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# Cloning, Expression, and Characterization of a Novel Methylglyoxal Synthase from *Thermus* sp. Strain GH5

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**Abstract** A gene encoding methylglyoxal synthase from *Thermus* sp. GH5 (TMGS) was cloned, sequenced, overexpressed, and purified by Q-Sepharose. The TMGS gene was composed of 399 bp which encoded a polypeptide of 132 amino acids with a molecular mass of 14.3 kDa. The $K_m$ and $k_{cat}$ values of TMGS were 0.56 mM and 325 ($s^{-1}$), respectively. The enzyme exhibited its optimum activity at pH6 and 75°C. Comparing the amino acid sequences and Hill coefficients of *Escherichia coli* MGS and TMGS revealed that the loss of Arg 150 in TMGS has caused a decrease in the cooperativity between the enzyme subunits in the presence of phosphate as an allosteric inhibitor. Gel filtration experiments showed that TMGS is a hexameric enzyme, and its quaternary structure did not change in the presence of phosphate.

**Keywords** Cloning · Purification · Methylglyoxal synthase · *Thermus* sp. GH5 · Cooperativity

**Abbreviations**

| | |
|---|---|
| DHAP | Dihydroxyacetone phosphate |
| MG | Methylglyoxal |
| MGS | Methylglyoxal synthase |
| TMGS | *Thermus* sp. GH5 methylglyoxal synthase |

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## Introduction

Methylglyoxal synthase (MGS) is an enzyme that catalyzes conversion of dihydroxyacetone phosphate (DHAP) to phosphate and pyruvaldehyde (the enole of methylglyoxal) which subsequently tautomerizes to form methylglyoxal (MG), nonenzymatically [1]. MG, having cytotoxic effects in millimolar quantities [2–4], affects the de novo synthesis of proteins and nucleic acids and is shown that it can be important for the uncoupling of anabolism and catabolism [3–6]. In mammals, MG is implicated in the development of hypertension and diabetic complications [6–8]. Thus, for detoxification in bacteria, MG is metabolized to D-lactic acid via glyoxalase system [9–11] and is converted to R-1, 2-propandiol (a commodity chemical), by glycerol dehydrogenase and aldehyde reductase (Fig. 1). The former pathway can provide a shunt around low ATP-yielding steps in glycolysis [9, 12], and the latter has an industrial importance [13–15]. Therefore, MGS can have physiological and industrial significance. Phosphate acts as an allosteric inhibitor of the enzyme. MGS was first purified from *Escherichia coli* [16] and thereafter found and characterized from *Pseudomonas saccharophila* [17], *Proteus vulgaris* [18], *Clostridium acetobutylicum* [19], *Saccharomyces cervisiae* [20], and goat liver [21].

To date, there has been no report on the characterization of an MGS from a thermophilic microorganism. In this study, we have isolated, sequenced, cloned, and expressed the gene encoding MGS from *Thermus* sp. GH5 which was isolated from Ghaynarcheh hot spring, north–west of Iran. We also present a characterization of the enzyme biochemical properties.

## Materials and Methods

### Chemicals

Restriction enzymes and T4 ligase were purchased from Fermentas (Vilnius, Lithuania). Oligonucleotides were synthesized by M.W.G company (Germany). Tryptone and yeast extract were obtained from Liofilchem (Roseto degli Abruzzi, Italy). Dihydroxyacetone phosphate was purchased from Sigma-Aldrich (USA). 2,4-Dinitrophenylhydrazine and other chemicals were obtained from Merck (Darmstadt, Germany).

### Bacterial Strains and Growth Conditions

*Thermus* sp. GH5 was isolated from Ghaynarcheh hot spring, north–west of Iran, and cultured in *Thermus* medium. *E. coli* BL21 (DE3; Stratagene) and pET21a (+) (Novagen,

