Purification and Properties of Thiosulfate Dehydrogenase from *Acidithiobacillus thiooxidans* JCM7814

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A key enzyme of the thiosulfate oxidation pathway in *Acidithiobacillus thiooxidans* JCM7814 was investigated. As a result of assaying the enzymatic activities of thiosulfate dehydrogenase, rhodanese, and thiosulfate reductase at 5.5 of intracellular pH, the activity of thiosulfate dehydrogenase was measured as the key enzyme. The thiosulfate dehydrogenase of *A. thiooxidans* JCM7814 was purified using three chromatographies. The purified sample was electrophoretically homogeneous. The molecular mass of the enzyme was 27.9 kDa and it was a monomer. This enzyme had cytochrome c. The optimum pH and temperature of this enzyme were 3.5 and 35°C. The enzyme was stable in the pH range from 5 to 7, and it was stable up to 45°C. The isoelectric point of the enzyme was 8.9. This enzyme reacted with thiosulfate as a substrate. The *K*<sub>m</sub> was 0.81 mm.

**Key words:** thiosulfate dehydrogenase; *Acidithiobacillus thiooxidans*

The sulfur-oxidizing bacterium *Acidithiobacillus thiooxidans*<sup>3</sup> (*Thiobacillus thiooxidans*) is an obligate chemolithoautotrophic bacterium with excellent acid tolerance, and it oxidizes inorganic sulfur compounds. The characteristics of *A. thiooxidans* are of interest from physiological and biochemical viewpoints. However, since it has been cultivated using solid elemental sulfur as a culture substrate, the bacterial growth rate and bacterial cell yield are small. So biochemical study of *A. thiooxidans* has delayed in comparison with the heterotrophs.

We used sodium thiosulfate as the energy substrate instead of elemental sulfur. The bacterial cells actively metabolize soluble sodium thiosulfate. The growth rate on sodium thiosulfate was higher than that on elemental sulfur. We established a culture method to obtain the large bacterial cells.<sup>2,3</sup> However, in spite of establishing the culture method, enzymatic researches of the thiosulfate-oxidizing enzyme of *A. thiooxidans* have not been done.

We studied the key enzyme of the thiosulfate oxidation in *A. thiooxidans*, and it was proved that thiosulfate dehydrogenase was the key enzyme in the thiosulfate oxidation. The largest portion of thiosulfate dehydrogenase was recovered from the periplasm in the cell.

Despite the partial purification and properties of the thiosulfate-oxidizing enzymes being reported in *Acidithiobacillus ferrooxidans* (*Thiobacillus ferrooxidans*),<sup>4</sup> *Thiobacillus thioparus*<sup>5</sup> and *Thermithiobacillus tepidarius* (*Thiobacillus tepidarius*),<sup>6</sup> only a few reports on the purification of thiosulfate dehydrogenase have been done in *Acidiphilium acidophilus* (*Thiobacillus acidophilus*)<sup>7</sup> and *Thiobacillus* sp. W5.<sup>8</sup> However, the properties of the thiosulfate dehydrogenase from *A. thiooxidans* have not been clarified. Therefore, we describe the purification and enzymatic characterization of thiosulfate dehydrogenase from this bacterium in this study.

**Materials and Methods**

Microorganism and culture. The strain used in this study was *Acidithiobacillus thiooxidans* JCM7814 (*Thiobacillus thiooxidans* JCM7814 or S3<sup>9</sup>), which was isolated by Wakao et al.<sup>9</sup> The medium was composed of: Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O, 5.0 g; KH<sub>2</sub>PO<sub>4</sub>, 3.0 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.25 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g; and Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.75 mg in 1.0 liter of distilled water, and the pH was adjusted to 5.0 by 4 N NaOH. The medium was sterilized by autoclaving at 115°C for 15 min. Two ml of each seed culture was inoculated into four 500-ml shaking flasks containing 100 ml of medium. The flasks were incubated at 30°C for 4 days on a reciprocal shaker. The cultured medium (400 ml) was transferred into a jar fermentor containing 3 liters of medium. The cultivation was done at 30°C for 48 hr. Culture was agitated at 400 rpm and aerated with 0.5vvm. The pH for the culture and the concentration of thiosulfate were maintained at 5.0 and 5 g/liter by feeding with a mixture of 1.53 M potassium carbonate and 1.5 M sodium thiosulfate.

