

## Research Paper

## Expression, Identification and Purification of *Dictyostelium* Acetoacetyl-CoA Thiolase Expressed in *Escherichia coli*

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### Abstract

Acetoacetyl-CoA thiolase (AT) is an enzyme that catalyses the CoA-dependent thiolytic cleavage of acetoacetyl-CoA to yield 2 molecules of acetyl-CoA, or the reverse condensation reaction. A full-length cDNA clone pBSGT-3, which has homology to known thiolases, was isolated from *Dictyostelium* cDNA library. Expression of the protein encoded in pBSGT-3 in *Escherichia coli*, its thiolase enzyme activity, and the amino acid sequence homology search revealed that pBSGT-3 encodes an AT. The recombinant AT (r-thiolase) was expressed in an active form in an *E. coli* expression system, and purified to homogeneity by selective ammonium sulfate fractionation and two steps of column chromatography. The purified enzyme exhibited a specific activity of 4.70 mU/mg protein. Its N-terminal sequence was (NH<sub>2</sub>)-Arg-Met-Tyr-Thr-Thr-Ala-Lys-Asn-Leu-Glu-, which corresponds to the sequence from positions 15 to 24 of the amino acid sequence deduced from pBSGT-3 clone. The r-thiolase in the inclusion body expressed highly in *E. coli* was the precursor form, which is slightly larger than the purified r-thiolase. When incubated with the cell-free extract of *Dictyostelium* cells, the precursor was converted to the same size to the purified r-thiolase, suggesting that the presequence at the N-terminus is removed by a *Dictyostelium* processing peptidase.

Key words: acetoacetyl-CoA thiolase, *Dictyostelium discoideum*, cloning; protein purification, protein processing.

### Introduction

Thiolases are known to present as functionally various forms in prokaryotes and eukaryotes. They are divided into two different groups,  $\beta$ -ketoacyl-CoA thiolase (KT, degradative) and acetoacetyl-CoA thiolase (AT, biosynthetic). The former plays a key role in the  $\beta$ -oxidative degradation of fatty acids, whereas the latter catalyses the condensation of 2 molecules of acetyl-CoA to form acetoacetyl-CoA, or the CoA-dependent thiolysis of acetoacetyl-CoA. In eukaryotes, there are five types of thiolases, which are distinguished by their functions and subcellular loca-

lization; mitochondrial KT and AT, peroxisomal KT and AT, and cytosolic AT. Mitochondrial AT regulates the levels of acetoacetyl-CoA and acetyl-CoA and also is involved in the ketone body metabolism in mitochondria. Cytosolic AT catalyses the first reaction of the mevalonate biosynthesis pathway to yield acetoacetyl-CoA, and the formed acetoacetyl-CoA enters steroid biosynthesis pathway in peroxisomes. It is considered that peroxisomal AT found in yeast [1] and rat liver [2] catalyses the first reaction of peroxisomal cholesterol and dolichol synthesis [2]. Like

this, ATs play very important functions in cells. In human, it is known that deficiency of mitochondrial AT, known as  $\beta$ -ketothiolase deficiency, causes a disease having the error of isoleucine and ketone body metabolism. Patients with this disorder have keto-acidotic attacks, and some patients die or have neurological sequelae. AT is an important one of the enzymes which are implicated in human diseases.

The cellular slime mold *Dictyostelium discoideum* is a simple eukaryote which is used as a model organism to study the macromolecular events coincident with and necessary for development. In the life cycle, there are two distinct stages of growth and differentiation. The amoebae grow vegetatively as single cells undergoing cell division under the food supply. Development can be synchronized and separated from vegetative growth, and upon starvation, vegetative growing amoeba cells undergo differentiation to form a fruiting body containing a stalk and spores. Previously, we isolated and identified several developmentally regulated genes during spore germination [3-6] or vegetative growth [7, 8]. During the course of our study on their genes, a unique partial cDNA clone was isolated, which has homology to known thiolases in Protein Database. So, we carried out the screening of its full-length cDNA clone. In this paper, we report cDNA cloning and the purification of the recombinant protein expressed in *E. coli*, and that the protein encoded in the cDNA clone is *Dictyostelium* AT. Also, we describe the processing of *Dictyostelium* AT precursor protein.

