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Purification, characterization, sequencing and molecular cloning of a novel cysteine methyltransferase that regulates trehalose-6-phosphate synthase from *Saccharomyces cerevisiae*

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ABSTRACT

Background: In *Saccharomyces cerevisiae* methylation at cysteine residue displayed enhanced activity of trehalose-6-phosphate synthase (TPS).
Methods: The cysteine methyltransferase (CMT) responsible for methylating TPS was purified and characterized. The amino acid sequence of the enzyme protein was determined by a combination of N-terminal sequencing and MALDI-TOF/TOF analysis. The nucleotide sequence of the CMT gene was determined, isolated from *S. cerevisiae* and expressed in *E. coli*. Targeted disruption of the CMT gene by PCR based homologous recombination in *S. cerevisiae* was followed by metabolite characterization in the mutant.
Results: The purified enzyme was observed to enhance the activity of TPS by a factor of 1.76. The 14 kDa enzyme was found to be cysteine specific. The optimum temperature and pH of enzyme activity was calculated as 30 °C and 7.0 respectively. The K_m V_{max} and K_{cat} against S-adenosyl-L-methionine (AdoMet) were 4.95 μM, 3.2 U/mg and 6.4 s^{-1} respectively. Competitive inhibitor S-Adenosyl-L-homocysteine achieved a K_i as 10.9 μM against AdoMet. The protein sequence contained three putative AdoMet binding motifs. The purified recombinant CMT activity exhibited similar physicochemical characteristics with the native counterpart. The mutant, Matα, *cmt*:: *kan*^{r} exhibited almost 50% reduction in intracellular trehalose concentration.
Conclusion: A novel cysteine methyltransferase is purified, which is responsible for enhanced levels of trehalose in *S. cerevisiae*.
General significance: This is the first report about a cysteine methyltransferase which performs S methylation at cysteine residue regulating TPS activity by 50%, which resulted in an increase of the intercellular stress sugar, trehalose.

1. Introduction

Trehalose is an important multifunctional, non-reducing disaccharide found in nature [1]. It is accumulated during periods of nutrient starvation, desiccation and heat shock [2]. This sugar serves as a stabilizer of cellular structures under stress conditions besides having an exceptional capacity to protect biological membranes and enzymes from the adverse effects of freezing or drying [3]. In *Saccharomyces cerevisiae*, trehalose pathway involves the transfer of glucose from uridine diphosphate glucose (UDPG) to glucose-6-phosphate (G-6-P) to form trehalose-6-phosphate (T-6-P) and uridine diphosphate (UDP) [4]. This step is catalyzed by the enzyme trehalose-6-phosphate synthase (TPS). Trehalose-6-phosphate is subsequently dephosphorylated in the next step by trehalose-6-phosphate phosphatase (TPP) to yield inorganic phosphate and trehalose [5].

Post-translational modifications of proteins like methylation play an important role in regulating protein functions [6]. Protein methylation has an important role in cellular signaling events [6]. Biological transmethylation reactions utilizing S-adenosyl methionine (AdoMet) as a methyl donor have attracted the attention of many biochemists. The reaction can be catalyzed by three major categories. N-methylation involved methylation of arginine, lysine, histidine side chains. O-methylation of either the internal carboxyl group of glutamate and isoaspartate residues or the C-terminal of the cysteine residue and S-Methylation of either cysteine or methionine residues [7,8].

Regulation of trehalose metabolism by Oxidized Adenosine (Adox) and AdoMet in *S. cerevisiae* was observed in this laboratory as evident from the previous report [9]. Recent reports from this laboratory have confirmed that methylation of trehalose-6-phosphate synthase (TPS) in the presence of AdoMet resulted in enhanced activity and subsequently increased trehalose production [10]. It was observed that the TPS methylation occurred at the cysteine residue thus suggesting a

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possible role of a methyltransferase enzyme in conducting the methylation reaction.

In order to better understand the molecular architecture of the enzyme and to gain an insight into its role in the cellular metabolism, the present work involved the purification, characterization and cloning of this novel cysteine methyltransferase responsible for the enhanced TPS activity.

2. Materials and methods

2.1. Materials

Trehalose-6-phosphate (T-6-P), uridine 5′-diphosphoglucose (UDPG), glucose-6-phosphate (G-6-P), S-adenosyl-L-methionine (AdoMet), phenylmethylsulphonyl fluoride (PMSF), benzamidine hydrochloride, 2-mercaptoethanol, Bradford reagent, Polyvinylidene difluoride (PVDF) membrane and Ponceau S solution were purchased from Sigma, USA. Polyacrylamide gel electrophoresis reagents and chemicals were obtained from BIORAD, USA. All the other chemicals and medium components used were of analytical grade and were purchased locally. For cloning experiments, kits used were obtained from QIAGEN and NOVAGEN, USA. GeneJET ™ Genomic DNA isolation kit was procured from Fermentas, India

2.2. Methods

2.2.1. Organism and culture conditions

The diploid Mat aα *S. cerevisiae* strain used in this work is the same as reported earlier [11]. Cells were grown in YPD medium till desired stage of growth, measured by taking absorbance at 660 nm (A_{660} ~ 20) [12]. Cells were harvested by centrifugation at 10,000 *g* at 4–5 °C for 10 min.

For cloning: *E. coli* DH5α and BL21(DE3) strains were donated by Dr. Saumen Datta, IICB. Cells were grown overnight at 37°C in LB medium for routine culturing.

2.2.2. Cell lysis and preparation of enzyme solution

Yeast cells grown in YPD medium up to A_{660} ~ 20 were harvested, washed and suspended in ice cold lysis buffer pH 7.0 (20 mM Tris–HCl, buffer containing 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM PMSF, 10% (w/v) glycerol, 0.1% Tween 40). The cells were lysed by passing twice through a FRENCH Pressure Cell Press (SLM Instruments, USA) at 18 000 psi. The homogenate was centrifuged at 10 000 ×*g* for 5 min. The supernatant containing CMT activity was designated as crude enzyme solution (Step 1 enzyme).

2.2.3. Enzyme assays

Three different assay methods were employed in order to confirm the activity of the cysteine Methyltransferase (CMT).

1. Primarily, the CMT activity was detected indirectly through a coupled assay involving the trehalose-6-phosphate synthesizing enzyme TPS (0.5 mg/ml) used as a substrate. TPS was purified following a published protocol from *S. cerevisiae* [12]. *S. cerevisiae* cells grown upto A_{660} ~ 19 (early stationary phase) were lysed and subjected to 30–50% ammonium sulphate fractionation. This was followed by HPGFLC on HiLoad 16/60 Superdex 200 and HPGFLC on Ultropak TSK G2000SW [12]. The purified TPS obtained was used as a substrate for CMT assay. TPS activity was found to be increased upon incubation with 0.1 mM AdoMet in the presence of CMT. TPS was incubated in the presence or absence of AdoMet for 20 min at 30°C. Following incubation, TPS activity was assayed at 37°C for 15 min using 5 mM UDPG and 5 mM G-6-P as substrates [12]. T-6-P formed was determined by anthrone color reagent after neutralizing all other sugars [12]. The difference in TPS activity with and without incubation with CMT and AdoMet was designated as the effect of CMT on TPS. This assay was used during all purification steps. One Unit (U) of CMT activity is defined as transfer of μmoles of methyl group to TPS per minute per ml of enzyme under assay condition. The specific activity of CMT was expressed as U/mg of protein.
2. A direct assay of CMT activity was performed with a SAM Methyltransferase Assay Kit, Cat—CBA096, Calbiochem, USA. The assay is a continuous enzyme coupled assay. The removal of the methyl group from SAM generates S-adenosylhomocysteine (AdoHcy), which is rapidly converted to S-ribosylhomocysteine and adenine by the included adenosylhomocysteine nucleosidase. This rapid conversion prevents the buildup of adenosylhomocysteine and its feedback inhibition on the methylation reaction. Finally, the adenine is converted to hypoxanthine, by adenine deaminase, which in turn is converted to urate and hydrogen peroxide. The rate of production of hydrogen peroxide is measured with a colorimetric assay by an increase in absorbance at 510 nm. Adenosylhomocysteine is used as a positive control. This assay protocol was used to determine the physicochemical properties of the purified protein. One unit (U) of methyltransferase was defined as transfer of μmoles of methyl group per minute per ml of enzyme at 37 °C. Here, AdoMet donate one methyl group to TPS thereby one molecule of AdoHcy was formed.
3. A vapor diffusion assay using radioactive [C^3H_3]-AdoMet was employed in order to further verify and quantitate CMT activity following a published protocol, with slight modifications [13]. The assay mixture (0.3 ml) consisted of phosphate–citrate–EDTA buffer 50 mM pH 6.0, Mixture of Cold and Hot S-adenosyl-L-methionine (methyl-3H) solution (Specific activity: 15 mCi, 200 μM, 20 μCi), and 0.5 mg/ml TPS as a substrate. The reaction was started with 15 μl of cysteine methyltransferase. The reaction mixture was incubated for 20 min at 30 °C. Reaction was terminated by 0.3 ml of 0.45 M KOH solution containing 4%SDS and 2% (v/v) methanol), pH 9.6. The radioactive base labile [3H] methanol vapors formed were measured after 18–24 h of incubation in a Liquid Scintillation Counter. Readings were expressed in counts per min per mg of protein [13].

