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# Establishment of Glutamine Synthetase of *Mycobacterium smegmatis* as a Protein Acetyltransferase utilizing Polyphenolic Acetates as the Acetyl Group Donors

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**Acetoxy Drug: Protein Transacetylase (TAase) mediating the transfer of acetyl group(s) from polyphenolic acetates (PA) to certain functional proteins in mammalian cells was identified by our earlier investigations. TAase activity was characterized in the cell lysates of *Mycobacterium smegmatis* and the purified protein was found to have $M_r$ 58,000. TAase catalysed protein acetylation by a model acetoxy drug 7,8-diacetoxy-4-methylcoumarin (DAMC) was established by the demonstration of immunoreactivity of the acetylated target protein with an anti-acetyllysine antibody. The specificity of the TAase of *M. smegmatis* (MTAase) to various acetoxycoumarins was found to be in the order DAMC > 7-AMC > 6-AMC > 4-AC > 3-AC > ABP. Also, the N-terminal sequence of purified MTAase was found to perfectly match with glutamine synthetase (GS) of *M. smegmatis*. The identity of MTAase with GS was confirmed by the observation that the purified MTAase as well as the purified recombinant GS exhibited all the properties of GS. The finding that purified *Escherichia coli* GS was found to have substantial TAase activity highlighted the TAase function of GS in other bacteria. These results conclusively established for the first time the protein acetyltransferase function of GS of *M. smegmatis*.**

**Key words: glutamine synthetase, Mycobacterium smegmatis, polyphenolic acetate, protein acetylation, transacetylase.**

Abbreviations: ABP, acetoxy benzopyran; 3-AC, 3-acetoxycoumarin; 4-AC, 4-acetoxycoumarin; 6-AMC, 6-acetoxy-4-methylcoumarin; 7-AMC, 7-acetoxy-4-methylcoumarin; CDNB, 1-chloro-2,4-dinitrobenzene; DAMC, 7,8-diacetoxy-4-methylcoumarin; GS, glutamine synthetase EC 6.3.1.2; GST, glutathione S-transferase; IPTG, iso thiopropyl galactoside; MSO, L-methionine-*S*-sulfoximine; MTAase, mycobacterial TAase; NOS, nitric oxide synthase; PA, polyphenolic acetates; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; TAase, transacetylase.

Acetyltransferases such as histone acetyltransferase catalyse the transfer of acetyl groups from acetyl CoA to the ε-amino group of specific lysine side chain of proteins. The general focus of studies on protein acetylation has been on its role in regulation of gene expression. Obviously, the protein acetylation has wider scope in influencing the biological action of functional proteins (*1*). The acetyl CoA-dependent acetyltransferases play an important role in mediating protein acetylation in the living cells (*2*). The knowledge on protein acetylation independent of acetyl CoA was restricted to the action of aspirin like drugs that would readily acetylate cyclooxygenase (*3*, *4*). Studies carried out in our laboratory identified a novel transacetylase (TAase) which catalyses the possible transfer of acetyl group from polyphenolic acetates (PA) to certain functional proteins such as glutathione S-transferase (GST), cytochrome P-450 reductase and nitric oxide synthase (NOS) leading to modulation of their catalytic activities (*5–7*) and associated physiological effects. An elegant procedure for the assay of TAase was developed utilizing the inhibition of cytosolic GST (Fig. 1) brought about by TAase catalysed acetylation by PA. Our earlier publications (*7–11*) have established the efficiency and accuracy of this assay procedure. Both the substrates namely, the target protein GST and the acetyl group donor PA were found to take part in the TAase-catalysed bimolecular reaction. Utilizing this assay procedure, TAase was purified from tissues like human placenta and rat liver and characterized as calreticulin, a calcium binding protein. TAase was partially purified for the first time from buffalo liver and the TAase catalysed acetylation of a target protein GST by 7,8-diacetoxy-4-methylcoumarin (DAMC) was established by MALDI-TOF and LC-MS/MS (*12*, *13*). PA was found effective in the inhibition of cytochrome P-450-mediated activation of mutagens (*14*) and inhibition of ADP-induced platelet aggregation (*7*).

