Cytosolic iron superoxide dismutase is a part of the triacylglycerol biosynthetic complex in oleaginous yeast

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A novel multienzyme complex for the biosynthesis of triacylglycerol in oleaginous yeast has been identified recently in the cytosol and characterized [Gangar, Karande and Rajasekharan (2001) J. Biol. Chem. **276**, 10290–10298]. Screening the library of *Rhodotorula glutinis* with an oligonucleotide probe derived from the N-terminal sequence of one of the protein components in the complex (21 kDa protein) resulted in the isolation of a 0.7 kb cDNA. Nucleotide sequence analysis revealed that the isolated gene codes for superoxide dismutase (SOD). Atomic absorption spectroscopy and inhibition assays showed that this cytosolic SOD

utilizes Fe as its cofactor. Enzymic assays, immunoprecipitation and cross-linking experiments revealed that SOD is a part of the triacylglycerol biosynthetic complex, which could protect the substrate and the complex from oxidative damages. These results indicate for the first time the presence of iron-containing SOD in a soluble form in yeast.

Key words: acyl-carrier protein, fatty acid activation, iron superoxide dismutase, *Rhodotorula glutinis*, triacylglycerol biosynthetic complex.

INTRODUCTION

Superoxide dismutases (SODs, superoxide:superoxide oxidoreductase; EC 1.15.1.1) are essential enzymes in the cellular defence mechanism which catalyse the dismutation of O_2^- to H_2O_2 and O_2 [1,2]. Four different types of SODs are known, each containing a different metal ion at the active site. Iron-containing SOD (FeSOD), found in prokaryotes, and manganese-containing SOD (MnSOD), found in mitochondria as well as in prokaryotes, are highly similar in sequence [3] and crystal structure [4], whereas copper- and zinc-containing SOD (CuSOD/ZnSOD), found in cytosol of eukaryotes, has very little similarity to other SODs. A previous study [5] has identified nickel-containing SOD (NiSOD) in *Streptomyces griseus* and *S. coelicolor*. A subclass of SOD exists which is capable of utilizing either Mn or Fe as the catalytic metal ion and termed as combialistic SOD [6]. *Candida albicans* expresses a different SOD that utilizes Mn as cofactor and is active in cytosol [7], and *Anabaena* PCC 7120 contains a MnSOD which is localized in both cytoplasm and thylakoid membranes [8].

Rhodotorula glutinis is an oleaginous yeast that accumulates 50 % triacylglycerol (TAG)/cell dry weight [9]. We have reported earlier the presence of a cytosolic 10 S multienzyme TAG biosynthetic complex (TBC) in this yeast [10]. The complex consists of acyl-carrier protein (ACP), acyl-ACP synthetase, lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase and diacylglycerol acyltransferase, with molecular masses of 21, 35, 32, 48 and 66 kDa respectively [10]. The multienzyme complex is capable of synthesizing TAG from lysophosphatidic acid and acyl-CoA. Moreover, it can accept fatty acid as acyl donor in the presence of ATP and magnesium [11]. A fatty-acid preference study indicated that it accepts polyunsaturated fatty acids (PUFAs) with higher affinity than saturated fatty acids. When wild-type *R. glutinis* was grown on low-glucose medium, the cells failed

to accumulate TAG and there was a significant reduction in cell growth, suggesting a role for TAG in cell growth [12].

In the present study, we report that the protein encoded by the 21 kDa component of TBC has SOD activity. The gene encoding SOD was cloned and sequence analyses revealed that the isolated gene is a member of the MnSOD/FeSOD family. Purification followed by extensive characterization showed that the protein utilizes Fe as cofactor and exists as a part of the 10 S TBC.

EXPERIMENTAL

Materials

R. glutinis was obtained from the Institute of Microbial Technology (Chandigarh, India; Microbial Type Culture Collection 1151). Ampicillin, tetracycline, kanamycin, sarcosyl, diaminobenzidine, lysophosphatidic acid, DEAE-cellulose and Mops were obtained from Sigma. cDNA library construction kit was from Stratagene. [$1-^{14}C$]Palmitoyl-CoA (51 mCi/mmol), [$1-^{14}C$]oleic acid (55 mCi/mmol), [^{35}S]protein-labelling mix and [^{32}P]dATP/dCTP (30 Ci/mmol) were obtained from PerkinElmer Life Sciences. Restriction enzymes, deoxynucleotidyl terminal transferase (TdT), *Taq* polymerase, low-melting agarose, dNTPs, Sephadex G-10 and G-50, EDTA, isopropyl β -D-thiogalactopyranoside (IPTG), 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside ('X-Gal') and all other molecular biology reagents were from Amersham Biosciences. Silica-TLC plates were from Merck. 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,

Abbreviations used: ACP, acyl-carrier protein; IEF, isoelectric focusing; IPTG, isopropyl β -D-thiogalactopyranoside; ORF, open reading frame; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; TAG, triacylglycerol; TBC, TAG biosynthetic complex; TdT, deoxynucleotidyl terminal transferase.

