Non-organellar Acyl Carrier Protein from Oleaginous Yeast Is a Homologue of Ribosomal Protein P2*

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Summary

Acyl carrier protein (ACP) is responsible for carrying the growing fatty acid chain from one enzyme active site to the next during fatty acid biosynthesis. Here we report the identification, purification, immunocytochemical localization and cloning of ACP from the oleaginous yeast, *Rhodotorula glutinis*. The soluble fraction of this organism can synthesize triacylglycerol and is able to accept acyl group from ACP for the synthesis. The ACP, cloned from the system, showed a significant similarity with ribosomal protein P2. Expression and characterization of the recombinant protein showed that the ACP was acylated in vitro. The recombinant protein was post-translationally modified as it was observed in $^{14}$Cβ-alanine labeling and matrix assisted laser desorption mass spectroscopic analysis. Site directed mutants were generated to identify a serine residue responsible for phosphopantetheinylation and found that mutation of serine 59 to alanine abrogated the fatty acylation ability of the protein. These results demonstrate that a novel modification of ribosomal protein P2 allows it to act as an acyl carrier protein and participate in acylation reactions.
Introduction

Acyl carrier protein (ACP) of E. coli is a small, acidic protein containing a prosthetic group 4’-phosphopantetheine to which fatty acyl chains are linked as thioester. ACP acts as a carrier for substrates and intermediates from the active site of one enzyme to the other in the fatty acid synthase [1], non-ribosomal polypeptide synthetases [2] and polyketide synthase [3]. ACP is also involved in phospholipid synthesis [4, 5], membrane bound oligosaccharide synthesis [6] in E. coli and host-specific lipochitin oligosaccharide signal molecule synthesis in Rhizobium leguminosarum [7]. Several isoforms of ACP have been identified in plant chloroplasts and are shown to be involved in specific fatty acid synthesis during seed development [8]. In Neurospora crassa [9] and bovine heart [10] ACP was identified in mitochondria as a subunit of the respiratory NADH:ubiquinone oxidoreductase complex. In Saccharomyces cerevisiae, nuclear encoded and 19 kDa molecular mass ACP has been reported in mitochondria [11, 12] but this ACP is not part of the respiratory complex [13]. No distinct low molecular mass non-organellar ACP has been identified in eukaryotes and this could be due to the presence of fatty acid synthase multifunctional enzyme (type-I). Eukaryotic ACP possesses characteristic organellar import sequence in front of the known core sequence [12]. The overall three-dimensional structure and the amino acid sequences around the phosphopantetheine-binding site of ACP are well conserved [14, 15].

A soluble triacylglycerol biosynthetic complex (TBC) from oleaginous yeast was isolated and the individual components were characterized [16]. The complex consists of ACP, acyl ACP synthetase, lysophosphatidic acid (LPA) acyltransferase, phosphatidic acid phosphatase, diacylglycerol (DAG) acyltransferase [16], and superoxide dismutase 1

1ACP, acyl carrier protein; CoA, coenzyme A; DAG, diacylglycerol; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; LPA, lysophosphatidic acid; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; Ni-NTA, nickel-nitrilotriacetic acid; PPTase, phosphopantetheine protein transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAG, triacylglycerol; TBC, triacylglycerol biosynthetic complex; TRITC, trimethylrhodamine isothiocyanate.
The acyl ACP synthetase in the multienzyme complex activates free fatty acids to acyl ACP in an ATP dependent manner and the synthesized acyl ACP is used as a substrate by TBC [18]. Mutation in any of the acyltransferases present in TBC or alteration in nutritional content leads to decreased synthesis of triacylglycerol in *Rhodotorula glutinis* [19].

Here we report the identification, purification and immunolocalization of a non-organellar ACP from oleaginous yeast that is an integral part of TBC. Cloning, expression and subsequent characterization of the protein reveals that it has a sequence homology to ribosomal protein P2. The protein is post-translationally modified to accept free fatty acid and the acyl ACP formed can act as acyl group donor in acylation reactions. Alteration of serine 59 to alanine by site directed mutation abolishes the post-translational modification and therefore the fatty acid esterification ability is lost in the ACP.
Experimental Procedures

Materials – *Rhodotorula glutinis* was obtained from the Institute of Microbial Technology, Chandigarh, India (Microbial Type Culture Collection 1151). Ampicillin, tetracycline, kanamycin, sarcosyl, ACP (*E. coli*), diaminobenzidine, LPA, DEAE cellulose, MOPS, benzoylated dialysis tubing, lyticase, poly-L-lysine and [14C]β-alanine (49 mCi/mmol) were obtained from Sigma Chemical Company (St. Louis, MO). cDNA library construction kit was from Stratagene (La Jolla, CA). [1-14C]Palmitoyl CoA (51 mCi/mmol), [1-14C]oleic acid (55 mCi/mmol), [α-32P]dATP/ dCTP (30 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Foster City, CA). Restriction enzymes, deoxynucleotidyl terminal transferase, Pfu polymerase, low melting agarose, deoxy- nucoetidyl triphosphates, Sephadex G-10, G-50, EDTA, isopropyl-β-D-thiogalactopyranoside (IPTG), X-GAL and all other molecular biology reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). DNA purification kit and nickel-nitrilotriacetic acid (Ni-NTA) matrix were from Qiagen (Valencia, CA). Silica-thin layer chromatography plates were from Merck (Rahway, NJ). Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-rabbit antibodies were from Bangalore Genei, Bangalore, India. All other reagents were of analytical grade.

Growth conditions – Yeast cells were grown in malt-yeast extract medium (pH 7.0) containing 0.3% yeast extract, 0.5% peptone, 0.3% malt extract supplemented with 1% glucose with aeration at 30°C. Alternatively, cells were grown in minimal medium supplemented with 0.67% yeast nitrogen base, 0.2% amino acids (with or without aspartate) and 1% glucose. Cell density was determined by light scattering at 600 nm (one $A_{600\text{ nm}} = 9 \times 10^7$ cells).
Metabolic labeling of *R. glutinis* cells with [14C]β-alanine – *R. glutinis* cells were grown in complete minimal medium (containing all the amino acids) till early log-phase (15 h) and then transferred to aspartate less minimal media to which [14C]β-alanine (10 µCi/ml culture) was added and incubated further for 12 h. Cells were centrifuged, washed and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 5% sucrose and 1 mM phenylmethysulfonyl fluoride), and glass bead lysis was performed. Cytosol and TBC were prepared [16] and resolved on 7% native-PAGE and 15% SDS-PAGE followed by fluorography.

