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原著論文

Analyzing Malodorous Compounds and Characteristics of Methanethiol Decomposing Bacteria

Hiroshi SATO[1)*], Susumu TAKAKUWA[2)] and Yukihiko NAKASHIMA[3)]
([1)]Dept. of Pharmacy, Faculty of Pharmaceutical Sciences, Nagasaki International University, [2)]Dept. of Food and Nutrition, Kyoto Women's University, [3)]Faculty of Pharmaceutical Sciences, Fukuoka University, *Corresponding author)

悪臭成分の分析とメタンチオール分解菌の特徴

佐　藤　　博[1)*]，高　桑　　進[2)]，中　島　幸　彦[3)]
([1)]長崎国際大学 薬学部 薬学科、[2)]京都女子大学 家政学部、[3)]福岡大学 薬学部 薬学科、*連絡対応著者)

要　旨

メタンチーオールは硫黄系有機化合物であり、ppb のオーダーでも感知できる強い臭気を有する。また、毒性を有し水溶性である。したがって、水溶液中のメタンチオールを簡便に測定することは非常に重要である。

これまでの研究で、水溶液中のメタンチオール濃度を測定するために既存の分析方法を改良して本研究に応用できることを報告している。本分析法は、5,5ジチオビス（２ニトロ安息香酸、DTNB）がメタンチオールと反応した生成物を 412nm の吸光度で測定するというエールマン反応に基づくものである。メタンチオールの濃度は、吸光度に比例し、検量線にて容易に定量できた。そして、微生物分解によるメタンチオール濃度の減少量を測定することにより、これらに適応可能かの評価を行い、水溶液中のメタンチオールを測定することが有効であると判断できた。

本研究では、土壌から分離した菌を有効に利用することにより、脱臭商品や効率的な脱臭装置の開発が期待される。そこで、分離した TOS−35 などのメタンチオール分解菌のフィージビリティスタディを行った結果、脱臭に関して可能性がある事が確認できた。

キーワード

エールマン反応、悪臭成分、フィージビリティスタディ

Abstract

Methanethiol is a corrosive gas with a strong odor, and has the poisoning action resembled to hydrogen sulfide. It has the environmental toxicity and is soluble in water; therefore, it is very important to develop an easily measurable method for methanethiol in aqueous solutions. In this series of research, a fast and convenient analytical method was developed and applied to determine the concentration of methanethiol in aqueous solutions. It is based on the Ellman reaction, a reaction of methanethiol with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and the measurement of the absorbance of the product, namely quinoid anion, at 412 nm. The concentration of methanethiol was directly proportional to the absorbance at 412 nm, and a linear standard calibration curve was obtained in a certain range of concentration. In this research, an applicability of the new measurement method for the screening of methanethiol degrading bacteria was evaluated and it was found to be effective for measuring methanethiol in aqueous solution. As a result of the screening, TOS−35 (*Alcaligenes faecalis*) demonstrated the highest ability of decomposing methanethiol and two feasibility studies were done in order to test the effectiveness of this bacterium.

Key words

Ellman reaction, malodorous compounds, feasibility studies

1. Introduction

In this series of research, methods for sampling and analyzing malodorous compounds were examined and the malodorous compounds were analyzed systematically.[1] As a part of this research, an advantageous method for analyzing major malodorous compound (methanethiol[2]) was investigated by using the Ellman reaction to determine methanethiol (MT) in the liquid phase.[3] The method was applied for screening MT decomposing bacteria and *Alcaligenes faecalis* was found to be highly effective in decomposing MT.[4),5)] In the research, the characteristics and application of this bacterium for microbial deodorization were investigated.

Some researchers, like Kanagawa and Mikami,[6] had purified the enzyme in MT and reported the results; however, they had not done it in details due to the instability of the enzymatic activity. From now on, by using the strain with high MT decomposing activity, from the point of not only the strain of the enzyme but also the level of enzyme, the characteristics of MT decomposing bacteria must be examined further.

2. Materials and Methods

Reagents and chemicals

Standard reagents were purchased from Wako Pure Chemical Industries (Japan) and Tokyo Kasei Kogyo Co. (Japan). All other chemicals used were commercially available and of chemically pure grade. Distilled water, purified with a Milli-QSP system (Millipore, Milford, MA, U.S.A.), was used for the preparation of all aqueous solutions.

