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# Purification and Characterization of Methyl Mercaptan Oxidase from *Thiobacillus thioparus* for Mercaptan Detection

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**Abstract** Methyl mercaptan oxidase was successfully induced in *Thiobacillus thioparus* TK-m using methyl mercaptan gas, and was purified for the detection of mercaptans. The purification procedure involved a DEAE (diethylaminoethyl)-Sephacel, or Superose 12, column chromatography, with recovery yields of 47.5 and 48.5%, and specific activities of 374 and 1240.8 units/mg-protein, respectively. The molecular weight of the purified methyl mercaptan oxidase was 66.1kDa, as determined by SDS-PAGE. The extract, from gel filtration chromatography, oxidizes methyl mercaptan, producing formaldehyde, which can be easily detected by the purpald-coloring method. The optimized temperature for activity was found to be at 55°C. This enzyme was inhibited by both $NH_4Cl$ and $(NH_4)_2SO_4$, but was unaffected by either KCl or NaCl at less than 200 mM. With $K_2SO_4$, the activity decreased at 20 mM, but recovered at 150 mM. In the presence of methanol, full activity was maintained, but decreased in the presence of glycerin, ethanol and acetone 43, 78 and 75%, respectively.

*Keywords*: methyl mercaptan oxidase, *Thiobacillus thioparus*, purpald, enzyme purification

## INTRODUCTION

According to a statistical report, more than 25 million Americans suffer the embarrassment of diseased bad breath, better known as halitosis, which is unperceivable by the afflicted [1]. The annual market value of mouthwash is $850 million. Many dentists use instruments like the halimeter to quantify halitosis [2]. However, this apparatus is not feasible for daily diagnosis due to its prohibitively high cost. Therefore, a simple and accurate sensor for the detection of halitosis would be useful. Halitosis is mainly attributed to the presence of sulfur compounds, including hydrogen sulfide, dimethyl mercaptan and methyl mercaptan (MM) [3]. The concentration of oral methyl mercaptan is between 4 and 20 nmol/L, but these levels can increase 4 fold if bleeding lesions are present [4].

MM is oxidized by the catalytic effect of methyl mercaptan oxidase (MMO) producing formaldehyde, hydrogen peroxide and hydrogen sulfide [5-7]. These products can be easily quantified by several colorimetric methods, as reported previously [8-11]. The oxidation of MM can be described as follows.

$$CH_3SH + O_2 + H_2O \rightarrow H_2S + HCHO + H_2O_2$$

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We previously reported the isolation and purification of MMO from *Thiobacillus thiooxidans* and *Rhodococcus rhodochrous* [12,13]. In this study we used *Thiobacillus thioparus* for the induction of MMO by supplying with MM gas as its substrate. *T. thioparus* is a chemolithoautotroph, with an optimal pH for growth between 6.5 and 7.0. It has been reported that the strain had a high removal efficiency for malodorous volatile sulfuric compounds, such as dimethyl sulfide, dimethyl disulfide and methyl mercaptan [15]. A list of organic sulfur oxidizing microorganisms is shown in Table 1 [16]. The induced enzyme, MMO, was isolated, purified and characterized.

## MATERIALS AND METHODS

### Chemicals and Bacterial Strain

Purpald (Aldrich Chemical Co., St. Louis, WI, USA) was used as a color indicator for measuring the enzyme activity. Methyl mercaptan gas (4,800 ppm in nitrogen) was purchased from Duk-Yang Gas Co (Korea). The low range protein molecular markers (Bio-Rad, Hercules, CA USA) were used as protein molecular weight standards. For FPLC (fast protein liquid chromatography) operation, DEAE(diethylaminoethyl)-Sephacel and Superose 12 column were purchased (Pharmacia LKB, Uppsala, Sweden). *Thiobacillus thioparus* TK-m was obtained from the Deutsche SammLung von Mikroorganismen (DSMZ)