## Materials and methods

### Bacterial strains, media, and vectors

*D. discoideum* strain AX-3 was grown axenically in HL5 medium [9] supplemented with 100  $\mu$ g/mL of streptomycin at 22°C on a reciprocal shaker (150 rpm). *E. coli* strains, DH-5 $\alpha$  and XL-1 blue, were used for subcloning and were grown in Luria Bertani (LB) medium at 37°C. Plasmids pBluescript SKII(-) (Stratagene) and pTrc99a (Pharmacia Biotech) were used for subcloning and as an expression vector in *E. coli*, respectively. *E. coli* JM105 was used for expression of the recombinant thiolase (r-thiolase), according to the manufacturer's protocols.

### Isolation of cDNA clone

*Dictyostelium*  $\lambda$ zap cDNA library (kindly provided by Dr. Herbert L. Ennis, Columbia University) was screened using digoxigenin-labeled CT-7 cDNA (see Results) as a probe. Two large positive clones isolated, pBSGT-3 and pBSGT-23, were analyzed. The nucleotide sequences were determined by the dideoxy chain termination method [10] using Sequenase

II kit (US Biochemical, USA). The DNA sequence data were analyzed using MacDNASIS (Hitachi Software Engineering, Japan).

### Construction of plasmid pTrc-thio

To amplify the open reading frame (ORF) of pBSGT-3, two oligonucleotide primers, 5'-thiof (5'-CGCGCCATGGTTTCGGGCCTTTCAAAAG-3') and 3'-thior (5'-CGCGGATCCTTATAATTTTCTAAAC-3') were designated. These primers contained *Nco*I and *Bam*HI sites (underlined), respectively, and the bold-type and italic letters indicate the initiation and stop codons, respectively. PCR was performed on pBSGT-3 DNA as a template using these primers, as described previously [11]. The amplified PCR product was double-digested with *Nco*I and *Bam*HI, and inserted into the corresponding sites downstream of *trc* promoter in pTrc99A to yield plasmid pTrc-thio. *E. coli* JM105 was transformed with pTrc-thio to obtain a transformant (pTrc-thio).

### Purification of SGT3 protein (r-thiolase)

*E. coli* transformant (pTrc-thio) was cultured in 500 mL of LB medium (+50  $\mu$ g/mL ampicillin) containing 0.1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) at 37°C for 10 h. Cells harvested by centrifugation (5,000  $\times$  g for 10 min) were suspended in Buffer A (10 mM potassium phosphate buffer (pH7.5), 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 5  $\mu$ g/mL phenylmethanesulfonyl fluoride (PMSF) and 10% glycerol), and disrupted by sonication with an Ultrasonic disruptor (Model UD-201, TOMY, Japan). The homogenate was centrifuged at 12,000  $\times$  g for 15 min to remove cell debris and the resulting supernatant (crude extract) was used for purification of the r-thiolase. All procedures were done at 0-4°C. (1) Ammonium sulfate fractionation: The crude extract was brought to 30% saturation with powdered ammonium sulfate and stored on ice for 30 min. After the precipitate was removed by centrifugation (12,000  $\times$  g, for 30 min), the resulting supernatant was brought to 70% saturation. After standing on ice for 30 min, the precipitate was collected by centrifugation, and then dissolved in Buffer A, followed by dialysis against Buffer A overnight. (2) DEAE-cellulofine A-500 column chromatography: The 30-70% ammonium sulfate fraction was applied on a DEAE-cellulofine A-500 column (2  $\times$  22 cm, Seikagaku-Kogyo, Japan) equilibrated previously with Buffer A. Proteins were eluted with a linear gradient from 0 to 0.4 M NaCl in Buffer A (200  $\times$  200 mL). The thiolase activity was detected in the flowthrough fraction, and the enzyme fractions were pooled. (3) Hydroxyapatite column chromatography: The pooled enzyme fraction was applied on a

Bio-Gel HTP column (2 × 10 cm, Bio-Rad) equilibrated previously with Buffer A. Proteins were eluted by step-wise elution of 50, 100, 150, and 200 mM potassium phosphate buffer (pH7.5) (each 100 mL). The enzyme fractions eluted with 150 mM potassium phosphate buffer were pooled, and concentrated by ammonium sulfate precipitation (80% saturation).

### Thiolase activity assay

Thiolase activity was determined by measuring the decrease in absorbance at 303 nm at 30°C according to the method of Davis et al.[12]. The reaction mixture (a total volume of 1 mL) contained 0.1 M Tris-HCl (pH 8.3), 25 mM MgCl<sub>2</sub>, 50 mM KCl, 20 nM acetoacetyl-CoA and 20 nM CoA. The molar absorption coefficient of 16.9 mM<sup>-1</sup> for acetoacetyl-CoA was used under the assay conditions. One unit of the thiolase activity was defined as the amount of enzyme that catalyses the cleavage of 1 μmol of acetoacetyl-CoA per min.