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The nucleotide sequence for the iron superoxide dismutase gene from *Rhodotorula glutinis* has been deposited in the DDBJ, EMBL, GenBank[®] and GSDS Nucleotide Sequence Databases under the accession number AF434197.

supplemented with 1 % (w/v) glucose with aeration at 30 °C. Cell density was determined by absorbance measurements at 600 nm and then converted into colony-forming units ($1 A_{600} = 9 \times 10^7$ cells).

Construction of cDNA library and cloning

Yeast cell culture (1 litre) was grown for 21 h, and total RNA was isolated using the guanidium isothiocyanate method [13]. mRNA was purified from total RNA using an oligo(dT)–cellulose column. The cDNA library was constructed from the purified mRNA using the cDNA library construction kit according to the manufacturer's instructions. The vector used for constructing the library was a pBK-CMV ZAP expression vector [14]. The cDNA was size-fractionated on a Sepharose CL-4B drip column, and fragments larger than 500 bp were directionally ligated into *Eco*RI and *Xho*I sites. The cloned vector was packaged in Gigapack packaging extract provided by the manufacturer and propagated in host XL-1 blue MRF' *Escherichia coli* cells. The titre of the amplified library was 10^{11} pfu (plaque-forming units)/ml.

The N-terminal sequence of the 21 kDa protein of TBC was obtained by microsequencing the protein transferred on to a PVDF membrane after Edman degradation. Protein sequencing was performed using an Applied Biosystems gas-phase sequencer at the protein sequencing facility of Rockefeller University. The nucleotide probes were generated from the protein sequence and radiolabelled at the 3' end using TdT and [α - 32 P]dATP. Filters carrying 1×10^5 clones from *R. glutinis* cDNA library were hybridized with the radiolabelled probe. The positive λ ZAPII phage plaques, identified by autoradiography, were purified further through two rounds of screening using the same probe. Three positive clones were obtained, and *in vivo* excision was performed using a helper phage (ExAssist). The clones obtained were sequenced by the dideoxy chain-termination method using a dye terminator cycle sequencing kit (PerkinElmer Life Sciences) with a DNA sequencer (Applied Biosystems Model 377; PE Applied Biosystems, Foster City, CA, U.S.A.).

Expression of full-length SOD gene in *E. coli*

The isolated SOD cDNA was transformed into *E. coli* JM109 cells and induced with 0.5 mM IPTG for 4 h. The crude cell lysate was sonicated and run on an SDS/12 % (w/v) polyacrylamide gel. Untransformed cell lysate was used as control.

Antibody production

The crude cell lysate obtained from the JM109 *E. coli* cells overexpressing full-length SOD was resolved by two-dimensional gel electrophoresis, and the spot corresponding to the overexpressed protein was excised from the gel and soaked overnight in PBS to remove SDS. The crushed gel pieces, containing 250 μ g of protein, were emulsified in Freund's complete adjuvant and were injected subcutaneously into a rabbit. Three booster doses, each containing 125 μ g of protein emulsified in Freund's incomplete adjuvant, were administered at three weekly intervals. Ten days after the last injection, blood was collected, and serum was separated and stored at -20 °C.

Enzyme assay

An in-gel SOD assay was performed as described in [15]. The samples were run on a 7 % (w/v) native polyacrylamide gel

at 4 °C. The gel was soaked in a solution containing 0.025 % Nitro Blue Tetrazolium and 0.01 % riboflavin for 20 min in the dark. The gel was then soaked in a solution containing 0.01 % *N,N,N',N'*-tetramethylethylenediamine for 5 min and exposed to intense light. The SOD-active band appeared white in the dark-blue background of the gel. A SOD solution assay was performed as described previously [16]. In brief, the inhibition of autoxidation of pyrogallol in alkaline pH (8.0) was measured as a function of time. The rate of inhibition was determined and 50 % inhibitory concentration was calculated. The amount of enzyme required to obtain 50 % inhibition was considered as one unit of SOD activity.