Ellman reaction with MT with DTNB

Ten mM 5,5′-dithiobis (2-nitrobenzoic acid), 3-carboxy-4-nitrophenyl disulfide (DTNB, Sigma), was dissolved in 100 mM phosphate-buffered saline (pH 7.0)−0.85% NaCl (PBS) and it was used as a reactive reagent. The concentration of MT in the liquid phase was determined using Ellman method[7] modified by Grassetti and Murray,[8] and Ishiguro.[9] The reaction mixture in the vial was filtered through a Millipore filter (pore size : 0.45 μm), and 20 μl of 10 mM DTNB added to 3 milliliters of the filtrate and vortexed. The absorbance of the mixture at 412 nm was measured with a spectrophotometer. A quantitative curve was obtained using sodium methylsulfide as a standard. A linear relationship was observed between 0 and 2.5 mM MT under the analytical conditions explained above.

Analytical method for gas and liquid phase

Ellman method was used for the liquid phase and GC method was used for the gas phase. The analysis of the concentration of methanethiol in the gas phase was carried out in order to inspect whether the concentration of methanethiol was measured precisely by Ellman method. First, the sample gas was bubbled into the aqueous solution in a vial and equilibrated. Then, after 10 minutes and also after 24 hours methanethiol in the gas phase was measured by a gas chromatography with a FPD detector. Hydrogen sulfide in the gas phase was also measured after 10 minutes and after 6 hours. Methanethiol-and hydrogen sulfide-decomposing activity by the bacteria are indicated with the residual amount of methanethiol and hydrogen sulfide in the gas phase.

Determination of MT decomposing activities with Thiobacillus thioparus TK-m

As MT-degrading bacteria, *Thiobacillus thioparus* TK–m, which Kanagawa and Mikami[6] isolated, was used. After cultured bacteria were centrifuged, the aggregate was washed in 100 mM PBS (pH 7.0) twice, suspended in 50 ml of 100 mM PBS in a 100 ml vial, and used as test bacteria. Subsequently, 5,000 ppm MT gas (Japan Air Liquid) was diluted 50-fold with synthetic air to prepare 100 ppm MT gas. The gas was placed in the vial, and bubbling was performed for 10 minutes at a flow velocity of 100 ml/min. MT in the liquid phase in the vial was serially measured using Ellman reaction, and MT in the gaseous phase was serially measured using GC. GC (HP5890 : Hewlett Packard) was performed using a DB–17 column (J&W) under the following conditions : flow velocity, 1 ml/min ; detector, FPD (200℃) ; and infusion orifice temperature, 150℃.

Determination of concentration of sulfur-containing compounds

Volatile sulfur-containing compounds were analyzed by GC using a Flame photometric detector (FPD) as a detector.[10] Hydrogen sulfide and methanethiol at a concentration of 100 ppm in nitrogen were used as standard gases for the preparation of our calibration curves.[11]

Feasibility study

The feasibility study was conducted with real food (Fig. 1). Five sets of the followings were prepared : 1) vegetable (cabbage and bean-sprout 2) fish and 3) vegetable and fish. Each item was placed in a 500 ml sized glass container and 4 various microorganisms and commercial odor absorbent were sprayed on each : 1) TOS–35 2) IFO 12697 3) Commercial product A 4) Commercial product B and one set was left as a control. A smell indicator was used to measure the residual concentration of gas in the glass container.