### Processing assay of r-thiolase precursor

*E. coli* transformant (pTrc-thio) was cultured in 100 mL of LB medium (+50 μg/mL Amp) at 37°C overnight in the presence of 1 mM IPTG. Cell pellet (0.9 g wet weight) collected by centrifugation was suspended in 8 mL of Buffer A and disrupted by sonication. The homogenate was centrifuged at 10,000 × g for 15 min and the pellet (inclusion body) was obtained. The inclusion body was washed with 10 ml of 1% Triton X-100/10 mM EDTA, and then dissolved in 1.5 mL of Buffer A containing 8 M urea, followed by dialysis against Buffer A containing 4M urea at 4°C for 12 h. The solution was again dialyzed against Buffer A (- urea) for 10 h, three times. Finally, the soluble r-thiolase precursor protein was obtained. *D. discoideum* strain AX-3 was grown axenically in HL5 medium up to a density of ~ 6.0 × 10<sup>6</sup> cells/mL at 22°C on a reciprocal shaker (150 rpm). Cell-free extract from *Dictyostelium* cells was prepared as described previously [11]. For analysis of r-thiolase protein processing, the cell-free extract (15 μg protein) was added to the reaction mixture containing r-thiolase precursor (2.5 μg protein) in 50 mM potassium phosphate buffer (pH 6.7) and incubated at 37°C. The processing protein products were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [13] and visualized by immunostaining with anti-thiolase antibody, as described for Western blot analysis.

### Preparation of antibody against r-thiolase

The anti-thiolase antibody was prepared by Hokkaido System Science Co. (Sapporo, Japan). One female rabbit was immunized with the purified

r-thiolase. The antiserum obtained was tested by ELISA and Western blot analysis. Antibody was purified from the antiserum as described [14].

### Western blot analysis

To compare the molecular masses of the r-thiolase in the inclusion body expressed in *E. coli* and the purified r-thiolase, and to confirm processing of the r-thiolase precursor, samples were separated by 12.5% SDS-PAGE, electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed using anti-thiolase antibody and anti-rabbit IgG-alkaline phosphatase (AP) (Sigma, USA) as primary and secondary antibodies, respectively, according to the method described previously [15].

### Analytical methods

The protein concentration was measured by the method of Lowry et al. [16] using bovine serum albumin as a standard. The molecular mass of the expressed or the purified r-thiolase was estimated by SDS-PAGE. An electrophoresis calibration kit (Pharmacia Biotech) was used for the estimation of the molecular mass as standard proteins. Protein sequencing of the purified r-thiolase was carried out on a protein sequencer (Model PPSQ-10, SHIMADZU, Japan) according to the method described previously [17].

### Nucleotide sequence accession number

The nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank databases under the accession number **AB212872**.

## Results

### Cloning of the full-length cDNA

In a previous study of the *Dictyostelium* ubiquitin gene [7], we isolated a unique partial cDNA clone CT-7, by chance, which was not related to the ubiquitin clones. However, the deduced amino acid sequence of CT-7 clone was similar to known thiolases. So, we carried out cloning of cDNA from λzap cDNA library using this CT-7 cDNA as a probe, and several cDNA clones were isolated. Out of them, two large cDNA clones, pBSGT-3 and pBSGT-23, were analyzed. pBSGT-3 clone was a full-length one of 1,353 bp long containing an ORF of 1,245 bp, whereas pBSGT-23 had an insert of 1,746 bp long containing an intron-like AT-rich sequence. When PCR amplification was performed with *Dictyostelium* genomic DNA as a template using 5'-thiof and 3'-thior as primers, the PCR product was 1.6 kbp long, which was larger by about 300 bp than the ORF of pBSGT-3. The nucleotide sequence of the PCR product was identical to

that of pBSGT-23, indicating that pBSGT-23 is an artificial clone containing a single intron of 271 bp between the codon (AAT) of Asn<sup>12</sup> and the codon (GTA) of Val<sup>13</sup> in the ORF. Genomic Southern analysis suggested that the SGT3 gene is a single copy in the *Dictyostelium* genome (data not shown).

