A Microbial Sensor for Highly Sensitive and Automatic Determination of Sulfite in Foods and Food Materials: 
Kinetic Study of the Sulfite Dehydrogenase-catalyzed Reaction in the Microbial State of Thiobacillus thiooxidans*

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A highly sensitive and automatic microbial sensor apparatus has been constructed with Thiobacillus thiooxidans and employed for sulfite analysis of food materials in practical fields including the starch industry, thus we tried to obtain enzymatic bases on the T. thiooxidans-catalyzed reaction. Based on the spectrophotometric observation of ferricyanide and the proportional dependency of the initial velocity on the concentration of T. thiooxidans, the sulfite oxidation was confirmed to be a microbial dehydrogenase-catalyzed reaction, of which the optimal pH is 7.5. The kinetic parameters, the Michaelis constant \( K_m \) and the maximum velocity \( V \) were evaluated to be \( 1.0 \pm 0.1 \) mM and \( 0.065 \pm 0.032 \) mM/min/mg protein, respectively. The concentration of ferricyanide was proportional to the initial velocity in the range of 0 to 2 mM. These results prove that the microbial sensor apparatus developed is satisfactory to determine the enzymatic bases for sulfite oxidation.

Sulfite and its salt in some different forms have been widely used in various kinds of food and food materials including starch, saccharides and their derivatives for preservation, bleaching, antioxidation treatment and other industrial purposes. The level of sulfite addition for such materials is controlled under food hygiene regulations.\(^{1)}\) For the determination of total sulfite concentration in foods and food materials, modified Rankine’s method has been generally employed in the practical fields of most industrial countries including Japan.\(^{2,3)}\) However, in the Rankine’s method, very sophisticated skill and much labor time must be taken for actual operation, especially some processes such as distillation. Under these existing circumstances, some new techniques having a simple and rapid operation for the determination of sulfite have been much anticipated.

Recently, a highly sensitive, convenient, and automatic apparatus for the determination of sulfite has been constructed by our research group using a microbial sensor with T. thiooxidans. It has been actually employed in many practical fields including the starch industry. In this microbial sensor apparatus, a new device, some membrane, fixed with T. thiooxidans in the microbial state, is employed for the main part. T. thiooxidans is known to be a typical sulfur-oxidizing bacterium (obligately chemolithotrophic) which oxidizes some inorganic sulfur compounds, such as sulfite and its salts, to get energy for growth.\(^{5)}\) Nakamura et al. have found and purified the enzyme sulfite dehydrogenase, which catalyzes sulfite oxidation to sulfate, whereas another enzyme system has been found from T. thioparus and T. denitrificans, in which two enzymes, adenosine 5’-phosphosulfate (APS) reductase and adenosine 5’-trihydrogen diphosphate (ADP) sulfurylase, are concerned in the sul-
Here, the authors have taken much notice of T. thiooxidans, of which sulfite dehydrogenase catalyzes specifically for a substrate sulfite, and obtained an idea to make a new device for determination of sulfite using an oxygen electrode which measures oxygen consumed by the sulfite oxidation reaction. In previous studies, the determination of sulfite in various foods and food materials is successfully carried out using a microbial sensor apparatus constructed by the authors, of which the results are in good agreement with those obtained using Rankine’s method. Thus, it was shown that the microbial device is simple, reliable, rapid without distillation, and more sensitive than Rankine’s method. However, no basic examinations from the viewpoint of enzyme-catalyzed reaction have been carried out for the microbial sensor device developed. Thus, in this study, we tried to characterize the sulfite dehydrogenase-catalyzed reaction in the microbial state of T. thiooxidans and obtain basic data on the enzymatic properties of the microbial sensor developed.