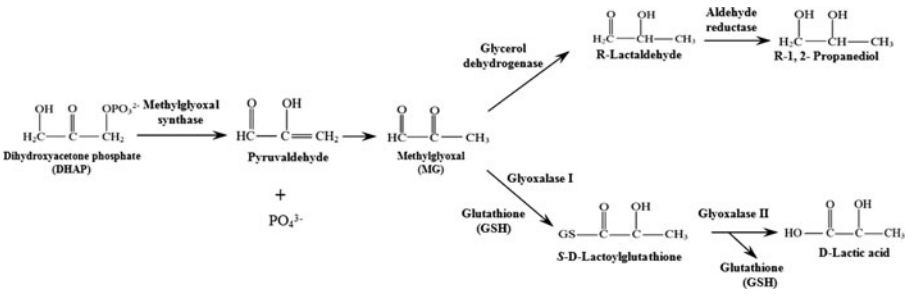


**Fig. 1** Metabolic pathways from DHAP to 1,2-propanediol and D-lactic acid

USA) were employed for protein expression. *E. coli* were cultured in Luria-Bertani medium.

## DNA Manipulation, PCR Amplification, 16S rDNA and MGS Gene Sequencing

Genomic DNA from *Thermus* sp. GH5 was prepared using DNA extraction kits. Universal forward (5′-AGTTTGATCCTGGCTCAG-3′) and reverse (5′-GGC/TTACCTTGTTAC GACTT-3′) primers were used to amplify and sequence the 16S rDNA. Forward and reverse primers were designed for the MGS gene on the basis of *Thermus thermophilus* HB8 MGS gene sequence. In both cases, genomic DNA of *Thermus* sp. GH5 was applied as the PCR template. The amplification products were purified using PCR purification kit (Roche, Germany) and then sequenced by M.W.G Company (Germany).

## Construction of Expression Plasmid

The TMGS gene was amplified from genomic DNA by PCR using the following primers: forward (5′-GGAATTCCATATGCGAGCCCTTGCCCTCATTG-3′) and reverse (5′-CGGAAGCTTCTATTGGCCCTGGGGGGTTTG-3′) to introduce the flanking *NdeI* and *HindIII* restriction sites (the underlined bases specify the *NdeI* and *HindIII* restriction sites in forward and reverse primers, respectively). The resulting fragment (399 bp) digested with *HindIII* and *NdeI* and ligated into similarly digested pET 21a(+) vector using T4 DNA ligase. Sequence integrity was confirmed by DNA sequencing.

## Expression and Purification of Methylglyoxal Synthase

In order to produce TMGS with the consequent molecular mass of 14.3 kDa, *E. coli* strain BL21 (DE3) was transformed by the constructed expression plasmid. The cells carrying recombinant plasmids were grown in 500 ml of Luria-Bertani medium supplemented with ampicillin at 37°C. TMGS production was initiated by the addition of isopropyl-β-thio galactopyranoside, once the cultures reached an optical density of 0.4 to 0.7 at 600 nm. After 16 h, the cells were harvested by centrifugation at 8,000×*g* for 20 min at 4°C and resuspended in a lysis buffer containing 50 mM imidazole-HCl buffer (pH6.5) and 1 mM phenylmethylsulphonyl fluoride, a protease inhibitor.

The suspension was subjected to sonic disruption, and the cell debris was discarded by centrifugation at 20,000×*g* for 20 min at 4°C. The supernatant underwent heat shock (70°C) for 15 min, and the precipitated proteins were removed by centrifugation (40,000×*g*, 25 min at 4°C). The resulting supernatant was dialyzed against 20 mM Tris buffer, pH8.0, and applied to Q-Sepharose column (15 cm×1 cm). Protein was eluted with a linear gradient of NaCl (0–1 M) prepared in 20 mM Tris buffer (pH8.0). Chromatography was performed by FPLC (Amersham Pharmacia, Sweden). The flow rate was set at 3 ml/min, and fractions containing the MGS activity were collected. SDS-PAGE was performed in 15% polyacrylamide gel by the method of Laemmli [22]. The gels were stained with Coomassie brilliant blue R-250.