### Comparison of amino acid sequence of SGT3 protein with those of known thiolases

The deduced amino acid sequence of SGT3 predicted a protein of 414 amino acids with a molecular mass of 43,445. Its isoelectric point (pI) was 8.43. The amino acid sequence of SGT3 protein showed a significant degree of homology to other known thiolases. We compared the deduced amino acid sequence of SGT3 with those of thiolases from other organisms. The SGT3 protein showed 54% (73%), 52% (72%), and 42% (67%) sequence identity (similarity) to ATs of *Saccharomyces uvarum* [18], rat mitochondria [19], and *Zoogloea ramigera* [20], respectively. Also, it showed 38% – 47% (59% – 65%) identity (similarity) to KT of *Alcaligenes eutorophus* [21], rat mitochondria [22] and peroxisome [23], human peroxisome [24], and *E. coli*

[25], respectively. The comparison results revealed that SGT3 protein is more closely identical to AT than KT, suggesting that pBSGT-3 clone encodes a *Dictyostelium* AT. So, we refer to SGT3 protein as *Dictyostelium* AT (Ddthiolase).

The amino acid sequence of Ddthiolase was aligned with those of several representative ATs from other organisms described above. Two cysteine and one histidine residues, which are important for the catalytic reaction and the enzymatic activity, are highly conserved among the thiolases of different species [26, 27]. The three residues correspond to Cys<sup>110</sup>, Cys<sup>400</sup>, and His<sup>370</sup> in the Ddthiolase sequence. The sequence around Cys<sup>110</sup> at positions 107 – 115 is in good agreement with the highly conserved active site in other thiolases, which was proposed as active sites [21] (Fig. 1). In Ddthiolase, Lys residue resides at the last position of the active site. A basic amino acid residue at this position is specific for AT, whereas KT has a Gln residue at this position [25], except for KT of *Alcaligenes eutorophus* [21]. These facts support that Ddthiolase is an AT.

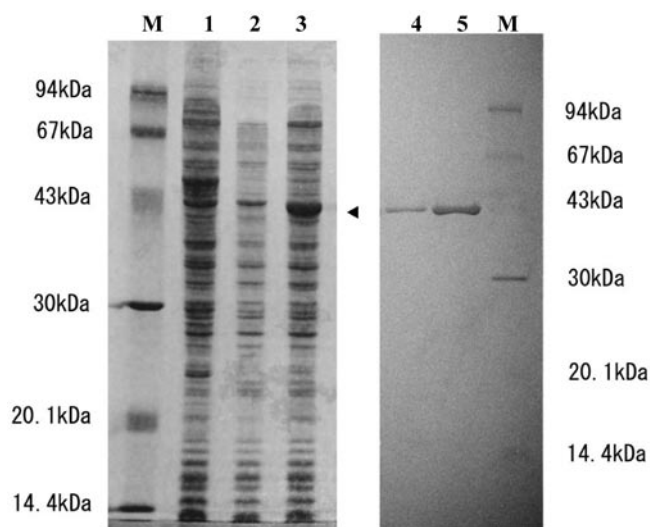
DdThiolase	107–115	N K V <b>C</b> S S G M <b>K</b>
ZRAT	86–94	N Q L <b>C</b> G S G L <b>R</b>
SUAT	88–96	N K V <b>C</b> A S A M <b>K</b>
RMAT	120–128	N K V <b>C</b> A S G M <b>K</b>
AEKT	85–93	N K V <b>C</b> G S G L <b>K</b>
RMKT	89–97	N R L <b>C</b> G S G F <b>Q</b>
RPKT	120–128	N R Q <b>C</b> S S G L <b>Q</b>
ECKT	88–96	N R L <b>C</b> G S S M <b>Q</b>
HPKT	120–128	N R Q <b>C</b> S S G L <b>Q</b>
Consensus		* . . * . * . . .

**Figure 1.** Comparison of active site around the conserved cysteine residue. The putative active site of Ddthiolase is aligned with the homologous region of known thiolases. The numbers indicate the positions of amino acids shown in this alignment. The conserved cysteine residues are boxed. The conserved residues specific for ATs and KTs are highlighted on black and gray backgrounds, respectively. The consensus sequence is indicated underneath. Asterisks (\*) and dots (.) indicate the identical and similar residues, respectively. Abbreviations used: DdThiolase, *Dictyostelium* AT (in this paper, DDBJ/EMBL/GenBank accession No. **AB212872**); ZRAT, *Zoogloea ramigera* AT (**J02631**) [20]; SUAT, *Saccharomyces uvarum* AT (**X07976**) [18]; RMAT, rat mitochondrial AT (**NM017075**) [19]; AEKT, *Alcaligenes eutorophus* KT (**J04987**) [21]; RMKT, rat mitochondrial KT (**X05341**) [22]; RPKT, rat peroxisomal KT (**J02749**) [23]; ECKT, *Escherichia coli* KT (**J05498**) [25]; HPKT, human peroxisomal KT (**X12966**) [24].