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Preparation of cell-free extract. Cells were harvested from culture broth by centrifugation at 11,000 × g for 15 min. The harvested cells were washed with distilled water. The washed cells (3 g dry mass) were suspended in 0.05 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 μM PMSF. The cell suspension was disrupted by sonication at 20 kHz for 5 min at 0°C, and then passed twice through a French pressure cell at 1,500 kg/cm². The homogenate was centrifuged at 15,000 × g for 30 min at 4°C. The supernatant was used as the cell-free extract.

The cell-free extract was ultracentrifuged at 144,000 × g for 60 min at 4°C. The supernatant was used as the crude enzyme for enzyme purification.

Enzyme assay. Thiosulfate dehydrogenase enzyme activity was measured spectrophotometrically following the reduction of ferricyanide. The reaction mixture contained 140 mM potassium phosphate buffer (pH 6.0), 0.5 mM potassium ferricyanide, 18 mM sodium thiosulfate and enzyme solution (0.1 ml). The final volume was 3.0 ml. The reaction was started by addition of substrate. The reduction of ferricyanide was following by measuring the absorbance at 420 nm. Enzyme activity was expressed as the amount of enzyme that reduced 1 μmol-ferricyanide per hour.

Rhodanese activity was measured by the thiocyanate formation. Activity was expressed as the amount of enzyme that formed 1 μmol of thiocyanate in 60 min. Thiosulfate reductase activity was measured by the hydrogen sulfide formation. Activity was expressed as the amount of enzyme that formed 1 μmol of sulfide in 60 min.

Measurement of protein. Protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.

Enzyme purification. The crude enzyme was added the ammonium sulfate to a final concentration of 1 M. The sample was put on a phenyl-Sepharose column (1.9 × 14 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 μM PMSF (referred to as buffer A) with 1 M (NH₄)₂SO₄ added. Unbound protein was removed by washing the column with the equilibrated buffer until the absorbance returned to its initial level. After the column was washed, adsorbed proteins were eluted by a linear gradient between 200 ml of equilibrated buffer and 200 ml of buffer A containing 50% ethylene glycol. Fractions containing enzyme activity were pooled and then dialyzed against buffer A to remove ammonium sulfate.

A CM-cellulose column (1.9 × 14 cm) was equilibrated with buffer A. The dialyzed sample was put on this column. Then the column was washed by 3 volumes of buffer A until the absorbance returned to its initial level. After the column washing, proteins were eluted by a linear gradient between 200 ml of buffer A and 200 ml of buffer A containing 0.5 M NaCl. Active fractions containing enzyme activity were pooled and dialyzed against buffer A.

The dialyzed sample was put on a hydroxylapatite column (1.5 × 11 cm) equilibrated with buffer A. The column was washed by buffer A. After the column washing, proteins were eluted by a linear gradient between buffer A and 0.5 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 μM PMSF. Active fractions were pooled and regarded as the purified sample.

All purification steps were done at 4°C. Purified enzyme was stored at 4°C.

Native polyacrylamide gel disc electrophoresis. Native-PAGE was done under acidic conditions (pH 4.5) by the method of Reisfeld et al. in 10% gel. Gels were stained with Coomassie brilliant blue R-250.

Measurement of molecular mass. (1) Gel filtration method. A Superose 12 HR 10/30 was used for the measurement of molecular mass of the native enzyme. The buffer used for equilibration was 0.05 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 10 μM PMSF and 0.2 M NaCl. Molecular masses of the marker protein used were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

(2) SDS-polyacrylamide gel electrophoresis. SDS-PAGE was done in 12.5% gel. Molecular masses of the calibration protein were aldolase (39.2 kDa), triose phosphate isomerase (26.6 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

Heme staining. Gel was stained as described by Thomas et al.