MATERIALS AND METHODS

**Thiobacillus thiooxidans.** A microorganism, T. thiooxidans NCI11773, was produced using the following modified procedures of the fermentation method described by Nakamura et al. The medium was composed of: Na₂S₂O₃·5H₂O, 5.0 g; KH₂PO₄, 3.0 g; (NH₄)₂SO₄, 2.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.25 g; FeSO₄·7H₂O, 0.01 g; and Na₂MoO₄·2H₂O, 0.75 mg in 1 L of distilled water. Each 1.0 mL of seed culture was incubated with two 1-L flasks containing 100 mL of medium. The flasks were incubated at 30°C for 72 h on a reciprocal shaker, and 10 mL of the cultured medium (200 mL) was transferred into two 1-L flasks containing 100 mL of medium. The flasks were incubated at 30°C for 48 h on a reciprocal shaker. The cultured medium (200 mL) was transferred into a jar fermentor containing 3 L of medium. The cultivation was performed at 30°C for 48 h. The culture was agitated at 300 rpm and aerated with 1.5 v.v.m. The pH was automatically maintained at 3.0 by the addition of 5% (w/v) sodium hydroxide. After fermentation, the microbe T. thiooxidans was washed several times with a 0.1 M phosphate buffer (pH 5.5) using a centrifuge at around 8000 rpm and was then suspended in the buffer to use as preparation for the microbe-catalyzed reaction.

**Substrate.** Disodium sulfite, a guaranteed grade, was purchased from Katayama Chemical Co., Ltd. and used without further purification as the first substrate. Potassium ferricyanide, a guaranteed grade, was obtained from Nacalai Tesque Inc. and employed without further purification as the second substrate.

**Chemicals.** Tris (hydroxymethyl) aminomethane, a surfactant Triton X-100, and EDTA Na₂·2H₂O were purchased from Nacalai Tesque Inc. and used without further purification. Cytochrome c, a biochemical grade used, was the product of Wako Pure Chemicals Co., Ltd. Other chemicals and reagents commercially available were also of guaranteed grade and used without further purification.

**Assay of the microbial T. thiooxidans-catalyzed reaction.** The enzymatic reaction was measured spectrophotometrically on the basis of spectrum change that occurred by ferricyanide reduction using the modified procedures of the assay method of Nakamura et al. as described in RESULTS AND DISCUSSION. A Tominaga centrifuge type-A and a Shimadzu UV-1600 spectrophotometer were used for these assay procedures. The plastic-made centrifuge tubes with screw caps were purchased from Bio-bik Ina Optika Co., Ltd. Based on the initial velocity obtained from the slope of the time curves, the kinetic parameters and their standard deviation were calculated by means of an NEC 9801 personal computer with the software Bio-Graph (Kyoto Soft Co.).

RESULTS AND DISCUSSION

**Assay of the microbial T. thiooxidans-catalyzed reaction.**

In the assay method previously described, reasonable time curves could not be obtained because the enzymatic reaction was not properly stopped at an adequate reaction time to observe a reaction proceeding as a function of time, time...
course or time curve. So, in this study, we first examined at first how to stop the enzymatic reaction adequately; that is, using acid, alkali, cooling treatments and others. Heating and centrifuging procedures were found to give good reaction time-curves, thus the assay was performed as follows, as summarized in Fig. 1: at first, 0.50 mL of 0.2 M McIlvaine buffer, pH 7.5, 0.25 mL of 1 mM potassium ferricyanide, 0.25 mL of 0.5% (w/v) Triton X-100, 0.25 mL of distilled water, and the microbe (T. thiooxidans) suspension were mixed in a 2 mL plastic tube with a screw cap. A substrate solution, 0.25 mL sulfite, was added to start the enzyme-catalyzed reaction at 25°C. After an adequate time interval, the reaction mixture in the tube was kept in boiling water for 1 min to stop the reaction, and centrifuged at 15,000 × g for 10 min. Finally, absorbance of the supernatant was determined at 420 nm, of which the change is refers to reduction of ferricyanide due to the sulfite dehydrogenase-catalyzed reaction in the microbial state of T. thiooxidans. These assay procedures on the sulfite dehydrogenase-catalyzed reaction in the microbial state of T. thiooxidans are summarized in Fig. 1.

**Effects of concentration of the T. thiooxidans suspension on the velocities of the enzyme-catalyzed reaction.**

Reaction time curves were obtained using the assay procedures described above, and some examples are shown in Fig. 2, where the absorbance at 420 nm decreases with reaction time observed in
the range of 0 to 10 min, and is on a straight line, which gives the initial velocity of the reaction. The effects of concentration of the T. thiooxidans suspension on the initial velocities were examined for the substrate sulfite. The results are shown in the Fig. 2-inset, where the concentration of the T. thiooxidans suspension stands for absorbance at 660 nm. The initial velocities evaluated from the time curves are plotted against the concentration of the T. thiooxidans, clearly indicating that the dependency is linear in the range of the microbial concentration examined; that is, the velocity is proportional to the concentration of the T. thiooxidans.