For the inhibition assays, one unit of enzyme was incubated with appropriate concentrations of the inhibitors at 4 °C for 1 h and the solution assay was performed as mentioned above. For immunoinhibition, one unit of enzyme was preincubated with anti-SOD antibodies at 1:100 dilutions for 3 h on ice and the solution assay was performed as described previously [16].

Dissociation of SOD from the TBC

TBC was treated with 5, 10 and 15 mM SDS for 1 h on ice and run on a 7 % native polyacrylamide gel followed by an in-gel SOD assay. The active band was cut and the protein was eluted from the gel. Alternatively, the purified TBC was run in isoelectric focusing (IEF) gel (pI range 3–10) and an in-gel assay was performed on IEF gel. The active protein was eluted from the gel. The purity of the eluted protein was checked by SDS/PAGE followed by silver staining.

Determination of metal content of SOD

The metal content of the gel-purified SOD from TBC as well as overexpressed SOD was determined by atomic absorption spectrophotometry (Z6100, polarized Zeeman atomic absorption spectrophotometer, Hitachi). Commercially available *E. coli* MnSOD and FeSOD were used as controls.

Western blotting

Proteins were separated on either a native or SDS/polyacrylamide gel and transferred on to a nitrocellulose membrane. SOD antibodies were used at a dilution of 1:1000 in 0.5 % gelatin in PBS containing 0.05 % (v/v) Tween 20.

Immunoprecipitation

Late-exponential-phase *R. glutinis* cells were labelled with 30 μ Ci/ml [35 S]protein-labelling mix for 3 h at 32 °C. The labelled cells were lysed and centrifuged at 10 000 *g* for 15 min to obtain the lysate. The lysate was precleared with normal rabbit serum and was incubated with primary antibodies for 1 h, followed by sequestration of immunocomplex with Protein A–agarose beads. The proteins were analysed by gel electrophoresis followed by autoradiography.

Cross-linking of proteins

The purified complex (5 μ g) was mixed with disuccinimidyl suberate to a final concentration of 0.5 mM in a total volume of 50 μ l. Cross-linking was performed for 1 h at 4 °C. The reaction was stopped by the addition of 5 μ l of 0.25 M Tris/HCl (pH 7.5). Cross-linked products were resolved by SDS/PAGE (5 % gel)

and electroblotted on to a nitrocellulose membrane. The membrane was probed with anti-ACP and anti-SOD antibodies for the detection of the cross-linked products.

RESULTS

Cloning of gene encoding 21 kDa protein of TBC

To obtain the full-length cDNA clone encoding a 21 kDa protein, the oleaginous yeast cDNA library was screened with the oligonucleotide degenerate primer 5'-TACAACAAG-ATCCCIGCTGTICTCCCTAAGCTCCCTTCGCITACAAC-3', designed based on the N-terminal amino acid sequence of the protein, NH₂-YNKIPAVLPKLPFAYN-CO₂H. The nucleotide probe was radiolabelled at the 3' end using TdT and [α -³²P]dATP. A large number of positive clones were isolated in the screen, and the plasmids from clones that contained the longest inserts (0.7 kb) were subjected to nucleotide sequencing. The complete primary structure of a cDNA (732 bp) is shown in Figure 1. Analysis of the nucleotide sequence revealed an open reading frame (ORF) of 191 amino acids, with consensus Kozak translation initiation sequence (GCCATGG) and translation initiating at the ²⁵ATG²⁷ codon. The 3'-untranslated region contained a non-canonical polyadenylation signal (AATAC) near the polyadenylated tail, indicating that the isolated clone represented full-length cDNA. The calculated molecular mass of the ORF is 20.9 kDa, close to that estimated by SDS/PAGE. When the sequence of N-terminal amino acid was aligned with the deduced amino acid sequence, there was a complete match, confirming that the cloned cDNA encodes for the 21 kDa protein (Figure 1). A hydropathy plot of the predicted protein suggested the absence of any transmembrane domains (results not shown). When the deduced amino acid sequence of 21 kDa was examined for a number of structural motifs, we identified a potential Asn-linked glycosylation site at ⁸⁰NHTL⁸³ and a casein kinase II phosphorylation site at ¹⁸⁷SLSE¹⁹⁰. In addition, there were six protein kinase C-dependent phosphorylation sites and two putative N-myristoylation sites, ⁷⁶GGHINH⁸¹ and ⁹⁶GQFPSS¹⁰¹.