## Enzyme Activity Assay and Protein Yield Determination

Methylglyoxal synthase activity was measured in a reaction mixture containing 0.4 ml of 50 mM imidazole buffer (pH6.5), 25 μl DHAP (15 mM), and 16.8 ng of enzyme at 60°C for 5 min. Then, 0.1 ml of the reaction mixture was added to a test tube containing 0.9 ml

distilled water and 0.33 ml 2,4-dinitrophenylhydrazine (0.1% 2,4-dinitrophenylhydrazine in 2 M of HCl). After incubation in 30 °C for 15 min, 1.67 ml of 10% (*w*/*v*) NaOH was added. The absorbance at 550 nm was measured by UV/Vis spectrophotometer (Unicam, England) after a further 15 min incubation at room temperature. Under these conditions, 1 μmol of MG has the absorbance of 16.4. Accordingly, all measured absorbances were converted to micromoles of produced MG [16, 23, 24]. In order to determine kinetic parameters, measurements were carried out using different substrate concentrations, and the final concentration of the enzyme was determined to be 34 ng/ml. Different concentrations of DHAP were used as the blanks in order to determine the activity. Steady-state kinetic parameters were determined, and experimental data were analyzed graphically by Michaelis-Menten curve and numerically using Lineweaver–Burk equation. Hill coefficient was calculated from the following equation:

$$\log[v/(V_{\max} - v)] = n\mathrm{H}\log[\mathrm{S}] - \log(\mathrm{K}') \quad (1)$$

where $v$ and $V_{\max}$ are velocity and maximal velocity of the enzyme, respectively, and $n$H is the Hill coefficient. $K'$ is related to $K_m$, but also contains terms related to the effect of substrate occupancy at one site on the substrate affinity of other sites. According to this equation, the value of $n$H can be achieved by plotting $\log[v/(V_{\max}-v)]$ against log[S].

Protein concentration was measured by Bradford method [25] using bovine serum albumin as the standard.

### Molecular Mass Determination

The quaternary structure of TMGS was determined by analytical gel filtration using Sephadex G-100 column (100 cm×1.6 cm). The running buffer contained 50 mM imidazole (pH6.5), having a flow rate of 0.6 ml/min. The column was calibrated with the following standards: catalase (250 kDa), rabbit aldolase (150 kDa), BSA (66 kDa), and lysozyme (14 kDa). The $k_{av}$ value was calculated for each protein and plotted against the log of its molecular mass. The equation $k_{av} = (V_e - V_o)/(V_t - V_o)$, represents partition coefficient, where $V_e$ is the elution volume, $V_o$ is the void volume, and $V_t$ is the total volume of the packed beds. The void volume of the column was determined by using glutathione dehydrogenase.

### Nucleotide Sequence Accession Numbers

The obtained 16S rDNA and TMGS gene sequences have been deposited in gene bank under the accession numbers of DQ973297 and EU744585, respectively.

## Results

### Biochemical Properties and 16S rDNA Analysis of Bacterium

Biochemical tests and 16S rDNA sequence analysis were carried out for the identification of the GH5 strain. The results showed that the strain is capable of producing yellow pigment and showing oxidase activity. GH5 strain could degrade gelatin and grow at 85 °C. In addition, it was able to grow at pH4.5 to 9.5 and utilize D-glucose, D-xylose, D-mannitol, sucrose, lactose, D-raffinose, D-rhamnose, D-arabinose, glycerol, L-arginine, L-phenylalanine, L-trypophan, L-glycine, and L-cystein. With respect to biochemical tests and according

to Bergey's Manual Systematic Bacteriology [26], the strain was identified as *Thermus* sp. GH5.

Ribosomal RNAs, being conserved among all organisms, are critical elements in protein synthesis. For this strain, PCR amplification was performed, and the resulting fragment was sequenced. The PCR product was 1,300 bp in length, but was edited to 1,174 bp, subsequently. Multiple sequence alignment with 16S rDNA sequences of other strains obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) was carried out utilizing ClustalW version 2, and the phylogenetic tree was constructed according to neighbor-joining method. Multiple alignment and phylogenetic tree exhibited that *Thermus* sp. GH5 is closely related to *Thermus brockianus* (data not shown). However, for the definitive classification, other characterization experiments, especially DNA–DNA hybridization should be accomplished in the future.