2.2.4. Growth curve and CMT activity during growth in S. cerevisiae

Activity of CMT was monitored along the growth curve of *S. cerevisiae* [12]. Aliquots were collected at different hours of growth. Growth was measured at A_{660} nm and CMT activity was measured based on TPS activity enhancement protocol as mentioned above.

2.2.5. Purification of native CMT

Solid ammonium sulphate was slowly added to the chilled supernatant of crude enzyme solution (Step 1), with constant stirring to reach a saturation of 30%. The solution was kept in ice overnight and was then centrifuged at 100 000 ×*g* for 30 min. The pellet containing little activity was discarded and the supernatant was brought to 50% saturation with solid ammonium sulphate, kept in ice for overnight and again centrifuged at 100 000 ×*g* for 30 min. The protein pellet was collected and dissolved in HPGFLC buffer to yield step-2 enzyme.

The enzyme solution from Step 2 was applied to high performance gel filtration liquid Chromatography (HPGFLC) using Protein Pak 300 SW column (Waters, USA). HPLC buffer, pH 7.0 (Tris–HCl 50 mM, containing 0.1 mM Benzamidine hydrochloride, 0.1 mM PMSF, 0.5 mM 2-mercaptoethanol, 0.5% (w/v) glycerol, 0.1% (w/v) and Tween 40) was used as mobile phase at a flow rate of 0.5 ml/min. A_{280} was monitored using a detector obtained from Waters, USA. Fractions containing CMT activity were pooled and concentrated by lyophilization.

The above mentioned enzyme was re-chromatographed in the same column using same mobile phase as mentioned above. Active fractions were pooled and concentrated by lyophilization to yield Step 4 enzyme.

2.2.6. Native and SDS Polyacrylamide gel electrophoresis

Non denaturing PAGE of Step-4 CMT enzyme (20 μg) was carried out at pH 7.0 on a 7.5% polyacrylamide gel using 8 × 8 × 1.5 mm gel slab using mighty small gel apparatus obtained from Hoefer, USA.

Electrophoresis was carried out at a constant current (20 mA/slab) according to the guidelines in the technical bulletin provided by the manufacturer. The single protein band was visualized on staining with Coomassie Brilliant Blue R-250 (Pierce, USA).

Denaturizing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified Step 4 protein was carried out at pH 8.5 on a 15% resolving gel same as above. 2-Mercaptoethanol (5%) and ethylenediaminetetraacetic acid (EDTA; 1 mM) were added to the sample buffer as a solubilizing agent. Low molecular weight markers obtained from GE Healthcare (Code RPN 755), USA, were loaded and run in parallel lanes and gels were stained with Coomassie Brilliant Blue R250 (Pierce, USA) by shaking overnight at 37 °C.

2.2.7. Molecular weight estimation

Molecular weight was determined by three methods as described below.

1. Molecular weight of the purified enzyme protein was estimated by HPGFLC from a plot of log of molecular weight versus K_{av} (Partition coefficient) values of Low molecular weight standards as Chymotrysin, Ribonuclease A, Albumin, Ovalbumin and Blue Dextran (GE Healthcare, USA). The chromatographic conditions were same as mentioned above. $K_{av} = (V_e - V_o)/(V_t - V_o)$, V_e = elution volume of solute, V_o = void volume of column, V_t or total column volume.
2. Molecular weight was estimated by SDS-PAGE from a plot of R_f (relative migration values) versus log of molecular weight of LMW gel electrophoresis markers [Genei, India].
3. Molecular weight estimation was performed by MALDI TOF analysis of whole protein using an Applied Biosystems Q10 4800 MALDI TOF/TOF™ analyzer using 4000 series explorer software for acquisition and GPS explorer software, version 3.6 for analysis.

2.2.8. Substrate specificity of CMT

Substrate specificity of the enzyme was measured using various substrates. The concentration of all the substrates used in the assay was 0.1 mM which is above the saturation concentrations of these substrates viz, Purified TPS, Gelatin, Adrenocorticotropic Hormone (ACTH), Glucose-6-phosphate dehydrogenase (G6PD), Ovalbumin, Ribonuclease A, Peroxidase, Lipase, Chymotrysin and Albumin. The purified enzyme was incubated with each substrate for 20 min at 30°C and assayed for the CMT activity using the methyltransferase assay kit. The total number of cysteine residues in each substrate was calculated from the protein sequence at the NCBI database.

In another set of experiment TPS was digested with CNBr [14]. The peptides generated were separated using Protein Pak 60 column (Waters, India) and subjected MALDI TOF analysis with similar conditions as described above. These peptides were subjected to MALDI TOF analysis with same specifications as mentioned above. The sequences of the peptides screened individually were worked out by matching them with the TPS enzyme sequence using Expasy software. These peptides were used as substrate to study CMT activity.

2.2.9. Product characterization of CMT catalyzed reaction by amino acid analysis

Enzymatic reactions were carried out with TPS and AdoMet, in the presence or absence of CMT enzyme. The reaction was incubated for 20 min and the products were analyzed for the presence of S-methyl L-cysteine. Acid hydrolysis of the sample was performed in a PICO-TAG Work Station according to the technical bulletin provided by the manufacturer and analyzed using a HPLC fitted with amino acid analysis column (Waters, USA) [12]. Standard amino acid mixtures as well as standard S-methyl L-cysteine were also injected for reference. The L-cysteine and methylated L-cysteine peaks as observed from the chromatogram was analyzed by ESI MS Q-TOF (Micromass-Waters, USA).

2.2.10. Effect of metal chelators on CMT activity

Purified CMT enzyme was pre-incubated with specific concentrations of different compounds in 50 mM Tris–HCl buffer (pH 7.0) for 30 min at 30 °C. Assay was started with the addition of the substrate. Residual activities of the pre-incubated samples were measured. Values were expressed in percentage and CMT activity of a sample identically pre-incubated but in absence of any compound was considered as 100%.

2.2.11. Effect of inhibitors on CMT activity

Purified CMT enzyme was pre-incubated with specific concentrations of different inhibitors in 50 mM Tris–HCl buffer (pH 7.0) for 30 min at 30 °C. Assay was started with the addition of the substrate. Values were expressed in percentage and CMT activity of a sample identically pre-incubated but in absence of any compound was considered as 100%. Effect of methylation inhibitor, AdoHcy was studied using varying concentration as 0, 1, 2, 5, 10, 15 μM. The determination of apparent K_m was calculated. The lines in the plot were fitted by linear regression analysis. The intersection point of lines (K_i value) was determined graphically.

2.2.12. Analysis of N-Terminal amino acid sequence

The purified protein was spotted on a PVDF membrane following the method suggested by the manufacturer [15]. The transferred protein bands on PVDF were stained and identified with Ponceau S solution. The identified protein bands of interest were excised for analysis of N-terminal sequence. The desired bands were analyzed by N-terminal sequencing using Model 491 Procise protein/peptide sequencer from Applied Biosystems, USA. The sequence obtained from N-terminal sequencer was matched with the database of NCBI (National Center for Biological Information) using BLAST (Basic Local Alignment Search Tool).