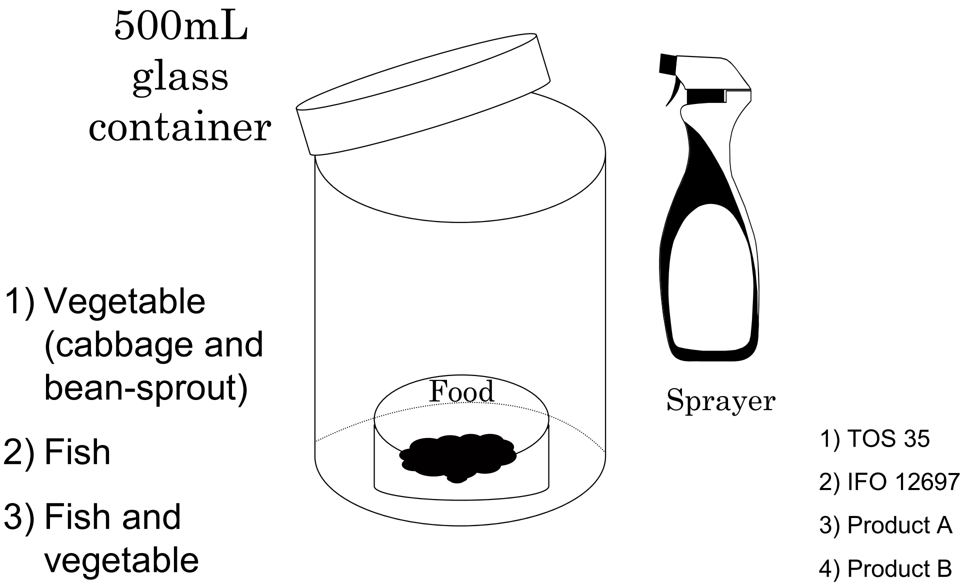

Fig. 1　Feasibility study

3. Results and Discussion

Scheme of Ellman reaction with MT and DTNB

In order to measure the concentration of MT in liquid phase, Ellman reaction was used. The scheme of Ellman reaction with MT and DTNB is shown in Figure 2. (1) shows the methanethiol separating hydrogen and methanethiol ion in the aqueous solution. At (2), methanethiol ion reacts with DTNB compound. Solution changes its color to yellow after the reaction as shown by (3) and (4). Then, by using spectrophotometer, the concentration of methanethiol in the aqueous solution can be measured.

Applications

Figure 3 shows the parallel between the time-course of the degradation of methanethiol by *T. thioparus* TK–m. Left graph shows the time course of the liquid phase. Right graph shows the time course of the gas phase. As a result, Ellman method was very useful for measuring the liquid phase ; however, the analysis showed that fairly precise data about the degradation of methanethiol

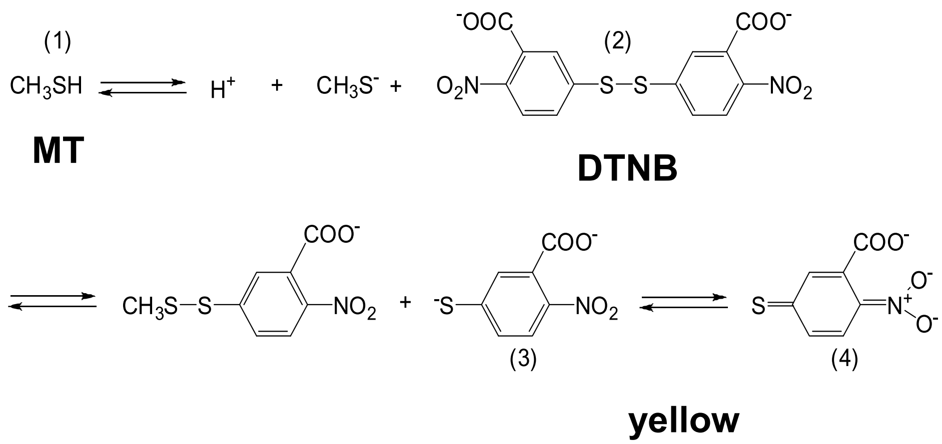

Fig. 2 Scheme of Ellman reaction with Methanethiol and DTNB

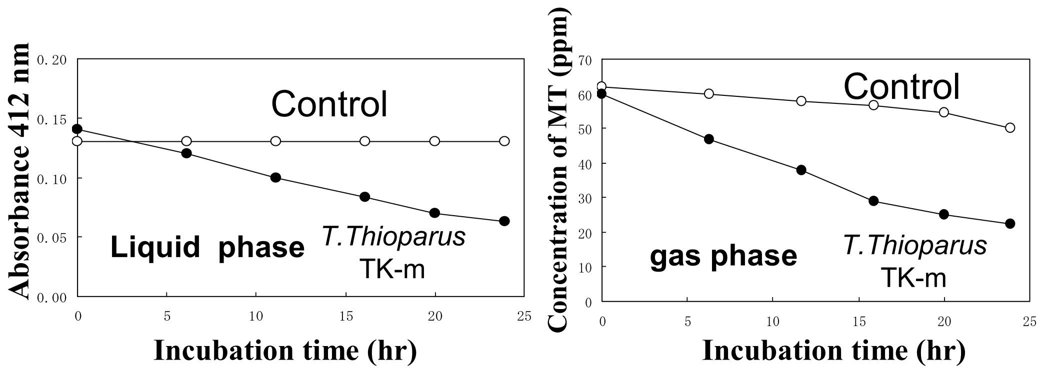

Fig. 3 Time course of the degradation of Methanethiol by *T. thioparus* TK–m
Methanethiol in the liquid phase (left graph) was determined by the Ellman reaction and Methanethiol in the gas phase (right graph) was determined by GC.

could be obtained by measuring the gas phase alone. Nonetheless, Ellman method is more advantageous than GC with respect to simple pretreatment, sample treatment, and measurement time. Therefore, the quantification of MT in the liquid phase using Ellman reaction may be an alternative method replacing GC.