The results of the spectrophotometric examinations indicate that decrease in absorption at 420 nm caused by the microbial suspension of T. thiooxidans refers to the conversion of ferricyanide, Fe$^{3+}$ form, to ferrocyanide, Fe$^{2+}$ form (data not shown). Thus, based on the following observations; velocity dependency on the microbe concentration, absorption spectrophotometry and the basic data on the purified enzyme preparation, the experiments described below were carried out under the assumption that the absorption change produced by the microbial state of T. thiooxidans refers to the sulfite dehydrogenase-catalyzed reaction.

The optimal pH for the sulfite dehydrogenase-catalyzed reaction.

pH-Dependence of the sulfite dehydrogenase-catalyzed reaction in the microbial state of T. thiooxidans was examined to obtain the optimal pH in the range of 5.0 to 8.5, where two kinds of buffers were used, McIlvaine buffer for pH 5.0 to 8.0 and KH$_2$PO$_4$-Na$_2$B$_4$O$_7$ buffer for pH 8.0 to 8.5. From the reaction time curves, the initial velocities $v$'s were evaluated and plotted against pH as illustrated in Fig. 3, indicating that the optimal pH for the sulfite dehydrogenase-catalyzed reaction in the microbial state is 7.5, thus the experiments described below were carried out at pH 7.5. Over pH 8.5, the microbe T. thiooxidans was destroyed (dissolved) and the enzyme-catalyzed reaction could not be observed by the assay procedures developed.

Optimal pH has been analyzed for some enzyme preparations purified from different microbial samples with a substrate sulfite as follows, on sulfite oxidases, pH 8.3 for T. denitrificans and 8.0 for T. thioparus,$^{8,9}$ and on sulfite dehydrogenase, pH 7.5 for T. thiooxidans$^{7}$; thus, the optimal pH obtained here, 7.5, is a reasonable value for this microbial enzyme system. On the other hand, the optimal pH of biosensor using the microbe T. thiooxidans has been evaluated to be 6.0,$^{24}$ which is not the same but a little different from the value (7.5) obtained here. At this stage, there is no idea to explain this difference, but immobilization of the microbe to a glass electrode (sensor) could be one of the reasons.

Effects of the concentration of a substrate sulfite on the initial velocities of the enzyme-catalyzed reaction.

The time curves on the sulfite dehydrogenase-catalyzed reaction were observed under various concentrations of a substrate sulfite. Some typical examples are illustrated in Fig. 4, where 0 to 10 mM of substrate were examined using the assay procedures described. The initial velocity $v$ was obtained from each time curve and plotted against the concentration of substrate $[S]_o$ as shown in Fig. 4-inset, giving a good saturation curve on $v$-[S]$_o$. Based on the $v$-[S]$_o$ plot, the kinetic parameters, the Michaelis constant $K_m$ and the maximum velocity $V$ can be evaluated using the two kinds of
linear plot. For example, a linear plot $[S]_o / v$ against $[S]_o$ gives the kinetic parameters for the sulfite dehydrogenase-catalyzed reaction in the microbial state of *T. thiooxidans* as follows; $K_m$ 1.0 ± 0.1 mM and $V$ 0.065 ± 0.032 mM/min/mg, where ± means the standard deviation. In this study, the molar activity $k_o$ could not be evaluated because the concentration of the enzyme in the reaction mixture cannot be actually obtained. Using the steady-state kinetics, the $K_m$ value has been evaluated for the enzyme preparations purified from some different bacterial samples with sulfite as the substrate; for example, 0.5 mM for *T. denitrificans* and 88 μM for *T. thioparus*. On *T. thiooxidans*, some kinetic studies have been carried out: the Michaelis constant $K_m$ for tetrathionate decomposition (oxidation) is evaluated to be 2.4 mM, and an attempt was made to improve the substrate specificity for sulfite oxidation by heat treatment; however, the $K_m$ value on the sulfite dehydrogenase-catalyzed reaction was not reported. Thus, $K_m$ is reasonable to apply in the microbial state *T. thiooxidans*-catalyzed reaction for the sulfite sensor device.