2.2.13. Amino acid sequencing

The purified protein was subjected to trypsin, chymotrypsin and CNBr digestion [11,16,14]. Peptides were separated using HPLC with Symmetry C18 reverse phase column (Waters, India). Two solvents used were Solvent A (Water containing 0.1% TFA) and Solvent B (Acetonitrile containing 0.1% TFA). Peptide was eluted by a 60 min linear gradient to reach from 100%A to 100% B. Flow rate was maintained at 1 ml/min. A_{214} and A_{280} were monitored as stated earlier. Peptides were pooled and concentrated by lyophilizing. Approx. 0.5 μl of peptides was spotted on an α-Cyano-4-hydroxy-cinnamic acid matrix. Analysis of the sample was performed by MALDI TOF with same specifications as mentioned above. Sequences of peptides were identified using Expasy proteomics and sequence analysis tool software comparing with the yeast database.

Tryptophan, tyrosine and cysteine were estimated to validate the amino acid sequence obtained.

Estimation of tryptophan and tyrosine content was determined spectrophotometrically [17].

Cysteine content was additionally determined colorimetrically after reduction with sodium boro hydride using 5, 5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent (DTNB) in the presence of 8.0 M urea [18]. Control tubes containing cysteine 50 nmol or bovine serum albumin 0.025 mg and the enzyme sample containing 0.1 mg protein were taken in 2 ml reaction mixture, color was developed and were read at 412 nm.

2.2.14. Identification of CMT gene

Total RNA was isolated from ~2×10^7 yeast cells using RNA extraction kit (RNeasy Mini Kit, Cat No. 74104, Qiagen) according to the manufacturer's instruction. EMBOSS backtranseq was used to reverse translate the amino acid sequence obtained from MALDI TOF/TOF analysis into a nucleotide sequence based on the codon usage of *S. cerevisiae*. Based on this primary nucleotide sequence the PCR primers were designed by introduction of NdeI and XhoI restriction sites to facilitate cloning.

Isolated total RNA (4 μg) was subjected to reverse transcription in order to obtain cDNA using SuperScript III One Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA) following the

manufacturer's instruction. This kit is a one step formulation by which both cDNA synthesis and PCR amplification can be performed in a single tube using gene specific primers. In short, the first round of PCR amplification was carried out with first-strand cDNA as the template. The second round of PCR was carried out with the first-round product as a template. The PCR was performed by using a DNA Thermal Cycler (Takara) with cycling conditions as follows: 1 cycle: 50°C for 15–30 min, denaturation at 94 °C for 2 min, extension at 94 °C for 15 s, followed by annealing for 30 s at 60 °C, 68 °C for 1 min/kb for 40 cycles, and finally a 5 min extension at 68 °C. PCR products were electrophoresed by a 0.8% agarose gel and visualized after staining with ethidium bromide.

DNA or the PCR product was sent to Xcelris Genomics, India for sequencing. Sequence similarity search was performed with BLAST. A final set of primers were designed based on the experimentally obtained nucleotide sequence.

2.2.15. Transformation of E. coli cells with CMT gene

The CMT gene was amplified by RT-PCR with a gene specific primer pair. The 5′ primer contained a NdeI restriction site while the 3′ primer contained the XhoI restriction site. The 400 bp amplicons were cloned into pET-28a(+) (Novagen), a vector designed for expression of N-terminal 6× His tagged protein. *E. coli* competent cells were prepared chemically using $CaCl_2$. The plasmids were introduced into *E. coli* strain DH5α and transformants were selected on LB plates supplemented with kanamycin (50 μg/ml). The recombinant plasmids were isolated from *E. coli* DH5α strains using the QIAprep Spin Miniprep Kit (Calalogue no. 27104, Qiagen). The recombinant plasmid carrying the CMT gene was transformed into chemically competent *E. coli* BL21(DE3) for protein expression. The cmt gene is further sequenced from the plasmid to further verify the nucleotide sequence. DNA was sent to Xcelris Genomics, India for sequencing. Nucleotide sequence similarity with the previous deduced sequence was performed with BLAST. The sequence data of CMT enzyme have been deposited in the NCBI GenBank database under accession no. JX072966.

2.2.16. Expression and purification of recombinant CMT

Overnight culture of transformed *E. coli* BL21(DE3) cells were grown till they reached A_{600} = 0.4–0.6. The cells were harvested and resuspended in fresh LB medium. Recombinant protein expression was induced by adding 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) (Promega, USA) and grown for an additional 4 h. Cells were harvested by centrifugation and all purification procedures were carried out at 4 °C in 25 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl and 5% Glycerol. The cells (0.62 g wet weight) obtained from a 50-ml culture were disrupted with Bugbuster Ni-NTA His Bind purification Kit, Novagen. After centrifugation (16,000 ×*g* for 20 min), the supernatant was applied to a His-Tag Nickel NT Bugbuster column (Novagen) equilibrated with buffer supplemented with 20 mM imidazole. The enzyme was eluted with a step-wise gradient of 20–200 mM imidazole. Fractions showing activity, which eluted with 200 mM imidazole, were collected, dialysed against the buffer and used as the enzyme for characterization. The protein was subjected to SDS-PAGE analysis and visualized with Coomassie Blue R-250.

2.2.17. Characterizations of native and recombinant protein

2.2.17.1. Michaelis–Menten kinetic parameters. Michaelis–Menten kinetic parameters for the activity of the native CMT enzyme (nCMT) as well as recombinant CMT enzyme (rCMT) were determined from substrate saturation assays by using various concentrations of the substrate. Values for the maximum velocity and half-saturation coefficient (K_m) were determined by plotting the substrate concentration versus the initial velocity of each reaction and subjecting the data to non-linear regression analysis (with the statistical software package Sigma Stat). Enzyme turn over number K_{cat} was also determined.

2.2.17.2. Determination of temperature and pH optima. Optimum pH was determined by measuring enzyme activity at different pH ranging from 5.0 to 9.5 using the following buffer systems: 0.05 M sodium acetate buffer (pH 5.0 to 5.5), 0.05 M sodium phosphate buffer (pH 6.0 to 6.5) and 0.05 M Tris–HCl buffer (pH 7.0 to 9.5). The pH corresponding to maximum activity was taken as the optimum pH. Optimum temperature was determined by measuring enzyme activity at various temperatures (10 °C to 80 °C) for 15 min at pH 8.5. The temperature corresponding to maximum activity was taken as the optimum temperature.

2.2.17.3. Amplification of the KanMX cassette for the CMT gene replacement. The disruptants of CMT gene were generated by PCR-mediated gene replacement [19] using the pUG6 vector obtained from Euroscarf, Germany. The plasmid contains the *kanMX* cassette from Tn903 that confers resistance to the aminoglycosid antibiotic G418 (Geniticin) of transformed yeasts. The deletion cassette conferring Geniticin resistance was obtained by performing a PCR on plasmid pUG6-del using the primers 5′ATCCATTGCAAAGTGCAGCAATGGAGCCCGCAGTATCTGCGCCT GCCGGCCAGCTGAAGCTTCGTACG 3′ and 5′TACATAGACGACATATTACT CTACTTTTGGGCGTAATAACTTTCGTCGGGCCACTAGTGGATCTG 3′.

2.2.17.4. Transformation of yeast cells. Yeast cells were transformed with the obtained PCR product by the lithium acetate method [20]. For selection of G418 resistant transformants, cells were grown for 4 h at 30 °C before being plated, respectively, onto YPD plates containing G418. Transformants appeared after 36–48 h of incubation.

2.2.17.5. Detection of gene disruption event by PCR. Transformed colonies were taken directly from a YPD + G-418 plate and the genomic DNA was isolated from the G418-resistant transformants using GeneJET™ Genomic DNA isolation kit, Thermo Scientific, India. The success of replacement of the CMT gene with the amplified modules was verified by PCR analysis of the genomic DNA using primers specific for CMT gene.