### Expression of the SGT3 protein (r-thiolase)

To determine whether or not SGT3 protein exhibits thiolase activity, we prepared *E. coli* transformant (pTrc-thio) under the control of a *ptrc* promoter. When the transformant was cultivated in the presence of 1 mM IPTG, a 43-kDa protein was strongly expressed (Fig. 2, lane 3), whose molecular mass was in a good agreement with that calculated from the ORF of pBSGT-3. But, no thiolase activity was detected in the supernatant or the precipitate (inclusion body) fractions prepared from the transformant. When the inclusion body was solubilized with 8 M urea and then activated according to the general method [28] as described in Materials and methods, the thiolase activity was expressed with a specific activity of 0.3 mU/mg protein. Endogenous thiolase activity was not detectable under the conditions used. These results suggest that SGT3 protein is a thiolase.



**Figure 2.** SDS-PAGE analysis of SGT3 protein expressed in *E. coli* and the purified r-thiolase. Samples were applied on 12% SDS-PAGE gel and after electrophoresis, the gel was stained with coomassie brilliant blue (Quick-CBB, Wako, Japan). Lane 1, crude extract of *E. coli* (without plasmid); lane 2, crude extract of *E. coli* transformant (pTrc99a); lane 3, crude extract of *E. coli* transformant (pTrc-thio) with IPTG induction (1 mM IPTG, for 3 h); lanes 4 and 5, 1 and 3  $\mu$ g of the purified r-thiolase, respectively; lane M, protein size markers. Protein expressed in *E. coli* transformant is indicated by an arrow head.

### Purification and characterization of the r-thiolase

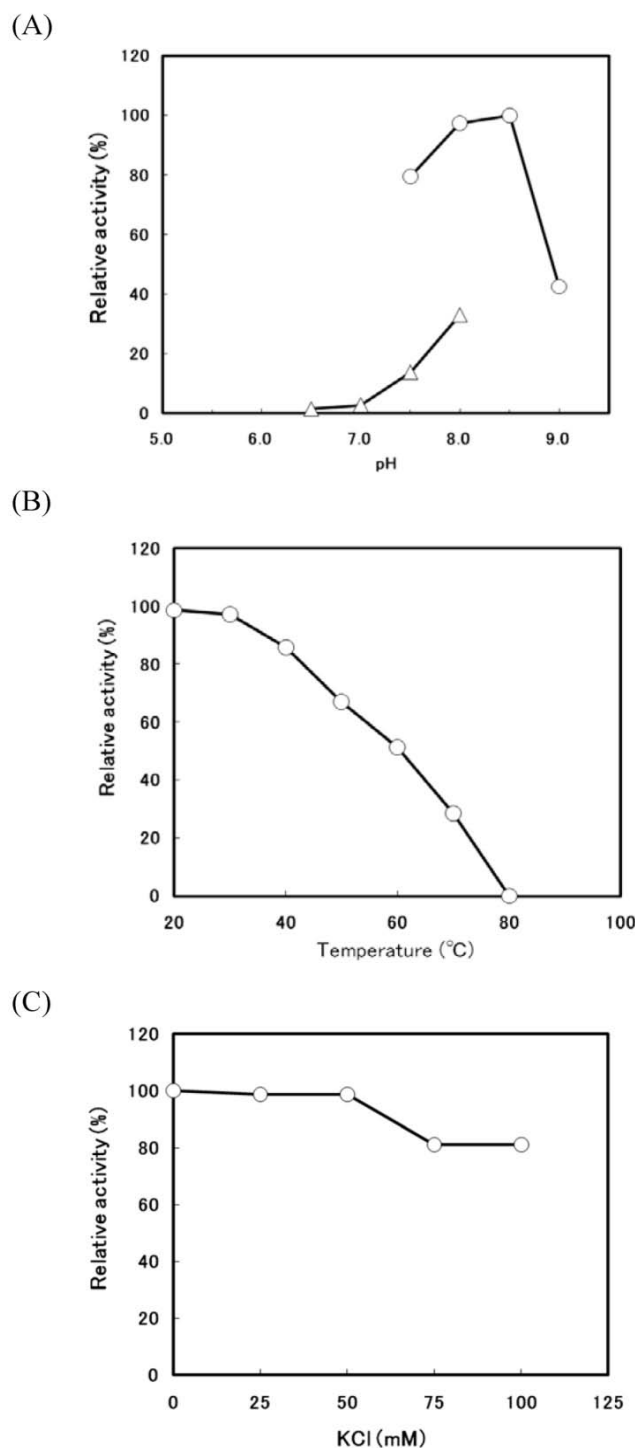
When the transformant (pTrc-thio) was cultivated at 37°C in the low concentration (0.1 mM) of IPTG, a large amount of thiolase activity was detected