![Fig. 4. Time curves of the sulfite dehydrogenase-catalyzed reaction.](image)

![Fig. 5. Dependency of $K_{3Fe(CN)_6}$ concentration on the sulfite dehydrogenase-catalyzed reaction.](image)

**Effects of ferricyanide concentration on the enzyme-catalyzed reaction.**

Dependency of the $K_{3Fe(CN)_6}$ concentration on the *T. thiooxidans*-catalyzed reaction was examined in the range of 0 to 2 mM of ferricyanide using the assay procedures developed, and time curves were obtained to evaluate their initial velocities. The results are illustrated in Fig. 5, where absorbance at 420 nm is plotted against reaction time under the various concentrations of ferricya-
nide. Every plot gives good linearity for the evaluation of initial velocity \( v \), thus the initial velocities \( v \) obtained are plotted against the concentration of ferricyanide as seen in Fig. 6, clearly showing that the ferricyanide concentration examined is very proportional to the initial velocity of the \( T. thiooxidans \)-catalyzed reaction. Therefore, in this study, the enzyme-catalyzed reactions were observed under a concentration of ferricyanide within 2 mM.

A calibration curve between \( A_{420} \) and the ferricyanide concentration was examined on the basis of absorbance at time 0 of the reaction curves illustrated in Fig. 5. The plots of \( A_{420} \), which are obtained from the values on the vertical line in Fig. 5, against the ferricyanide concentration are not shown here, but it was confirmed that \( A_{420} \) is proportional to the ferricyanide concentration. Thus, in this study, the concentrations of ferricyanide were quantitatively determined using absorbance at 420 nm.

On the other hand, cytochrome \( c \) was also examined for possible use; that is, effective or not as a second substrate in this microbial enzyme system, because in some bacterial systems, cytochrome \( c \) is actually effective as a second substrate.\(^8\)\(^9\)\(^0\) Using cytochrome \( c \) in place of ferricyanide, we tried to observe the sulfite dehydrogenase-catalyzed reactions; however, the enzyme-catalyzed reaction could not be observed by the assay procedures. In this state, we assumed that cytochrome \( c \) would not contact the enzyme sulfite dehydrogenase in \( T. thiooxidans \) through the cell wall of this microbe. Thus, the enzyme could be situated at the inner part of the cell, maybe at the plasma membrane in the organelle. We must try to observe the enzyme-catalyzed activity in the cytosol fraction without the cell wall of \( T. thiooxidans \) in the near future.

The authors sincerely thank Prof. Dr. Amano and Dr. Nakamura of Yamanashi University for giving many suggestions on this study, and Rakuto Kasei Industrial Co., Ltd. and Daiwa Kasei K.K. for the production of \( T. thiooxidans \).

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(Received January 24, 2000; Accepted March 2, 2000)

食品中残存亜硫酸の高感度自動測定
バイオセンサ装置
—硫黄酸化細菌の菌体による触媒反応の基盤解析—
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Thiobacillus thiooxidans を使った亜硫酸の高感度自動測定装置が、著者らの研究グループによって開発され、澱粉産業を含む食品工業界において食品およびその素材に残存する亜硫酸の定量に供され、多くの実績を上げている。しかしながら、本センサの酵素学的な解析はこれまでまったくされてこなかった。そこで、菌体と酵素反応基盤を確立しようとした。まず、菌体を酵素試料とする assay 法を確立し、次に、反応初速度が T. thiooxidans の濃度に比例すること、およびフェリシアン化カリウムの分光光度法による解析等から、菌体を用いた触媒反応は亜硫酸脱水素酵素に依存すると考えて、実験を行った。菌体酵素触媒反応の至適 pH は 7.5 であった。したがって、本センサー装置は pH 7.5 で操作するのが適切と考えられる。また、基質亜硫酸に対する速度論量として、Michaelis 定数 K_m は 1.0±0.1 mM, また最大速度 V は 0.065±0.032 mM/min/mg であった。以上の結果から、本センサ装置は亜硫酸脱水素酵素（sulfite dehydrogenase）触媒反応基盤を満たしており、亜硫酸分析装置として妥当なことと判断された。