Absorption spectrum of thiosulfate dehydrogenase. The oxidized form of the enzyme was prepared by the addition of 0.3 mM potassium ferricyanide. The reduced form of the enzyme was prepared by the addition of a few grains of sodium dithionite. The absorption spectra were measured from 400 nm to 700 nm. The enzyme concentration was 0.10 mg/ml.

Isoelectric focusing. Isoelectric focusing for enzyme preparation was done by the method of Wrigley.

Stoichiometry. Thiosulfate dehydrogenase was incubated in a mixture containing 47 mM citric acid, sodium citrate buffer (pH 3.5), 0.5 mM potassium fer-
ricyanide and 0.5 mM sodium thiosulfate by the method described by Meulenberg et al. Samples were taken at 5 minute intervals to determine thiosulfate and tetrathionate concentration. Thiosulfate and tetrathionate were measured by the method of Kelly et al.

### Results

**Key enzyme of thiosulfate oxidation**

To discover the key enzyme in thiosulfate oxidation in *A. thiooxidans* JCM7814, the activities of three enzymes associated with thiosulfate oxidation, thiosulfate dehydrogenase, rhodanese, and thiosulfate reductase, were assayed using cell-free extracts at the intracellular pH 5.5 of *A. thiooxidans*.

The specific activities of thiosulfate dehydrogenase, rhodanese, and thiosulfate reductase were 3,616, 0.033, and 0.002 μmol/hr·mg, respectively. In conclusion, the key enzyme of thiosulfate oxidation in *A. thiooxidans* JCM7814 was thiosulfate dehydrogenase.

**The location of thiosulfate dehydrogenase**

Cell-free extract was ultracentrifuged at 144,000 × g for 60 min and collected the supernatant as the soluble fraction, and the pellet as the membrane fraction. The enzyme activity of the soluble fraction obtained from cell-free extract (100%) was 75%. In contrast, that of the membrane fraction obtained from the cell-free extract was only 5%. The specific activity of soluble fraction was 5.4 μmol/hr·mg, but that of the membrane fraction was 1.0 μmol/hr·mg. Therefore, most of thiosulfate dehydrogenase activity from *A. thiooxidans* JCM7814 was presented in periplasm.

Consequently, 144,000 × g supernatant was used as crude enzyme solution for thiosulfate dehydrogenase purification of *A. thiooxidans* JCM7814.

**Purification of thiosulfate dehydrogenase**

Thiosulfate dehydrogenase of *A. thiooxidans* JCM7814 was purified by using phenyl-Sepharose, CM-cellulose, and hydroxylapatite column chromatographies. A summary of the purification of thiosulfate dehydrogenase is shown in Table 1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity [μmol/hr]</th>
<th>Total protein [mg]</th>
<th>Specific activity [μmol/ hr·mg]</th>
<th>Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>144,000 × g supernatant</td>
<td>3,301.2</td>
<td>671.6</td>
<td>4.9</td>
<td>100.0</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1,696.8</td>
<td>49.9</td>
<td>34.0</td>
<td>51.4</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>1,317.7</td>
<td>6.8</td>
<td>193.5</td>
<td>39.9</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>913.2</td>
<td>4.6</td>
<td>196.8</td>
<td>27.7</td>
</tr>
</tbody>
</table>

Finally, purified enzyme resulted in an approximately 40-fold purification with a recovery of 27.7%.

![Fig. 1. Polyacrylamide Gel Electrophoresis of the Purified Thiosulfate Dehydrogenase.](image)

Polyacrylamide gel disc electrophoresis (PAGE) was done under acidic conditions (pH 4.5). The gel was stained with Coomassie brilliant blue R-250 (Fig. 1). One protein band was detected in the gel. Therefore this enzyme was electrophoretically homogeneous.

**Estimation of molecular mass of the purified enzyme**

The molecular mass of the purified enzyme was calculated to be 27 kDa by a gel filtration method. The molecular mass of denatured protein was estimated to be 27.9 kDa by a SDS-PAGE method (Fig. 2B).