BLAST search for the deduced amino acid sequence in all the available databases for the isolated cDNA clone revealed that

the protein is identical with mitochondrial Fe/Mn subtype SOD across the species. The deduced amino acid sequence shared more than 25% identity with the SOD of *Ganoderma microsporum* (38%), human (33%), maize (31%), *Saccharomyces cerevisiae* (29%) and *E. coli* (25%). In addition, we used the ClustalW program to obtain an optimized multiple sequence alignment (Figure 2) and the isolated protein showed an overall identity in the N-terminal region with known FeSOD/MnSODs, but no such identity was observed towards the C-terminus. Among the conserved metal-binding sites, His^{28/33} and His⁸¹ were present, but C-terminal Asp¹⁶⁵ and His¹⁶⁹ were absent in the isolated clone. This sequence was submitted to GenBank® with the identification as Fe²⁺ SOD (accession no. AF434197).

Southern-blot and phylogenetic analyses of the isolated SOD

R. glutinis genomic DNA was digested with *Eco*RI or *Sac*I, and the fragments were separated on a 0.8% agarose gel and transferred on to nylon membrane. The blots were then probed with radiolabelled full-length SOD cDNA. Digestion with *Eco*RI (Figure 3B, lane 1) yielded a strong single 2 kb hybridizing band with SOD cDNA, whereas a different hybridization pattern was obtained on digestion with *Sac*I (Figure 3B, lane 2). Three bands were obtained: one at less than 500 bp, others were at 1.5 and 6 kb. These results suggested that the SOD gene is a member of a low-copy multigene family. A dendrogram showing the phylogenetic relationship of *R. glutinis* SOD to SODs from many different organisms is shown in Figure 3(C). This protein falls under the class of yeast and fungi SOD and is related to *S. cerevisiae* and *G. microsporum* in the same way.

Expression of SOD in *E. coli*

The full-length *R. glutinis* cDNA in a pBK-CMV expression vector containing a *lac* promoter was used to transform *E. coli* cells (JM109) as described in the Experimental section. The transformed bacteria were induced with 1 mM IPTG, cell extracts were prepared from the induced bacteria and assayed for SOD activity. It was found that there was no increase in SOD activity in *E. coli* JM109 cells transformed with the recombinant plasmid

GGCACCAGGGGCGACGACTCGAGCCATGGCCGCTATAACAAGATCCCTGCTGTTCTCCC	60
M A A Y N K I P A V L P	12
GAAGCTTCCCTTCGCGTACAACGCTCTGGAGCCGCCATTTCCTCCCAGATCATGGAGCT	120
K L P F A Y N A L E P A I S S Q I M E L	32
CCACCACTCGAAGCACCCACGCCACCTACGTCGCCAACTTCAACAAGGCCACGAGGACAT	180
H H S K H H A T Y V A N F N K A H E D I	52
CCAGGCTGCTTCGAGGCCAGGACATCAAGAAGCAGATTGCCCTCCAGGCCACCGTCAA	240
Q A A S Q A Q D I K K Q I A L Q A T V K	72
GTTCAACGGCGGTGGCCACATCAACACACCTCTTCTGGGAGAACCTCGCCCCCAGTC	300
F N G G G H I N H T L F W E N L A P Q S	92
GCAGGGCGGCGGCCAGTTCCTTCGTCGGCAAGCTCCACGACCAAGTCCAGCAGGACTT	360
Q G G G Q F P S S G K L H D Q V Q Q D F	112
CGCGGTCTCGACGGCTTTGAAGAAGCCGTCACGCGCTGCCCTCGGTATCCAGGGAT	420
G G L D G F E E G R Q R R C P R Y P G I	132
CTGGATGGGCTGGCTCGGGGTACAACCCGACGACCAAGAACCTTGAAGGCTGTCTCGACC	480
W M G L A R G T T R R P R T L R L S R P	152
GCAACCAGGGACCCGCTTCTCGGCTACGTTCCCCCGGTCGGCATTGACATGTGGGAGCAC	540
Q P G T R F S A T F P R S A L T C G S T	172
GCTTACTACATCGACTACAAGAAGTCAAGGCCTCGTACCTCGAAGTCTCTCGGAGGCCATA	600
L T T T S T T R S Q G L V P R S L S E A *	191
AGTCTCGCCCTCCCTTCTCGAGCCGCTTGAAGGAAGGAAGGAATGCGCTTGAA	660
CCCATGTAGTACGCGAAAGTCGAAATACGAAATCCCCTCAGTCGTTGCAAAAAAAAAA	720
AAAAAAAAAAAA	732