## Primary Structure of MGS and Expression of the Recombinant Enzyme

MGS gene from *Thermus* sp. GH5 was isolated and sequenced. The results showed that the MGS gene has an open reading frame of 399 bp. This nucleotide sequence encodes a polypeptide with 132 amino acids and a molecular mass of 14.3 kDa. A significant similarity was observed between the amino acid sequences of MGS from *Thermus* sp. GH5 and its counterpart from *T. thermophilus* HB8 (90%). The comparison of TMGS and *E. coli* MGS (a well-studied MGS) showed 66% similarity. Sequence analysis revealed that amino acids 143–152 which form the C-terminal helix in *E. coli* MGS are not present in TMGS (Fig. 2). Previous investigation showed that, among the amino acids involved in the helix, Arg 150 has a role in transmitting allosteric changes [27]. Saadat and Harrison suggested that the formation of a salt bridge between Asp 20 and Arg 150 in the presence of phosphate in the neighboring subunit could be a pathway for transmitting allosteric changes through subunits of *E. coli* MGS (*E. coli* MGS is a hexameric enzyme) [27]. In the other pathway, Pro 92, Arg 107, and Val 111 may have a role in transmitting allosteric information among subunits in *E. coli* MGS [27]. These amino acids are conserved in all three aligned sequences (Fig. 2). In *E. coli* MGS, Asp 71, Asp 101, and His 98 are involved in catalysis [1, 28, 29] and are conserved likewise.

To study the enzymatic properties of TMGS, its gene was cloned in the expression vector pET 21a (+) and expressed. The recombinant protein was purified from the crude extract by heat shock and anion exchange chromatography (Q-sepharose column). The purification results are summarized in Table 1. The enzyme was purified 7.30-fold, with the

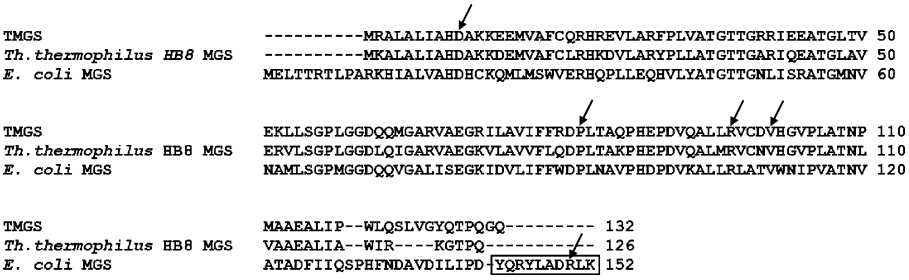


**Fig. 2** Amino acid sequence alignment of TMGS with *T. thermophilus* HB8 MGS and *E. coli* enzyme. Results showed a 90% and a 66% similarity, respectively. TMGS and *T. thermophilus* HB8 MGS do not possess the amino acids that are located within the *box* in *E. coli* MGS. Amino acids marked by *arrows* have a role in transmitting allosteric changes among subunits in MGS [27]

**Table 1** Purification of recombinant TMGS from *E. coli* Bl21 (DE3).

| Purification step | Total protein (mg) | Total activity (μmol/min) | Specific activity (μmol/min/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 60 | 49,260 | 821 | 1 | 100 |
| Heat shock | 19 | 42,104 | 2,216 | 2.69 | 85.4 |
| Q-sepharose | 2 | 12,000 | 6,000 | 7.30 | 24.3 |

recovery yield of 24.3%. Samples from each step were subjected to SDS-PAGE. This analysis of SDS-PAGE showed a 14.3 kDa polypeptide as the expressed gene and confirmed the purity of TMGS in the final purification step (Fig. 3).

## Kinetic Studies of MGS and Allosteric Behaviors

The purified recombinant protein was tested for activity utilizing direct assay of MGS [16, 23]. Methylglyoxal synthase can convert DHAP to an enediol and inorganic phosphate ($P_i$). Thereafter, the enediol is converted to MG, nonenzymatically. The Michaelis-Menten and Linweaver-Burk curves for MGS were determined using imidazole-HCl buffer pH6.5 (Fig. 4a). The enzyme represented $K_m$ and $k_{cat}$ of 0.56 mM and 325 $s^{-1}$, respectively (Table 2). Both parameters are higher than those obtained from the *E. coli* enzyme (Table 2). In contrast, phosphate is the allosteric inhibitor of both TMGS (Fig. 4b) and the *E. coli* enzyme [1, 16]. In the presence of phosphate, a difference is observed between the Hill coefficients of *E. coli* MGS and TMGS, which are 3.4 and 1.5, respectively (Table 2, Fig. 5).