2.2.17.6. CMT activity and trehalose estimation in the CMT mutant strains. CMT was assayed in the mutant strains using methyltransferase assay kits. Trehalose was measured following published protocol by using purified Acid trehalase (AT) in our laboratory [11] from both wild type as well as mutant strains of *S. cerevisiae*.

3. Results

3.1. Growth curve and CMT activity during growth in S. cerevisiae

CMT activity seemed to increase from mid logarithmic phase (after 10 h) and peaked at late log phase (at 30 h) after which it was seen to fall gradually (Fig. 1). The maximum specific activity of CMT was observed to be 1.6 U/mg protein at A_{660} ~ 20. A_{660} values were observed to increase with time till 40 h after which it displayed a tendency towards the stationary phase during the last observations.

3.2. Purification of CMT

Purification of the present enzyme was achieved in four steps (Table 1). During ammonium sulfate fractionation, 30–50% fraction recorded maximum CMT activity which was designated as step 2 purified enzyme. Step 2 enzyme was then injected in the Protein Pak 300 SW column where 4 peaks were detected. Out of which activity of CMT was eluted out at 22 min (data not shown). Chromatographic profile of Step 3 enzyme showed a single major peak eluting at 22–24 min (Fig. 2). The specific activity was detected as 10.2 U/mg in the single peak with 99.02 purification fold and yield of 50.25% (Table 1).

Active CMT at 22.6 min in the HPGFLC column was determined to be ~14 kDa from column calibration and SDS PAGE (Fig. 3). Non denaturing gel electrophoresis of Step 4 enzyme showed a single band confirming the

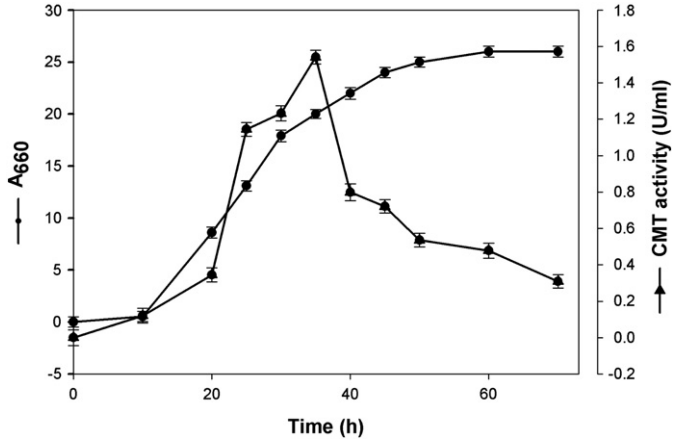

Fig. 1. Growth curve and CMT activity during growth of *S. cerevisiae*. Growth curve was monitored and measured at A_{660} nm. Specific activity of CMT was measured at different phases of growth by measuring enhancement of TPS activity as mentioned in methodology Section. X-axis denotes Time of cultivation (h) and Y-axis denotes Absorbance at A_{660} nm. Secondary Y-axis denoted the increase in TPS activity as CMT activity. All experiments were conducted in triplicate and data represented are average of three sets of observation, which did not vary more than ±5% represented by error bars.

purity of the enzyme (Fig. 3). Molecular weight estimation by MALDI TOF analysis of whole protein also confirmed the molecular weight of the enzyme as 13.6 kDa (Supp Fig. 1). The purified CMT was observed to increase TPS activity by a factor of 1.76.

3.3. Characterization of the novel enzyme confirmed as cysteine methyltransferase substrate specificity of the enzyme

CMT activity was assayed using different substrates such as enzymes (Lipase, Chymotrypsin, peroxidise, ribonuclease A, and G6PD), proteins (Albumin and ovalbumin) and peptides of TPS obtained by digestion with CNBr to observe whether they could act as a potential acceptor of the methyl group for the purified enzyme (Table 2). A much higher 19.3 U/mg CMT activity was observed with Albumin containing 35 cysteine residues. Low activity of 0.81 U/mg. was observed with glucose-6-P dehydrogenase as a substrate which inherently contains only one cysteine residue. Interestingly no activity was observed with ACTH and Gelatin.

Peptides containing one cysteine residue were found to display lower CMT activity as 1.01 U/mg versus 1.9 U/mg activity with peptides containing two cysteine residues. Peptides lacking cysteine residue were devoid of CMT activity (Table 3).

3.4. Product characterization of CMT catalyzed reaction by amino acid analysis

The S-methyl L-cysteine Standard (135 Da) gave a peak at 8.0 min whereas L-cysteine standard (121 Da) peaked at 14.3 min during HPLC elution. AdoMet incubated TPS displayed peak at 8.0 min with L-cysteine peak area of 28.1% and S-methyl cysteine peak area of 71.9%. In the presence of CMT, L-cysteine peak area was observed to be decreased to 4.1% and S-methyl cysteine peak area increased to 96.9%

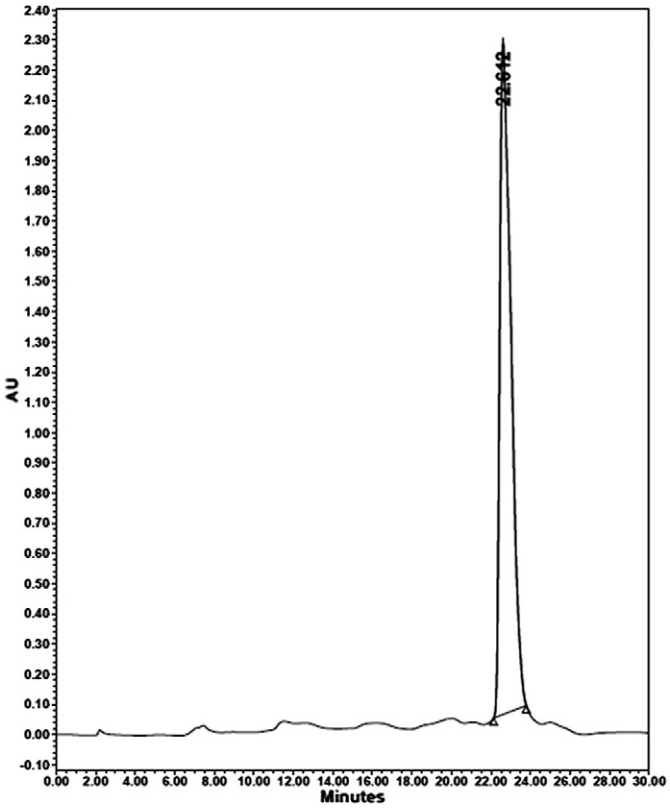

Fig. 2. Final step purification of nCMT on Protein Pak 300 SW column. Enzyme solution obtained from step 3 was separated by using HPGFLC column Protein Pak 300 as described in the text.

(Table 4). In both cases, the 8.0 min peak was collected and analyzed by ESI-MS. The peak displayed a molecular weight of 135 Da (data not shown).

3.5. Effect of Metal ions and inhibitor on CMT activity

Among the common metal co-factors tested $ZnCl_2$ displayed enhanced activity of CMT (Table 5). The activity of the purified enzyme was observed to be enhanced in the presence of EGTA and EDTA and decreased in the presence of Sulfosalicylic acid as well as Sodium Azide (Table 5). AdoHcy was seemed to competitively inhibit the activity of CMT as apparent from the constant V_{max} and increased K_m values. Inhibitor dissociation constant (K_i) value for AdoHcy was calculated as 10.9 μM (Supp Fig. 2).

3.6. Amino acid sequencing of CMT enzyme

N terminal sequencing of the pure enzyme detected around 18 amino acids in the following order 'MIHCKVQQWSPQYLRLPA'. Different spectrums were generated after CMT was digested with trypsin, chymotrypsin and CNBr. The complete amino acid sequence as derived from MALDI TOF MS/MS analysis displayed around 131 amino acid residues (Fig. 4). The protein is rich in Leucine, Serine and Threonine. The

Table 1
Purification table.

