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CHARACTERIZATION OF METHYLGLYOXAL SYNTHASE IN *SACCHAROMYCES CEREVISIAE*

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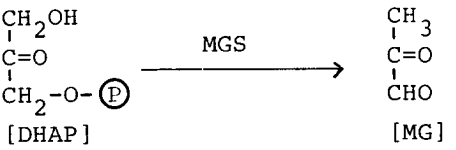
Methylglyoxal synthase in *Saccharomyces cerevisiae* was purified approximately 300 folds from cell extracts with 20 % of activity yield. During purification procedures, polymorphyic behaviours of the enzyme were observed. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis and consisted of a single polypeptide chain of M_r=26,000. The enzyme was most active at pH 9.5-10.5 and strictly specific to dihydroxyacetone phosphate with Km=3 mM. Phosphoenolpyruvate, glyceraldehyde-3-phosphate, orthophosphate and thiol compouds were potent inhibitors of the enzyme.

Methylglyoxal synthase (MGS) catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to methylglyoxal (MG) [Eq. 1] and is, therefore, an important enzyme in providing an alternative catabolic fate of triose phosphates formed during glycolysis.

[Eq. 1]



[DHAP] [MG]

MGS has been purified from *Escherichia coli* (1), *Proteus vulgaris* (2) and from *Pseudomonas saccharophila* (3) and

Abbreviations: G-6-P, Glucose-6-phosphate, G-1-P, Glucose-1-phosphate; F-1,6-dP, Fructose-1,6-diphosphate; 3-PGA, Glyceraldehyde-3-phosphate; 1,3-DPG, 1,3-Diphosphoglycerate, 2-PG, 2-Phosphoglycerate; 3-PG, 3-Phosphoglycerate; PEP, Phosphoenolpyruvate, DTT, Dithiothreitol; 2-ME, 2-Mercaptoethanol; GSH, Glutathione(reduced); Pi, Orthophosphate; PPi, Pyrophosphate; PMSF, Phenylmethylsulfonyl fluoride

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significant contribution on the enzyme to triose phosphates catabolism has been suggested. We have also been studying on the enzymes involved in the diversion of triose phosphates to pyruvate via MG in yeast cells and now report the properties of MGS from *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Chemicals DHAP and glyoxalase I were purchased from Sigma Chemical Co., St Loius MO, U.S.A.

Purification procedures The cells of *S. cerevisiae* DKD-5D-H (a leu2-3 leu2-112 trp1 his3) were grown in a medium comprising 2 % glucose, 1 % yeast extract and 2 % peptone (pH 5.0) with aeration at 30 °C for 24 h. The pastes of washed cells were broken on Dyno-Mill and cell extracts were prepared in Tris-PMSF [10 mM Tris-HCl (pH 7.3) containing 0.05 mM PMSF]. All purification procedures were at 4 °C. After removal of cell debris, the supernatants were loaded on a column (5 cm x 60 cm) of DEAE-Cellulose and was eluted with an increasing gradient of KCl (0-1 M in Tris-PMSF, total volume 4 liter). Active fractions were pooled concentrated using Amicon PM10 Membrane and were then applied to a column (4.5 cm x 120 cm) of Sephadex G-150. The active fractions eluted with Tris-PMSF were collected, concentrated as before and applied to a column (2.5 cm x 80 cm) of DEAE-Sepharose CL-6B. Upon elution with an increasing gradient of KCl (0-0.7 M in Tris-PMSF, total volume 1,000 ml), active fractions were combined, concentrated as before and dialyzed against Tris-PMSF overnight. The dialysate were applied to a column (1.5 cm x 12 cm) of hydroxylapatite and the enzyme was eluted with an increasing gradient of potassium phosphate buffer (pH 7.3) (1 - 500 mM, total volume 150 ml). The active fractions were collected, diluted with 2 volumes of Tris-PMSF and applied again to a column (1.5 cm x 10 cm) of DEAE-Cellulose. The enzyme was eluted with gently increasing gradient of KCl (0 - 100 mM in Tris-PMSF, total volume 50 ml). Active fractions were collected, concentrated as before and dialyzed against 10 mM Tris-HCl buffer (pH 7.3) overnight.

Enzyme assay Two methods (A and B) were used to assay MGS as described by (1). In method A, the reaction mixture contained 2.0 mM DHAP, 100 mM Tris-HCl buffer (pH 7.3) and enzyme. After termination of the reaction, MG in reaction mixture was determined enzymatically using glyoxalase I. In method B, the MGS reaction was conducted as above. MG formed in reaction mixture was converted to hydrazone by incubating with 2,4-dinitrophenylhydrazine and the color intensity after alkalization was measured at 555 nm. Protein was determined by the method of Lowry et al. (4) using bovine serum albumin as standard.