Figure 1 Nucleotide and deduced amino acid sequence of 21 kDa polypeptide

Nucleotide sequence of the isolated cDNA (upper line) is presented with its deduced amino acid sequence (lower line). Nucleotides are numbered (boldface) in the 5' to 3' direction and amino acids are numbered from N-terminus starting from the first methionine residue. Putative polyadenylation signal is underlined. * indicates the stop codon of the ORF. The first 20 amino acid residues (shown underlined) were matched with the N-terminal sequence obtained from the 21 kDa protein of TBC.

Figure 2 Multiple sequence alignment of 21 kDa protein

The deduced amino acid sequence of the isolated clone was used to search the public database (SwissProt) using BLAST algorithm. The first nine significant hits were SOD from many different organisms (*E. coli*, *S. cerevisiae*, *G. microsporium*, maize, pea, capsicum, human, bovine and rabbit). Multiple sequence alignment was performed with these polypeptide sequences with the isolated clone using the ClustalW algorithm. -, gaps introduced to obtain the best alignment; *, conserved residues in all sequences in the alignment; :, conserved substitutions; ., semi-conserved substitutions. Three most conserved domains present among all the SODs are marked as I, II and III; among them motifs I and II are present in the isolated clone, but motif III is absent.

as compared with untransformed cells (results not shown). The SDS/PAGE profile demonstrated the presence of recombinant protein in transformed cells as compared with untransformed control (Figure 3D). Recombinant SOD is of a higher molecular

mass (26 kDa) when compared with the wild-type, because of the insertion of a few amino acids at the N-terminus from the parent vector. Western-blot analysis of the transformed JM109 cells with cross-reactive anti-ACP antibodies (for explanation

Figure 3 Southern-blot analysis, dendrogram and expression of isolated SOD

(A) Genomic DNA of *R. glutinis* was digested with *EcoRI* (lane 1) or *SacI* (lane 2) run on a 1 % (w/v) agarose gel. (B) Southern hybridization was performed on the same gel (in A) using the α - ^{32}P -labelled SOD cDNA as a probe. (C) Dendrogram of the aligned proteins from Figure 2. The length of the lines connecting different proteins is a measure of their sequence relatedness. (D) SOD cDNA (in pBK-CMV vector) was transformed into *E. coli* JM109 cells and induced with 0.5 mM IPTG for 4 h and cell lysates were run on an SDS/12 % polyacrylamide gel. Lane 1, uninduced *E. coli* cell lysate; lanes 2, 3, soluble and particulate fraction of the induced *E. coli* cell lysate respectively; lane M, molecular-mass markers. (E) Western-blot analysis of the same gel with anti-SOD antibodies. Arrowhead indicates a 26 kDa protein in gel as well as in Western-blot analysis.

see the Discussion section) showed an intense signal at approx. 26 kDa, and no signal was detected in the untransformed cells (Figure 3E).

SOD is a part of TBC

To demonstrate whether the TBC has SOD activity, cytosol and purified complex were used for the native in-gel SOD activity assay. Surprisingly, we found only one active SOD band in the cytosol, which runs exactly at the same position with respect to TBC. Purified TBC also showed a single SOD-active band in the native gel (Figure 4A). To confirm the presence of SOD in TBC, polyclonal antibodies raised against the overexpressed recombinant SOD were used to probe the cytosol and purified TBC under native (Figure 4B) and denaturing conditions (Figure 4C). The antibodies recognized the complex in the native immunoblot (Figure 4B) and a single band of 21 kDa protein from cytosol and complex in the SDS/polyacrylamide gel immunoblot (Figure 4C). These results indicate that TBC has SOD activity.