Steps	Total protein (mg)	Total methyltransferase enzyme activity (U)	Specific activity (U/mg)	Yield	Purification fold
Cell lysate (Step 1)	278.1 ± 0.8	28.85 ± 0.3	0.103 ± 0.001	100	1
Ammonium sulphate precipitation (30%–50%) (Step 2)	21.85 ± 0.7	24.65 ± 0.2	1.12 ± 0.002	85.44	10.8
HPGFLC on Protein Pak 300 SW (Step 3)	3.99 ± 0.6	20.1 ± 0.2	5.03 ± 0.09	69.6	48.83
HPGFLC on Protein Pak 300 SW (Step 4)	1.42 ± 0.3	14.5 ± 0.1	10.2 ± 0.07	50.25	99.02

Purification was performed as described in the text. Values represented are average of three sets of purification data.
One unit (U) of CMT is defined as one unit increase in TPS activity per ml of enzyme per minute under assay condition.

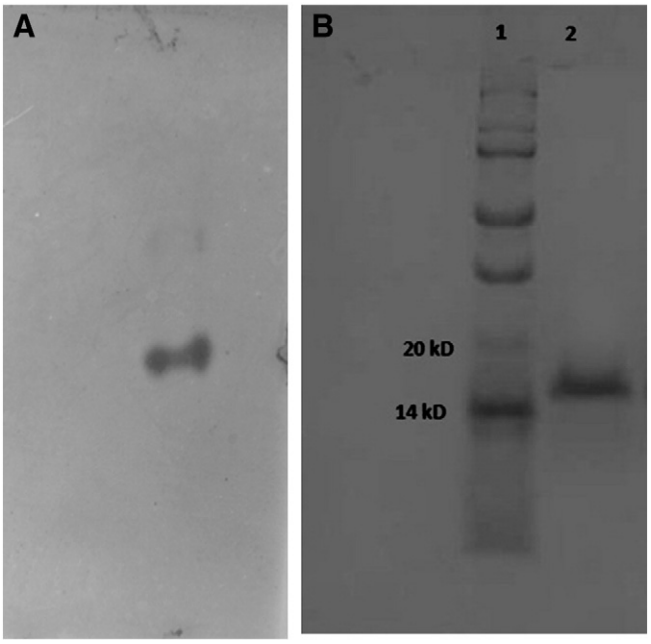

Fig. 3. Native and SDS Gel Electrophoresis of nCMT. (A)Non denaturing PAGE was carried out as described in the text. Step 4 enzyme was run on the gel. (B) SDS PAGE was carried out as mentioned in the text. Low molecular weight markers were run in Lane 1 obtained from Genei, India.

sequence obtained was matched with the database of NCBI (National Center for Biological Information) using BLAST (Basic Local Alignment Search Tool) against Non-Redundant databases (Each protein sequence is presented only once). BLAST revealed some similarity with the protein sequences of known methyltransferase from various organisms (Supp Table 1). Highest similarity was observed with Histone methyltransferase from *S. cerevisiae* (IU2Z_A).

Further validation of the amino acid sequence was performed as mentioned above in which tryptophan, tyrosine and cysteine were observed to be 2, 8, 3 residues in the protein. These results were matched with the derived amino acid sequence thus validating the sequence.

3.7. Cloning of CMT in E. coli

The primary nucleotide sequence of CMT was deduced with the help of MALDI-TOF/TOF analysis based on EMBOSS backtranseq. This sequence was verified further by sequencing the cloned cmt gene from both plasmid as well as pcr product by Xcelris Genomics, India and submitted in GENBANK under accession no. JX072966 since all the obtained sequence displayed ~98% similarity. Nucleotide BLAST of CMT sequence with the complete genome of *S. cerevisiae* (S288c) displayed a 6.8×10^{-11} E-value located at chromosome IV.

Using RT PCR, cDNA was synthesized from isolated mRNA of *S. cerevisiae*. The putative CMT gene was ampliflied from cDNA using primers based on deduced protein sequence by MALDI TOF/TOF analysis. The primers used were 5′ ATCCATTGCAAAGTGCAGCAATGGAGC CC 3′ and 5′ TACATAGACGACATATTACTCTACTTTTGGGCG 3′. Primers were designed based on the nucleotide sequence with (restriction enzyme sequence at 3′/5′ end) and utilized for amplifying the gene product by PCR. Insertion of the gene of interest into the pET-28a vector and successful transformation was obtained in *E. coli* DH5α. All the transformed clones had acquired the desired plasmid containing insert of approximately 400 bp and hence further studied (Supp Fig. 3). The target enzyme of interest was expressed as N-terminal 6× His tagged fusions in *E. coli* BL21(DE3) and induced by IPTG. The supernatant resulting from high speed centrifugation was subjected to SDS-PAGE and proteins were visualized by Coomassie Blue (Fig. 5). In the absence of IPTG very faint band observed in Lane 2 of the supernatant. Induction of the cells in IPTG resulted in production of more prominent ~14 kDa band in Lane 3. CMT was purified to homogeneity by His-TAG column with Ni-NTA resin (Novagen). The soluble protein was applied to the column and after thorough washing in 20 mM imidazole, the column was eluted batch wise with various concentration of imidazole. Lane 4 displayed the fraction passing through Ni column. Lane 5 displayed fraction with 50 mM imidazole. Lane 6 displayed fraction with 200 mM imidazole that migrated to give a ~14 kDa single band. Molecular weight of the rCMT was also verified using MALDI TOF analysis and single peak of 13.68 KDa was observed and was found in accordance with the nCMT (Supp Fig. 4). rCMT was also subjected to trypsin digestion and the peptide mass fingerprint obtained was observed to be identical with that of the nCMT peptide mass fingerprint (data not shown).

3.8. Comparison of native and cloned CMT activity with TPS as a substrate

Purified native CMT increased the activity of TPS enzyme from 2.1 U/ml to 3.7 U/ml as compared to 2.1 U/ml to 4.1 U/ml by the recombinant CMT expressed in *E. coli*. The cloned CMT enzyme was observed to increase the TPS activity by a factor of 1.95. The native enzyme was observed to increase TPS activity by a factor of 1.76 as mentioned earlier (Fig. 6).

3.9. Kinetic parameters of native and cloned enzyme

Initial velocity (v) of the enzyme activity was seen to increase gradually with increasing concentration of AdoMet from 0.5 mM to 20 mM. K_m for AdoMet was determined to be 4.95 μM and V_{max} was determined to be 3.20 U/mg (Table 6). The K_m values were observed to be same for the cloned enzyme. The V_{max} value was observed to be slightly higher as 3.5 U/mg for cloned enzyme. The turn over number, K_{cat} was observed as 6.4/s and 7/s for native and cloned protein respectively.

3.10. Determination of temperature and pH optima for cloned and native enzyme

The purified CMT enzyme displayed a classical bell shaped curve for temperature optima (Supp Fig. 5). Highest CMT activity was observed at 30 °C with 3.7 U/mg activity. Very low activities of 0.2 U/mg were detected at 10 °C. Beyond 60 °C no activity of CMT was seen. The

Table 2
Study of various substrates on CMT activity.

S. no.	Substrate	Specific activity of CMT (U/mg)	No. of approx. cysteine residue
1	Albumin (BSA)	19.3 ± 0.12	35
2	Lipase	8.5 ± 0.16	14
3	Chymotrypsin	6.8 ± 0.31	10
4	Peroxidase	4.8 ± 0.35	9
5	Ribonuclease A	4.9 ± 0.09	8
6	Ovalbumin	4.2 ± 0.13	6
7	Trehalose-6-phosphate synthase (TPS)	3.55 ± 0.3	4
8	Glucose-6-phosphate dehydrogenase (G6PD)	0.81 ± 0.07	1

CMT assay was done as described in the text. Gelatin and ACTH were also used as a substrate along with the above mentioned substrates. All experiments were performed in triplicate.

Table 3
Study of various peptides generated from TPS on CMT activity.

