursor with a cleavable N-terminal targeting determinant [6] known as the peroxisomal targeting signal 2 (PTS2). The only two other known exceptions with an N-terminal extension with similar features to that of the rat 3-ketoacyl-CoA thiolase are the glyoxysomal citrate synthase from pumpkin and watermelon glyoxysomal malate dehydrogenase [7,8]. The nature of the PTS2-type signal has been most extensively analyzed in 3-ketoacyl-CoA thiolase. Studies in both mammalian and yeast cell systems have proven the essential role of the N-terminus for import of the 3-ketoacyl-CoA thiolase [9,10]. However, the processing of PTS2-type proteins, rat peroxisomal 3-ketoacyl-CoA thiolase, cucumber glyoxysomal 3-ketoacyl-CoA thiolase and watermelon glyoxysomal malate dehydrogenase [6,11,8], remains poorly understood and it is still unclear whether the processing is associated with import or if it is an event unrelated to import. In vitro import studies with the rat peroxisomal 3-ketoacyl-CoA thiolase into isolated peroxisomes have provided the first indications that processing of the 3-ketoacyl-CoA thiolase precursor is not necessarily coupled with import [12]. Thus, cleavage of the presequence could be a secondary proteolytic modification and it is therefore of interest to examine whether the precursor is able to fold and dimerize into an active enzyme. We therefore expressed the glyoxysomal 3-ketoacyl-CoA thiolase cDNA from oilseed rape in *E. coli* as well as a construct lacking the N-terminal targeting signal. Both recombinant proteins showed similar activity in extracts of *E. coli*, suggesting that the presequence does not prevent proper folding or assembly of the active enzyme. To our knowledge, this is the first report demonstrating enzymatic activity of the glyoxysomal 3-ketoacyl-CoA thiolase precursor.

2. Materials and methods

2.1. Constructs and E. coli expression of cDNA encoding 3-ketoacyl-CoA thiolase

The full-length glyoxysomal 3-ketoacyl-CoA thiolase was originally isolated by reverse transcription PCR on mRNA from etiolated *Brassica napus* seedlings, with primers flanking the start and stop codons [13]. PCR was used to introduce an *Nde*I restriction site at the initiation codon of the isolated 3-ketoacyl-CoA thiolase cDNA. The resulting PCR product was cloned in the pGEM-T overhang vector from Promega and sequenced. Subsequently the entire coding region was excised by *Nde*I and *Sal*I digestion and subcloned in the expression vector pT7-7, digested with *Nde*I and *Sal*I prior to ligation. This construct was sequenced from both ends with insert-flanking primers representing the vector sequence. A truncated version of the 3-ketoacyl-CoA thiolase cDNA lacking the codons for the first 35 amino acids of the N-terminus was made by PCR on the full-length cDNA using the matching forward primer 5′- CATATGGCTGGGGACAGTG-CTGCGT-3′ and the matching reverse primer 5′-GGAATTCTAAC-GAGCGTCCTTGGA-3′. The forward primer anneals at position 106–124 downstream from the start ATG of the full-length sequence and introduces an *Nde*I site at the 5′-end of the PCR product. The reverse primer flanks the stop codon region and introduces an *Eco*RI site. The resulting PCR product was ligated into the *Nde*I and *Eco*RI sites of the expression vector pT7-7. The expression vector alone or either of the two 3-ketoacyl-CoA thiolase constructs were used to transform the *E. coli* strain BL21(DE3). Expression was performed by growing transformed cells until OD_{600}=0.6 at 37°C and then adding IPTG to a final concentration of 1 mM. Cells were grown for another 2 h at 37°C, and then collected by centrifugation at 3000×*g* for 5 min. The cell pellet was dissolved and boiled in SDS-sample buffer [14] and proteins resolved on 10% SDS-PAGE [15].

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Full length:  CAT ATG GAG AAA GCG ATG GAG AGA CAA AGA GTT CTT CTT GAA CAT CTC
                M   E   K   A   M   E   R   Q   R   V   L   L   E   H   L

              CGT CCT TCT TCT TCT TCC TCT CAC AGC TTT GAA GGC TCT CTC TCT
                R   P   S   S   S   S   S   H   S   F   E   G   S   L   S

              GCT TCA GCT TGC TTG GCT GGG GCA AGT GCT ----------
                A   S   A   C   L   A   G   D   S   A
                            *

Truncated:                    CAT ATG GCT GGG GAC AGT GCT ----------
                                M   A   G   D   S   A
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Fig. 1. The full-length and the amino-terminal truncated version of the *Brassica napus* 3-ketoacyl-CoA thiolase. * indicates the conserved cysteine residue in the sequence. The introduced start codon in the truncated sequence is double underlined. Single underlining highlights the common regions in the full-length and truncated versions.