**Table 1.** Microorganisms that metabolize various organic sulfurs

| Strains | Sulfur compounds metabolized |
|---|---|
| *Pseudomonas sp.* | DBT |
| *Beijerinckia sp.* | DBT |
| *Rhizobium* | DBT |
| *Acinetobacter* | DBT |
| *Hyphomicrobium sp.* | MM, DMS, DMDS, DMSO, $DMSO_2$ |
| *Sulfololobus acidocaldarius* | DBT, thianthrene, thioxanthene |
| *Thiocystis sp.* | DMSO, $DMSO_2$ |
| *Thiobacillus sp.* | DMS |
| *Brevibacterium sp. DO* | DBT |
| *Cunninghamella elegans* | DBT |
| *Pseudomonas acidovorans* | MM, DMS, DMDS, DMSO, $DMSO_2$ |
| *Rhodococcus rhodochrous IGTS8* | DBT, sulfides, sulfones, mercaptans, sulfoxides |
| *Rhodococcus erythropolis* | DBT |
| *Corynebacterium sp. SY-1** | DBT, DMS, DMSO, DBSO, sulfonates, $CS_2$ |
| *Desulfovibrio desulfuricans M6* | DBT, BDS, BS, BT, thios, crude oils |
| *Desulfotomaculum orients* | DBT |
| *Thermodesulfobacterium commune* | DBT |

* (reidentified as *Rhodococcus*)
BDS: benzyldisulfide; BS: benzylsulfide; DBSO: dibenzylsulfoxide; DBT: dibenzothiophene; DMDS: dimethyl disulfide; DMS: dimethyl sulfide; DMSO: dimethyl sulfoxide; $DMSO_2$: dimethyl sulfone; MM: methyl mercaptan

## Media and Fermentation Conditions

*T. thioparus* was cultivated in a growth medium containing (g/L): $Na_2S_2O_3 \cdot 5H_2O$, 2.5; $KH_2PO_4$, 2.0; $K_2HPO_4$, 2.0; $Na_2CO_3$, 0.4; $NH_4Cl$, 0.4; a trace metal solution, 3.0 mL and a vitamin solution, 3.0 mL. The trace metal solution consisted of EDTA·2Na, 50; $ZnSO_4 \cdot 7H_2O$, 11; $CaCl_2$, 5; $MnCl_2 \cdot 4H_2O$, 2.5; $CoCl_2 \cdot 6H_2O$, 0.5; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 5 and $CuSO_4 \cdot 5H_2O$, 0.2 adjusted to pH 6.0 with NaOH. The vitamin solution contained (in mg/L) thiamine·HCl, 10; riboflavin, 20; pyridoxine, 10; nicotinic acid, 20; p-aminobenzoic acid, 10; calcium pantothenate, 20; biotin, 1 and cyanocobalamin, 1, and was adjusted to pH 7.0 with NaOH [5]. Flask cultivations were carried out at 30°C and 150 rpm in shaking incubator. The growth characteristics were observed from 500 mL flasks containing 100 mL of the growth medium, and the mass cultivation was carried out in 10 liter distillated water bottles (Nalgene Co., USA) containing 4 liters of the growth medium. Normal air was supplied at 150 mL/min until the growth stationary phase was reached, it was then substituted for 4,800 ppm MM in nitrogen at 20 mL/min for 40 h for the induction of the MMO [12,13]. The cell growth was determined by measuring the optical density of the culture media at 600 nm ($OD_{600}$) using an UV spectrophotometer 8562A (Hewlett Packerd, USA).

