Biotechnol. Bioprocess Eng. 2000, 5: 465-468

Isolation and Purification of Methyl Mercaptan Oxidase from *Rhodococcus rhodochrous* for Mercaptan Detection

Sang-Joon Kim[1], Hyun-Jae Shin[2], Yeu-Chun Kim[1], Dae-Sil Lee[2], and Ji-Won Yang[1]*

[1] *Department of Chemical Engineering, KAIST, Taejon 305-701, Korea*
[2] EnzBank, Inc., 309 BVC, KRIBB, Taejon 305-333, Korea

Abstract Methyl mercaptan oxidase was successfully induced from *Rhodococcus rhodochrous* IGTS8 using methyl mercaptan gas and purified to homogeneity for the detection of mercaptans. The purification procedure involved DEAE-Sephacel and Superose 12 column chromatography with recovery yields of 85.8 and 83.3%, and a specific activity of 92.7 and 303.4 units/mg-protein, respectively. The molecular weight of purified methyl mercaptan oxidase was determined to be 64.5 kDa by SDS-PAGE. The extract from gel filtration chromatography oxidizes methyl mercaptan to produce formaldehyde, which can be easily detected by the purpald-coloring method. Optimum temperature for activity was achieved at 60°C. This enzyme was inhibited by both K_2SO_4 and NaCl at concentration of less than 100 mM and recovered to original activity at concentration of 200 mM. In the presence of methanol, the activity decreased by 33%.

Keywords: methyl mercaptan oxidase, *Rhodococcus rhodochrous*, purpald, enzyme purification

INTRODUCTION

According to a statistical report, 65% of U.S. adults have a chronic bad breath symptom [1] and people more than 25 million suffer the embarrassment of diseased bad breath, called halitosis. For this reason the domestic mouthwash market reached about $ 5 million in 1997 and continues to increase. Many dentists use instruments like the halimeter to quantify halitosis [2]. However, this apparatus is not adequate for daily diagnosis because of its high cost. Therefore, a simple and accurate sensor to detect halitosis is needed. Halitosis is mainly attributed to the presence of sulfur compounds including hydrogen sulfide, dimethyl mercaptan, and methyl mercaptan (MM) [3]. Orally, the concentration of methyl mercaptan is about 4 to 20 nmol/L. When the bleeding lesions are present, the concentration increases by a factor of 4 [4].

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

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

We previously reported the isolation and purification of MMO from *Thiobacillus thiooxidans* [12]. In this study we used *Rhodococcus rhodochrous* [13] to induce MMO by supplying with MM gas as a substrate, and the resulting enzyme was isolated, purified and characterized.

*Corresponding author
Tel: +82-42-869-3964 Fax: +82-42-869-3910
e-mail: jwyang@kaist.ac.kr

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). Low range protein molecular markers from Bio-Rad (Hercules, CA, USA) were used as protein molecular weight standards. For FPLC operation, DEAE-Sephacel and Superose 12 column were purchased from Pharmacia LKB (Uppsala, Sweden). *Rhodococcus rhodochrous IGTS8* was obtained from the American Type Culture Collection (ATCC).

Media and Fermentation Conditions

R. rhodochrous was cultivated in a medium containing (g/L): KH_2PO_4, 2.44; $Na_2HPO_4 \cdot 12H_2O$, 14.05; Na_2SO_4, 0.028; NH_4Cl, 2; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.001; and $FeCl_3$, 0.0006 (g/L); and glycerol 10 mL. Fermentation was carried out at 30°C and pH 6.7. To observe growth characteristics, 500-mL flasks containing 100 mL of the medium were used, and for mass cultivation

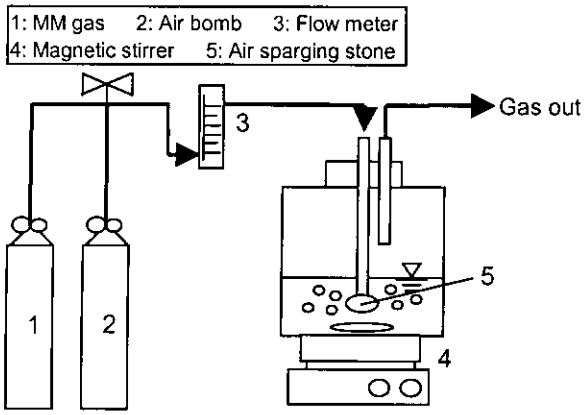

Fig. 1. Schematic diagram of a large-scale cultivation system.