**Enzyme assays** – Acyl ACP synthetase was assayed and ACP was quantified as described (20). The reaction mixture consisted of 0.1 M Tris-HCl (pH 8.0), 0.4 M LiCl, 5 mM ATP, 5 mM MgCl₂, 1 mM DTT, 0.2% Triton-X-100, 25 µM ACP from *R. glutinis* or *E. coli*, [1-14C]oleate (100 mM; 0.5 µCi) and *E. coli* acyl ACP synthetase (25 mU). The reaction mixture was incubated at 30°C for 30 min and spotted on Whatman 3MM filter discs and washed thrice with chloroform: methanol: acetic acid (3:6:1, v/v) followed by drying. The radioactivity associated with filter discs was determined in liquid scintillation counter using toluene based scintillation cocktail.

LPA and DAG acyltransferases and phosphatidic acid phosphatase activities are collectively represented as TAG synthase (16). This activity was performed by monitoring the incorporation of [14C]oleoyl CoA (100,000 dpm) or [14C]oleoyl ACP (35,000 dpm) into TAG in the presence of the acyl acceptor, LPA. TAG synthase assay mixture consisted of 5-25 µg enzyme, 20 µM acyl acceptor, 50 µM LPA in a total volume of 100 µl. The reaction was carried out at 30°C for 30 min and stopped by lipid extraction [21]. Lipids were separated on silica-TLC plates using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v) and visualized by staining with iodine vapor. The spot corresponding to TAG was scraped and radioactivity was measured in a liquid scintillation counter.
**Purification of acyl carrier protein** – Acyl carrier protein was purified from *R. glutinis* as described [22]. Late-logarithmic phase cells (35 g wet weight) were centrifuged and resuspended in 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride and 100 mM leupeptin. The cells were lysed using glass beads (0.45 - 0.6 mm) in the absence of detergent and the lysate was precleared by centrifugation at 300 × g for 15 min. Lysate was extracted with isopropanol followed by DEAE cellulose chromatography and acid precipitation. The purity of the protein was monitored on 15% SDS-PAGE and isoelectric focusing gel electrophoresis. The purified ACP was used as the substrate for *E. coli* acyl ACP synthetase.

**Antibody generation and purification** - Antisera were produced against the purified ACP as described earlier [16]. The IgG fraction of the polyclonal antibodies was purified by passing the antisera through protein A agarose column after adjusting the pH of the antibody preparation to 8.0 [23]. The column was washed with 100 mM Tris-HCl (pH 8.0) followed by 10 mM Tris-HCl (pH 8.0). The bound antibodies were eluted with 100 mM glycine (pH 3.0) and the pH was immediately brought back to neutral by the addition of 1 M Tris (pH 8.0). Immunoglobulin fractions were identified by separating them in 10% SDS-PAGE. The titer and the specificity of antibodies were determined by direct and inhibition ELISA [24]. This purified fraction was used for further analysis.

The peptide CY-VVGAQPFGGARGS corresponding to DAG acyltransferase was conjugated to BSA using m-maleimidobenzoyl-N-hydroxysuccinimide ester, and antisera were raised as described (18). The immunoglobulin fraction from the antisera was precipitated with 33% saturation of ammonium sulfate, the precipitate was dialyzed extensively against phosphate-buffered saline (PBS) and purity was monitored in 10% SDS-PAGE. This immunoglobulin fraction was used for further use. Protein was estimated by the method of Bradford [25] using bovine serum albumin as the standard.
Purification of acyl ACP synthetase from E. coli- Acyl ACP synthetase from E. coli was partially purified [26]. Overnight grown E. coli (JM109) cells were lysed by passing through a French pressure cell (Aminco) at 16000 p.s.i. The lysate was centrifuged at 10,000 × g for 20 min. MgCl₂ was added to the supernatant to make a final concentration of 10 mM and centrifuged at 80,000 × g for 90 min. The resulting pellets were suspended uniformly in 50 mM Tris-HCl (pH 8.0), and NaCl and MgCl₂ were added to reach a final concentration of 0.5 M and 10 mM, respectively. The solution was stirred for 15 min and centrifuged at 80,000 × g for 90 min. The membrane pellet was suspended in 50 mM Tris-HCl, pH 8.0 and Triton-X-100 was added to a final concentration of 2%. The suspension was then centrifuged at 80,000 × g for 90 min. ATP was added to the supernatant to make a final concentration of 5 mM and then placed in a 55°C water bath for 7 min. The solution was cooled and centrifuged at 15,000 × g for 20 min and the resultant supernatant was used as the source of the enzyme.

Double immunofluorescence – Logarithmic phase (21 h) R. glutinis cells were fixed with 4% paraformaldehyde for 20 min followed by 4% formaldehyde for 60 min. The fixed cells were washed three times with 0.1 M phosphate buffer (pH 5.9) and resuspended (10⁷ cells/ml) in 1.2 M sorbitol and lyticase was added to a final concentration of 100 µg/100 µl of cells. Cells were incubated at 30°C for 6 h to obtain spheroplasts. The cells were washed, resuspended and plated onto a poly-L-lysine pretreated slides. The slides were treated with ice-cold methanol for 6 min and acetone for 30 s.

Slides were first treated with purified anti-ACP antibodies (1:100 dilution) followed by TRITC conjugated goat anti-rabbit secondary antibodies. Slides were washed thoroughly with PBST and PBS and treated with goat anti-rabbit immunoglobulin (1:100 dilution) to block the unoccupied sites of primary antibodies. Anti-DAG acyltransferase antibodies (IgG fraction) were added in 1:100 dilution followed by FITC conjugated goat anti-rabbit secondary antibodies. The slides were viewed in a
confocal laser-scanning microscope (Leica, TCS SP, Heidelberg, Germany) for localization.

Purification of acyl ACP – Acyl ACP formed in the reaction mixture was purified through DEAE-Sepharose column chromatography followed by blue-Sepharose column chromatography [27]. DEAE (1 ml) column was made and equilibrated with 20 mM Tris-HCl (pH 8.0). Acylation reaction product was loaded onto the column and washed with 10 ml of 20 mM Tris-HCl (pH 8.0). The column was then washed with 50% isopropanol in 20 mM Tris-HCl (pH 8.0) to remove the unreacted fatty acid. Bound protein was eluted with 0.5 M NaCl and 1 ml fractions were collected. Acyl ACP containing fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 8.0). Fractions were checked for radioactivity in case of radioactive assay or run on a 15% SDS-PAGE followed by silver staining. Blue-Sepharose column (1 ml) was equilibrated with 20 mM Tris-HCl (pH 8.0) containing 2% Triton-X-100. The dialyzed fraction from DEAE column was loaded onto blue-Sepharose column and flow-through was collected and checked for acyl ACP.