in the soluble fraction. The r-thiolase was purified from the soluble fraction (crude extract) by the 30-70% saturated ammonium sulfate fractionation followed by two steps of column chromatography using DEAE-cellulofine and Bio-Gel HTP. The results of the purification of the r-thiolase are shown in Table 1. The enzyme was purified 42.7-fold with a yield of 24.4%. The purified enzyme had a specific activity of 4.70 U/mg protein and showed a single band on a 12.5% SDS-PAGE gel (Fig. 2, lanes 4 and 5), indicating that it is homogeneous. For the characterization of the purified r-thiolase, the effects of pH, temperature, and salt on the enzyme activity were examined. The activity was highest at pH 8.0 – 8.5, and it decreased significantly under conditions below pH 7.0 and above pH 9.0 (Fig. 3A). The enzyme was stable up to 40 °C, and its activity decreased to about 50% at 60 °C (Fig. 3B). To examine whether the thiolase activity is activated by KCl or not, we measured the enzyme activity in the presence of KCl. Fifty mM KCl did not activate the activity and also not inhibit. But, the activity was slightly inhibited (about 20% inhibition) by high concentration of KCl (Fig. 3C).

**Table 1.** Purification of recombinant *Dictyostelium* acetoacetyl-CoA thiolase

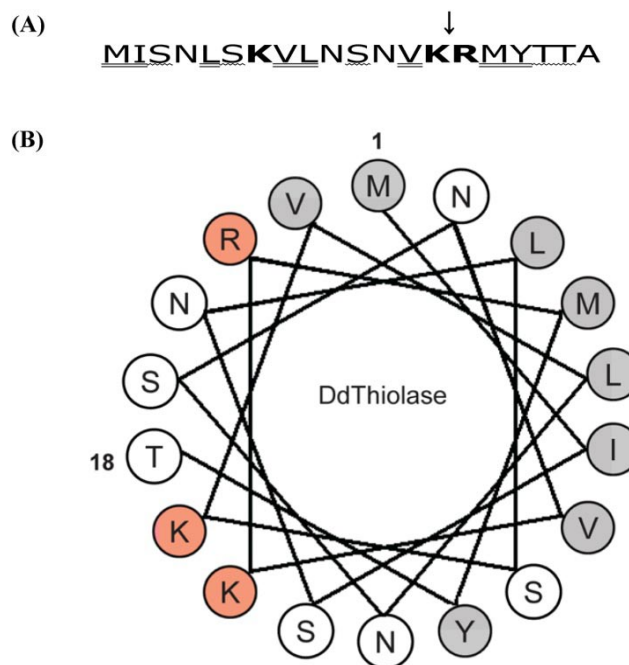
Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Fold (x)
Crude extract	455.5	52.0	0.11	100	1.0
30 - 70% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	292.8	32.5	0.11	62.5	1.0
DEAE-Cellulofine	21.3	19.4	0.91	37.3	8.3
Hydroxyapatite	2.7	12.7	4.70	24.4	42.7

Next, we determined the N-terminal sequence of the purified r-thiolase. As a result, it was found that the N-terminal sequence is (NH<sub>2</sub>)-Arg-Met-Tyr-Thr-Thr-Ala-Lys-Asn-Leu-Glu-, which corresponds to the sequence from positions 15 to 24 of Ddthiolase. This indicates that the N-terminal 14 residues were removed by an *E. coli* protease. The cleavage site is between Lys at position 14 and Arg at position 15, as shown in Fig. 4A. These results suggest that the N-terminal presequence of r-thiolase might be removed by *E. coli* protease OmpT, which cleaves the peptide bond between consecutive basic amino acid residues [29, 30].