These results together with the comparison of MGS amino acid sequences reveal remarkable points about the enzyme's cooperativity. Arg 150 which has proposed a role in transmitting allosteric changes in *E. coli* MGS [27] is not present in TMGS and *T.*

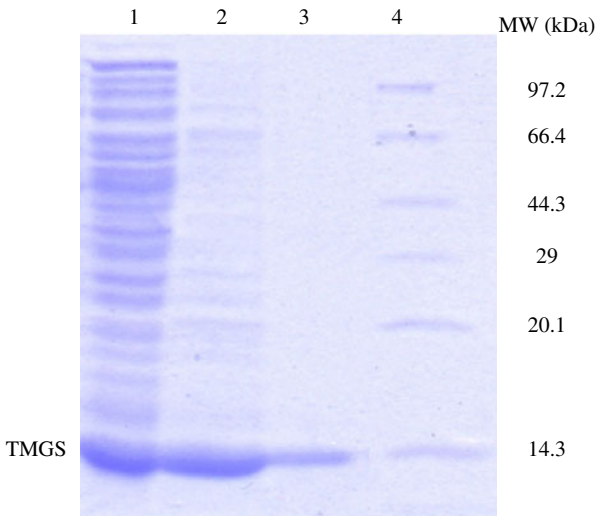


**Fig. 3** SDS-PAGE results for the crude extract (induced by IPTG), heat-shocked supernatant, and purified enzyme. *Lanes 1*, *2*, *3*, and *4* show the induction of MGS with IPTG in the crude extract, the heat-shocked supernatant of the crude extract, purified MGS, and molecular weight markers, respectively

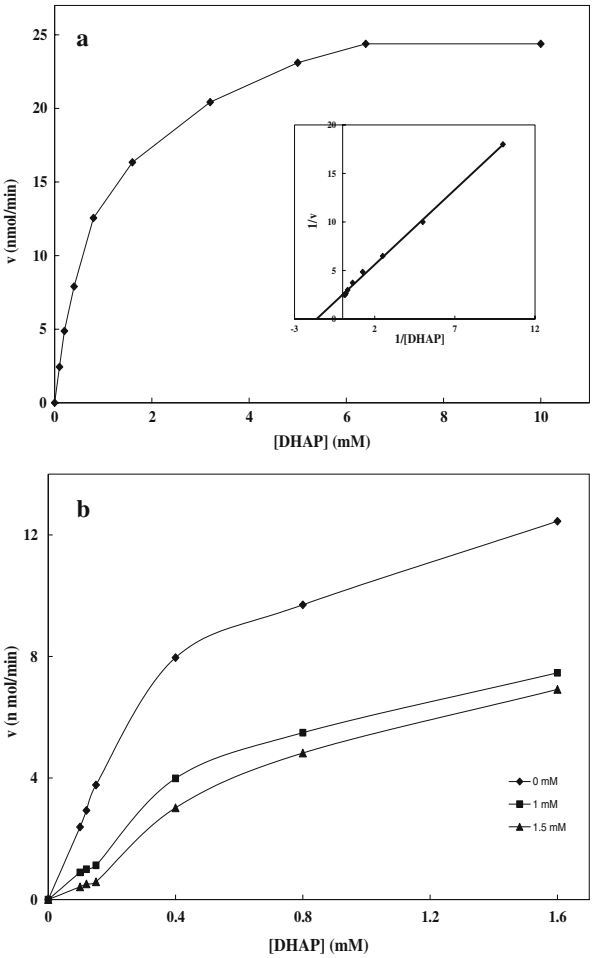


**Fig. 4** **a** Michaelis-Menten curve of TMGS. Using different concentrations of DHAP, Michaelis-Menten curve for TMGS was obtained. The *inset*: Lineweaver–Burk curve of TMGS in different concentration of DHAP. **b** The effect of phosphate on the kinetics of TMGS. Michaelis-Menten curve of TMGS is obtained in 0 mM, 1 mM, and 1.5 mM of phosphate and 0.1 mM to 1.6 mM of DHAP. In this range of DHAP concentration, allosteric inhibition induced by phosphate has been observed in the *E. coli* enzyme [1, 16]