**Properties of thiosulfate dehydrogenase**
Thiosulfate Dehydrogenase from A. thiooxidans

The enzyme was incubated for 4 days at 4°C in citric acid-sodium citrate buffer (●); potassium phosphate buffer (○). Residual activity of the enzyme was assayed at 35°C and pH 6.0.

Fig. 4. Effects of Temperature on the Activity of the Thiosulfate Dehydrogenase.
The enzyme activity was assayed at pH 6.0.

Fig. 5. Effects of pH on the Stability of the Thiosulfate Dehydrogenase.
The enzyme was incubated for 4 days at 4°C in citric acid-sodium citrate buffer (●); potassium phosphate buffer (○). Residual activity of the enzyme was assayed at 35°C and pH 6.0.

The pH optimum was studied by using two buffers in the pH range of 3.0 to 8.0. The pH optimum of the thiosulfate dehydrogenase was pH 3.5 (Fig. 3). The temperature in the reaction system of thiosulfate dehydrogenase was varied from 15°C to 50°C. The temperature optimum of thiosulfate dehydrogenase was investigated at pH 6.0 of phosphate buffer because sodium thiosulfate reacted chemically with ferricyanide at pH 3.5. The temperature optimum was 35°C (Fig. 4).

Enzyme was incubated for 4 days at 4°C in the pH range of 4.0 to 8.0. After 4 days, each sample was measured for residual activity. Consequently, this enzyme was stable in the pH range of 5.0 to 7.0 (Fig. 5). The thermal stability of the enzyme was investigated by incubation for 1 hour at various temperatures, and this enzyme was stable up to 45°C (Fig. 6).

The absorption spectra of oxidized and reduced forms of thiosulfate dehydrogenase were measured. The wavelengths of absorption peaks on the reduced form were 414.5, 521.0, and 550.0 nm (Fig. 7). The gel of SDS-PAGE was immersed a solution to detect the heme protein. One band stained as heme protein (Fig. 2(C)) was found at the same mobility as a CBB-staining band (Fig. 2(B)). Therefore this enzyme contained cytochrome c.

The course of the thiosulfate dehydrogenase reac-

Fig. 2. Sodium Dodecyl Sulfate (SDS)—Polyacrylamide Gel Electrophoresis of the Purified Thiosulfate Dehydrogenase.
SDS-PAGE was done in a 12.5% polyacrylamide gel containing 0.1% SDS. The molecular masses of the standard protein (A) were aldolase (39.2 kDa), triose phosphate isomerase (26.6 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). Purified enzyme (2 μg) was placed on the gel (B). Gel (A) and (B) were stained with Coomassie brilliant blue. Purified enzyme (20 μg) was placed on the gel (C). Gel (C) was stained to detect the heme according to Thomas et al. 27

Fig. 3. Effects of pH on the Activity of the Thiosulfate Dehydrogenase.
The enzyme activity was assayed at 35°C. The buffers used were ●, citric acid-sodium citrate buffer; ○, potassium phosphate buffer.

Fig. 4. Effects of Temperature on the Activity of the Thiosulfate Dehydrogenase.
The enzyme activity was assayed at pH 6.0.
Fig. 6. Effects of Temperature on the Stability of the Thiosulfate Dehydrogenase.

The enzyme was incubated for 1 hour at 0–65°C. Residual activity of the enzyme was assayed at 35°C and pH 6.0.

Fig. 7. Visible Absorption Spectra of the Oxidized (———) and the Reduced (— — —) Thiosulfate Dehydrogenase.

The enzyme was oxidized with potassium ferricyanide. The enzyme was reduced with sodium dithionite. The enzyme concentration was 0.10 mg/ml.

activity. Substrate inhibition by thiosulfate was detected at the range of substrate concentration over 20 mM. The apparent $K_m$ was estimated to be 0.81 mM by a reciprocal plotting on substrate concentration below 18 mM.