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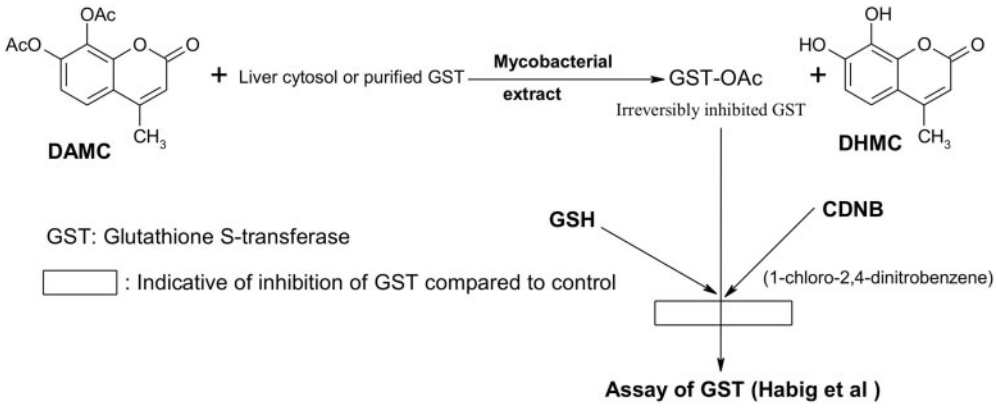


Fig. 1. **MTAase catalysed inhibition of GST.**

Having established the presence of TAase in the microsomes of several mammalian tissues and cells (*7*, *13*, *15*, *16*), an effort was made to look for the presence of TAase in a prokaryotic organism. The present investigation describes the identification and purification to homogeneity of TAase from lysates of *Mycobacterium smegmatis*. This report documents for the first time *M. smegmatis* glutamine synthetase (GS) as a protein acetyltransferase utilizing PA as an acetyl group donor.

## MATERIALS AND METHODS

*Materials*—Middlebrook's 7H9 broth was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). The synthesis of acetoxycoumarins was described in our earlier publications (*8–10*). Diaminobenzidine (DAB) system and goat anti-rabbit-HRP (horseradish peroxidase) conjugated secondary antibody were obtained from Bangalore Genie, (Bangalore, India). All molecular grade chemicals and restriction enzymes were purchased from New England Biolabs, UK and Fermentas, Canada. pGEM-T Easy vector system was purchased from Promega Inc., USA. Expression vector pTrc His was obtained from Invitrogen, USA. DNA Elution Kit was obtained from Biological Industries (Israel). All other chemicals and reagents were of high purity and were obtained from local suppliers.

*Organism and Growth Conditions*—The axenic cultures of *M. smegmatis* strain $VT_{301}$ were cultivated in Middlebrook's 7H9 medium. The media was prepared according to the manufacturer's instructions.

*Preparation of Cell Free Extract*—The mid log phase culture was centrifuged in the Sorvall centrifuge at 6,000*g* for 30 min at 4°C. The supernatant was discarded and the cell pellet was suspended in 0.1 M $NaH_2PO_4$ and sonicated using Misonix Sonicator (16 pulses, each followed by an interval of 1 min). The sonicated extract was centrifuged at 10,000*g* for 35 min and the supernatant was stored at −20°C.

*Assay of mycobacterial TAase—Mycobacterial TAase* (MTAase) was assayed routinely using DAMC as the first substrate (unless otherwise mentioned), and cytosolic GST as the second substrate. The assay mixture in a total volume of 0.8 ml consisted of 0.25 M phosphate buffer (pH 6.5), MTAase (1–10 μg protein), cytosol (12 μg protein) and DAMC (100 μM) and pre-incubated for 10 min, followed by the addition of 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) for the assay of GST (*17*). The percent inhibition of cytosolic GST under the conditions of the assay was considered proportional to MTAase activity (*6*).

*Purification of MTAase*—CM-Sepharose column was equilibrated with sequential washing of 200 mM, 100 mM, 75 mM, 50 mM and 10 mM potassium phosphate buffer (pH 7.4). The sonicated supernatant was loaded onto the CM-Sepharose column. The unbound protein obtained from CM-Sepharose was fractionated on DEAE-Sepharose column by a linear gradient of NaCl (0–0.5 M NaCl containing 1 mM EDTA, 1 mM DTT and 1 mM PMSF), the active fractions were pooled and further subjected to Q-Sepharose column chromatography. The column was washed with 10 mM potassium phosphate buffer of pH 7.4, to remove all the unbound protein and the MTAase was eluted by a gradient of NaCl (0–0.5 M NaCl containing 1 mM EDTA, 1 mM DTT and 1 mM PMSF). The individual fractions were dialysed against 10 mM potassium phosphate buffer, pH 7.4. The purity of MTAase was confirmed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The fractions bearing highest MTAase activity were stored at −20°C. The SDS–PAGE electrophoresis was carried out with discontinuous buffer system according to the standard procedure (*18*).