Sampling area of isolated bacteria

An analysis on soil samples from various areas and sources in Japan was done. A total of 272 heterotrophic bacterial strains were isolated from 71 soil samples by DMS-enrichment culture. The MT-decomposing activity of the isolated strains was examined and 28 strains exhibited this activity. None of the heterotrophic bacteria isolated from pigpen, cowshed and henhouse were capable of decomposing MT, yet, it is important to note that the bacteria that are capable of decomposing methanethiol are found in general environments such as park and riverside.

Decomposing activity level and generic assignment

Table 1 shows the comparison of methanethiol and hydrogen sulfide decomposing activity level among different bacterial genera. As the heterotrophic bacteria isolated from the soil samples were analyzed, the majority of them were *Bacillus* and *Pseudomonas*. Bacteria with high methanethiol decomposing level also showed high, or at least some, hydrogen sulfide decomposing activity. However, interestingly, some bacteria such as TOS–64 and TOS–182 showed the contradictory result.

Moreover, *Pseudomonas* and *Alcaligenes* like TOS–35 generally have very high methanethiol and hydrogen sulfide decomposing activity level. TOS–35 was found at the riverside. Once again, MT decomposing bacteria are found in general environments like riverside and forest.

Characteristics of TOS–35

Characteristics of TOS–35 are as follows: its shape is short rod, it is a gram negative bacterium, and its growth pH is 5.0–11.5. Al-

Table 1 Decomposing activity level and generic assignment

Strain	MT	HS	generic assignment	Strain	MT	HS	generic assignment
TOS–1	+++	+	*Bacillus*	TOS–38	+++	+	*Pseudomonas*
TOS–3	+++	++	*Bacillus*	TOS–52	+++	+++	*Pseudomonas*
TOS–5	+++	+	*Bacillus*	TOS–54	+++	++	*Pseudomonas*
TOS–7	+++	+	*Bacillus*	TOS–55	+++	+++	*Pseudomonas*
TOS–32	++	+	*Bacillus*	TOS–101	++	+	*Pseudomonas*
TOS–60	+	+	*Bacillus*	TOS–104	+	+++	*Pseudomonas*
TOS–64	+	+++	*Bacillus*	TOS–133	+++	+++	*Pseudomonas*
TOS–72	+	+	*Bacillus*	TOS–227	++	++	*Pseudomonas*
TOS–83	+	+	*Bacillus*	TOS–276	++	++	*Pseudomonas*
TOS–93	+++	++	*Bacillus*	TOS–283	++	++	*Pseudomonas*
TOS–182	+	+++	*Bacillus*	**TOS–35**	**+++**	**+++**	*Alcaligenes*
TOS–207	++	+	*Bacillus*	TOS-130	+++	+	*Enterococcus*
TOS–211	+	+	*Bacillus*				
TOS–213	++	+	*Bacillus*				
TOS–230	++	+++	*Bacillus*				
TOS–254	+	+	*Bacillus*				

Abbreviations for activities are as follows : MT, methanethiol decomposition ; HS, hydrogen sulfide decomposition. Symbols indicate the decomposing
activities (%) : +, less than 49% ; ++, 50–89% ; +++, more than 90%

so, interestingly, this bacterium emits strawberry-like smell and it means this bacterium could be used for deodorization and also possibly as an ingredient of air fresheners.

MT oxidase specific activity of TOS–35

Figure 4 shows the correlation between the cell growth and MT oxidase specific activity level of TOS–35. First, both components did not show any changes in their traits ; however, as the growth curve started increasing steadily, the specific activity level started to increase as well. Then, when the cell growth hit its maximum level, the specific activity also reached its highest level. Also, the pH of the solution at the beginning was 7, yet, as it was measured again after 6 hours, it was 8. It proves that as it grows, TOS–35 secretes some kind of substance that increases the pH level. Next, the MT oxidase activity level of TOS–35 in various solutions with different pH levels was examined. As a result, TOS–35 showed the highest activity level in the solution with pH level of 9.0 (Fig. 5).