## Enzyme Purification Procedure

Harvested cells were washed twice with 10 mM Tris/HCl, pH 8.2 and suspended in 15 mL of 10 mM Tris/HCl, pH 8.2 at 4°C, then sonicated 3 times for 5 min, at 3 min intervals. The cell debris was spun down by centrifugation at 17,000 *g* for 30 min at 4°C. The crude extract was purified by FPLC using a DEAE-Sephacel column (25 $\phi$ × 80 mm) with 500 mM KCl and 10 mM Tris/HCl, pH 8.2 as the gradient buffer. After collecting fractions showing activity, the eluate was concentrated using an Amicon ultrafiltration unit with a YM10 membrane filter. The extract was then passed through a Superose 10/30 column (25 $\phi$ × 300 mm). At each purification step, the total protein concentration was measured by the Bradford method [14], using bovine serum albumin a standard.

## Analyses

Enzyme activity was determined using the purpald/formaldehyde color reaction [8]. The substrate solution was prepared by bubbling the 4,800 ppm methyl mercaptan in nitrogen through a 10 mM potassium phosphate buffer, pH 7.5 for 3.0 h, to achieve a final concentration of 1.55 mM. A 34 mM purpald solution, in 2 M NaOH, was used as the coloring agent. The assay mixture of 1 mL, containing: 400 μL distilled water, 400 μL purpald and 200 μL of the MM solution, was placed in a 20 mL round-bottom screw-tapped test tube. The mixture was then shaken in a 20 mL rotary incubator at 36°C and shaken at 30 rpm for 20 min. On completion of the reaction the absorbance was measured at 550 nm An activity unit was defined as nmol of formaldehyde formed per min, for the conditions described above.

## SDS-PAGE

The protein molecular mass was determined by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), and staining was carried out using a silver staining kit (Pierce Co., Rockford, USA).

## Effect of Salts and Organic Solvents

A change in the enzyme activity was observed on the addition of $NH_4Cl$, $(NH_4)_2SO_4$, KCl, $K_2SO_4$, and NaCl in the range of 20 to 200 mM. The enzyme activity was also measured with the addition of 10 or 20% (v/v) organic solvents, including glycerin, methanol, ethanol and acetone. The enzyme solution used was obtained from gel filtration chromatography.

# RESULTS AND DISCUSSION

## Growth Characteristics

Flask cultivations were carried out to identify the growth characteristics of *Thiobacillus thioparus* TK-m

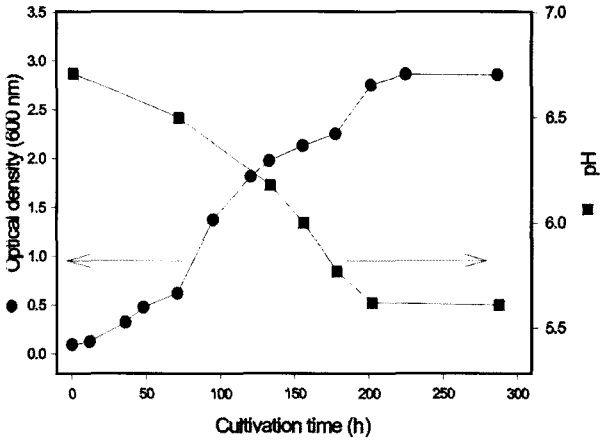


**Fig. 1.** Time course of the cultivation of *Thiobacillus thioparus* TK-m.

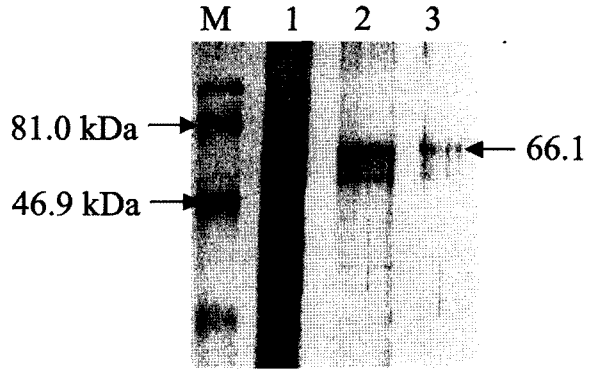


**Fig. 2.** Silver-stained SDS-PAGE of MMO. Arrows: molecular weight markers, lane: crude extract, lane 2: DEAE-Sephacel chromatography, and lane 3: after gel filtration chromatography.

