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Detection and Identification of Dihydrolipoamide Dehydrogenase as a Feroxamine Binding Protein from *Streptomyces coelicolor*

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As the first step to elucidate the physiological role of siderophore deferoxamine, the binding proteins in the cell of *Streptomyces coelicolor* were explored by an affinity chromatography. An affinity column was designed using feroxamine as an affinity ligand. As a result of chromatography, one major protein band at 51 kDa, along with traces of minor proteins, was detected in eluted fraction by analysis of SDS-PAGE. The 51k Da protein was identified as putative dihydrolipoamide dehydrogenase using peptide mass fingerprinting method.

Key words: Dihydrolipoamide dehydrogenase, Deferoxamine, *Streptomyces coelicolor*, Affinity column.

Siderophore is defined as a low-molecular compound secreted by microorganisms to uptake ferric ion efficiently under low iron stress. The ferric iron has a very low0solubility at neutral pH and therefore cannot be utilized by microorganisms. Siderophore chelates ferric ion with high affinity and establishes soluble complex that can be taken up via specific membrane transporter.[1] In the environments, it is critical to survive ferric deficient situation, since siderophore iron uptake system is very important function for soil bacteria including streptomycetes. It has been found that streptomycetes produce one group of structure-related siderophores including coelichelin,[2,3] foroxymithine,[4-5] and tsukubachelin[6].Deferoxamine was isolated from *Streptomyces pilous*[7] and has been the effective medical reagent to cure for hemochromatosis.[8] Interestingly, Yamanaka *et al*. reported previously that deferoxamine E produced by *Streptomyces griseus* stimulates growth and development of *Streptomyces tanashiensis*.[9] This finding suggests that deferoxamine E may have certain function involved in cell control system in *Streptomyces*. The ferric-siderophore uptake system via membrane transporter has been well studied. However, after the uptake into cell, how the ferric siderophore is metabolized is not clear. Feroxamine is highly hydrophilic small molecule which easily diffuses in bacterial cytosol, and there may be some binding protein which possibly plays a key role in development. The model streptomycete, *S. coelicoilor*, is also known to produce deferoxamine[10]. As a step to elucidate the function of deferoxamine in *S. coelicolor*, we conducted search for feroxamine-binding protein by use of an affinity column coupled to feroxamine. Here

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we describe exploration and identification for feroxamine binding protein in cell of *S. coelocolor*.

MATERIALS AND METHOD

Elution using affinity column coupled with deferoxamine

The 10 mg of $FeCl_3$ was added to deferoxamine (Sigma-Aldrich) solution (50 mg in 2 mL of distilled water) for conversion to feroxamine. The affinity column was prepared by coupling of feroxamine to 1 mL of the Affi-Gel 10 affinity supports (Bio-Rad) in 0.1M MOPS solution (pH 7.5). The gel was mixed at 4 °C for 2 h, and then washed with 7 M urea solution containing 1 M NaCl. *S. coelicolor* strain J1501 was cultured in ISP2 liquid medium at 27 °C for 5 days.0The cells were harvested by centrifugation, and washed with distilled water. Cellular proteins were extracted from the washed cells by subjecting the cells to ultrasonication in 10% SDS Tris buffer. The proteins extracted were precipitated with trichloroacetic acid. The precipitated proteins were dissolved at the concentration of 10 mg/mL in 0.1M Tris buffer (pH 7.0). The protein solution (2 mL) was loaded onto the affinity column, followed by elution with 2 mL each of distilled water and 0.1N NaOH solution in this order.

Analysis of SDS polyacrylamide gel electrophoresis

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition (Laemmli, 1970). Gels were consisted of a 4% stacking gel and a 10% resolving gel. Electrophoresis was carried out until the dye front reached the bottom of the gels. Gels were stained with Coomassie Brilliant Blue (CBB) R purchased from Sigma.