*Native–PAGE*—The composition of resolving buffer, stacking buffer, electrode buffer and loading dye were used as described (*19*). The electrophoresis was carried out at 4°C. After Native–PAGE, the gel was stained by reverse staining. For 5 min, the gel was incubated in 1% (w/v) sodium carbonate. This was followed by incubation of the gel for 15 min in 0.2 M imidazole and washed with distilled water and further incubated for 40 s in zinc sulphate and finally washed twice with distilled water. The protein was visualized as transparent band against white background and photograph was taken against the black background.

*N-terminal Protein Sequencing*—The N-terminal sequencing of purified MTAase was carried out at

Table 1. **Purification of MTAase from *M. smegmatis*.**

| Steps | Total volume (ml) | Total protein (mg) | Total units | Specific activity | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|
| Crude | 350 | 35 | 9,800 | 280 | – | 100 |
| CM-Sepharose | 221 | 12.8 | 7,600 | 594 | 2.12 | 78 |
| DEAE-Sepharose | 69 | 3.1 | 5,500 | 1,774 | 6.30 | 56 |
| Q-Sepharose | 2 | 0.35 | 5,382 | 6,728 | 24.02 | 55 |

The fractions to be assayed for MTAase activity were pre-incubated with DAMC followed by the addition of CDNB and reduced glutathione in order to assay GST activity. The unit of MTAase was expressed in terms of percent inhibition of GST.

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*MTAase Catalysed Target Protein Acetylation by DAMC using an Anti-acetyllysine Antibody*—The acetylated lysine residues were detected by using anti-acetyllysine polyclonal antibody. Purified MTAase (50 μg) was incubated with GST (50 μg), DAMC (200 μM) and 10 mM phosphate buffer (pH 7.2) and incubated for 30 min at 37°C in water bath. After the completion of reaction, sample buffer (loading dye) was added to the reaction mixture to stop the reaction. This reaction mixture was used to detect the acetylated protein using western blot.

For western blot, electrophoretically separated proteins were transferred onto nitrocellulose sheets at 300 mA for 3 h at 4°C. Non-specific sites on the nitrocellulose sheet were blocked with blocking reagent. Primary antibody dilution was prepared in TBS containing 1% bovine serum albumin (BSA) and incubation was carried out at 4°C for 12 h with shaking at intervals. The nitrocellulose sheets were extensively washed with TBST (TBS with 0.05% Tween). Goat anti-rabbit-HRP (horseradish peroxidase) conjugated secondary antibody, appropriately diluted in TBS was then added and an incubation of 1 h at room temperature was carried out. The sheets were washed extensively and antibody-reactive moieties were visualized with DAB system. After 5 min incubation reagents were drained, and sheets were kept in water as described in the earlier publication (*11*).

*MTAase Activity in Escherichia coli GS*—The MTAase activity was assayed in commercially obtained *E. coli* GS (Sigma Chemical Co., USA) as described above.

*GS Activity*—Purified MTAase and the purified recombinant protein were assayed for GS activity as described (*20*). Kinetic constants were determined varying the ATP concentration (1–50 mM). The inhibition of GS activity by L-methionine-*S*-sulfoximine (MSO) was assessed as described (*21*).

*Cloning of the GS Gene*—Suitable primers with restriction sites BamH1 in the forward primer and EcoR1 in the reverse primer were designed to amplify the full-length gene. The forward and reverse primers designed for amplification were as: FP, 5′-CCAGTAAA GGATCCCACTCAGTG-3′ (23-mer, $T_m = 62.4$°C); RP, 5′-G CGAGCCGAATTCCACGTGCTAGTAGTA-3′ (28-mer, $T_m = 68.1$°C).Genomic DNA was isolated by the method of Bose *et al*. (*22*). PCR was carried out using genomic DNA isolated from *M. smegmatis* using Gradient PCR BioRad I thermal cycler. The conditions for amplification were: an initial denaturation step at 95°C for 5 min followed by 36 cycles of 30 s denaturation at 95°C, 1 min annealing at 48°C and extension of 1 min at 72°C. The final cycle was followed by strand extension at 72°C for 5 min. The eluted 1.5 kb amplicon was first cloned in pGEM-T Easy Vector. The recombinant gene was excised and then ligated to pTrc His A expression vector at BamH1 and EcoR1 site. The reading frame of clone (pTrc GS) was confirmed by sequencing (AB1 prism version 2.1, Microsynth).