To determine whether the 21 kDa SOD was held with the other polypeptides of the TBC by physical interactions, the exponential-phase *R. glutinis* cells were labelled metabolically with [^{35}S]methionine, followed by immunoprecipitation with antibodies raised against 26 kDa overexpressed protein as well as cross-reactive anti-ACP antibodies (Figure 4D). Normal rabbit serum and no antibodies were used as controls. The immunoprecipitates were resolved by SDS/PAGE (13 % gel),

Figure 4 SOD is a part of 10 S TBC in oleaginous yeast

(A) Cytosol (lane 1) and purified TBC (lane 2) was run on a 7 % native polyacrylamide gel, and in-gel SOD assays were performed as described in the Experimental section. SOD-active band was seen both in cytosol and TBC. (B) Polyclonal antiserum in rabbit was raised against the recombinant SOD and used to probe cytosol (lane 2) and TBC (lane 3) under native conditions. Lane 1, preimmune sera control. Antisera recognized both TBC and cytosol. (C) Cytosol (lane 1) and purified TBC (lane 2) was run on an SDS/12 % polyacrylamide gel and Western-blot analysis was performed with the antisera. SOD was detected in both cytosol and TBC. (D) [^{35}S]Met-labelled *R. glutinis* lysate was immunoprecipitated with anti-SOD (overexpressed 21 kDa protein) antibodies (lane 3) as well as anti-ACP (lane 4) antibodies. The immunoprecipitates were analysed by SDS/PAGE (13 % gel) and proteins were visualized by autoradiography. Lane 1 is control with no primary antibodies and in lane 2, normal rabbit serum was used instead of primary antibodies. (E) The presence of SOD in the purified TBC was observed by cross-linking the TBC with disuccinimidyl suberate. The cross-linked product was run on 5 % SDS/polyacrylamide gel, followed by Western-blot analysis probed with anti-SOD antibodies (lane 2). Anti-ACP antibodies were used as a positive control (lane 3) and uncross-linked cytosol was used as negative control (lane 1).

followed by autoradiography. Both the antibodies pulled down the TBC, but six proteins were visible in autoradiograph. In our earlier observation by SDS/PAGE (12 % gel), we observed only five polypeptides precipitating together [10]. Here, we could see the appearance of a new protein at the molecular mass of approx. 11 kDa. This discrepancy could be due to the complete running out of the protein (11 kDa) along with the tracking dye by SDS/PAGE (12 % gel), whereas in these experiments, the precipitates were resolved by SDS/PAGE (13 % gel). The identity of the 11 kDa protein is not yet known, but it could possibly be an ACP.

TBC was subjected to chemical cross-linking using a homobifunctional cross-linker, disuccinimidyl suberate. The cross-linked product was run on an SDS/5 % polyacrylamide gel, transferred on to a nitrocellulose membrane and probed with anti-SOD and anti-ACP antibodies (Figure 4E). It was observed that the cross-linked product migrated at approx. 200 kDa, confirming that the 21 kDa SOD was held to TBC by protein-protein interaction and not by lipid-protein interaction.

Purification of SOD from the TBC

The purified complex was pretreated with 5, 10 and 15 mM SDS for 1 h on ice and SOD activity was monitored by alkaline pyrogallol assay, which found that there was only 25 % inhibition of activity with 15 mM SDS treatment (Figure 5A). The treated

Figure 5 Dissociation of SOD from TBC

(A) SOD activity was determined after the treatment with SDS. TBC was treated with various concentrations of SDS for 1 h on ice and alkaline pyrogallol assay was performed. (B) TBC was treated with different concentrations of SDS and resolved on 7% native polyacrylamide gel, followed by Western-blot analysis with anti-SOD antibodies. (C) TBC was resolved on IEF gel and in-gel SOD assay was performed. (D) SOD was purified on IEF gel and was eluted. The eluted protein was resolved by SDS/PAGE (12% gel), followed by silver staining. Molecular-mass markers are indicated on the left. (E) The presence of SOD in IEF eluate was confirmed by Western-blot analysis with anti-SOD antibodies.

TBC was run on a 7% native polyacrylamide gel and an in-gel SOD assay as well as Western-blot analysis was performed. It was observed that 10 mM SDS dissociated the SOD from the complex in an active form, and the dissociation was further confirmed by Western-blot analysis (Figure 5B). SOD was purified from TBC by dissociating the TBC on IEF gel (pI range 3–10). TBC was separated into distinct components on IEF gel and an in-gel assay showed that SOD remained active under these conditions (Figure 5C). The SOD-active band (pI 8) was eluted from the gel pieces. The presence of SOD in the eluted fraction was checked by the solution assay and in-gel SOD assay. The summary of purification of SOD is given in Table 1. We achieved 616-fold purification with 14% recovery of the protein. The purified protein showed a single band after SDS/PAGE (12%

Table 1 Purification of SOD from *R. glutinis*

Exponential-phase oleaginous yeast cells from 1 litre of culture were lysed with glass beads and the lysate was used for purification as described in the Experimental section. Assays were performed by alkaline pyrogallol method.