2.2. Purification and N-terminal sequencing

Induced 3-ketoacyl-CoA culture (1 l) was lysed by sonication and the 100 000×*g* supernatant was loaded on a Q-Sepharose column in 20 mM bis-Tris, pH 7.4. Proteins were eluted with a 0–300 mM NaCl gradient. The 3-ketoacyl-CoA thiolase containing peak was further fractionated by Superdex 200 gel filtration in 100 mM Tris-HCl, 50 mM KCl, 25 mM MgCl, pH 7.5. The resulting thiolase-containing fraction was separated by SDS-PAGE and blotted onto a PVDP membrane. The N-terminal sequence was determined using an Applied Biosystems gas-phase sequencer model 470A equipped with an online PTH-amino acid detector, model 120A.

2.3. Activity measurements

Induced *E. coli* culture (50 ml) was pelleted by centrifugation and resuspended in 1 ml of reaction buffer containing 100 mM Tris-HCl, pH 8.1, 25 mM $MgCl_2$ and 50 mM KCl. Cells were lysed by sonication for 4×1 min on ice. The crude lysate was then centrifuged at 100 000×*g* for 20 min in a Beckman Desk-top Ultracentrifuge. The resulting supernatant was split into two halves. One half was used as enzyme source without further treatment in one set of experiments. The other half was subjected to partial purification by incubation with 1 ml Q-Sepharose (Pharmacia) equilibrated in the reaction buffer. The supernatant was collected after 1 min incubation and used as the enzyme source in another set of experiments. The enzyme assay was set up in a silica cuvette containing 100 mM Tris-HCl buffer, pH 8.1, 50 mM KCl, 25 mM $MgCl_2$, 50 μM CoA and 10 μM acetoacetyl-CoA in a final volume of 1 ml. The thiolytic activity was determined by following the breakage of acetoacetyl-CoA, measured by the decrease in absorption of the enol form at 302 nm [16]. One unit of activity is defined as the amount of enzyme that converts 1 μmol acetoacetyl-CoA/min. Calculations were made assuming a molar extinction coefficient of 17 000 M^{-1} cm^{-1} for acetoacetyl-CoA at 302 nm. Background activity was measured in induced cultures transformed with the expression vector alone.

3. Results and discussion

The cleavage of rat 3-ketoacyl-CoA thiolase and watermelon malate dehydrogenase precursors has been identified to

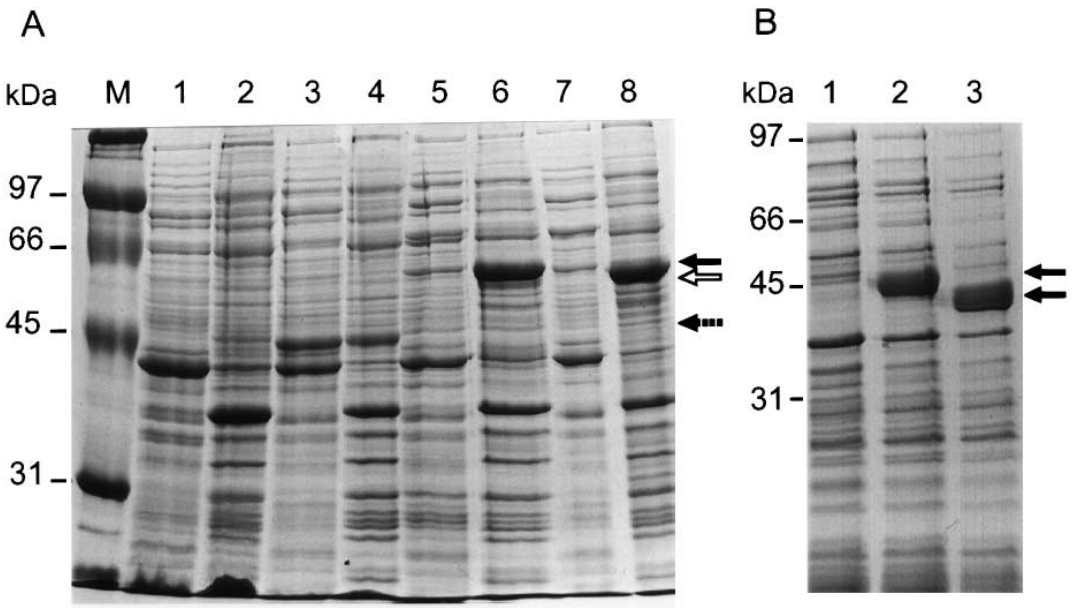

Fig. 2. A: SDS-PAGE of soluble (tracks 1,3,5,7) and insoluble (tracks 2,4,6,8) polypeptides of *Brassica napus* 3-ketoacyl-CoA thiolase (tracks 3,4), isocitrate lyase (tracks 5,6) and malate synthase (tracks 7,8) synthesized in *E. coli*. Tracks 1,2 are extracts from *E. coli* transformed with the expression vector without insert. The closed, open and dashed arrows indicate the position of isocitrate lysase, malate synthase and 3-ketoacyl-CoA thiolase polypeptides, respectively. B: Partially purified 3-ketoacyl-CoA thiolase from induced *E. coli* cultures. Track 1: Expression plasmid without insert. Track 2: Full-length polypeptide. Track 3: Amino terminal truncated polypeptide. The arrows indicate the position of the two polypeptides.