*Expression and Purification of Recombinant GS*—GS was expressed in *E. coli* (Prill) by induction at 1 mM iso thiopropyl galactoside (IPTG) for 6 h at 37°C. The recombinant protein was purified from supernatant using Ni-NTA His Bind Resin according to the manufacturer's instructions. Protein concentration was determined by the method of Bradford (*23*) using BSA as standard.

## RESULTS AND DISCUSSION

*Identification of TAase in M. smegmatis*—Our earlier research work revealed that PA could acetylate certain proteins such as GST mediated by a protein acetyltransferase (Fig. 1), identified in several mammalian tissues as calreticulin, a $Ca^{2+}$-binding protein in the endoplasmic reticulum lumen of eukaryotes (*11*, *15*). Our area of interest extended from the eukaryotic tissues to the establishment of similar TAase activity in prokaryotic cells as well. The preliminary studies indicated considerable TAase activity in the crude cell lysate of *M. smegmatis*, which was found to reach maximum in the log phase of the growth.

*Purification of MTAase*—MTAase was purified to homogeneity using Q-Sepharose chromatography with 55% yield (Table 1) and it was found to be $M_r$ 58,000 on SDS–PAGE (Fig. 2a). It was also observed that the purified protein demonstrated robust activity of TAase. To confirm the purity of the protein further it was resolved on a Native–PAGE under non-denaturing conditions (Fig. 2b). The protein eluted from Native gel gave TAase activity comparable to the Q-Sepharose fraction as discussed above. The eluted protein appeared as a single band of $M_r$ 58,000 on SDS–PAGE.

*Structure Activity Relationship for Acetoxycoumarin: MTAase*—MTAase catalysed reaction was characterized by examining the specificity of TAase for various acetoxycoumarins. MTAase catalysed reaction is bimolecular in nature. The TAase activity was measured by varying the concentration of acetoxycoumarin, keeping the cytosolic protein (GST) constant (Table 2). DAMC possessing the two acetoxy groups was found to be more effective substrate for MTAase compared to the mono-acetoxycoumarins [7-acetoxy-4-methylcoumarin (7-AMC), 6-acetoxy-4-methylcoumarin (6-AMC), 4-acetoxycoumarin (4-AC) and 3-acetoxycoumarin (3-AC)] as evident by the