**Figure 3.** Effects of pH, temperature, and salt on the activity of the r-thiolase. **(A)** Optimum pH for the activity of the r-thiolase. The enzyme activity was measured under the standard enzyme assay conditions, except that the following buffers were used; 50 mM potassium phosphate buffer (pH 6.5 – 8.0, open triangles) and 50 mM Tris-HCl buffer (pH 7.5 – 9.0, open circles). The relative activity is expressed as the percentage of the maximum activity attained under the assay conditions used. **(B)** Thermostability of the r-thiolase. After the purified r-thiolase (0.52  $\mu$ g, 2.45 mU) was incu-

bated at the indicated temperatures for 10 min with 10 mM potassium phosphate buffer (pH 7.5), the remaining activity was measured under the standard enzyme assay conditions. The remaining activity is expressed as the percentage of the original enzyme activity. **(C)** Effect of KCl. Using the purified r-thiolase (0.52  $\mu$ g, 2.45 mU), the activity was measured under the standard enzyme assay conditions at the KCl concentrations indicated. The relative activity is expressed as the percentage of the activity obtained in the absence of KCl.

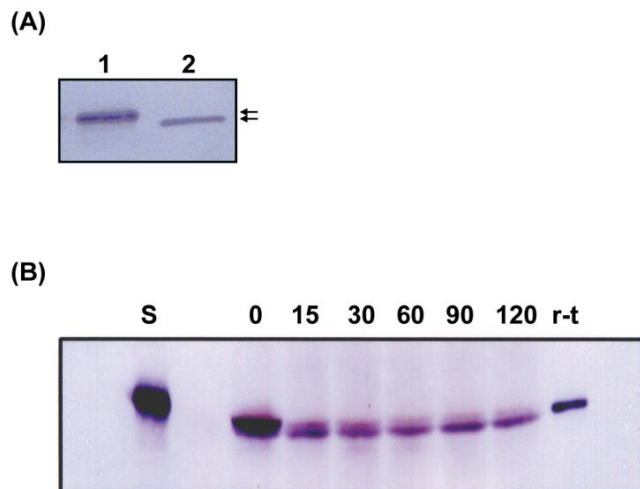


**Figure 4.** Characteristics of the N-terminal sequence of Ddthiolase. **(A)** The first 20 amino acid sequence of Ddthiolase. Positively charged, hydroxylated, and hydrophobic residues are indicated by bold face, wavelined, and double-underlined, respectively. A downward arrow indicates cleavage site by *E. coli* protease OmpT, which has specificity for the paired basic residues. **(B)** Helical wheel analysis. Residues 1–18 of the N-terminal sequence of Ddthiolase are plotted circularly in an ideal  $\alpha$ -helix, with 3.6 residues per helical turn. Positively charged and hydrophobic residues are represented by red and gray circles, respectively. The other residues are indicated open circles. The numbers 1 and 18 indicate the first Met and the 18<sup>th</sup> Thr, respectively.

### Processing of the r-thiolase precursor

When the molecular size of the r-thiolase in the inclusion body was compared with that of the purified r-thiolase, the former was slightly larger than the latter (Fig. 5A). This result suggests that the r-thiolase

might be a precursor form carrying still the N-terminal presequence. So, the r-thiolase isolated from the inclusion body was incubated at 37°C with the *Dictyostelium* cell-free extract. With increase of incubation times, the r-thiolase was converted to the smaller molecule, which size is the same to that of the purified r-thiolase (Fig. 5B). This result suggests that the r-thiolase in the inclusion body is a precursor with the N-terminal presequence, which is removed by a *Dictyostelium* processing peptidase.



**Figure 5.** Western blot analysis. **(A)** Samples were subjected to SDS-PAGE, and Western blot analysis was performed using anti-thiolase antibody. Lane 1, inclusion body (10 µg protein); lane 2, the purified r-thiolase (0.5 µg protein). The positions of thiolase precursor and r-thiolase were indicated by arrows. **(B)** Time course of processing of the r-thiolase precursor. The r-thiolase precursor (2.5 µg protein) was mixed with the cell-free extract (15 µg protein) prepared from *Dictyostelium* cells and incubated at 37°C for the time indicated in figure. Processing peptides were subjected to SDS-PAGE followed by Western blot and immunostaining using anti-thiolase antibody and anti-rabbit IgG-AP. Numbers indicate incubation times (min) of the reaction. Lanes S and r-t contain substrate (r-thiolase precursor) alone and the purified r-thiolase, respectively.