In-Gel Trypsin-digestion

The protein band was cut with a clean scalpel blade, transferred to a microcentrifuge tube and cut into small pieces. Gel pieces are washed for 2 h with 200 µL of wash solution consisted of water/MeOH/acetic acid (at the ratio of 5/4/1). The gel pieces were washed with 200 µL of acetonitrile and dehydrated by incubation for 5 min at room temperature. In-gel digestion was carried out with Sequencing Grade Modified Trypsin (10 µg/mL in 50 mM ammonium bicarbonate, Promega). After overnight incubation at 37 °C, an equal volume of acetonitrile was added and the peptide mixtures were extracted by shaking for 10 min at room temperature. The resulting supernatant was transferred to a fresh microcentrifuge tube. Peptides in the gel pieces were further extracted first by adding 50 µL of 5% formic acid and shaking for 20 min and then 50 µL of 100% acetonitrile for another 20 min. The supernatant was then collected and combined to the first fraction. The extract was dried in a vacuum centrifuge and stored at -80 °C until use for analysis.

MALDI-TOF-MS analysis

Matrix-assisted laser desorption/ ionization (MALDI) time of flight (TOF) mass spectra were recorded on Reflex II (Bruker Daltonik GmbH, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid as the matrix with irradiation of an N_2 laser.

RESULTS AND DISCUSSION

Feroxamine was coupled with affinity support, Affi-Gel 10 affinity supports, in 0.1M MOPS buffer. The whole protein extract from *Streptomyces coelicolor* was loaded onto the affinity gel. The flow-through fraction was followed by elution with diluted water and 0.1N NaOH solution in this order. Each fraction was separately collected in conical tube and analyzed by SDS-PAGE with CBB staining. As a result, one thick band of protein (approximately 51 k Da) was detected in diluted water and 0.1N NaOH fractions (lanes 3 and 4 with arrow in Fig. 1). We used peptide mass fingerprinting (PMF) method to identify the protein.[11, 12] Briefly, the band was cut out from gel and digested by trypsin. The peptide derived from digested protein was extracted by 50% acetonitrile and subjected to MALDI-TOF-MS analysis.[13] On the Mascot web search, the protein was identified by matching with expected peptide fragments generated by trypsin digestion. The ion peaks were observed by MALDI-TOF-MS analysis (Table 1). As a result of Mascot web search, the protein was identified as putative dihydrolipoamide dehydrogenase (gene bank accession number, CAB51264). The expected molecular weight of the dihydrolipoamide dehydrogenase was 51365 Da (matched with the molecular weight deduced by SDS-PAGE), and calculated pI was 5.92. Thus, the

Table 1. Observed ion peaks on MALDI-TOF-MS spectrum

Observed ion peak	Expected sequence	Residues
1366.95	ALLHAGEVADQSR	80-92
1194.84	TLPGLEIDGNR	172-182
1101.79	FHLGTFFQK	254-262
1446.06	EFEAEVLLVAIGR	279-291
1849.27	GPVSQGLGYEENGVATDR	292-309
1228.82	GFVLVDEYMR	310-319
1217.83	TVPVDYDGVFR	357-367
1832.30	VTYCHPEVASVGLTEAR	368-384
1311.90	DGAVVGVHMVGDR	423-435

protein was identified as dihydrolipoamide dehydrogenase.

Dihydrolipoamide dehydrogenase (DLD) is one component in a complex of three enzymes and catalyzes the oxidation of a dihydrolipoyl residue covalently bound to a component protein. DLD oxidizes dihydrolipoate bound to a lysine residue of the complex with mediation of a flavin. DLDs normally function as integral components of the pyruvate, 2-oxoglutarate, and branched chain 2-oxoacid dehydrogenase multienzyme complexes [14-16] and of the glycine cleavage system.[17] Therefore, DLDs play a significant role in metabolism and energy production. In the present study, we found that feroxamine had an affinity to putative dihydrolipoyl dehydrogenase, which was produced at high level in *S. coelicolor*. Taking into account the critical role of dihydrolipoyl dehydrogenase in energy production, it is possible that feroxamine affects the function of dihydrolipoyl dehydrogenase in *Streptomyces*, leading to the enhanced growth and development, although further study is needed to confirm this hypothesis. The fact that the affinity column chromatography specifically bound DLD may be explained by structural similarity of the substrate