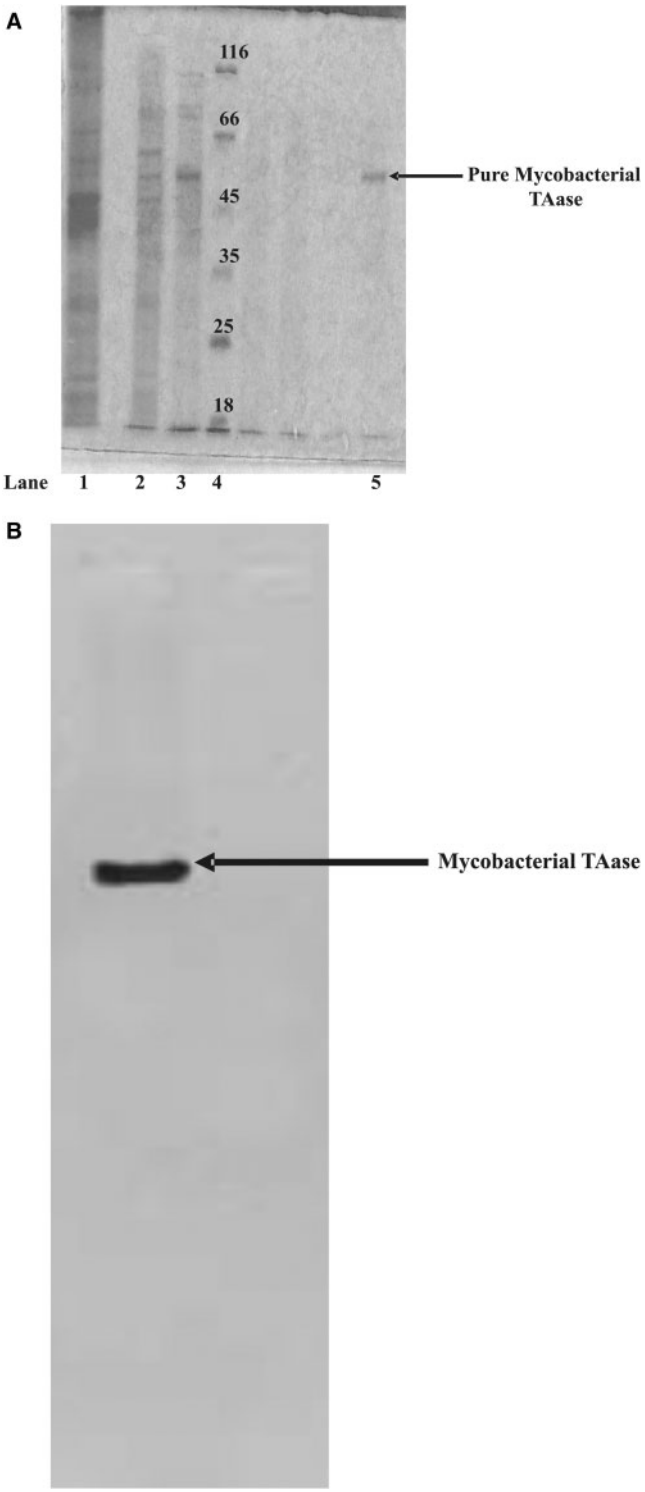


Fig. 2. **SDS–PAGE of MTAase (A) and characterization of purified MTAase by Native–PAGE (B).** (A) SDS–PAGE of MTAase was carried out in different stages of purification. Lane 1, crude mycobacterial extract; lane 2, protein eluted from CM-chromatography; lane 3, fraction of DEAE chromatography having highest TAase activity; lane 4, $M_r$ standards (β-galactosidase 116,000 $M_r$, BSA 66,000 $M_r$, ovalbumin 45,000 $M_r$, lactate dehydrogenase 35,000 $M_r$, REase 25,000 $M_r$, β-lactoglobulin 18,000 $M_r$, lysozyme 14,000 $M_r$); lane 5, MTAase eluted after Q-chromatography (58,000 $M_r$). (B) Protein was loaded on Native–PAGE and stained with zinc sulphate (negative staining) at 4°C. The protein bands were cut and eluted at 4°C in 10 mM potassium phosphate buffer (pH 7.2), MTAase activity was measured.

TAase activity. It is also clear from the results that negligible MTAase activity was observed when 4-AC and 3-AC were used as the substrates. The acetoxy group at C3 of 3-AC was found to be clearly distant from the oxygen heteroatom compared to the acetoxy group at C7 of 7-AMC when the structures were optimized by semiempirical calculations (*8*). This observation leads to the logical conclusion that the proximity of the acetoxy group on the benzenoid ring of the coumarin moiety to the oxygen heteroatom confers on the MTAase a high degree of acetyl group transfer capability. The absolute requirement of the carbonyl group in the pyran ring of polyphenols was confirmed by the observation that the purified MTAase failed to transfer the acetyl group of acetoxy benzopyran (ABP) when used as a substrate (which lacks the pyran carbonyl group). MTAase catalysed reaction exhibited linearity with respect to the concentration of the substrates PA and cytosolic GST. It was observed that the inclusion of iodoacetamide in the assay mixture resulted in the inhibition of MTAase activity (Fig. 3).

*Demonstration of MTAase Catalysed Acetylation of GST by Western Blot using an Anti-acetyllysine Antibody*—Efforts were made to demonstrate the MTAase catalysed acetylation of functional protein using an anti-acetyllysine antibody. GST was separately incubated with DAMC along with purified MTAase followed by separation of the modified protein by SDS–PAGE. A single band at $M_r$ 26,000 (Fig. 4) indicated the acetylation of GST; whereas, the control samples comprising of receptor protein and DAMC did not show any acetylation, further confirming the enzymatic nature of the process.