Acyl ACP (500 µl) sample was taken and 100 µl of 0.5 N NaOH was added to it and the sample was incubated at 100°C for 15 min. Reaction mixture was cooled to room temperature and neutralized. Lipids were extracted and resolved on a silica-TLC, using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) as the solvent system followed by autoradiography.

Modification of sulfhydryl group by iodoacetate or iodoacetamide – ACP (wild type or mutant) was taken as 2 mg/ml solution in 50 mM Tris (pH 8.5), 0.15 M NaCl, 5 mM EDTA. Iodoacetate or iodoacetamide was added to the reaction mixture to a final concentration of 50 mM and incubated for 2 h at room temperature [28]. Excess reagent was removed by dialysis and protein was used for enzymatic assay and MALDI mass analysis.
Western blotting – Proteins were separated by either native or SDS-PAGE and transferred onto a nitrocellulose membrane for immunoblotting. ACP antibodies were used at a dilution of 1:2000 in western blot analysis and at 1:1000 dilution for cDNA library screening.

Ribosome Preparation – Exponential phase R. glutinis cells were pelleted down and resuspended in equal volume of ice-cold 2 × buffer A (20 mM Tris (pH 7.4), 16 mM MgCl₂, 0.1 M KCl, 12 mM 2-mercaptoethanol and 0.2 mM EDTA) and lysed with glass beads. Lysate was centrifuged at 10000 × g for 15 min and the resulting supernatant was centrifuged at 20000 × g for 30 min. The supernatant was loaded onto 10-40% linear sucrose density gradient and centrifuged for 17 h at 20000 × g. The pellet containing polysome was collected and used for analysis. All the operations were performed at 4°C (29).

Cloning, expression in E. coli and purification of recombinant ACP – cDNA library was constructed using the cDNA library construction kit and pBK-CMV ZAP expression vector [17]. cDNA library was screened using the purified polyclonal antisera raised against R. glutinis ACP. The positive λZAPII phage plaques were excised using a helper phage (ExAssist) and sequenced in both directions using T7 and T3 primers in a DNA sequencer (Applied Biosystems Model 377).

For expression of recombinant ACP in E. coli, a 369 bp DNA fragment containing the coding sequence of ACP was generated by PCR amplification of the cDNA clone using forward primer (5’ - TCTATTCAGCTCCATATGAAGCACGTCGCC – 3’) and reverse primer (5’ - GGGGAGAGGCGAGAATTCTCATTAGTCGAAGAG – 3’). The forward primer contains a NdeI site followed by the beginning of open reading frame (ORF); the reverse primer contains an EcoRI site and a stop codon after the ORF. PCR (1 min denaturation at 94°C, 1 min annealing at 55°C and 45 s elongation at 72°C) was performed using Pfu
polymerase for 30 cycles with 10 mM of each primer in a final volume of 100 µl. The purified PCR product was digested with NdeI and EcoRI and ligated directionally in predigested pET 21a (Novagen) vector. The construct was transformed into E. coli BL21 (DE3) cells and the insert was sequenced. The transformed E. coli cells were induced with 0.5 mM IPTG for 4 h and the cell lysate was resolved on 15% SDS-PAGE to look for the expression.

Recombinant ACP was purified by Ni-NTA affinity column chromatography. Cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl (buffer A). Cells were disrupted by passing through a French pressure cell at 16000 p.s.i. The supernatant (10000 × g) was allowed to bind to Ni-NTA matrix. The column was washed with buffer A containing 20 mM imidazole. The bound protein was eluted with NaCl containing 250 mM imidazole. One milliliter fractions were collected and checked for the presence of protein in 15% SDS-PAGE followed by silver staining. Fractions containing ACP was pooled, dialyzed extensively using benzoylated dialysis tubing, concentrated and used for further assays.

*Site directed mutagenesis* – Wild type ACP template (80 ng), sense and antisense primers (25 pmol each) were added to PCR tubes containing 0.2 mM dNTP’s, 1 mM MgSO₄, 2.5 mU Pfu polymerase and 1× reaction buffer. Amplification was carried out using the following conditions: denaturation of the template at 94°C for 4 min followed by 20 cycles at 94°C for 45 s (denaturation), 52°C for 1 min (annealing) and 72°C for 6 min (extension). The reaction was continued for another 20 min at 72°C to complete the extension. The sequences of sense and anti-sense primers are described in detail in Table II. The PCR amplified product was treated with DpnI at 37°C for 1 h to digest the methylated template and transformed to E. coli DH5α strain. The presence of mutations was confirmed by sequencing the plasmid.
Matrix assisted laser desorption ionization mass spectroscopic analysis – Two microgram of WT or mutant ACP was resolved in 15% SDS-PAGE and visualized by Coomassie blue staining. The band of interest was excised from the gel, placed in a acid washed tube and dried under vacuum. Then the gel pieces were washed twice with 50 µl of water for 30 min. Then the gel pieces were alternately dehydrated and rehydrated in acetonitrile and 100 mM ammonium bicarbonate, respectively, till they became white. Trypsin (80 ng/ml in 25 mM ammonium bicarbonate, pH 8.5) was added to the dry gel pieces and allowed to stand for 45 min on ice. The excess trypsin was removed and the gel pieces were soaked in minimum amount of 25 mM ammonium bicarbonate (pH 8.5) and incubated overnight at 30°C. Tryptic fragments were extracted by 50% acetonitrile/0.1% trifluoroacetic acid, dried and suspended in 2.5-dihydroxybenzoic acid and applied to MALDI sample plate. MALDI mass spectroscopic analysis was performed on a Kratos PCKompact Seq 1.2.2 mass spectrometer in linear mode. The masses obtained were compared with the predicted mass of the tryptic fragments obtained from PeptideMass (http://www.expasy.org/tools/peptide-mass).
Results

Identification of an ACP – Acyl carrier protein contains a 4’-phosphopantetheine prosthetic group, attached to a conserved serine residue as a post-translational modification. Phosphopantetheine is synthesized from aspartate in a two-step reaction and β-alanine is an intermediate in this pathway (1). To show the presence of ACP in soluble fraction, wild type R. glutinis cells were metabolically labeled with [14C]β-alanine in aspartate less medium, and cytosol was resolved on a 13% SDS-PAGE. A small ~14 kDa protein was found to be labeled (Fig. 1A). In another set of experiments, incorporation of [14C]β-alanine in TBC was monitored by isolating TBC from the labeled cells and resolving the purified TBC on SDS-PAGE (Fig. 1A). These experiments suggested that ~14 kDa polypeptide that was labeled by β-alanine could be soluble ACP.