*thermophilus* HB8 MGS. Accordingly, decrease in TMGS's cooperativity might be the consequence of this loss, showing the critical role of Arg 150 in transmitting allosteric changes in *E. coli* MGS [27]. On the contrary, Pro 92, Arg 107, and Val 111 are conserved in *E. coli* MGS, TMGS (Pro 82, Arg 97, and Val 101), and *T. thermophilus* HB8 MGS. These amino acids participate in the other suggested pathway for transmitting allosteric changes in *E. coli* MGS [27] and might be responsible for the observed allosteric inhibition by phosphate in TMGS (Fig. 4b).

**Table 2** Kinetic parameters comparison between TMGS and *E. coli* MGS.

| MGS | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $n$H[a] | pH optimum | Temperature optimum (°C) | Number of subunits | Molecular mass (kDa) | Amino acids per subunit |
|---|---|---|---|---|---|---|---|---|
| *Thermus* sp. GH5 | 0.56 | 325 | 1.5 | 6 | 75 | 6 | 85.8 | 132 |
| *E. coli*[b] | 0.2 | 220 | 3.4 | 7.5 | 30[c] | 6 | 101.5 | 152 |

[a] Hill coefficient for TMGS was obtained in the presence of 1.5 mM of phosphate.

[b] Kinetic parameters for *E. coli* obtained from [1, 16].

[c] Temperature optimum of *E. coli* MGS has not been reported previously, but was assayed in 30°C [1, 16].

## Effects of pH and Temperature on MGS Activity and Enzyme Stability at Different Temperatures

To determine the optimum pH of MGS, enzymatic activity was measured in the mix buffer of different pH values. TMGS showed an optimal activity at pH6 (Fig. 6a, Table 2). Regarding other enzymes reported from *E. coli*, *P. vulgaris*, *P. saccharophila, C. acetobutylicum*, with optimum pH values of 7.5, 7.7, 8.2, and 7.5, respectively [16–19], *Thermus* sp. GH5 enzyme had the lowest pH optimum among them. Enzyme activity in different temperatures showed that the enzyme had an optimum activity at 75°C (Fig. 6b, Table 2). For determining thermal stability, the enzyme was incubated in different temperatures for 15 min and immediately cooled on ice. The remaining activity was then measured at 60°C. The enzyme maintained its stability up to 75°C (Fig. 6c).

## Molecular Mass Determination and the Quaternary Structure of MGS

The quaternary structure of MGS in 50 mM imidazole-HCl buffer (pH6.5) was estimated by analytical gel filtration chromatography. Figure 7 summarizes the results obtained from calibrated G-100 Sephadex indicating a molecular mass of 89 kDa for MGS that is

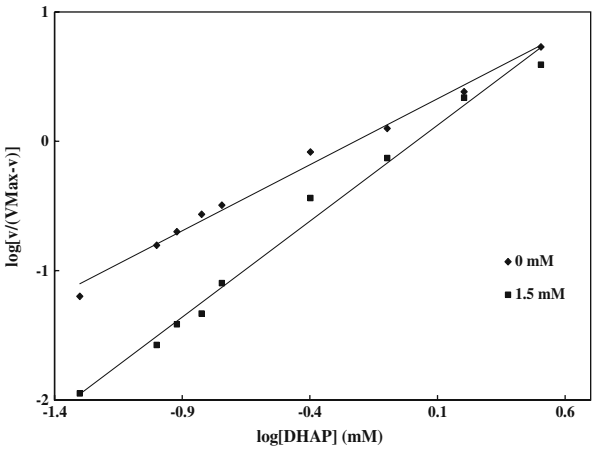


**Fig. 5** Hill plot for TMGS. Hill plot was drawn according to the equation mentioned in Materials and Methods section and Hill coefficients of 1.0 and 1.5 were obtained for TMGS in the presence of 0 mM and 1.5 mM of phosphate, respectively