Mass spectrometric analysis of the acetylated target protein such as GST revealed that the peptide maps covering 97% of the rat GST (GST3-3) sequence, and the fragmentation pattern revealed that the 6-lysines (Lys-51, -82, -123, -181, -191 and -210) were acetylated (*12*, *13*). Similarly, the autoacetylation (*24*) of CRTAase and CRTAase-mediated acetylation of neuronal NOS (*25*) by the model PA (7,8-diacetoxy-4-methyl coumarin) revealed the presence of 9 and 12 acetylated lysines, respectively.

*Establishment of the Identity of MTAase with GS*—In-depth studies were conducted to decipher the protein identity of MTAase. Since the prokaryotes do not possess CRT, MTAase purified from *M. smegmatis* was subjected to N-terminal sequencing in order to establish its identity. The N-terminal amino acid sequence (**AEKTSDDIFKLI**) of MTAase when aligned with non-redundant Swiss Prot Database revealed 100% identity with N-terminal sequence of GS of *M. smegmatis*. The purified MTAase indeed was found to have GS activity (Table 4) and the kinetics of GS activity of MTAase revealed $K_m$ and $V_{max}$ values of 3.2 mM and 47 U, respectively with regard to ATP concentration which is very close to the reported value of $K_m$ 2.43 mM and

Table 2. **Comparison of specificity of MTAase for various PA.**

| Test compounds | MTAase activity (U) |
|---|---|
| DAMC | 250 |
| 7-AMC | 200 |
| 6-AMC | 133 |
| 3-AC | Poor substrate |
| 4-AC | Poor substrate |
| ABP | Poor substrate |

PA (test compound) was separately pre-incubated with MTAase and the activity was determined in order to assess the relative specificities. The unit of MTAase is expressed in terms of the percent inhibition of GST under the experimental conditions.

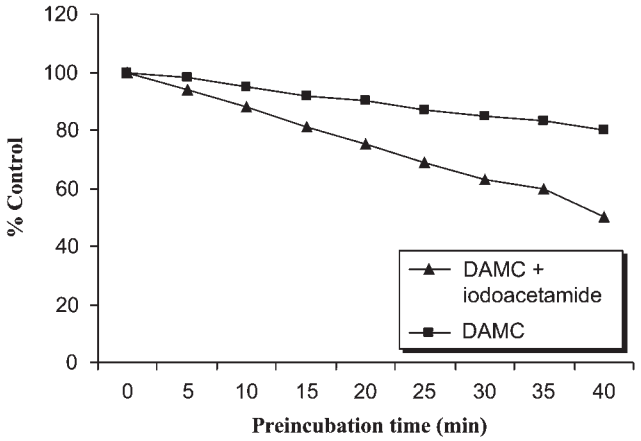


Fig. 3. **MTAase-mediated mechanism based inhibition of cytosolic GST by DAMC: effect of iodoacetamide.** Purified MTAase (5 g protein), DAMC (100 μM) and cytosol (12.5 μg) were incubated for 10–40 min and assayed for MTAase activity. Concentration of iodoacetamide was $10^{-2}$ M. Values are mean of three observations with variation <5%.

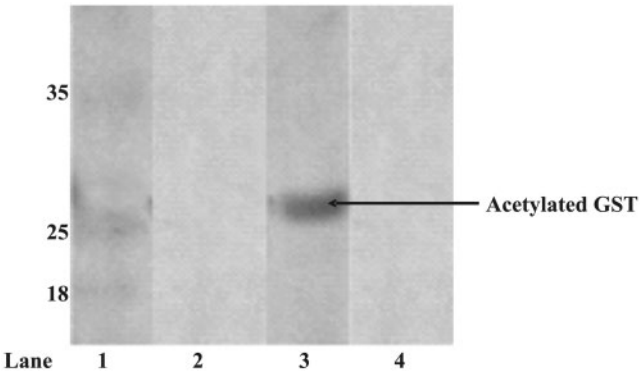


Fig. 4. **MTAase catalysed acetylation of GST by DAMC using an anti-acetyllysine antibody.** Western blot was carried out using polyclonal antibody against an anti-acetyllysine. Lanes: 1, pre-stained $M_r$ standards; 2, control (GST + DMSO); 3, acetylated GST (GST + MTAase + DAMC); 4, control (GST + DAMC).