To estimate the amount of cellular ACP associated with other components of TBC, cytosol was resolved on a 7% native-PAGE and 1-cm fractions were cut from the resolving gel. Protein was eluted and ACP and TAG synthase activities were measured as described in detail in Experimental Procedure. It was observed that 63% of the total ACP activity was associated with TBC and the remaining activity was found in free form (Fig. 1B). Western blot analysis of cytosol with anti-ACP antibodies, after separating in 7% native-PAGE, showed two reactive bands, one at the place of TBC and other band at around 14 kDa (Fig. 1C).

Purification of ACP – Acyl carrier protein was purified from R. glutinis by treating the cell lysate with isopropanol where ACP partitioned into the isopropanol supernatant. The ACP was then adsorbed onto DEAE cellulose followed by elution with increasing concentrations of salt. The acidic nature of ACP was used to selectively precipitate the protein. ACP was purified to 8-fold with a recovery of only 0.5% (Table I), which could be due to the loss of activity during purification. The activity of ACP was determined by estimating the formation of acyl ACP and specific activity of the purified protein was
calculated to be 1.7 nmol/min/mg protein. The purified ACP showed an aberrant mobility on SDS-PAGE, which could be due to the highly acidic nature of the protein (Fig. 1D). The pI was determined to be ~4 by isoelectric focusing (data not shown). Polyclonal antisera were raised against the purified ACP and IgG fraction was purified by protein-A agarose column. In western blot analysis with cytosol, the purified antibodies recognized a polypeptide of 14 kDa (Fig. 1E). The antiserum was titrated against 1 µg of immobilized ACP. The 50% inhibitory binding was found at 1:600 dilution of the antibodies. Inhibition ELISA was performed using the same dilution of antiserum and different concentrations of the purified ACP. The binding (50%) was achieved with 2.6 µM protein (Fig. 1F).

**Immunocytochemical localization of ACP in TBC** – To determine whether ACP colocalizes with other components of TBC, double immunostaining experiments were performed. This involves the probing of the spheroplasts of oleaginous yeast cells with anti-ACP and anti-DAG acyltransferase antibodies. Confocal analysis revealed a substantial colocalization of both the proteins in cytosol (yellow color in Fig. 2, panel c).

**Cloning of acyl carrier protein** – cDNA expression library was screened with antibodies raised against the purified ACP. After screening approximately 1 × 10^5 plaque-forming units, three strong positive signals were obtained. All the clones were excised and propagated into *E. coli* XLOLR strain; plasmids were prepared and subjected to nucleotide sequencing. Sequences obtained were identical in all three with a few base pair changes. The primary structure of the cDNA is depicted in Figure 3. The isolated clone contained a full-length cDNA of 565 nucleotides with a 64-bp 5’-untranslated region. Analysis of the nucleotide sequence revealed an open reading frame of 110 amino acids with translation initiation at 65ATG 67 codon. The calculated molecular mass of the protein is 11.3 kDa and the isoelectric point is 3.9. Hydropathy plot of the predicted protein revealed that this clone does not have any transmembrane domain.
We used PROSITE domain search for structural motifs and found a potent protein kinase C phosphorylation site $^{59}$SKK$^{61}$, two casein kinase 2 dependent phosphorylation sites $^{18}$SAED$^{22}$ and $^{106}$SDDD$^{103}$, and three myristoylation sites, $^{69}$GAAPAA$^{74}$, $^{77}$GGAAAG$^{82}$, $^{78}$GAAAGG$^{83}$. BLAST search analysis for the deduced amino acid sequence in all the available databases suggested that the protein has more than 60% identity with ribosomal protein P2 from Cladosporium, Alternata, Saccharomyces, maize, rat and human. Multiple sequence alignment was carried out using ClustalW algorithm with the ACP clone using the above-mentioned ribosomal protein P2 and it was observed that the isolated ACP has an overall sequence homology with them (Fig. 4A). No significant homology was observed when multiple sequence alignment was carried out with different ACP sequences from *H. pylori*, Plasmodium, Pseudomonas, *E. coli*, *S. cerevisiae* and Brassica (Fig. 4B). When the most conserved region of the ACP was used to carry out a local alignment (LALIGN), we could see more than 70% identity with the isolated cDNA in the nucleotide level (data not shown). This sequence was submitted to GenBank™ with the identification as non-organellar ACP (Accession number AF434667).

**Expression of ACP** – The full-length *R. glutinis* ACP cDNA in pBK-CMV expression vector containing lac promoter was used to transform *E. coli* (JM109) and induced with IPTG to produce recombinant protein. Though the induction was observed in SDS-PAGE as well as in western blot, the level of expression was very low (data not shown). To obtain high level of expression, the ACP gene was PCR amplified from the parent vector (pBK-CMV) with gene specific primers and subcloned into pET 21a vector. The transformed *E. coli* BL21 (DE3) cells showed a very high level of expression upon induction with 0.5 mM IPTG as compared to uninduced control (Fig. 5A). *E. coli* BL21 (DE3) cells expressing ACP were simultaneously induced and metabolically labeled with [14C]β-alanine. Cell lysate from [14C]β-alanine labeling reaction was resolved on a 13% SDS-PAGE and the recombinant protein was found to be radiolabeled after fluorography (Fig.
Uninduced cell lysate showed the labeling of the endogenous *E. coli* ACP. Western blot analysis with anti-ACP antibodies (generated against the recombinant protein) confirmed the identity of both the proteins (Fig. 5C). Western blot analysis was performed with ribosome using the same antisera and it recognized a low molecular weight protein (Fig. 5C).

**Purification and functional characterization of recombinant ACP** – Wild type ACP, overproduced in *E. coli* Bl21 (DE3) using T7 RNA polymerase expression system, induced by IPTG. Most (~80%) of the overexpressed protein was found to be in insoluble inclusion body. The remaining overexpressed protein in soluble fraction was purified by Ni-NTA affinity chromatography (Fig. 6A).

To determine the acylation capacity of the recombinant wild type protein, purified rACP was subjected to *in vitro* acylation assay using *E. coli* acyl ACP synthetase and radiolabeled oleic acid. The amount of acyl ACP formed (pmol/min) was comparable to that of native protein. Acyl ACP synthetase or ACP alone did not show any significant acylation (Fig. 6B).

The acylation reaction in ACP is an unique phenomenon as it involves the formation of a thioester linkage between the thiol group of phosphopantetheinylated protein and the acyl group. To understand the specificity of the acylation, purified rACP was pretreated with thiol group blockers, such as iodoacetamide and iodoacetic acid and acylation reaction was performed after complete removal of the excess reagent. Iodoacetamide treatment inhibited the acylation capacity to 80%, whereas iodoacetic acid treatment left back around 35% acylation activity. This difference could be attributed to the fact that iodoacetamide binds thiol group irreversibly whereas iodoacetic acid binds reversibly (Fig. 6C).