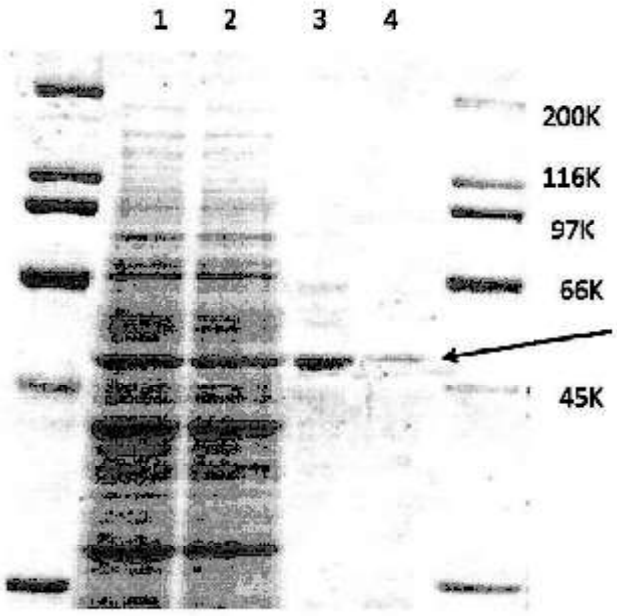

Fig. 1. SDS-PAGE analysis of protein fractions; Lane 1, cellular protein fraction; lane 2, flow through fraction; lane 3, fraction eluted with distilled water; lane 4, fraction eluted with 0.1 N NaOH

of the enzyme to deferoxamine as shown by the box with the shadow (*N*-butylpropionamide-sturucture) in Fig. 2.

DLD had been isolated from *Streptomyces seoulensis*, with five separation steps including several column chromatographs.[18] Ajith *et al.* purified DLD with DNA affinity magnetic beads, which were designed from DNA binding site of dnrO gene upstream, however the yield in the separation procedure was not sufficient.[19] In the present study, we successfully separated DLD with only one step of affinity column chromatography. This method therefore may be an efficient biochemical tool to purify DLD.

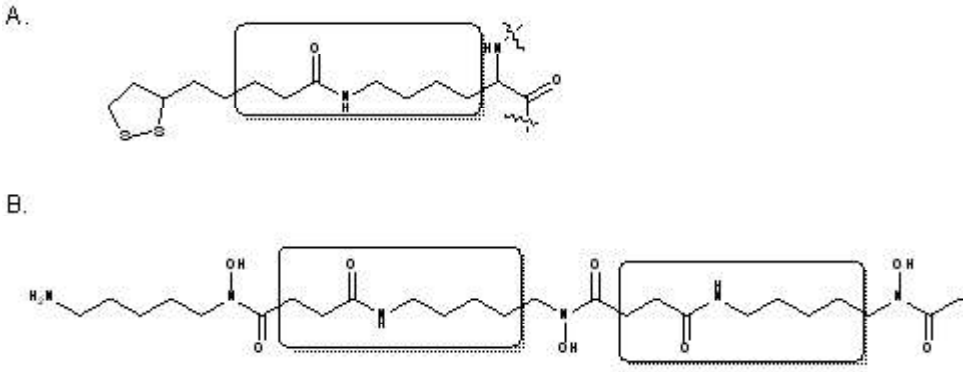

Fig. 2. Chemical structures of lipoic acid attached to N6 amide residue of Lys in substrate protein (A) and deferoxamine (B)

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