	Specific activity (units · min ⁻¹ · mg ⁻¹)	Total activity (units/min)	Yield (%)	Purification (-fold)
Cytosol	10.7	68 210	100	1
Native polyacrylamide	3145.8	25 166	36.89	294
IEF gel	6593.6	9890	14.50	616

Figure 6 Characterization of SOD

(A) SOD activity in *R. glutinis* was determined at the indicated time points. Equal numbers of cells were obtained at different times of growth. TBC was purified and run on 7% native polyacrylamide gel and SOD in-gel assay was performed. (B) *R. glutinis* cytosol was treated with 0, 20 and 40 mM sodium azide (lanes 1, 2 and 3 respectively) and run on 7% native polyacrylamide gel and assayed for in-gel SOD activity. (C) For comparison, in-gel SOD activity was performed in *E. coli* JM109 cell lysate that was treated with 0 and 20 mM sodium azide (lanes 1 and 2 respectively). (D) Atomic absorption spectra of purified SOD are represented. Upper panel: spectra obtained for iron; lower panel: spectra for manganese. (E) Cross-reactivity of the anti-SOD antibodies was checked with *R. glutinis* cytosol (lane 1), commercially available *E. coli* MnSOD (lane 2), *E. coli* FeSOD (lane 3) and bovine FeSOD (lane 4). Molecular-mass markers are indicated on the left.

gel) followed by silver staining (Figure 5D). Western-blot analysis confirmed that the purified protein was indeed SOD (Figure 5E).

The distribution of SOD activity in mitochondria and cytosol was also determined, and most (70%) of the activity was found in cytosol. Western-blot analysis of the mitochondrial fraction with anti-SOD antibodies showed a cross-reactive band having the same molecular mass (results not shown).

Characterization of SOD

The SOD activity in *R. glutinis* was monitored as a function of growth. At each time point of growth, the same number of cells (8×10^7) was taken out, TBC was purified and an in-gel SOD assay was performed with all the samples (Figure 6A). It was observed that the oleaginous yeast had active SOD even at the early phase of growth, but it increased reasonably in the late-exponential phase (28 h). This activity profile was found to match with the rate of synthesis and accumulation of TAG in *R. glutinis* [10].

To determine whether this SOD is sensitive to sodium azide, purified TBC was preincubated with 20 and 40 mM sodium azide (Figure 6B) for 30 min on ice. On running a 7% native gel followed by an in-gel SOD assay, it was observed that this SOD was insensitive to azide. As a positive control, *E. coli* JM109 cell lysate was treated with 20 mM azide and it was found that most of the activities were inhibited (Figure 6C). This observation indicated that *E. coli* SOD is sensitive to azide.

In the immunoinhibition assay, TBC was preincubated with anti-SOD antibodies as well as anti-ACP antibodies at a 1:100 dilution for 3 h on ice and the solution assay for SOD was

Table 2 Inhibition of SOD activity

Purified TBC was used as SOD source and solution assays were performed using alkaline pyrogallol.

	Specific activity (units · min ⁻¹ · mg ⁻¹)	Inhibition (%)
No inhibitor	3149 ± 17	0
Antibodies		
Anti-SOD	1824 ± 13	58
Anti-ACP	1918 ± 7	61
Fe/Mn inhibitors		
5 mM H ₂ O ₂	1383 ± 15	44
10 mM EDTA	1572 ± 10	50
20 mM EDTA	0.00	100
Cu/Zn inhibitors		
5 mM KCN	3136 ± 12	0
10 mM DDC	3151 ± 21	0

performed. It was found that both the antibodies were capable of inhibiting 60 % of SOD activity (Table 2).

To determine the cofactor requirement of SOD, TBC was treated with two different classes of inhibitors (Table 2). KCN and diethyl dithiocarbamate are specific inhibitors of Cu/Zn subtype of SOD, and EDTA and H₂O₂ are specific for Fe/Mn type SOD. Neither 5 mM KCN nor 10 mM diethyl dithiocarbamate could inhibit the SOD activity. On the other hand, 5 mM H₂O₂ treatment showed 50 % inhibition of SOD activity and 20 mM EDTA inhibited the activity completely. Atomic absorption spectroscopy with purified and overexpressed SOD revealed that it had 0.71 mol of iron/mol of protein and very negligible amount of Mn in it. The characteristic spectra for Fe and Mn are shown in Figure 6(D).