Determination of molecular mass The relative M_r of native enzyme was determined on calibrative column of Sephadex G-150. Proteins were eluted with 10 mM Tris-HCl buffer (pH 7.3). Polyacrylamide gel electrophoresis (PAGE) in the presence or absence of SDS was also used for M_r estimation and was performed as in (5).

RESULTS AND DISCUSSION

The purification procedures of MGS are summerized in Table 1. The enzyme was obtained as a single active peak on DEAE-Cellulose (Fig. 1-A) and Sephadex G-150 (Fig. 1-B) column chromatographies. However, when the enzyme was chromatographed on DEAE-Sepharose CL-6B (Fig. 1-C), two active peaks (I and II) were appeared. Further chromatography of a small portion of Peak II on Sephadex G-75 column (Fig. 1-D) resulted in the dissociation of the activity into three different peaks (III, IV and V) and M_rs of Peaks IV and V were estimated to be 55,000 and 26,000, respectively. All the reaction products of Peaks I, II, III, IV and V were identified enzymatically to be MG using glyoxalase I. To avoid the separation of activity, Peak II was further fractionated on ion-exchange columns of hydroxylapatite and DEAE-Cellulose. Final MGS preparation was homogeneous on PAGE (12.5 %) (Fig. 2, Lane F) and the enzyme after treatment with SDS/DTT migrated as a single protein band of M_r=26,000 on SDS(0.1 %)/PAGE(12.5 %) (Fig. 3, Right, Lane C). However, when the enzyme was applied to calibrative

Table 1
Summary of purification of MGS from S. cerevisiae

Step	Total protein (mg)	Specific activity[a] (nmole/min/mg-protein)	Total activity (nmole/min)	Yield (%)
1. Cell extracts	5250	0.185	971.3	100
2. DEAE-Cellulose	1220	0.663	808.9	83.3
3. Sephadex G-150	230	2.74	630.2	64.9
4. DEAE-Sepharose CL-6B	45.6	13.8	629.3	64.8
5. Hydroxylapatite	17.2	28.8	495.4	56.0
6. DEAE-Cellulose	3.32	53.6	177.9	18.3

[a] Activities were determined by method B.

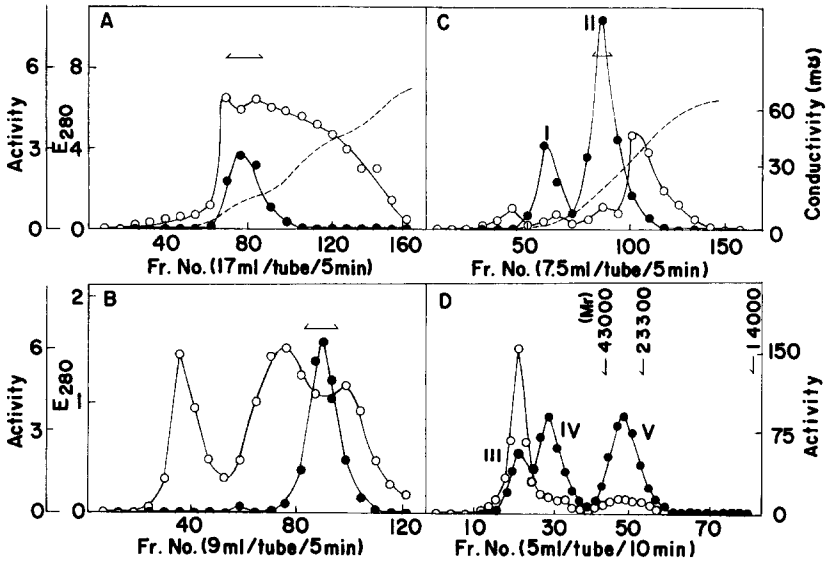


Fig. 1. Elution pattern of MGS on column chromatographies
A, DEAE-Cellulose; B, Sephadex G-150; C, DEAE-Sepharose CL-6B; D, Sephadex G-75. In Fig. D, the arrows indicate the relative elution positions of Egg albumin (M_r=43,000), Trypsin (M_r=23,300) and Lysozyme (M_r=14,300). Bars in all Fig.s indicate the active fractions collected for purification. Activities were determined by Method B and are expressed as nmole of MG formed per min per ml of eluate. -o-, $E_{280\ nm}$; -●-, Activity; ---, Conductivity

column of Sephadex G-150, two protein peaks (P_1 and P_2) with MGS activities (Fig. 3, Left) were found and M_r of P_2 was estimated to be 53,000, approximately twice of that of the purified