Enzyme activity of thiosulfate dehydrogenase was measured by using potassium ferricyanide and horse heart cytochrome c as an electron acceptor. As a result, potassium ferricyanide was able to be an electron acceptor, but horse heart cytochrome c did not react as an electron acceptor.

Polyacrylamide gel electrofocusing of purified enzyme was done. The isoelectric point was found to be 8.9 on the gel. Therefore it was obvious that thiosulfate dehydrogenase was a basic protein.

Discussion

The pathway of thiosulfate oxidation is an important process to obtain energy in *A. thiooxidans* JCM7814. The enzyme activities of thiosulfate dehydrogenase, rhodanese, and thiosulfate reductase were assayed to know the key enzyme on the oxidation of thiosulfate. We found that thiosulfate dehydrogenase was key enzyme among three kinds of enzyme at pH 5.5 of intracellular pH in *A. thiooxidans*. Rhodanese and thiosulfate reductase did not have enzyme activities at pH 5.5. The optimum pHs of rhodanese and thiosulfate reductase were 10.

Thiosulfate dehydrogenase in *A. thiooxidans* was first purified to a homogeneous state. The molecular weight of native thiosulfate dehydrogenase agreed that of denaturated thiosulfate dehydrogenase. Therefore, the enzyme had a monomer structure with a molecular mass of 27.9 kDa. The structure of
thiosulfate dehydrogenase from *A. thiooxidans* in our study was very different from that of other thiosulfate dehydrogenases from some sulfur-oxidizing bacterium. Thiosulfate dehydrogenase from *Thiobacillus thiooxidans* had a monomer structure with the molecular mass of 115 kDa, but the molecular weight was higher than that from *A. thiooxidans*. The thiosulfate dehydrogenase from *Thiobacillus* sp. W5 was a tetramer containing two different subunits with molecular masses of 33 and 27 kDa. Among two subunits from *Thiobacillus* sp. W5, the molecular mass of the subunit of 27 kDa was agreed with the molecular mass of 27 kDa from *A. thiooxidans* in our study. Consequently, the thiosulfate dehydrogenase from *A. thiooxidans* was a unique and simpler structure.

The absorption spectrum of the reduced form of enzyme had the absorption peak at 550 nm. This result suggested that a protein similar to cytochrome c was included. In addition, there was detected the heme staining band at the position equal to the protein band in the SDS-PAGE gel. It suggested that the enzyme had the heme c protein. The containing of cytochrome c in the thiosulfate dehydrogenase is also reported in other sulfur-oxidizing bacterium. There was cytochrome c in a subunit from *A. acidophilus* and *Thiobacillus* sp. W5.

The optimum pH of the enzymatic activity of *A. thiooxidans* resembled the results for other Thiobacilli. Optimum pHs of thiosulfate dehydrogenase of acidophilic Thiobacilli ranged from 3.0 to 5.5. This is a property of periplasm localization enzymes in acidophilic Thiobacilli. The enzyme was stable up to 45°C, and 60% of activity was retained from pH 5.0 to 7.0. In addition, 30% of activity was kept in storage at 4°C for 30 days. The stability of thiosulfate dehydrogenase from *A. thiooxidans* was different from the thiosulfate dehydrogenase of low enzyme stability from *A. acidophilus*. The enzyme from *A. thiooxidans* was stable in the long term at 4°C.

The isoelectric point of this enzyme was 8.9. It was shown that the enzyme was a basic protein. The isoelectric point and molecular weight were different from isoelectric point (10.6) and molecular weight (12,600) of the cytochrome c from *A. thiooxidans*. Thiosulfate dehydrogenase from *A. thiooxidans* did not agree with the cytochrome c from this bacterium. Probably this enzyme is the protein contained the heme c protein.

The enzyme reacted with thiosulfate as a substrate. This enzyme had high substrate specificity. The *Km* (0.81 mm) for thiosulfate was at the same affinity level as the *Km* of thiosulfate dehydrogenase from *A. ferrooxidans*.

### References