S. no.	TPS peptide mass (kD)	Sequences	Methyltransferase specific activity (U/mg)
1	4	HKSIPFEELISLYAVSDVCLVSSTRDGM	1.01 ± 0.09
2	6	EVFLNEHPEWRGKVVLVQVAVPSRGDVEEYQYLRS VVNELVGRINGQFGTVEFVPIHFM	–
3	11	EVFLNEHPEWRGKVVLVQVAVPSRGDVEEYQYLRSVVN ELVGRINGQFGTVEFVPIHFMHKSIPFEELISLYAVSDVCLVSSTRDGM	0.98 ± 0.08
4	11.7	NLVSYEYIACQEEKKGSLILSEFTGAAQSLNGAIIVNPWNTDDLSDAIN EALTLPDVKKEVNWEKLYKYISKYTSAFWGENFVHELYSTSSSSTSSSATKN	–
5	19.4	LRVKIHEKQLQNVKVGWFLHTPFPSSEIYRILPVRQEILKGVLSCDLVGFHT YDYARHFLSSVQRVLNVNTLPNGVEYQGRFVNVGAFPIGIDVDKFTDGLK KESVQKRIQQLKETFKGCKIIVGVDRLDYIKGVPQKLHAM	1.97 ± 0.1

CMT assay was done as described in the text. The sequences of the peptides were deduced from ExPaSY sequence predictor tool matching against TPS sequence obtained from NCBI database. All experiments were performed in triplicate.

recombinant enzyme displayed overall similar optimum temperature of 30 °C but with a high activity of 5.5 U/mg. The pH optimum was recorded at pH 7.0 with 3.4 U/mg activity (Supp Fig. 6). The recombinant enzyme also displayed pH optima at 7 with a higher activity as 4.2 U/mg.

3.11. CMT gene disruption by PCR based method

Detection of *cmt* gene disruption was verified by PCR method. Analysis of genomic DNA displayed a significant difference in the size of the PCR products generated from control type and mutant strains (Fig. 7). The control or wild type strain displayed an amplified product of 400 bp and the mutant strains displayed a 1000 bp product.

3.12. CMT activity and trehalose estimation in knock out mutant strains

CMT activity was not detected in the mutant strains (data not shown). Trehalose concentration was observed as 0.93 μmol/μg wet weight in the wild type strain as compared to 0.51 μmol/μg wet weight in the cmt mutant of *S. cerevisiae* (Fig. 8).

4. Discussion

This study is the continuation of a previous work that shed light on the possible regulation of trehalose metabolism by methylation in *S. cerevisiae* [10]. The key observation was that, TPS displayed enhanced enzyme activity in its methylated form and the site of methylation was probably at the cysteine residue. This enhancement in TPS activity was reflected in the elevated levels of trehalose in the cell [10]. The study thus triggered the need to identify the enzyme that was responsible for methylation of TPS and subsequent effect on trehalose metabolism. Hence, efforts were directed towards the purification and characterization of this cysteine methyltransferase enzyme. In order to strengthen the knowledge about this enzyme sequencing of this enzyme as well as cloning was further carried out.

Methylation is an important chemical modification that is capable of altering the structural and functional aspects of cellular components like protein or DNA [6,21]. Methylation has a primary role in gene expression by mediating local histone modifications as well as ensuring the fidelity of replicated DNA during post replication DNA repair [22,23]. But methylation is also a key instrument in regulating protein function [22]. Methylation reactions may be of transmethylation type where methyl group form one residue is simply transferred to another residue or it may be AdoMet Dependant, where methyl group from AdoMet is transferred to a substrate [7]. Methylation at specific amino acid residues, like lysine, arginine, histidine (N-methylation), glutamate or isoaspartate (C-methylation), cysteine or methionine (S-methylation) etc. may act as a switch either to initiate or up-regulate the activity of a cellular protein [7]. The two well studied cysteine methyltransferase from yeast *S. cerevisiae* are, STE14 and MGT1 [22,23]. While STE14 is a C terminal Isoprenylcysteine methyltransferase, MGT1 is methyltransferase involved in DNA repair [22,23].

STE14 facilitates AdoMet-dependant carboxyl methylation of the *S. cerevisiae* CAAX (C is Cys, A is aliphatic, and X can be one of several amino acids) proteins a-factor, RAS1, and RAS2. A series of three sequential modifications occur at the C-Terminal CAAX Sequence—isoprenylation of the cysteine residue is followed by the proteolytic cleavage of the AAX residues and finally the a-carboxyl methylation of the previously formed isoprenylcysteine. The prenyl group is critical for membrane association of these CAAX proteins and although not entirely essential for survival, methylation does assist in its membrane association.

The second known cysteine methyltransferase is involved in the DNA repair pathway. MGT1 transfers the methyl groups from the abnormal bases O^6-methylguanine (O^6MeG) and O^4-methylthymine (O^4MeT) to a cysteine residue in itself resulting in production of S-methylcysteine. The S-methylcysteine is not subsequently demethylated and the MTase MGT1 is inactivated by the act of repair.

Both these cysteine methyltransferases serves very specific functions in *S. cerevisiae* employing very different mode of action. While STE14 catalyzes methylation of a C-terminal cysteine residue, a prior prenylation step is necessary for the enzyme function. MGT1 catalyzes the transfer of the methyl group from O6MeG and O4MeT to a cysteine residue in itself resulting in subsequent deactivation of the enzyme. The cysteine methyltransferase presented in this study catalyzes methylation of an enzyme involved in trehalose metabolism, trehalose-6-Phosphate Synthase. The methylation is AdoMet dependant and does not require any pre-processing of the target residue, thus differing from STE14. Methylation upregulates TPS activity and does not cause

Table 4
Product characterization of CMT enzyme.

Sample	Cysteine		Methyl cysteine	
	Elution time	Peak area	Elution time	Peak area
Standard L-cysteine	14.3	924,1859	Absent	–
Standard L-cysteine methyl ester	Absent	–	8.0	2,3872,437
TPS + AdoMet	14.3	663,590 (28.1%)	8.0	1,697,227 (71.9%)
TPS + AdoMet + CMT	14.3	279,584 (4.1%)	8.1	6,441,329 (95.9%)

Amino acid analysis was determined by HPLC in a PICO-TAG work station according to the technical bulletin provided by the manufacturer (Waters) (Chromatogram not shown). The percentage was determined from the total peak area eluted at 14.3 and 8.0 min.

Table 5
Effect of chelators, inhibitors and metal ions.

Chemical added	Concentration (mM)	CMT activity (U/mg)	Residual activity (%)
None	–	2.23	100
EDTA	2.5	2.99	134
EGTA	2.5	2.93	131
Sulfosalycylic acid	2.5	1.45	65
Sodium azide	2.5	1.33	59
Citric acid	2.5	1.69	75
$ZnCl_2$	5	3.1	153
$MgCl_2$	5	2.23	110

CMT assay was done as described in the text by methyltransferase assay kit. Sets containing purified CMT were pre-incubated with the chemicals for 15 min at 37 °C. All experiments were performed in triplicate. CMT assay was also done with other metal ions $CdCl_2$, $FeSO_4$, $CuCl_2$ but no change in activity was detected.

self-deactivation in the lines of MGT1. The novelty of the studied enzyme lies in the fact that it is a potent post-translational modifying methyltransferase that brings about increase in the levels of a key metabolite, trehalose, when its concentration is crucial for cell survival [1].

There are reports about purification of protein farnesyl cysteine carboxyl methyltransferase (PFCCMT) and Homocysteine Methyltransferase from bovine brain, and rat liver respectively [24,25]. However, to the best of our knowledge cysteine methyltransferase is not reported from any organism. Thus for the first time, the purified cysteine methyltransferase illustrates the fact that methylation is occurring at the cysteine residue from *S. cerevisiae*.

Due to the novelty of this enzyme there was no known assay protocol to define CMT activity. CMT activity was thus detected by monitoring the increase in TPS activity and this assay protocol was the basis of CMT purification. After the purified CMT was achieved a more direct assay protocol was followed using the methyltransferase assay kit. Further confirmation of CMT activity was performed with the radioactive assay protocol using the radioactive AdoMet.