To determine the cross-reactivity of *R. glutinis* SOD with other SODs, Western-blot analysis was performed with commercially available *E. coli* MnSOD and FeSOD, and bovine FeSOD (Figure 6E). Cytosol from oleaginous yeast was used as a positive control. From Western-blot analysis, we observed that antibodies against *R. glutinis* SOD cross-reacted with *E. coli* SOD, but not with bovine SOD. These results indicated that the isolated SOD is a near relative of bacterial FeSOD/MnSOD, but not of mammalian FeSOD.

DISCUSSION

TAG biosynthesizing machinery in *R. glutinis* is present in cytosol as a multienzyme complex. The 21 kDa protein of this complex cross-reacted with polyclonal antisera raised against purified ACP. Based on this cross-reactivity, we assumed earlier that 21 kDa protein could be an ACP [10,11]. Cloning and sequence analyses of the gene encoding 21 kDa protein revealed that it is an FeSOD. The reason for cross-reactivity of anti-ACP antibodies with 21 kDa protein is not very clear. SOD may share a common and/or identical epitope with ACP, which was recognized by the polyclonal antibodies in Western-blot analyses. *In silico* analysis of the sequence data suggested that this is not a classical cytosolic SOD, as it showed similarity towards mitochondrial FeSOD/MnSOD, mostly at the N-terminal region. Even in the absence of two of the conserved residues required for metal binding, the protein showed activity and the activity was insensitive to azide. Several lines of evidence (in-gel assays and Western-blot analyses, immunoprecipitation, immunolocalization

and chemical cross-linking studies) support the presence of this enzyme in cytosol as part of TBC.

To protect against the damaging effect of oxygen radicals, most eukaryotes express a cytoplasmic CuSOD/ZnSOD and a mitochondrial MnSOD that convert the superoxide anion into H₂O₂. FeSOD is well-known in bacteria and parasites [17–19]. Existence of FeSOD in eukaryotic system is reported only from plant species such as *Arabidopsis thaliana* and *Nicotiana plumbaginifolia* [20]. We report here for the first time the presence of cytosolic FeSOD in a yeast system. All the reported FeSODs are of mitochondrial origin. Sequence analyses suggested that the isolated enzyme from *R. glutinis* does not contain any mitochondrial import signal sequence. A hydropathy-plot analysis also shows that this protein is cytosolic in nature. Atomic absorption spectroscopy and a solution assay with different inhibitors showed clearly that *R. glutinis* SOD utilizes Fe as cofactor. An in-gel SOD assay with a post-mitochondrial fraction showed only one SOD activity. One cross-reactive band of the same size was observed in the mitochondrial fraction. We therefore conclude that oleaginous yeast contains only one cytosolic SOD that falls in the FeSOD category.

Reactive oxygen species are generated during the normal respiratory metabolism, but their damaging effects are suppressed normally by antioxidants. SOD, along with other enzymes, protects cells from oxidative damage of cellular protein and lipids; however, SOD is considered to be the first line of defence against oxygen toxicity. Baker's yeast, *S. cerevisiae*, contains two different SODs: SOD1, an abundant cytosolic CuSOD/ZnSOD [21], and SOD2, an MnSOD present in the mitochondria [22]. A fraction of CuSOD/ZnSOD is reported to be present in the mitochondrial intermembrane space [23]. Moreover, CuSOD/ZnSOD in *S. cerevisiae* showed its involvement in Zn metabolism [24]. Mutants deficient in cytosolic CuSOD/ZnSOD were used to study the role of various oxygen species generated during the peroxidation of unsaturated fatty acids in *S. cerevisiae* under anoxia, and it was observed that these mutants became highly sensitive to oxidative stress [25].

What could be the reason for the association of SOD with TAG biosynthesis in oleaginous yeast? TBC contains an active acyl-ACP synthetase, which leads to the alternative activation of fatty acid by formation of acyl-ACP. The fatty-acid specificity of acyl-ACP synthetase was comparable with that of accumulation of PUFAs in TAG *in vivo* [9,11]. The soluble TAG biosynthetic machinery prefers PUFA and the presence of SOD as a part of TBC could protect the soluble TBC and the fatty acid from reactive oxygen species damage.

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