a 10-L distillated water bottle (Nalgene Co., USA) filled with 4 L of the medium. As shown in Fig. 1, the screw cap was bored to fit the inlet and outlet gas tubes. An air sparging stone was connected to the end of inlet gas tube and submerged in the medium. Samples were taken from the valve located on the bottom and analyzed. Normally air was supplied at 150 mL/min until growth reached the stationary phase, when it was substituted with 4,800 ppm of MM in nitrogen at 20 mL/min for 40 h to induce MMO. Cell growth was determined by measuring the optical density of the culture media at 600 nm (OD_{600}) using an UV spectrophotometer 8562A (Hewlett Packard, USA).

Enzyme Purification Procedure

Harvested cells were washed twice and suspended in 15 mL of 10 mM Tris/HCl, pH 8.2 at 4°C, and sonicated at 3 min interval for 15 min. Cell debris was removed 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 × 80 mm) and 500 mM KCl in 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 part of this extract was then applied to a Superose 10/30 column (25 × 300 mm). At each purification step the total protein concentration was measured by the Bradford method; bovine serum albumin was used as the standard.

Analyses

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

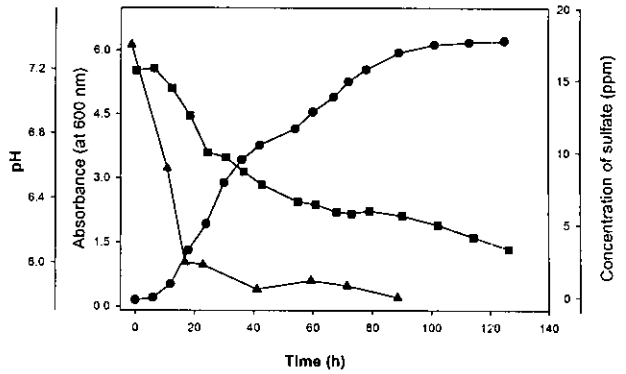

Fig. 2. Time course of the cultivation of *Rhodococcus rhodochrous* IGTS8. ●, cell growth; ■, pH; ▲, sulfate.

SDS-PAGE

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

Effect of Salts and Organic Solvents

A change of 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. Enzyme activity was also measured on the addition of 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

The flask cultivation was carried out to identify the growth characteristics of *Rhodococcus rhodochrous* IGTS8 and to determine when the effective induction of methyl mercaptan oxidase (MMO) was initiated. Sulphate was taken up by the cells after 30 h and cultivation the OD_{600} was 5.7 after 80 h. During the cultivation, the pH decreased from 7.2 to 6.1, probably because the carbon dioxide produced by the oxidation of glycerin was rapidly transformed to carbonic acid (HCO_3^-) at around pH 7.0 (Fig. 2). After 40 h, however, the pH was 6.5 and had stabilized because carbon dioxide was being produced in inverse proportion to the carbonic acid [14]. Consequently, MM was being introduced at

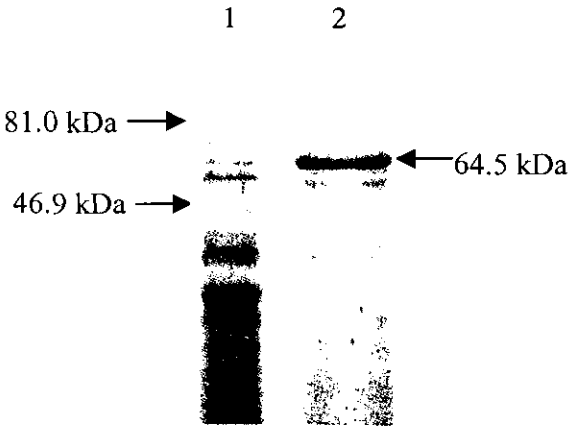

Fig. 3. Silver-stained SDS-PAGE of MMO. Arrows: molecular weight markers, lane 1: crude extract, and lane 2: after DEAE-Sephacel chromatography.