The acylated ACP was purified from the reaction mixture by passing it through DEAE-Sepharose followed by blue-Sepharose column chromatography. The purified acylated product was resolved onto a 15% non-reducing SDS-PAGE followed by silver
staining. The acylated ACP binds SDS better and runs at a faster rate in polyacrylamide gel electrophoresis as compared to ACP (Fig. 6D).

The purified [14C]oleoyl ACP was subjected to alkaline hydrolysis and the extracted lipids were separated on a TLC followed by autoradiography. The alkaline hydrolyzed product was identified as fatty acid (data not shown).

Site directed mutagenesis and analysis of mutant proteins – Based on the sequence analysis of isolated clone, all 8 serine residues present in the protein (Fig. 7A) were subjected to site directed mutagenesis and substituted with alanine. All the mutant proteins were overexpressed in E. coli BL21 (DE3) cells, induced with IPTG and purified through Ni-NTA affinity chromatography. In western blot analysis, anti-ACP antibodies recognized all the mutant proteins and there were no apparent mobility difference in the mutant proteins as compared to wild type (data not shown).

The acylation abilities of all the mutant proteins were assessed by the in vitro acylation assay using E. coli acyl ACP synthetase. Replacement of ser-59 with alanine reduced the acylation capability of the protein to 10-15%. Other mutations (S12A, S17A, S19A, S40A, S64A, S67A and S100A) did not show any significant change in their acylation pattern as compared to wild type ACP (Fig. 7B). All the mutants were analyzed for the presence of post-translational modification by their capability of getting labeled by [14C]β-alanine. No labeled protein band corresponding to rACP was detected in S59A mutant whereas all other mutants showed the similar profile as wild type (Fig. 7C).

Wild type and S59A mutant proteins were subjected to acylation reaction using [14C]oleic acid and the acylated product was separated in a 15% SDS-PAGE in the presence and the absence of 2-mercaptoethanol followed by fluorography. The acylated wild type protein showed a strong signal in fluorography, which disappeared upon 2-mercaptoethanol treatment, whereas mutant protein did not show any signal (Fig. 7D).
TAG synthesis with acyl ACP – To determine the functional role of rACP, $[^{14}C]$oleoyl ACP was purified and used it as a substrate in the TAG synthase assay. Different enzyme sources were used in the assay. Among them only *R. glutinis* cytosol and purified TBC produced triacylglycerol from acyl ACP as acyl donor, whereas neither *R. glutinis* membranes nor developing peanut cytosol and its membranes could accept acyl ACP (Fig. 7E).

MALDI-MS analysis of wild type and mutant ACP – Mass spectroscopic analysis was carried out to identify the post-translationally modified serine residue in ACP. Recombinant ACP (wild type and/or S59A mutant) was digested with trypsin before and after acylation and the protein was analyzed by MALDI mass spectroscopy (Fig. 8). Phosphopantetheinylation increased the mass by 340 Da and acylation with oleic acid increased the mass of the same fragment further by 282 Da. We analyzed the tryptic fragment that showed a shift in 340 Da and after acylation that shifted by the mass of fatty acyl group. One of the major peaks obtained by *in silico* analysis (1159 Da, peak C) fitted with these criteria and corresponds to the peptide fragment $^{50}$DVNEVIAEGSK$^{60}$. In wild type protein, peak C is not detectable as it showed a shift to peak A that has a mass of 1511 (352 Da shift). After acylation, peak A shifted to peak B showing a mass of 1786 Da. This tryptic fragment has a single serine residue (Ser-59). We did not observe any evidence of phosphopantetheinylation in any other tryptic fragment. The MALDI-MS analysis of S59A mutant showed the presence of peak C but peaks A and B were not detectable, confirming that this fragment contains the post-translationally modified serine residue.
Discussion

Acyl carrier protein is a part of 10S soluble multienzyme complex involved in TAG biosynthesis in \textit{R. glutinis}. ACP accepts free fatty acid and serves as acyl donor in acyltransferase reactions in TBC. Quantitation of ACP and immunocytochemical studies with antibodies raised against DAG acyltransferase and ACP provided evidence for the association of ACP with other components of TBC. Cloning and characterization of this non-organellar ACP from oleaginous yeast was achieved. The following observations revealed the presence of a functional ACP in \textit{R. glutinis}: 1) The gene encoding for ACP was isolated and overexpressed in \textit{E. coli}. A fraction of expressed protein was post-translationally modified and found capable of accepting free fatty acid. 2) MALDI-MS analysis of recombinant ACP confirmed the presence of post-translational modification. 3) Site directed mutagenesis of all the serine residues in recombinant ACP was performed and it was found that Ser-59 is indeed involved in post-translational modification.

Sequence analysis of the isolated clone for ACP showed that this protein shares a good percentage of homology with ribosomal protein P2. P2 is an acidic ribosomal protein involved in ribosomal stalk formation and is present as a part of the 60S ribosomal subunit of all eukaryotic cells [30]. There are two pools of P2 proteins, cytosolic and ribosomal. Cytosolic protein is not phosphorylated; upon phosphorylation, the protein gets translocated to ribosome [31]. Western blot analysis of \textit{R. glutinis} ribosome with anti-ACP antibodies showed a strong signal around 14 kDa suggesting the presence of a cross-reactive protein. Possibly the cytosolic pool of unphosphorylated P2 protein could behave as an acyl carrier protein in oleaginous yeast. It was observed that another ribosomal protein L7 was found to be copurified with the lipid inclusion bodies obtained from oleaginous bacteria like \textit{Rhodococcus opacus} PD360 and \textit{Rhodococcus ruber}. The significance of this association is not clear [32].
Overexpression of rACP in *E.coli* cells is mildly toxic resulting in a retarded growth rate. Normally, ACP exists in apo- and holo-forms. Under the inducible expression system, most of the ACP was present as apo-form, which was shown to be a potent inhibitor of glycerol-3-phosphate acyltransferase [33]. Phosphopantetheine:protein transferase (PPTase) is responsible for the transfer of 4'-phosphopantetheine from CoA to the hydroxyl group of serine residue of acyl carrier protein [34]. During overproduction of ACP in bacterial system, the enzymatic activity of PPTase becomes limiting to convert all the apo-ACP to the holo-ACP form. This was evident from the $[^{14}C]\beta$-alanine labeling experiment with the recombinant protein where a moderately intense signal in fluorography was observed, even though the expression level was very high. The recombinant protein was overproduced in *E. coli* system and most of the protein was found in inclusion bodies. The soluble fraction, which was used to purify the recombinant protein, contained mostly the holo form of the protein, as post-translationally modified peptide fragment was found predominantly in MALDI-MS analysis. Metabolic labeling of yeast cells with $[^{14}C]\beta$-alanine could possibly incorporate the label both in CoA and ACP. Hence, it is difficult to draw a correlation between the amount of label added and the amount incorporated into recombinant ACP in these experiments.