Table 1
Activity of recombinant thiolase produced in *E. coli*

Enzyme source	Thiolase		Truncated		Control	
	Exp. 1 (U/mg)	Exp. 2 (U/mg)	Exp. 1 (U/mg)	Exp.2 (U/mg)	Expl (U/mg)	Exp. 2 (U/mg)
Crude extract	0.133	0.20	0.118	0.23	0.0033	0.0020
Q-Sepharose	-	0.17	-	0.31	-	0.0024

occur close to a cysteine, which is conserved in all PTS2 proteins including the *Brassica napus* 3-ketoacyl-CoA thiolase [17,8]. Consequently, we deleted the first 35 N-terminal residues including the conserved cysteine from the full-length *Brassica napus* cDNA encoding glyoxysomal 3-ketoacyl-CoA thiolase (Fig. 1). The two cDNAs were ligated into a unique *Nde*I restriction site of the pT7-7 expression vector. This cloning strategy ensured expression of the inserted 3-ketoacyl-CoA thiolase reading frames, without residues encoded by the vector.

About half of these two *E. coli* expressed plant 3-ketoacyl-CoA thiolase polypeptides were found to be soluble as determined by SDS-PAGE (Fig. 2A, tracks 3 and 4). In contrast, two other glyoxysomal enzymes from *Brassica napus*, malate synthase (EC 4.1.3.2, Accession number: Y13357) and isocitrate lyase (EC 4.1.3.1, Accession number: Y13356), were both almost exclusively in the insoluble fraction (Fig. 2A, tracks 5–8). The differences in the solubility of the three proteins does not reflect large differences in their homology to the corresponding *E. coli* enzymes since, the amino acid identity of the three plant proteins to the *E. coli* enzymes are approximately 40% in each case. Both full-length and truncated 3-ketoacyl-CoA thiolase were expressed to a high level in the bacterial host. Full-length 3-ketoacyl-CoA thiolase appeared as a dominant polypeptide of 53 kDa, which is in good agreement with the calculated molecular mass of 48.7 kDa (Fig. 2B, track 2). The truncated 3-ketoacyl-CoA thiolase appeared on an SDS-PAGE gel as a prominent protein with a slightly lower molecular mass (Fig. 2B, track 3).

Activity of full-length and truncated 3-ketoacyl-CoA thiolase was measured in freshly prepared *E. coli* extracts in two sets of identical experiments (Table 1). In the first experiment, full-length and truncated 3-ketoacyl-CoA thiolase showed 40 and 36 times the activity of the background, respectively. In the second experiment the activity of truncated 3-ketoacyl-CoA thiolase was found to be 115-fold the background, while full-length 3-ketoacyl-CoA thiolase activity was determined to be 100 times the background. Both constructs had comparable specific activities demonstrating that the presence of the presequence does not prevent proper folding and assembly of the precursor polypeptide in *E. coli*.

To confirm that the full-length 3-ketoacyl-CoA thiolase had not been proteolytically modified by *E. coli*, this recombinant protein was purified and the N-terminal sequence was determined. The sequence obtained was found to be MEKAMERQRV which perfectly matches the predicted sequence of the 3-ketoacyl-CoA thiolase presequence (Fig. 1) [13]. Thus, we concluded, that the 3-ketoacyl-CoA thiolase precursor form indeed was enzymatically active, suggesting that cleavage of the N-terminal targeting sequence was not essential for proper folding and function of the enzyme. A similar observation was recently reported for the watermelon gMDH [18].

The function of the 3-ketoacyl-CoA thiolase N-terminus as a targeting signal is well documented in mammals and yeast. In plants, a clear correlation between import and cleavage has yet to be proven. Cleavage, however of gMDH and 3-ketoacyl-CoA thiolase is assumed to occur during or after the translocation across the glyoxysomal membrane [11,8]; based on our data, further analyses are needed to determine whether this processing occur before or after assembly of the active enzyme dimer. In yeast, processing of PTS2 proteins like alcohol oxidase, 3-ketoacyl-CoA thiolase and amine oxidase does not take place and heterologous expression of watermelon gMDH leads to the import into yeast peroxisomes without cleavage of the pre-sequence[19]. Since yeast peroxisomal 3-ketoacyl-CoA thiolase is a dimer with the disordered NH2-terminal containing the the PTS2 signal exposed to the solvent [20] it is not surprising that the *Brassica napus* glyoxysomal 3-ketoacyl-CoA thiolase with the PTS2 signal present can fold into an active enzyme.

We conclude, that the 3-ketoacyl-CoA thiolase presequence does not prevent proper folding and function of the enzyme.

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