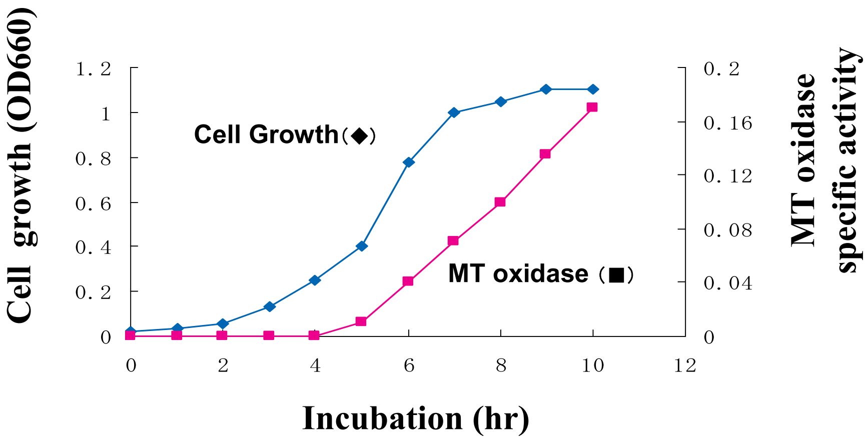

Fig. 4 The growth curve and Methanethiol oxidase specific activity of TOS–35

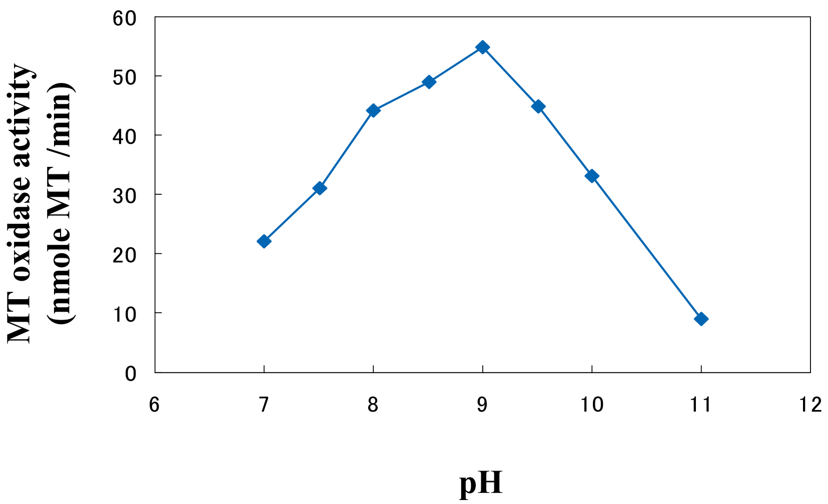

Fig. 5 Optimum pH level for oxidase activity of TOS–35

In order to isolate MT decomposing enzyme from TOS–35 that showed the highest MT decomposing activity, the bacterium was destroyed with using ultrasonication and enzyme activity of cell free extract was measured ; however, after isolation, MT decomposing activity level became lower and got unstable. Therefore, since this enzyme was considered to be membrane-bounded, the research was conducted by using suspension which contained cells after washing with phosphate buffer (pH 7.0). At this point, there is no further information about this bacterium and it is still to be investigated.

Result of the feasibility study

Odor of each container was evaluated one by one after 10 minutes, 60 minutes and then 3 days and it was rated according to the standardized evaluation scale. As a result, TOS–35 was found to be effective for vegetable and fish group and vegetable only group; however, it was not effective for the fish-only group. It explains that TOS–35 can decompose sulfur compounds but cannot or has a weak ability to decompose trimethylamine which is contained in fish (Table 2). TOS–35 showed very high level of decomposition for hydrogen sulfide and MT ; however, it did not show

Table 2 Examination of Deodorant

1 : very weak～ 5 : very strong

Samples		TOS35	IFO12697	A	B	Control
vegetable＋fish						
after	10 min	1	4	1	4	4
	60 min	5	4	2	5	5
	3 days	3	4	1	4	5
vegetable						
after	10 min	1	3	1	4	4
	60 min	5	4	2	5	5
	3 days	3	4	1	4	5
fish						
after	10 min	3	4	4	4	4
	60 min	5	5	5	5	5
	3 days	5	5	5	5	5

*Commercial product A : bioC, Commercial product B : BSEodoclean

Table 3 Examination of deodorant

Examination of deodorant (unit : ppm)

Deodorant	TOS35	A	Control
Hydrogen sulfide	＜0.1	0.5–1.0	1.5–2.5
Total-MT	＜0.25	1.0	1.7
Ammonia	13	3	19
Amine	10	3	19
Sensory test	3	3	5
Evaluation	○	○	

1 : very weak～ 5 : very strong
*Commercial product A : bioC

much result with ammonia and amine (Table 3).

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