Recombinant ACP was readily acylated using *E. coli* acyl ACP synthetase and this acylation was inhibited by pretreatment of the protein with alkylating agents. This confirmed that acylation happened via the thiol group and resulted in thio-ester linkage. The anomalous mobility of ACP in SDS-PAGE can be explained by considering the high acidic charge on the protein that prevents binding of SDS to the protein. Upon acylation, more SDS binds with ACP and acylated ACP runs faster than the non-acylated form in SDS-PAGE. The difference in mobility was very clear when purified acyl ACP was resolved in non-reducing SDS-PAGE.

All the known ACP sequences contain a conserved serine that was post-translationally modified by the attachment of phosphopantetheine to it [14].
Surprisingly, we could not locate any conserved serine in our clone upon multiple sequence alignment, but the overexpressed ACP is post-translationally modified and capable of undergoing fatty acylation. There is only one ACP (ORF5 of Nod locus) reported from Azorhizobium caulinodans, which does not have a conserved serine, but involves in acylation reactions [35]. Site directed mutagenesis of the recombinant protein and MALDI-MS analysis after tryptic digestion clearly showed that Ser-59 could be the site of post-translational modification in R. glutinis. Sequence analysis showed that the flanking residues of Ser-59 are highly charged (57EGSKKL62). The conserved motif of phosphopantetheinylation in any other ACP is DSLD, where the charged residues flank the serine. The PPTase in E. coli is known to recognize the conserved motif for the transfer of phosphopantetheine [34]. However, our expression studies indicated that the expressed ACP was post-translationally modified even when the conserved motif is not present in the protein. The identification of non-organellar ACP has significant implications for the understanding of TAG biosynthesis and regulation in oleaginous yeast.
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Footnotes

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The acyl carrier protein gene sequence is available in database with accession number AF434667.

Key words : Acyl carrier protein, Triacylglycerol, Triacylglycerol biosynthetic complex, Ribosomal protein, Site directed mutagenesis, Post-translational modification.
Figure Legends

Figure 1. Identification and purification of soluble ACP from *R. glutinis*. A, Early log phase *R. glutinis* cells, grown in aspartate less minimal media, were metabolically labeled with [14C]β-alanine (10 μCi/ml culture) for 12 h. Incorporation of label was monitored by resolving cytosol (50 μg) (lane 2) and purified TBC (10 μg) (lane 3) in native and SDS-PAGE followed by fluorography. Lane 1 represents the labeling in Luria-Bertani media. B, The cytosol was separated on a 7% native-PAGE, and the proteins were eluted from the gel pieces and assayed for ACP. The gel-eluted fractions were also assayed for the incorporation of labeled free fatty acids into triacylglycerol in the presence of LPA as acyl acceptor and these are represented as TAG synthase activities. C, Cytosol was electrophoresed on 7% native-PAGE and transferred to a nitrocellulose membrane that was probed with anti-ACP antibodies. The antibodies recognized two bands under non-denaturating conditions. D, Purification profile of ACP was determined by resolving the samples from each purification step on a 13% SDS-PAGE followed by silver staining. Lane 1 represents isopropanol supernatant; lane 2, DEAE cellulose eluate; lane 3, acid precipitation step; lane 4, molecular weight markers. Approximately 10 μg of protein was loaded in each lane. E, Purified ACP (2.5 μg) from *R. glutinis* (lane 2) was resolved in a 15% SDS-PAGE and western blot was performed with the anti-ACP antibodies. Normal rabbit serum was used as a control (lane 1). F, Displacement of binding of ACP antibodies to immobilized ACP by the free ACP as determined by competitive inhibition ELISA. ACP was coated in a microtitre plate (1 μg/well) followed by blocking the unoccupied sites. Varying concentrations of ACP (0.1 to 1 μM) was incubated with ACP antibodies at a dilution that showed 50% of maximum binding to 1 μg of the immobilized ACP.
Figure 2. Double immunofluorescence staining and confocal images of *R. glutinis.* Cells were processed for immunostaining using anti-ACP (Ab1) and anti-DAG acyltransferase (DAGAT) antibodies (Ab2) as described under “Experimental Procedures” and analyzed by confocal microscopy. Cytosolic staining was observed with both the antibodies (panels a and b) and superimposition of images (panel c) reveals the colocalization of ACP and DAGAT in yellow. Cells treated with either only anti-DAGAT (panels e-g) or only anti-ACP (panels i-k) or no primary antibodies (m-o) were used as controls. Panels d, h, l and p showed the phase contrast (PC) images of *R. glutinis* cell spheroplasts.

Figure 3. Nucleotide and deduced amino acid sequence of ACP. The nucleotide sequence of isolated ACP clone (upper line) is presented with its deduced amino acid sequence (lower line). Nucleotides are numbered in the 5′–3′ direction and the amino acids are numbered from amino terminus starting from the first methionine residue. Asterisk indicates the stop codon of the ORF.

Figure 4. Multiple sequence alignment of isolated ACP. A. The conceptual translation product of the cDNA identified from TBC was used to search the SWISSPROT database. Six polypeptides [60S ribosomal protein P2 from Cladosporium (P42038); *S. pombe* (P08094); major allergen of Alternaria (P42037); rat (P02401); human (P05387) and maize (O24415) ribosomal proteins], which appeared as significant hits in BLAST search (scoring over 60) were multiply aligned with the cloned cDNA using ClustalW algorithm. B. Polypeptide sequences of different ACPs [*E. coli, S. cerevisiae, H. pylori, plasmodium, pseudomonas, brassica*] were obtained from public database (Swissprot) and were used to do a multiple sequence alignment with the isolated ACP clone using ClustalW algorithm. On the consensus line underneath each section of the alignment, in both panel A and B, asterisk indicates identical or conserved residues in all
sequences in the alignment; colon indicates conserved substitutions and dots denote semi-conserved substitutions.

Figure 5. Expression and characterization of ACP. A, ACP cDNA (in pET 21a vector) was transformed into *E. coli* BL21 (DE3) cells and induced with 0.5 mM IPTG for 4 h and cell lysates were run on a 15% SDS-PAGE. Lane 1 represents uninduced *E. coli* cell lysate and lane 2 represents the induced *E. coli* cell lysate. Lane M indicates molecular weight markers. B, [*14C*]β-Alanine (10 µCi/ml culture) was added to the *E. coli* BL21 (DE3) cells expressing ACP at the time of induction with IPTG. After 4 h of induction, the cell lysate (lane 2) was run on a 13% SDS-PAGE followed by fluorography. Lane 1 shows uninduced control. C, Western blot analysis with anti-ACP antibodies generated against the recombinant protein. Lane 1 represents uninduced *E. coli* cell lysate and lane 2 represents the induced *E. coli* cell lysate, lane 3 is *R. glutinis* cytosol and lane 4 is *R. glutinis* ribosomal fraction. Approximately 50 µg of protein was loaded in each of the lanes of all the panels.