## Discussion

When we started the study on Ddthiolase, its structure and function remained unknown. During the course of this thiolase work, the sequence of AT gene was reported from the *Dictyostelium* Genome Project [31]. Searches of the *Dictyostelium* database (<http://dictybase.org>) revealed that three single genes of AT (Dicty gene ID DDB\_G0271544), KT (DDB\_G0274339), and a putative thiolase (DDB\_G0269588) exist in the *Dictyostelium* genome.

The nucleotide sequence of pBSGT-23 is identical to that of the AT gene in the *Dictyostelium* database. *Dictyostelium* KT and the putative thiolase remain to be characterized.

The majority of mitochondrial proteins are synthesized in the cytosol as a precursor protein with an N-terminal mitochondrial targeting signal (MTS) sequence, which is a signal for import of proteins to mitochondria [32-34]. Although MTS sequence is different in length and in sequence, it is rich in positively charged, hydroxylated, and hydrophobic amino acids to form an amphiphilic  $\alpha$ -helix structure. As shown in Fig.4A, Ddthiolase has 3 positively charged, 5 hydroxylated and 8 hydrophobic amino acids in the N-terminal region. A helical wheel analysis of the first 18 residues of Ddthiolase shows that an amphiphilic  $\alpha$ -helix structure would be formed, although its amphiphilicity is slightly weak (Fig.4B), suggesting that the N-terminal sequence may function as a MTS to direct Ddthiolase to mitochondria. As shown in (Fig. 5B), the r-thiolase precursor was processed to the smaller form by *Dictyostelium* processing peptidase. Since Ddthiolase possesses a MTS sequence at the N-terminus as described above, this processing peptidase appears to be a mitochondrial processing peptidase (MPP), which cleaves the MTS presequence to make a mature protein. The cleavage site of Ddthiolase presequence by MPP is considered to be between Tyr at position 17 and Thr at position 18 from the tendency of the MPP cleavage site according to the "R-2/3 rule" [35, 36]. The correct cleavage site of Ddthiolase precursor remains to be identified.

Peroxisomal proteins have peroxisomal targeting signal (PTS) necessary for import of those proteins to peroxisomes. Two types of PTS are well known; PTS-1 is a tripeptide motif of Ser-Lys-Leu (SKL) sequence, which is located at the C-terminus [37], and PTS-2 is a nonapeptide motif of the consensus sequence (Arg/Lys)(Leu/Val/Ile)(Xxx)<sub>5</sub>(His/Gln)(Leu/Ala) [(R/K)(L/V/I)X<sub>5</sub>(H/Q)(L/A)] within the N-terminal region, where X indicates any amino acid [38]. Ddthiolase has Glu-Lys-Leu (EKL) similar to PTS-1 sequence at the C-terminus, suggesting that this thiolase may be localized to peroxisomes. Taken together, Ddthiolase appears to be a dual-localization enzyme containing MTS and PTS-1 sequences at the N- and C-terminus, respectively. The subcellular localization of Ddthiolase remains to be determined. It was reported that rat mitochondrial AT has both MTS and PTS-1 sequences at the N- and C-terminus, respectively [39]. Also, several proteins, such as 3-hydroxymethylglutaryl-CoA lyase [40], dienoyl-CoA isomerase [41], and malonyl-CoA decarboxylase [42], contain both N-terminal MTS and C-terminal



PTS-1. It is of interest to understand the dual-localization mechanism of proteins to mitochondria and/or peroxisomes.

In summary, when Ddthiolase was expressed as a soluble enzyme in an *E. coli* expression system, it was an active enzyme, of which the N-terminal presequence was cleaved off by *E. coli* protease. On the other hand, expressed as an insoluble enzyme in the inclusion body under the high expression conditions, the r-thiolase was a precursor protein with the N-terminal presequence, which might be protected by aggregating from the cleavage by *E. coli* protease. In *in vitro* studies of protein processing, precursor proteins are indispensable as substrates for processing peptidases. Generally, precursor proteins are labeled with radioactively amino acids using an *in vitro* transcription/translation kit and used as a substrate for processing peptidase, and the detection is dependent to autoradiography. As shown in this paper, however, if precursor proteins could be prepared using an *E. coli* expression system, *in vitro* further studies of signal peptidase or processing peptidase would be carried out using them as substrates by SDS-PAGE/Western blot and immunostaining using their antibodies.

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## Conflict of Interests

The authors have declared that no conflict of interest exists.

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