20 mL/min for 40 h, when carbon and sulphate source were exhausted.

The Enzyme Purification and Characteristics

A purification summary of the MMO induced under the above condition is shown in Table 1. An eluate from gel filtration remained a high specific activity. A distinct band was observed after DEAE-Sephacel column chromatography of molecular weight 64.5 kDa (Fig. 3), which was similar to that from *T. thiooxidans*, with the molecular weight of 68.1 kDa [12]. On the other hand, those from *Hyphomicrobium* EG and *T. thioparus*, induced with dimethyl sulfide (DMS) were 40-50 and 40 kDa, respectively [5,7]. According to these results, it appears that the properties of MMO will be different from strain types and substrates. Additionally, the optimal temperature of this enzyme was determined to be 60°C, which was relatively high compared to the growth temperature of 30°C.

Effect of Salts and Organic Solvents

The salt effect on enzyme stability is shown in Fig. 4. When the concentration of $(NH_4)_2SO_4$ was 100 mM the maximum enzyme activity increased 1.2-folds. For NH_4Cl, the activity decreased in proportion to the concentration. In the cases of K_2SO_4 and NaCl, the enzyme activity was inhibited until the concentration reached 20 mM and gradually recovered, and the enzyme was relatively unaffected by KCl. From *T. thioparus* TK-m a different salt effect was observed on MMO which was inhibited by 53% after adding either 10 mM $(NH_4)_2SO_4$ or 10 mM of NH_4Cl [5]. From this result 100 mM $(NH_4)_2SO_4$ or 100 mM NaCl would appear suitable for the enhancement of enzyme stability and activity.

When the purpald/formaldehyde color reaction is used in detection system, purpald and MMO should be coated on a detection membrane which is exposed to the mouth air. Purpald dissolved in a organic solvent is coated by evaporation: the MMO immobilized in advance contacts with the organic solvent [15]. Therefore, the choice of an adequate organic solvent is very important for the stabilization of enzyme activity. After adding acetone and ethanol by 10% (v/v), almost all enzyme activities disappeared. In the case of glycerin and methanol, the enzyme was inhibited by 77 and 67% (data not shown), and therefore, methanol was chosen as the organic solvent for purpald coating.

Table 1. Purification summary of methyl mercaptan oxidase from *R. rhodochrous* IGTS8

	Total units (nmol/min)	Total protein (mg)	Specific activity (units/mg-rotein)	Purification ratio	Recovery yield (%)
Crude extract	32.4	73.3	0.44	1	100
DEAE-Sephacel	27.8	0.30	92.7	210.7	85.8
Gel filtration	27.0	0.089	303.4	689.5	83.3

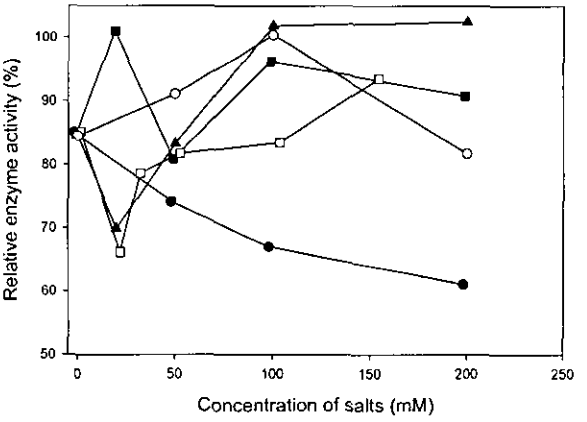

Fig. 4. Effect of salt concentrations on enzyme activity. ●, NH_4Cl; ○, $(NH_4)_2SO_4$; ■, KCl; □, K_2SO_4; ▲, NaCl.