Examination of CMT activity along the growth curve of *S. cerevisiae* revealed that it was highest at the A_{660} ~ 20 which was designated as the early stationary phase or the late log phase (Fig. 1). The significance of this finding lies in the fact that the CMT activity is found to peak at the time when TPS is present at its greatest concentration during the growth cycle as observed in previous reports [12]. Thus, all purification experiments were initiated at A_{660} ~ 20. The CMT enzyme purification reported here enriched activity 99.02 fold, with a final yield of 50.25% (Table 1). Reports from other workers regarding purification of PFCCMT from bovine brain displayed purification fold of 130 with 1.8% yield [24]. Homocysteine Methyltransferase purified from Rat Liver displayed purification fold of 170 with 7.5% yield [25]. HPGFLC and

>gi|459391947|gb|JX072966.4| *Saccharomyces cerevisiae* cysteine methyltransferase

1	MIHCKVQQWSPQYLRLPATG
21	YEELTLTLNTSMLARMPEEE
41	DQLFLVVGDSPSSTYVDWGE
61	DCNSTRYPSYDHCTHKILYL
81	GASAGTTRSKRLSATIGIRL
101	SKGTGSNNVLGTPMYLLYNE
121	MKTRIIESSK

General Features:

Total No. of amino acid-130

Coiled region-58.46%

Helix region-12%

Strand region- 29%

Theoretical pI- 6.82

Fig. 4. Complete amino acid sequence of CMT. The complete amino acid sequence is provided. Underlines regions are the AdoMet binding motifs of the sequence.

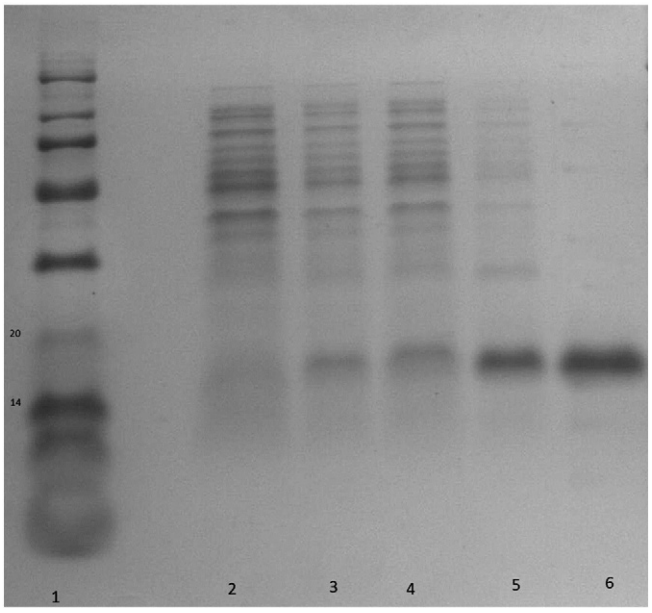

Fig. 5. Recombinant CMT Purification gel by Ni NTA column chromatography. Purification of His-Tag CMT expressed in *E. coli* Ni NTA column Chromatography. Crude extract fraction of BL21 harboring pET28 with insert was applied to a Ni-NTA column, as described in the Materials and methods section. Lane 1: Mol weight Marker Lane 2: Supernatant without IPTG, Lane 3: Supernatant with IPTG. Lane 4: Flowthrough from Ni-NTA column, Lane 5: Fraction eluted at 50 mM imidazole, Lane 6: Fraction eluted at 200 mM imidazole.

MALDI TOF analysis depicted a molecular weight of 13.68 kDa (Fig. 2). PFCCMT molecular weight was reported to be of 30 kDa [24]. The molecular weight of the active form of homocysteine methyltransferase purified from Rat Liver is reported to be 40 kDa [25]. The preparation was judged to be homogenous after native polyacrylamide gel electrophoresis yielding a single band (Fig. 3).

Substrate specificity assays were carried out in order to confirm the purified enzyme as cysteine methyltransferase (Table 2). Amino acid sequences retrieved from NCBI database revealed that cysteine content in the proteins used in this experiments are Albumin > Lipase > Chymotrypsin > Peroxidase > Ribonuclease A > Ovalbumin > TPS > G6PD. The CMT activity was obtained in accordance with the cysteine content of the proteins when used as substrate. When ACTH and Gelatin were used as substrates CMT activity was found to be nil which corresponded to the fact that both these proteins were lacking in cysteine residues. In a different experiment, TPS was digested with CNBr and the peptides generated were used as substrate for CMT (Table 3). The peptides containing cysteine residue displayed enzyme activity in

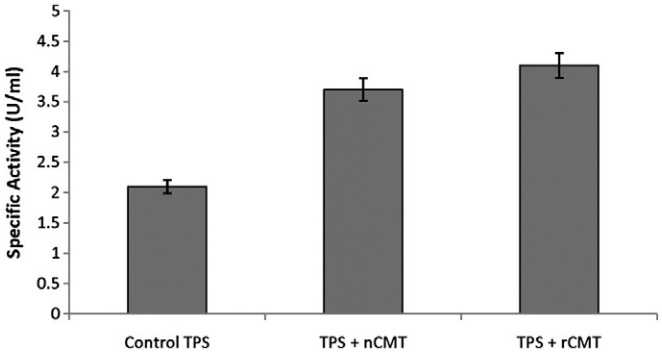

Fig. 6. Comparison of nCMT and rCMT activity on TPS as a substrate. Assay was carried out to study the effect of nCMT (Native CMT) and rCMT (Recombinant CMT) on TPS. The assay was performed following the TPS enhancement protocol. All the experiments were conducted in triplicate and the data represented are average of three sets of values, which did not vary more than ±5% represented by error bars.

Table 6
Effect of substrate concentration on reaction velocity.

Enzyme	Substrate	V_{max} (U/mg)	K_m (μM)	K_{cat} (s^{-1})
Native CMT	AdoMet	3.2	4.9	6.4
Recombinant CMT	AdoMet	3.5	4.9	7.0

Michaelis constant (K_m), maximum velocity (V_{max}) and turn over number (K_{cat}) for CMT activity measured at increasing concentration of AdoMet keeping trehalose concentration constant. Data given are average of three sets of values.

correspondence to the cysteine content. No activity was observed in relation to peptides that were devoid of any cysteine residues. Concluding from this data the purified enzyme can be designated as a cysteine methyltransferase as its activity predominantly corresponded with the cysteine content of the substrates and is not TPS specific.

Amino acid analysis was performed to identify the formation of S-Methyl L-cysteine when TPS was used as a substrate (Table 4). In the presence of CMT, S-Methyl L-cysteine peak area was observed to be enhanced whereas L-cysteine peak area decreased. This observation thus confirmed the formation of S-Methyl L-cysteine. ESI-MS analysis of the S-methyl cysteine peak confirmed the presence of an entity with molecular weight 135 Da which is 14 Da higher than the molecular weight of

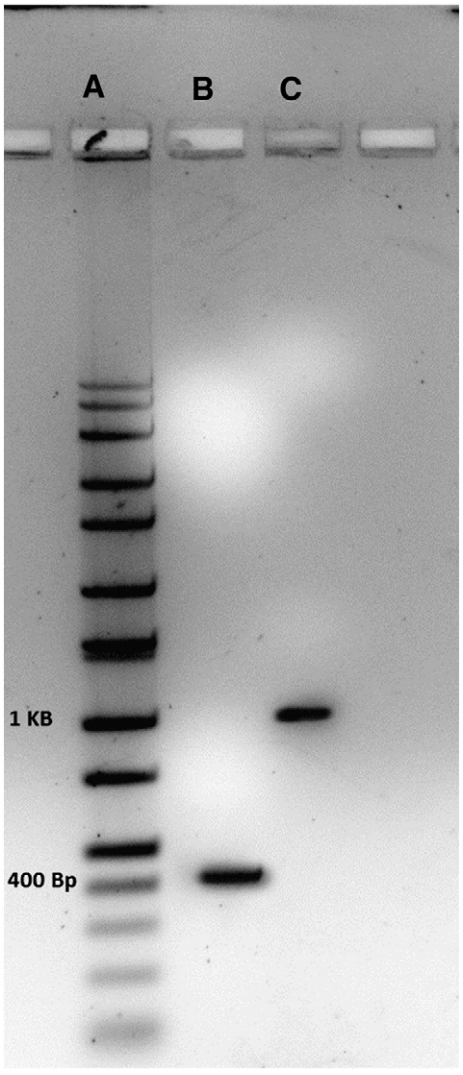

Fig. 7. Verification of knock out mutation by PCR analysis of genomic DNA. Genomic DNA was isolated from both CMT knock out mutants as well as wild type *S. cerevisiae* strains. PCR was carried out with gene specific markers. Lane A is Marker, Lane B have PCR product from wild type strain of ~400 bp size, Lane C have PCR product from CMT knock out mutants of 1 Kbp size.