a ıd to determine when methyl mercaptan was supplied f r the effective induction of methyl mercaptan oxidase ( MMO). During the cultivation, the pH decreased from 6 7 to 5.6, probably due to the oxidation of the thiosulf te ($S_2O_3^{2-}$) to the sulfate with the production of hyd ogen ions (Fig. 1). After 200 h, the optical density at 6 )0 nm, and the pH, were stabilized at 2.8 and 5.6, re- s ectively. Consequently, the MM was introduced to t e culture at 20 mL/min over a 40 h period, and at 200 h following the culture initiation the thiosulfate was t ought to be exhausted.

## E nzyme Purification and Characteristics

A summary of the purification of the MMO induced u ıder the specified condition is given in Table 2. The a tivity of eluate from the gel filtration remained high. T ıe reason for the higher recovery yield, following the g l filtration, compared to that following the DEAE-S phacel chromatography, was thought to be due to the p rtial inhibition of the activity caused by the 300 mM K Cl used during the DEAE-Sephacel chromatography. A d stinct band, with a molecular mass of 66.1 kDa, was o served following the gel-filtration column chromat graphy (Fig. 2), which was similar to that shown by b th the *Thiobacillus thiooxidans* and *Rhodococcus rhodoc rous,* which had bands for molecular mass bands of 6 .1 and 64.5 kDa, respectively [12,13]. Conversely, t ose induced by dimethyl sulfide, from *Hyphomic bium* EG and *T. thioparus,* were 40-50 and 40 kDa, re- s ectively [5,7]. According to these results, it appears that different strains, and substrates will affect the activities of MMO. Additionally, the optimum temperature for the induction of this enzyme was determined to be 55°C (Fig. 3), which was relatively high compared to the growth temperature of 30°C.

## Effect of Salts and Organic Solvents

The salt effect on the enzyme activity is shown in Fig. 4. When the concentrations of $(NH_4)_2SO_4$ and NaCl were 20 and 200 mM, the enzyme activity increased 1.1 and 1.2 times. With $NH_4Cl$, the activity decreased in proportion to the concentration. In the case of $K_2SO_4$, the enzyme activity was inhibited until the concentration reached 20 mM, but gradually recovered. The enzyme activity was relatively unaffected by KCl and NaCl. With the enzyme induced by dimethyl sulfide, in the *T. thioparus* TK-m, a different salt effect was observed for the MMO, with the activity being inhibited by 53% on the addition of either, 10 mM $(NH_4)_2SO_4$, or 10 mM $NH_4Cl$ [5]. Therefore, the MMO induced with MM was different from that with dimethyl sulfide though the same microorganism induced them. From this result, 20 mM $(NH_4)_2SO_4$, 150 mM KCl or more than 200 mM NaCl would appear suitable for the enhancement of the enzyme stability and activity.

When the purpald/formaldehyde color reaction is used as the detection system, purpald and MMO should be coated on a detection membrane that will be exposed to air from the mouth. Purpald, dissolved in an organic solvent, is coated by evaporation onto the detection

**T ıble 2.** Purification summary of methyl mercaptan oxidase from *T. thioparus* TK-m

| | Vol. (mL) | Total units (nmol/min) | Total protein (mg) | Specific activity [nmol $min^{-1}$ (mg protein)$^{-1}$] | Purification ratio | Yield (%) |
|---|---|---|---|---|---|---|
| Crude extract | 7.5 | 27.55 | 2.18 | 12.64 | 1 | 100 |
| DEAE-Sephacel | 5.0 | 13.09 | 0.035 | 374 | 29.6 | 47.5 |
| Gel filtration | 6.7 | 16.13 | 0.013 | 1240.8 | 98 | 48.5 |