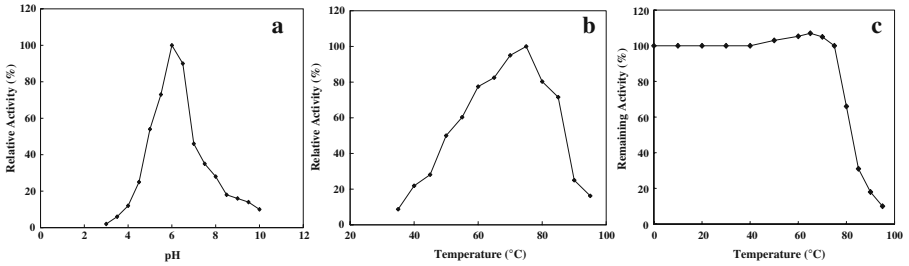


**Fig. 6** pH and temperature profiles and thermal stability of TMGS. **a** pH optimum for activity of TMGS determined using mix buffer adjusted to the desired pH. The mix buffer contained 50 mM of glycine-HCl (pH 2.2 to 3.8), succinic acid-NaOH (pH3.8 to 6), imidazole-HCl (pH6.2 to 7.8), tris-(hydroxymethyl)-aminomethane (pH7.1 to 8.9), and glycine-NaOH (pH8.6 to 10.6). **b** Relative activity of TMGS was determined by measuring the enzymatic activity at different temperatures in 50 mM of imidazole buffer, pH6.5. **c** For thermal stability determination, TMGS was incubated in the desired temperatures for 15 min in 50 mM of imidazole, pH6.5, and immediately cooled on ice. The remaining activity was then measured at 60°C

correlated to the hexameric form of this enzyme with molecular mass of 14.3 kDa for each monomer.

Changes in the oligomerization state by allosteric effectors occur in some allosteric enzymes [30–32]. To test whether the same event applies to MGS, the enzyme was run on G-100 column in the presence of 1 mM phosphate but the elution volume of the enzyme showed no change, which exhibited that the presence of phosphate has not changed the oligomerization state of the enzyme. This result supports what is discussed above, that the allosteric behavior of TMGS in the presence of phosphate is a result of transmitting allosteric changes between enzyme subunits through amino acids, and not a change in the oligomerization pattern. Thus, with respect to a previous study by Saadat and Harrison [27], it can be suggested that Pro 82, Arg 97, and Val 101 have a role in the transmitting allosteric changes in TMGS.

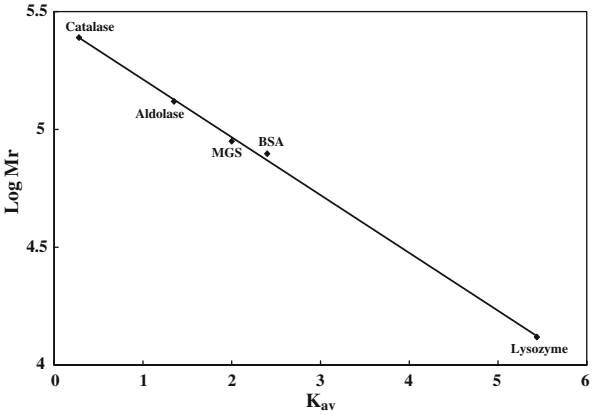


**Fig. 7** Molecular mass determination by gel filtration chromatography. Sephadex G-100 column was calibrated with catalase, aldoase, BSA, and lysozyme and then, TMGS was run. TMGS with $k_{av}$ of 2 has a calculated molecular mass of 89.12 kDa

## Conclusion

Biochemical tests and 16S rDNA sequencing analysis showed that GH5 strain belongs to *Thermus* sp. and is closely related to *T. brockianus.* This study is the first to characterize the biochemical properties of MGS (a key enzyme in production of 1,2-propandiol in microorganisms) from a thermophilic microorganism. This work supports the previous hypothesis [27] that Arg 150 is a critical amino acid for heterotropic cooperativity in MGS from *E. coli*. TMGS is a hexameric enzyme and phosphate (as an allosteric inhibitor) cannot change oligomerization state of the enzyme. Allosteric behavior of TMGS is the result of transmitting allosteric changes between enzyme subunits through amino acids. It has been estimated that Pro 82, Arg 97, and Val 101 play a role in generating the observed cooperativity in the presence of phosphate in TMGS. Study of these amino acids in detail is underway.