CONCLUSION

When all of the methyl mercaptan in the mouth air is oxidized by the enzymatic reaction, an identical concentration of formaldehyde would be produced. To detect this formaldehyde, we developed the purpald coloring method and tried to determine the detection threshold by eye at the nano-molar level. When formaldehyde was added to the test solution in the concentration range of 10-50 nmol, the absorbance at 550 nm increased proportionally [12]; the slope and correlation coefficient were 0.0105 and 0.99, respectively with a high degree of linearity. The violet color was distinctly visible at more than 30 nmol, and the purpald based coloring method is believed to have a sufficient sensitivity to quantify bad breath symptom.

Acknowledgements The authors appreciate the partial support given by the Hanmi Venture Capital Corporation.

REFERENCES

[1] Rosenberg, M. and C. A. G. McCulloch (1992) Measurement of oral malodors: current methods and future prospects. *J. Periodontol.* 63: 776-782.

[2] Hennet, P. R., B. Delille, and J. L. Davot (1998) Oral malodor measurements on a tooth surface of dogs with gingivitis. *Am. J. Vet. Res.* 59: 255-257.

[3] Quirynen, M., C. Mongaridini, and D. van Steenberghe (1998) The effect of a 1-stage full-mouth disinfection on oral malodor and microbial colonization of the tongue in periodontitis patients: A pilot study. *J. Periodontol.* 69: 374-382.

[4] Preti, G., G. R. Huggins, and J. Tonzetich (1978) Method of predicting and determining ovulation by monitoring the concentration of volatile sulfur-containing compounds present in mouth air. *US patent* 4,119,089.

[5] Gould, W. D. and T. Kanagawa (1992) Purification and properties of methyl mercaptan oxidase from *Thiobacillus thioparus* TK-m. *J. Gen. Microb.* 138: 217-221.

[6] De Bont, J. A. M., J. P. Dijken, and W. Harder (1981) Dimethyl sulphoxide and dimethyl sulphide as a carbon, sulphur and energy source for growth of *Hyphomicrobium* S. *J. Gen. Microb.* 127: 315-323.

[7] Suylen, G. M. H., P. J. Large, J. P. Van Dijken, and J. G. Kuenen (1987) Methyl mercaptan oxidase, a key enzyme in the metabolism of methylated sulphur compounds by *Hyphomicrobium* EG. *J. Gen. Microb.* 133: 2989-2997.

[8] Quesenberry, M. S. and Y. C. Lee (1996) A rapid formaldehyde assay using purpald reagent: Application under periodation condition. *Anal. Biochem.* 234: 50-55.

[9] Lee, C. H. and C. M. Tsai (1998) Quantification of bacterial lipopolysaccharides by the purpald assay: measuring formaldehyde generated from 2-keto-3-deoxyoctonate and heptose at the inner core by periodate oxidation. *Anal. Biochem.* 267: 161-168.

[10] Muginova, S. V., I. A. Veselova, and T. N. Shekhovtsova (1999) Kinetics and pathways of oxidation of *o*-phenylenediamine, 3,3-dimethoxybenzidine, and 3,3,5,5-tetramethylbenzidine with hydrogen peroxide, catalyzed with horse radish peroxidase immobilized on various supports. *Rus. J. Appl. Chem.* 72: 803-810.

[11] Muginova, S. V., N. A. Akovbyan, and T. N. Shekhovtsova (1999) Use of paper as a support for immobilized peroxidase in a test method for the determination of mercury(II) traces. *J. Anal. Chem.* 54: 569-573.

[12] Kim, S.-J., H.-J. Shin, D.-S. Lee, and J.-W. Yang (2000) Isolation and purification of methyl mercaptan oxidase from *Thiobacillus thiooxidans* for detection of mercaptans. *Kor. J. Biotechnol. Bioeng.* 15: 145-149.

[13] Kim, C. S. (1998) *Characterization of Dibenzothiophene Degradation by* Rhodococcus rhodochrous *IGTS8.* MS Thesis. KAIST. Taejon Korea.

[14] Borowitzka, M. A. and L. J. Borowitzka (1988) *Microalgal Biotechnology.* Cambridge University Press, NY, USA.

[15] Kwon, S. K. (1994) A study on the preparation of polyurethane diagnostic membrane for urine glucose test. *J. Kor. Ind. Chem.* 5: 975-980.

[Received July 11, 2000; accepted October 28, 2000]