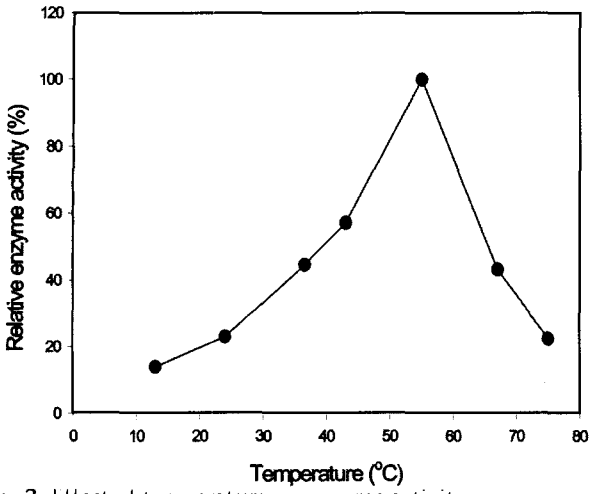


**Fig. 3.** Effect of temperature on enzyme activity.

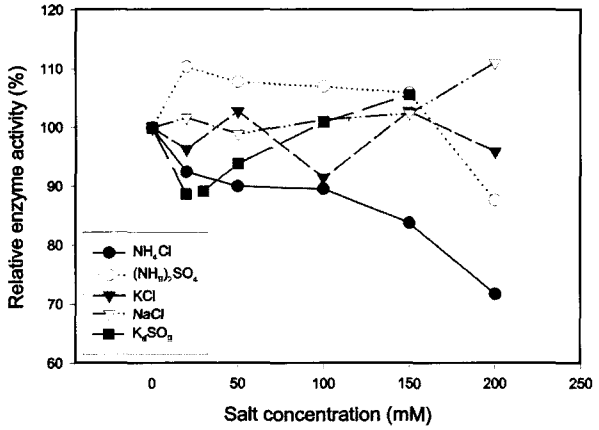


**Fig. 4.** Effect of salt concentrations on enzyme activity.

membrane: the MMO immobilized in advance by evaporation of an organic solvent containing MMO [17]. Therefore, the choice of an adequate organic solvent is very important because some organic solvents may inhibit the enzyme activity. After the addition of 10% acetone or ethanol, the enzyme was inhibited by 78 and 76%, respectively. With the addition of 20% (v/v) glycerin or methanol, the activity decreased by 43%, but 20% (v/v) methanol, almost all enzyme activity was preserved with no inhibition being apparent (data not shown). From these results, the MMO induced in the *T. thioparus* TK-m appears more stable against organic solvents than those induced in the *Thiobacillus thiooxidans* or the *Rhodococcus rhodochrous* [12,13]. From our result we would choose methanol as the organic solvent for purpald coating.

## CONCLUSION

In this study methyl mercaptan oxidase (MMO), for the use in a mercaptan sensor, was induced and isolated from *Thiobacillus thioparus* TK-m. When all the methyl mercaptan in mouth air is oxidized by the enzymatic reaction, about 10-50 nmol formaldehyde is produced. The amount of formaldehyde shows as a distinctively violet color due to its reaction with purpald. For the enhancement of the enzyme stability and activity, the MMO was characterized in the presence of various salts and at different concentrations. From the result, 20 mM $(NH_4)_2SO_4$, 150 mM KCl or more than 200 mM NaCl appeared suitable for maintaining the enzyme's activity. Additionally, in selecting the coating solvent to be used in the immobilization of the enzyme, onto the surface of matrix, the degree of inhibition of the MMO, by ethanol, methanol, acetone and glycerin, was investigated. Of the solvents tested, 20% methanol (v/v) was chosen due to its lack of enzyme inhibition. The results from this study might help increase understanding for the manufacture of enzyme linked biosensor in the detection of halitosis.

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