## References


1. Saadat, D., & Harrison, D. H. T. (1998). *Biochemistry, 37*, 100074–110086.
2. Booth, I. R., Ferguson, G. P., Miller, S., Li, C., Gunasekera, B., & Kinghorn, S. (2003). *Biochemical Society Transactions, 31*, 1406–1408.
3. Kalapos, M. P. (1999). *Toxicology Letters, 110*, 145–175.
4. Tötemeyer, S., Booth, N. A., Nicholas, W. W., Dunbar, B., & Booth, I. R. (1998). *Molecular Microbiology, 27*, 553–562.
5. Kalapos, M. P. (2008). *Drug Metabolism and Drug Interactions, 23*, 69–91.
6. Tempest, D. W., & Neijssel, O. M. (1984). *Annual Review of Microbiology, 38*, 459–486.
7. Chang, T., & Lingyun, W. (2006). *Canadian Journal of Physiology and Pharmacology, 84*, 1229–1239.
8. Wu, L. (2006). *Canadian Journal of Physiology and Pharmacology, 84*, 129–139.
9. Cooper, R. A. (1984). *Annual Review of Microbiology, 38*, 49–68.
10. Inoue, Y., & Kimura, A. (1995). *Advances in Microbial Physiology, 37*, 177–227.
11. Thornalley, P. J. (1990). *Biochemical Journal, 269*, 1–11.
12. Hopper, D. J., & Cooper, R. A. (1971). *FEBS Letters, 13*, 213–216.
13. Altaras, N. E., & Cameron, D. C. (1999). *Applied and Environmental Microbiology, 65*, 1180–1185.
14. Bennet, G. N., & San, K.-Y. (2001). *Applied and Environmental Microbiology, 55*, 1–9.
15. Cameron, D. C., Altaras, N. E., Hoffman, M. L., & Shaw, A. J. (1998). *Biotechnology Progress, 14*, 116–125.
16. Hopper, D. J., & Cooper, R. A. (1972). *Biochemical Journal, 128*, 321–329.
17. Cooper, R. A. (1974). *European Journal of Biochemistry, 44*, 81–88.
18. Tsai, P.-T., & Gracy, R. W. (1976). *Journal of Biological Chemistry, 251*, 364–367.
19. Huang, K.-X., Rudolph, F. B., & Bennett, G. N. (1999). *Applied and Environmental Microbiology, 65*, 3244–3247.
20. Murata, K., Fukuda, Y., Watanabe, K., Saikusa, T., Shimosaka, M., & Kimura, A. (1985). *Biochemical and Biophysical Research Communications, 131*, 190–198.
21. Ray, S., & Ray, M. (1981). *Journal of Biological Chemistry, 256*, 6230–6233.
22. Laemmli, U. K. (1970). *Nature (London), 227*, 680–685.
23. Cooper, R. A. (1975). *Methods in Enzymology, 41*, 502–508.
24. Wells, C. F. (1966). *Tetrahedron, 22*, 2685–2693.
25. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
26. Murray, R. G. E., Brenner, D. J., Bryant, M. P., Holt, J. G., Krieg, N. R., Moulder, J. W., et al. (1984). *Bergey's manual systematic bacteriology.* Baltimore: Williams and Wilkins.
27. Saadat, D., & Harrison, D. H. T. (1999). *Structure, 7*, 309–317.
28. Marks, G. T., Susler, M., & Harrison, D. H. T. (2004). *Biochemistry, 43*, 3802–3813.
29. Saadat, D., & Harrison, D. H. T. (2000). *Biochemistry, 39*, 2950–2960.
30. Ali, M. H., & Imperiali, B. (2005). *Bioorganic & Medicinal Chemistry, 13*, 5013–5020.
31. Kim, J., & Raushel, F. M. (2001). *Biochemistry, 40*, 11030–11036.
32. Levitzki, A., & Koshland, D. E. (1972). *Biochemistry, 11*, 247–253.