$V_{max}$ 50 U (*26*). This finding further substantiated the observation that the purified MTAase was endowed with GS function. The biochemical mechanism of GS has been widely studied (*21*). MSO, 4-*N*-hydroxy-L-2,

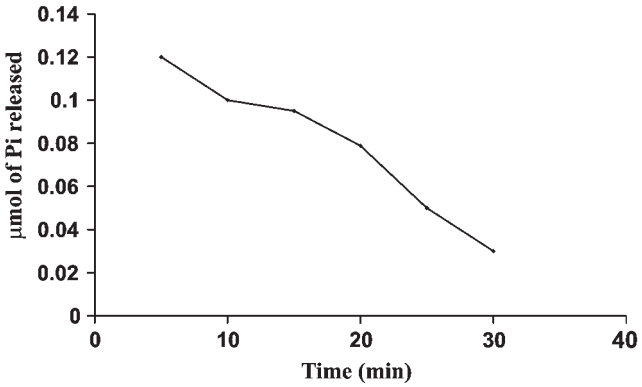


Fig. 5. **Inhibitory effect of MSO on GS activity.** MSO (75 mM) was incubated with MTAase and GS activity was assayed by addition of ATP (7.6 mM), glutamic acid (100 mM), ammonia (500 mM), $MgCl_2$ (5 mM), imidazole buffer (50 mM, pH 7.6) were pre-incubated at 37°C for 5–30 min and read at 660 nm. Values are mean of four observations with variation <5%.

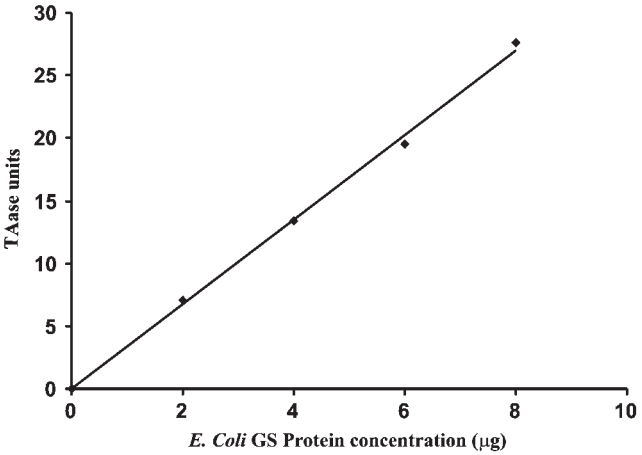


Fig. 6. **TAase assay in purified *E. coli* GS.** Effect of protein concentration: varying concentrations (2–10 μg) of purified *E. coli* GS were added in the pre-incubation mixture of TAase assay and the assay performed as described above. Values are mean of four observations with variation <5%.

4-diaminobutyric acid (NH-DABA), 2-amino-4-(hydroxy-methyl-phosphoryl) butanoic acid (phosphinothricin) are some of the well-established inhibitors of GS. MSO was found to effectively inhibit GS activity of MTAase (Fig. 5). It was thought interesting to examine whether the inhibitor of GS would exert any action on TAase function of MTAase. For this purpose, MSO was pre-incubated with MTAase followed by the assay of TAase as described under MATERIALS AND METHODS section. MSO a potent inhibitor of GS failed to inhibit MTAase catalysed reaction. This observation also highlighted that the TAase activity of MTAase is independent of the catalytic activity of GS. The fact that GS of other bacteria could exhibit TAase activity was apparent from the observation that the purified GS from *E. coli* (Sigma Chemical Co.) showed substantial activity of TAase (Fig. 6).