Figure 6. Purification and characterization of recombinant ACP. A, SDS-PAGE profile of expression and purification of recombinant ACP. Lane 1, crude extract obtained from French pressure cell lysis; lane 2, 10000 × g supernatant; lane 3, 10000 × g pellet; lane 4, flow through of Ni-NTA column; 5-8, eluates obtained from Ni-NTA column. A band, migrating at the higher molecular mass of the hexahistidine-tagged ACP, was present after purification (lanes 5-8). B, Ni-NTA purified rACP (100 pM) was used in *in vitro* acylation assay using enriched *E. coli* acyl-ACP synthetase and [*14C*]oleic acid. *R. glutinis* ACP and *E. coli* ACP were used as positive controls. Four independent experiments were performed in triplicate and mean ± SE has been represented. C, Purified rACP (100 pM) was treated with 10 mM iodoacetamide or 10 mM iodoacetic acid for 2 h, extensively dialyzed and acylation assay was performed. Percent specific activity was represented considering no treatment as 100% activity. D, Acyl ACP was
purified from a bulk cold acylation reaction and passed through DEAE column chromatography. The fractions (containing around 5 µg of protein) were checked in 15% non-reducing SDS-PAGE followed by silver staining. Lane 1, purified rACP; lane 2, reaction product loaded onto DEAE column; lane 3, flow through; lane 4, wash; lane 5, isopropanol wash; lanes 6-9 eluates. Acylated product runs with faster velocity in SDS-PAGE.

Figure 7. Schematic representation of active serine residue in rACP. A, The locations of 8 serine residues on rACP was indicated. The flanking sequence of the putative active serine residue was shown. B, All rACP mutant proteins were affinity purified and used for the acylation reaction using *E. coli* acyl ACP synthetase and [14C]oleic acid. C, [14C]β-Alanine (10 µCi/ml culture) was added to the *E. coli* BL21 (DE3) cells expressing S59A mutant ACP at the time of induction with IPTG. After 4 h of induction, the cell lysate (50 µg) was run on a 15% SDS-PAGE followed by fluorography. U represents uninduced cell lysate and I represents induced cell lysate. D, *In vitro* acylation reaction was performed using purified WT and S59A mutant ACP and the reaction product was run in a 15% SDS-PAGE in presence or absence of 2-mercaptoethanol. E, Acylation assay was performed with acyl ACP and the amount of TAG formed (pmol/min) was represented graphically. The values are mean ± SE of three independent experiments, each performed in triplicate.

Figure 8. Identification of the serine residue involved in phosphopantetheinylation in rACP using MALDI-MS. Wild type (WT) and S59A mutant ACP (2 µg) were resolved on a 15% SDS-PAGE under reducing conditions followed by Coomassie blue staining. WT acylated ACP (2 µg) was resolved on 15% SDS-PAGE under non-reducing conditions followed by staining with Coomassie blue. The stained ACP bands were excised from the gel and subjected to in-gel trypsin digestion. Tryptic fragments of A, WT non-acylated; B, WT acylated and C, S59A mutant ACP were
analyzed in MALDI mass spectroscopy. Fragment A contains the post-translationally modified peptide in wild type which actually shows 340 dalton shift from fragment C. Fragment B represents the acylated peptide obtained from WT protein which shows 280 dalton shift in the spectrum. Tryptic digested peptides of S59A mutant showed only fragment C. The tryptic peptide masses obtained experimentally were compared with the in silico data and represented at the bottom.
The logarithmic phase oleaginous yeast cells from 2-liter culture were lysed with glass beads and the lysate used for purification as described in Experimental Procedures.

| Volume          | Specific Activity | Total Activity | Yield | Purification |
|-----------------|-------------------|----------------|-------|--------------|
| ml              | nmol/min/mg       | nmol/min       | %     | -fold        |
| Cell free extract | 100              | 0.22           | 16.15 | 100          | 1.00         |
| Isopropanol supernatant | 150              | 0.38           | 10.18 | 63           | 1.73         |
| DEAE cellulose  | 30                | 0.43           | 2.71  | 17           | 1.96         |
| Acid precipitation | 0.55             | 1.7            | 0.075 | 0.46         | 7.73         |
TABLE II

*Primers used in site directed mutagenesis of the serine residues*

| Serine Position | Mutation | Primer                                      |
|-----------------|----------|---------------------------------------------|
| S12A            | T98G     | Sense: 5’-CCTCCTCGTCGCCCGCCGCCAACA-3’       |
|                 |          | Antisense: 5’-TGTTGCCGGCGACGAGGAGG-3’       |
| S17A            | T113G    | Sense: 5’-CGGCAACACCAGCGCCGCTCGCGGCGG-3’    |
|                 |          | Antisense: 5’-CGGGCGAGGGCCGCGCTGTTGCCG-3’   |
| S19A            | T119G    | Sense: 5’-CACCTCGCGCCGAGCGAAGCG-3’          |
|                 |          | Antisense: 5’-CGTCTCGGCCGCGGCGAGGTCG-3’     |
| S40A            | T182G    | Sense: 5’-GGAGCAGCTCGGCTGCTCATCA-3’         |
|                 |          | Antisense: 5’-TGATGAGCAACCGCAGCCGCTCC-3’    |
| S59A            | T239G    | Sense: 5’-TGCGGAGGGAGCCAAGAACGCTGCG-3’      |
|                 |          | Antisense: 5’-CGAGCTCTTGGTGCCTCGCGAAG-3’    |
| S64A            | T254G    | Sense: 5’-GAAGCTCTGCTGCCGCTCCCTCGG-3’       |
|                 |          | Antisense: 5’-CGGAGAGGCAGGACGAGCTTCC-3’     |
| S67A            | T263G    | Sense: 5’-TTCCGTCGGCGCCGCGCCGCGGCG-3’       |
|                 |          | Antisense: 5’-CGGAGAGGCAGGACGAGCTGCAA-3’    |
| S100A           | A361G    | Sense: 5’-GGATGAGGAAGGCGACGACGACA-3’        |
|                 | G362C    | Antisense: 5’-TGTCGTCGCTGCGCCGCTCCATCC-3’  |
Figure 1

A

7% Native PAGE

13% SDS-PAGE

B

C

kDa

200

66

29
Figure 1

[Image of SDS-PAGE gel with molecular weight markers and protein bands labeled 14 kDa.]