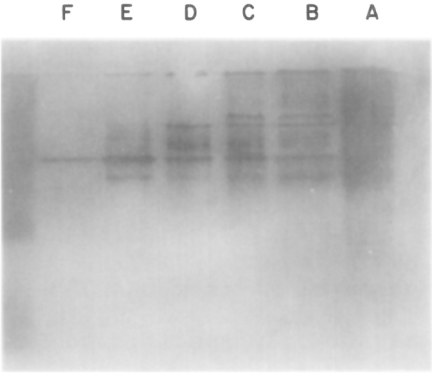


Fig. 2. Polyacrylamide gel electrophoresis of various samples in purification procedures
A, Crude cell extracts (50 μg); B, MGS after DEAE-Cellulose (50 μg); C, MGS after Sephadex G-150 (40 μg); D, MGS after DEAE-Sepharose CL-6B (30 μg); E, MGS after hydroxylapatite (30 μg); F, MGS after second DEAE-Cellulose (15 μg).

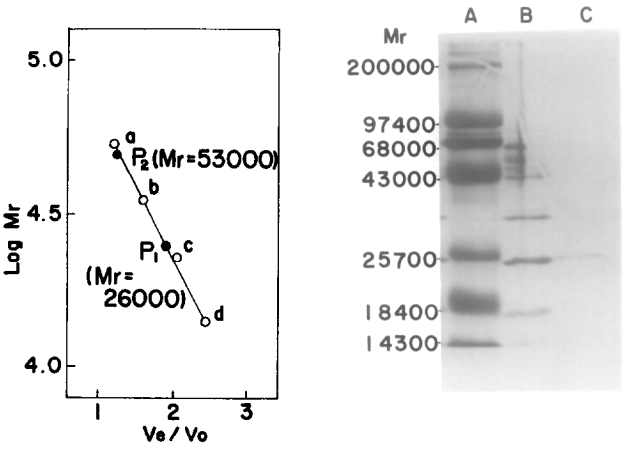
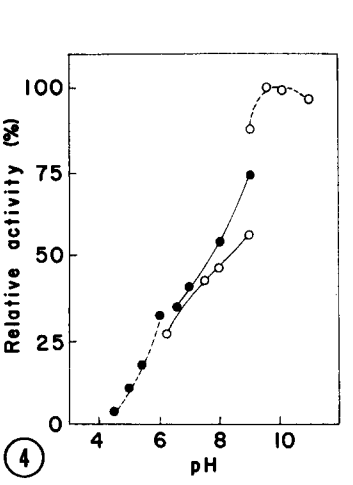


Fig. 3. Molecular mass of MGS
Left: Sephadex G-150 column chromatography of MGS. Ve(elution volume/Vo(void volume) was plotted versus log M_r. The position of MGS (P_1 and P_2) was shown by closed circles. Standard proteins used were: a, Pyruvate kinase (M_r=57,000); b, Glyceraldehyde-3-phosphate dehydrogenase (M_r=36,000); C, Trypsin (M_r=23,300) and d, Lysozyme (M_r=14,300). Light: SDS/PAGE of MGS. Sample after DEAE-Sepharose CL-6B (40 µg , Lane B) and purified MGS (10 µg, Lane C) were treated with SDS/DTT and analyzed on SDS(0.1 %)/PAGE(12.5 %). Standard proteins in Lane A were: Myosin(H-chain) (M_r=200,000); Phosphorylase B (M_r=97,400); Bovine serum albumin (M_r=68,000); Ovalbumin (M_r=43,000); α-Chymotrypsinogen (M_r=25,700); β-Lactoglobulin (M_r=18,400) and Lysozyme (M_r=14,300)

MGS (M_r=26,000). The M_rs of *E. coli* (1), *P. vulgaris* (2) and *P. saccharophila* (3) MGSs have been shown to be 67,000, 135,000 (dimeric form) and 67,000, respectively. However, no data as to the association /dissociation behaviours of the bacterial MGSs have been reported so far.

The enzymatic properties of yeast MGS were studied. The maximal activity was attained at the range pH 9.5 - 10.5 in 100 mM Glycine-KOH buffer (Fig. 4). Although crude extracts catalyzed the formation of MG from DHAP, 3-PGA or F-1,6-dP, the purified MGS was strictly specific to DHAP. 3-PGA, 1,3-DPG, 3-PG, 2-PG and PEP could not substitute for DHAP. When the activity of the purified MGS was measured at the various concentrations of DHAP, a sigmoidal relationship was obtained and Km of the MGS for DHAP was estimated to be 3.0 mM (Fig. 5-