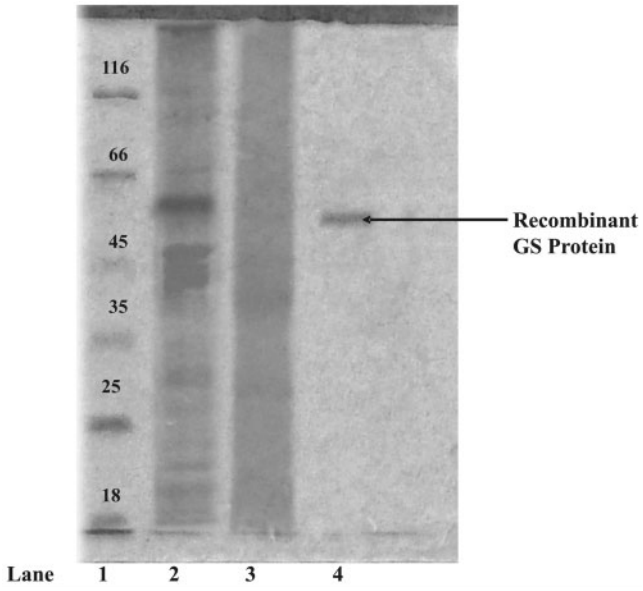


Fig. 7. **SDS–PAGE analysis of purified recombinant GS protein.** Lanes: 1, $M_r$ standards (β-galactosidase 116,000 $M_r$, BSA 66,000 $M_r$, ovalbumin 45,000 $M_r$, lactate dehydrogenase 35,000 $M_r$, REase 25,000 $M_r$, β-lactoglobulin 18,000 $M_r$, lysozyme 14,000 $M_r$); 2, induced protein (after sonication); 3, unbound protein; 4, purified recombinant protein (58,000 $M_r$).

Table 3. **Comparison of TAase activities of purified MTAase and recombinant GS.**

| Parameters | Purified MTAase | Purified recombinant GS |
|---|---|---|
| TAase activity (U) | 250 | 305 |
| Optimum pH | 7.0 | 7.0 |
| Optimum temperature (°C) | 37 | 37 |
| Effect of MSO | None | None |

MTAase was purified from *M. smegmatis* cultures and the identity of MTAase was confirmed as GS based on N-terminal sequence analysis. The *M. smegmatis* GS gene was cloned, expressed in pTrc-HisA and the recombinant protein was purified. Purified GS from *E. coli* (obtained from Sigma Chemical Co.) was also assayed for TAase activity. The TAase was assessed using DAMC as the substrate. TAase activity of purified and recombinant proteins were measured by varying the concentration of substrate (DAMC). Optimum temperature and pH were determined for the both the proteins and were found to be same. In both the cases, there were no effects of MSO (25–100 mM) on TAase activity. Values are mean of four observations with variation < 5%.

*Cloning and Characterization of GS*—The firm proof for the identification of the GS activity of MTAase necessitated the cloning of GS gene from *M. smegmatis*. GS gene was cloned using pTrc His A expression vector using IPTG as the inducer followed by purification of recombinant GS (rGS) on Ni-NTA column. rGS was found homogenous with $M_r$ of 58,000 (Fig. 7). MTAase catalysed protein acetylation was confirmed by the positive interaction of the acetylated target protein (acetylated GST) with an anti-acetyllysine antibody. Apart from the native GS of the *M. smegmatis*, the recombinant protein exhibited activities of TAase as well as GS and the values revealed close conformity with the purified protein (Tables 3 and 4). The mass spectrometric

Table 4. **GS function of MTAase: comparison with recombinant GS.**

| Parameter | Purified MTAase | Recombinant GS |
|---|---|---|
| $K_m$ (mM) | 3.2 | 3.59 |
| $V_{max}$ (U) | 47.61 | 51.28 |
| Optimum pH | 7.5 | 7.5 |
| Optimum temperature (°C) | 37 | 35 |
| Effect of MSO on GS activity | 86% | 97% |

Kinetic constants of purified as well as recombinant GS were measured by varying the concentration of the substrate (ATP). The concentration of MSO was 75 mM. Values are mean of four observations with variation < 5%.

details of the MTAase catalysed acetylated target protein by PA would be the subject matter of the future publication.

It is not unusual that a certain protein is endowed with more than one catalytic function as evident from the biochemical literature. Lately, nitrite reductase (NR) activity in mammalian tissues was found to be exhibited by several distinct proteins such as cytochrome P-450 reductase, haemoglobin, myoglobin and xanthine oxidase (*27–30*). Multi-functional proteins, many of which are the well-known metabolic enzymes, are lately designated as 'Moonlighting proteins' (*31*). In the light of such phenomenon, the TAase function of bacterial GS highlighted in our present article assumes importance. The study reported in this communication has unravelled the protein acetyltransferase function of GS of *M. smegmatis*, hitherto unknown.

## CONFLICT OF INTEREST

None declared.

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