13% SDS-PAGE

15% SDS-PAGE

14 kDa

F

Concentration (μM)

% Binding

[Graph showing concentration of ligand versus % binding, with a curve indicating a decrease in binding as concentration increases.]
Figure 3

```
GGCAGGGCTCCTCAGCATCTCTGCTCCTGACATTTCTGTTGCTTAAGGCTCTATGCT
CAAGATGAGACGCATCCGCTCGGCTACTCTCTTCTGCTGCTGCGGCCGAGCTCCTGCT
MKHVAAYLLLVSLAGNTSPT
GGCCAGGGACGTCAGAGAGGCCTCGCCGGCCGGCAGTCCTCCAGGGAGAGAGCCCT
AEVAKVLAADIDQADERL
CTCGGCCTTCATCAAGGACGCGGCGGCAAGGACGTCACAGGGCTTGCCTGCGGATC
SVIKEGKRVDVNEVIAEGS
CAAGAGCTCGTCCTCCCTCCCTCGGGGCGGCCGGCGGCCCCGCCGCTGCGCTGCGGCC
KKLASVPSGGGAAAPAAAAAGGA
TGCCGCTGCGGCTGCCGGAGGAGGGCTGAGGAAGGACAGCCCGCTGAGRAAGGATGAGGA
AAGGAEEKAEDKPAEKEDE
GAGGCAAGACGCATGGGCTCGGTCTTTGACCTAGCTCCTGTCTCGCTCTCCCTCT
SDDDMGFLFD*
CTCGGAACGCACAACTTCTCGGACCTTCTCACTGCGGACTTGCGGAAAGGATGTTGCT
540
GTTTGAGAGATCGATGGGATTGCGCTAGGGAAGCCCTTGAGGAAAGGGGGGTTGCT
TCTC
565
```
### Figure 4

| Organism       | Sequence                                                                 | Score |
|----------------|--------------------------------------------------------------------------|-------|
| Cladosporium   | MKYHAAYLLLGLAGNSPSAEIKTVLSV5GIDADEERLSSLLIKELEGKDINELISSGSQ               | 60    |
| Alternata      | MHKLLAYLLELGCGNTSPEAVKAVLESVGIEADSRLDKLISELEEGKDINELIASGE               | 60    |
| R. glutinis    | MHKVAAAYLLLVSAGNTSPEAOVKLVAADAQIDEERLSSLLIKELEGKDINELISSGSQ             | 60    |
| S. cerevisiae  | MKYLAAYLLTVGKDP5SADIESVLSVGEAESERTEILNELINGKDDELIAAGNE                 | 60    |
| Maize          | MKVIAAYLAVLGGNTSPTADDVKSISELVGEAEADEEKLFLTTLEKDITTEVIAACRE              | 60    |
| Rat            | MRVASYLAAALGGNASPKDIKKLD5VGEAADDELMKVISELNGKINEDVIAQG6G                 | 60    |
| Human          | MRVASYLAAALGGNASPKDIKKLD5VGEAADDELMKVISELNGKINEDVIAQGIG                | 60    |

| Organism       | Sequence                                                                 | Score |
|----------------|--------------------------------------------------------------------------|-------|
| Cladosporium   | KLASVPSGGSGAAPSAGAAAGG--ATEAAPEAAKEKEEE----SDDDMGFGLFD                   | 111   |
| Alternata      | KLASVPSGGAGAAASEGGAAGAGSAQAAEAPEAAKEESEE----SDDDMGFGLFD                 | 113   |
| R. glutinis    | KLASVPSGGAPAAAGG--AAAGG--AEEKEADEKPAEKDEE----SDDDMGFGLFD                | 110   |
| S. cerevisiae  | KLAVTPCAGG--ASAAPAAAGG--AAPAAEEAACEEEAEE----SDDDMGFGLFD                 | 110   |
| Maize          | RLSSVPSGGAIDMGAPAAGGAGGAAPGAEEAEEKEKEESEE----SDDDMGFGLFD                | 115   |
| Rat            | KLAVPAGGAVASAPPAAG--SAPAAEIKEEKEESEEEDDDMGFGLFD                           | 115   |
| Human          | KLAVPAGGAVASAAGPAAPGAAG--SAPAAEIKEEKEESEEEDDDMGFGLFD                     | 115   |

### Table B

| Organism       | Sequence                                                                 | Score |
|----------------|--------------------------------------------------------------------------|-------|
| Pylori         | ...................................................................................................... | 14    |
| Plasmodium     | ...................................................................................................... | 59    |
| Pseudomonas    | ...................................................................................................... | 15    |
| E. coli        | ...................................................................................................... | 15    |
| S. cerevisiae  | ...................................................................................................... | 60    |
| Brassica       | ...................................................................................................... | 13    |
| R. glutinis    | ...................................................................................................... | 49    |

| Organism       | Sequence                                                                 | Score |
|----------------|--------------------------------------------------------------------------|-------|
| Pylori         | ...................................................................................................... | 74    |
| Plasmodium     | ...................................................................................................... | 119   |
| Pseudomonas    | ...................................................................................................... | 75    |
| E. coli        | ...................................................................................................... | 75    |
| S. cerevisiae  | ...................................................................................................... | 120   |
| Brassica       | ...................................................................................................... | 61    |
| R. glutinis    | ...................................................................................................... | 108   |

| Organism       | Score |
|----------------|-------|
| Pylori         | 78    |
| Plasmodium     | 123   |
| Pseudomonas    | 78    |
| E. coli        | 78    |
| S. cerevisiae  | 125   |
| Brassica       | 109   |
Figure 7

A

\[ \text{NH}_2 \quad 12 \quad 17 \quad 19 \quad 40 \quad 59 \quad 64 \quad 67 \quad 100 \quad \text{COOH} \]

\[ \text{AEGSKKL} \]

B

| pnmol of Acyl-ACP formed/min |
|-----------------------------|
| WT  | S12A | S17A | S19A | S40A | S59A | S64A | S67A | S100A |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 0.20 | 0.21 | 0.21 | 0.22 | 0.20 | 0.18 | 0.23 | 0.25 | 0.22 |
Figure 7

C

D

E

Figure 7

C

D

E

Figure 7

C

D

E
Figure 8

| Peak | Mass In Silico (Dalton) | Mass Obtained (Dalton) |
|------|-------------------------|------------------------|
| A    | 1499                    | 1511                   |
| B    | 1781                    | 1786                   |
| C    | 1159                    | 1182                   |
Non-organellar Acyl carrier protein from oleaginous yeast is a homologue of ribosomal protein P2
Sumana Raychaudhuri and Ram Rajasekharan

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