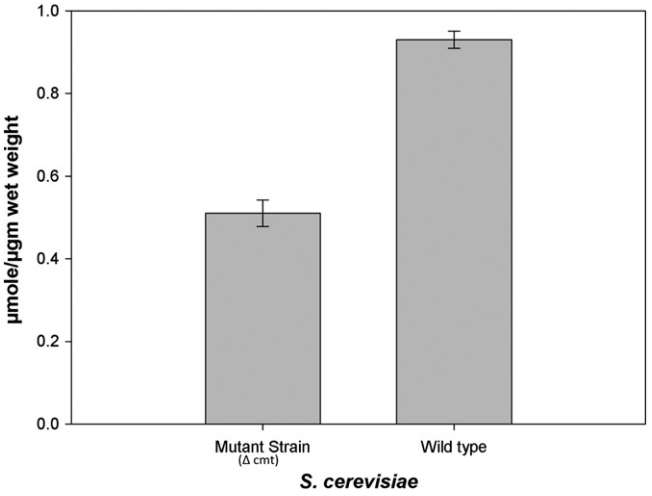

Fig. 8. Trehalose estimation in Wild type and CMT knock out mutant strain of *S. cerevisiae*. Trehalose was estimated as mentioned in the text. All the experiments were conducted in triplicate and the data represented are average of three sets of values, which did not vary more than ±5% represented by error bars.

L-cysteine—incidentally the change in molecular weight after substitution by a-CH_3 group also adds up to 14 Da. This parity in molecular weight difference establishes the production of methylated cysteine by the cysteine Methyltransferase performing S-methylation.

Metal ions function as enzyme co-factors either by binding to the actual catalytic site of the enzyme or by converting the enzyme into its active form without being directly involved in catalysis. During the present study, it was observed that though requirement for metal co-factor was not mandatory, in the presence of some metals like $ZnSO_4$ and $MgCl_2$ enhancement of activity was obtained (Table 5). There are reports of Zn + activating homocysteine methyltransferase in *S. cerevisiae* [26]. Metals like $CuCl_2$, $CoCl_2$ and $AgNO_3$ displayed some inhibitory effect on the enzyme activity. Increased CMT activity in the presence of EGTA and EDTA contributed to the fact that these two might be potent chelators of those metals which might be an inhibitor for CMT activity (Table 5). AdoHcy is well known for its competitive inhibition of all AdoMet dependent methyltransferases including present CMT enzyme. K_i was calculated by other workers for AdoHcy for various methyltransferase. K_i was observed to be as low as 0.08 μM for L-isoaspartyl methyltransferase from bovine brain [27]. Similarly, highest K_i was observed for Glycine methyltransferase as 35 μM from Rabbit liver [28]. K_i obtained for CMT was observed to be 10.9 μM which is considered to be in range among all the known methyltransferase.

The complete amino acid sequence consisted of a stretch of 131 amino acids confirming the molecular weight of ~14 kDa as mentioned above (Fig. 4). Also the number of tryptophan, tyrosine and cysteine residues obtained from spectrophotometric analysis matched with the predicted amino acid sequence obtained. The CMT exhibited similarity with other methyltransferases (Supp Table 1). BLAST analysis suggested the fact that CMT is homologous to an Histone methyltransferase, Glutamate methyltransferase of *S. cerevisiae* and *Streptococcus pseudoporcinus*. Incidentally, this enzyme was predicted to contain an AdoMet binding motif similar to other methyltransferases. Seemotif search tool was used to explore and visualize the sequence motifs. Three putative AdoMet binding motifs were recognized as $^{103}Gx^{105}G$, $E^{22}E^{23}$ and $^{38}E^{39}E^{40}E$. These motifs are also present at AdoMet binding site of Phosphatidylethanolamine N-methyltransferase (PEMT) [29]. Site-directed mutagenesis experiments demonstrated the requirement for these conserved motifs in PEMT specific activity [29]. Nucleotide BLAST of cysteine methyltransferase sequence with the complete sequence of *S. cerevisiae* (S288c) displayed a $6.8e^{-11}$ E value with the sequence of chromosome IV between 806,784 and 807,002 bp. This portion of the sequence is not yet annotated and hence any ORF has not been designated.

The amino acid sequence was further utilized to deduce the nucleotide sequence keeping in mind the codon usage in *S. cerevisiae*. The rationale behind this particular experiment was twofold—a) validate the deduced amino acid sequence from the MALDI-TOF MS/MS data, also b) clone the gene based on the deduced nucleotide sequence and subsequent expression.

The deduced CMT gene was cloned, expressed and purified (Supp Fig. 3, Fig. 5) and the recombinant enzyme was assayed for CMT activity. The fact that CMT activity was detected in the purified recombinant enzyme validated our claim that *S. cerevisiae* employs a unique cysteine methyltransferase in order to control trehalose levels during early stationary phase.

SDS PAGE and MALDI TOF analysis of rCMT revealed the fact that the molecular weight is in accordance with the nCMT purified by the conventional method (Fig. 5, Supp Fig. 4). The same physicochemical characteristics were also observed for both native and the recombinant enzyme. Under initial rate conditions, both the native enzyme and the recombinant enzyme displayed Michelis–Menten kinetics. Enzyme activity, Kinetic parameters, V_{max}, K_m (AdoMet) and K_{cat}, for both forms of CMT enzyme were determined. Although V_{max} was found to be slightly different for both enzymes, K_m (AdoMet) values were identical. Since V_{max} differed, K_{cat} for both native and recombinant varied slightly (Table 6). The difference in enzyme activity, V_{max} and K_{cat} might be due to the difference in homogeneity of the enzyme preparation. K_m for AdoMet was calculated by other workers for various methyltransferase [27]. K_m was observed to be as low as 0.3 μM for tRNA methyltransferase and as high as 100 μM for Glycine methyltranferase [28,30]. K_m of CMT was found to be in accordance with arginine methyltransferase [31] The temperature and pH optima were found to be in accordance with other methyltransferases viz, homocysteine S-methyltransferase from *S. cerevisiae* and protein-S-isoprenylcysteine O-methyltransferase from *Nicotiana tobaccum* (Supp Fig. 5, 6) [25,32].

Gene disruption experiments elucidated a direct relationship between CMT activity and trehalose levels in *S. cerevisiae* (Fig. 8). Although CMT is not TPS specific but as observed it can regulate TPS by methylating at the cysteine residue. TPS, being the key metabolite in the trehalose biosynthesis pathway in turn resulted in the enhancement of the intracellular levels of trehalose in the cell. The cmt mutants displayed lower levels of intracellular trehalose which corresponded to the fact that CMT can regulate trehalose levels in *S. cerevisiae*. These observations suggested that CMT plays an important role in protecting the organism from various stress conditions as well as contributes towards stationary phase survival by regulating trehalose levels in the cell (Fig. 8).

5. Conclusion

A unique and hitherto not reported methyltransferase was detected in *S. cerevisiae* which methylates at the cysteine residues of the trehalose synthesizing enzyme TPS and contributes towards elevated levels of trehalose in the cell as it enters the stationary phase. Native CMT was observed to increase the TPS activity by 1.76 factor whereas recombinant CMT increased the TPS activity by 1.96 factor. We believe that the cysteine methyltransferase is a key regulator of trehalose metabolism and a significant contributor towards stationary phase survival of *S. cerevisiae*. Adding to that is the novelty of the enzyme, that it is the first ever report of a Methyltransferase that selectively performs S-methylation at the L-cysteine residues in a protein.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.01.005.

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