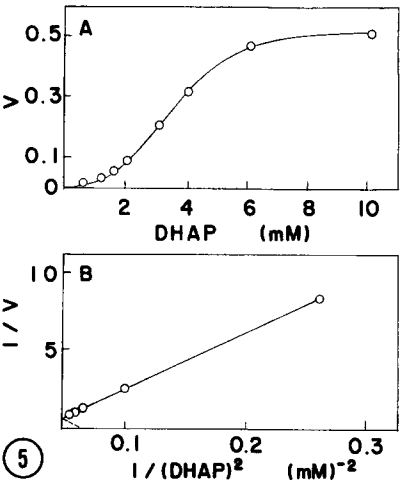


Fig. 4. Effect of pH on MGS activity
Enzyme activity was determined by Method A. Buffers (0.1 M) used were:--●-, CH_3COOH/CH_3COONa; -o-, Tris/HCl; -●-, Triethanolamine/HCl; --o--, Glycine/KOH

Fig. 5. Effects of substrate concentration
Velocity is expressed as μmole of MG formed per min per mg of protein and is determined by Method A. A: Substrate saturation curve of MGS reaction. B: Double reciprocal plots of velocity (arbitrary unit) versus square of DHAP concentration.

A). The sigmoidal curve could be converted into a linear relationship by plotting the reciprocal of the square of DHAP concentration (Fig. 5-B). The results in Fig. 5-A were also plotted according to the Hill equation (6), and a straight line with a slope of 4.0 was obtained. This suggested that the MGS has more than one DHAP binding sites. To check the involvment of Shiff base formation in MGS reaction [Eq. 1], the purified MGS was treated with sodium borohydride in the presence and absence of DHAP essentially according to the method of Grazi et al. (7). However, as in the case of *E. coli* MGS (1), no evidence for the formation of Shiff base was obtained, though the data are not shown here.

Effects of various chemicals on MGS activity were investigated. The enzyme activity was inhibited by Pi and PPi (Fig. 6). In case of *E. coli* MGS, approximately 0.1 mM of Pi has been reported to be enough to cause 50 % inhibition (1). However, yeat

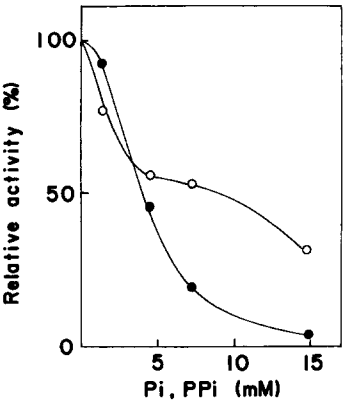


Fig. 6. Effect of Pi and PPi concentrations on MGS activity
The activity was determined by Method A in the presence or absence of Pi (●) or PPi (o).

MGS was rather insensitive to Pi concentration than *E. coli* MGS and about 4 mM of Pi concentration was required to attain 50 % inhibition. Intermediates in glycolysis showed inhibitory effects on MGS activity (Table 2). Among them, 3-PGA and PEP

Table 2
Effect of various compounds on MGS activity

Inhibitors		Relative activity[a] (%)
None		100
G-1-P	(8 mM)	68.3
G-6-P	(8 mM)	72.1
F-1,6-dP	(8 mM)	66.4
3-PGA	(8 mM)	21.7
1,3-DPG	(8 mM)	65.4
2-PG	(8 mM)	69.1
3-PG	(8 mM)	78.0
PEP	(8 mM)	20.7
Adenosine	(5 mM)	96.7
AMP	(5 mM)	80.5
ADP	(5 mM)	71.1
ATP	(5 mM)	65.5
DTT	(1 mM)	25.4
2-ME	(1 mM)	33.6
GSH	(1 mM)	21.8
L-Cysteine	(1 mM)	12.3

a Activities were determined by method A

were the most potent inhibitors. The MGS was also inhibited by thiol compounds such as GSH and L-cysteine (Table 2). Adenosine and adenosine nucleotides (AMP, ADP and ATP) were not so effective as inhibitors.

Thus, the results obtained suggested that the yeast MGS was different from MGSs from procaryotes in molecular weight and in sensitivity toward various inhibitory chemicals. Hopper and Cooper (1) discussed the function of *E. coli* MGS from a standpoint of channelling of triose phosphates catabolism and indicated that the Pi could regulate the diversion of triose phosphates to either 1,3-DPG or MG, since *E. coli* MGS was severely inhibited by Pi concentrations that were close to Km value for Pi as a substrate for 3-PGA dehydrogenase. The similar regulation postulated for *E. coli* MGS, however, is not applicable to the yeast, since yeast MGS is somewhat insensitive to Pi concentration. In addition to the inhibitory effects of glycolytic intermediates such as 3-PGA and PEP, the inhibition of yeast MGS by thiol compounds, especially by glutathione which is contained in yeast cells in a large quantity, should be taken into account as a main modifier of MGS activity. The regulation of activities in glycolytic enzymes by thiol/disulfide exchange has been postulated by Gilbert (8) as "The Third Messenger".

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