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Expression of spinach ferredoxin-thioredoxin reductase using tandem T7 promoters and application of the purified protein for *in vitro* light-dependent thioredoxin-reduction system

Yuki Okegawa, Ken Motohashi*

Department of Bioresource and Environmental Sciences, Faculty of Life Sciences, Kyoto Sangyo University, Kamigamo Motoyama, Kita-ku, Kyoto 603-8555, Japan

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ABSTRACT

Thioredoxins (Trxs) regulate the activity of target proteins in the chloroplast redox regulatory system. *In vivo*, a disulfide bond within Trxs is reduced by photochemically generated electrons via ferredoxin (Fd) and ferredoxin-thioredoxin reductase (FTR: EC 1.8.7.2). FTR is an αβ-heterodimer, and the β-subunit has a 4Fe–4S cluster that is indispensable for the electron transfer from Fd to Trxs. Reconstitution of the light-dependent Fd/Trx system, including FTR, is required for the biochemical characterization of the Trx-dependent reduction pathway in the chloroplasts. In this study, we generated functional FTR by simultaneously expressing FTR-α and -β subunits under the control of tandem T7 promoters in *Escherichia coli*, and purifying the resulting FTR complex protein. The purified FTR complex exhibited spectroscopic absorption at 410 nm, indicating that it contained the Fe–S cluster. Modification of the expression system and simplification of the purification steps resulted in improved FTR complex yields compared to those obtained in previous studies. Furthermore, the light-dependent Trx-reduction system was reconstituted by using Fd, the purified FTR, and intact thylakoids.

1. Introduction

In plant chloroplasts, the activity of many enzymes, including Calvin cycle enzymes, malate dehydrogenase, and ATP synthase, is regulated in a light-dependent manner. Such enzymes are known as redox-regulated proteins [1,2]. Thioredoxin (Trx) is a key enzyme for redox regulation in the chloroplasts. It reduces a disulfide bond of redox-regulated proteins by using electrons from the photosynthetic electron transfer pathway [3,4] and modulates their activities [5–7]. Ferredoxin-thioredoxin reductase (FTR) is a unique protein that supplies electrons from photochemically reduced ferredoxin (Fd) to Trx in the chloroplasts [8], unlike the cytoplasmic and mitochondrial Trx systems [9,10]. In plants, FTR functions as a heterodimer complex consisting of one α subunit (FTR-α; 13 kDa) and one β subunit (FTR-β; 13 kDa). The FTR-β is the catalytic subunit and has a 4Fe–4S iron-sulfur cluster [11], which is used to supply electrons to Trx [12,13].

Previously, spinach FTR was expressed in *Escherichia coli* by a dicistronic system, which led to the transcription of a single mRNA containing the *FTRA* (FTR-α) and *FTRB* (FTR-β) genes under the control of a single T7 promoter [14]. The expressed spinach FTR complex was purified using four different types of column chromatography, including ferredoxin-affinity chromatography. Cyanobacterial FTR was also produced using the same system [15]. Both FTRs were functional, but the spinach FTR was expressed at lower levels and was unstable compared with the cyanobacterial FTR [15,16]. The yield of spinach FTR complex was about 1.5 mg per liter of *E. coli* culture [14], whereas that of cyanobacterial FTR was 7–12 mg per liter of *E. coli* culture [15]. In this study, we modified an expression system for spinach FTR complex in *E. coli* by a tandem T7 promoter system and improved the purification steps by using three different types of column chromatography.

Abbreviations: CBB, Coomassie Brilliant Blue R-250; DTT, dithiothreitol; Fd, ferredoxin; FTR, ferredoxin-thioredoxin reductase; MDH, malate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SLiCE, seamless ligation cloning extract; Trx, thioredoxin.

* Corresponding author.
E-mail address: motohas@cc.kyoto-su.ac.jp (K. Motohashi).

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2. Materials and methods

2.1. Cloning of spinach FTRA and FTRB genes from spinach cDNA

Spinach *FTRA* (DDBJ/EMBL/GenBank, X78880) and *FTRB* (DDBJ/EMBL/GenBank, X77164) genes were obtained from the spinach cDNA library by polymerase chain reaction (PCR) using KOD-Plus-DNA polymerase™ (TOYOBO, Osaka, Japan), a derivative of DNA polymerase from *Thermococcus kodakaraensis* KOD1 [17]. The following oligonucleotides were used: for *FTRA*, 5′-aactgcagcatatggaagtagctttgaaatccgat-3′ (*Nde*I) and 5′-cgggatcctactctgctatgatttcgaat-3′ (*Bam*HI); for *FTRB*, 5′-aactgcagccatggtggaaccttcagacaagtccg-3′ (*Nco*I) and 5′-cggaattcacatgttcgctgtcacttccc-3′ (*Eco*RI). The restriction sites shown in parentheses are underlined in the sequence. The amplified DNA fragments were cloned into the *Nde*I and *Bam*HI sites of pET23a or *Nco*I and *Eco*RI sites of pET23d (Merck Millipore, Billerica, Massachusetts). The length of the cloned DNA inserts was confirmed by colony-PCR using the T7 promoter and T7 terminator sequences of the pET vectors [18], and the sequence of the inserted plasmid DNA obtained from the colony-PCR-positive clones was confirmed by DNA sequencing. Two expression vectors, Sp*FTRA*/pET23a and Sp*FTRB*/pET23d were used as controls for the individual expression of spinach *FTRA* or *FTRB*, respectively.

2.2. Construction of the spinach FTRAB co-expression system

The spinach *FTRAB* (*FTRA* and *FTRB*) co-expression system was constructed using the tandem T7 promoter system [19]. Briefly, the *Bgl*II-*Bam*HI fragment of Sp*FTRA*/pET23a was ligated to a *Bgl*II site within Sp*FTRB*/pET23d, and clones ligated in the correct direction were screened by colony-PCR [18]. The DNA sequence of the resulting Sp*FTRAB* tandem expression vector was confirmed by DNA sequencing. The Sp*FTRAB* expression plasmid under the control of tandem T7 promoters was named as Sp*FTRAB*-tandem/pET23d (Fig. 1). To compare with the tandem T7 promoter system for *FTRAB* co-expression, a single T7 promoter system for co-expression of *FTRA* and *FTRB* [14] was constructed by seamless ligation cloning extract (SLiCE)-mediated PCR-based site directed mutagenesis [20]. The spinach *FTRA* and *FTRB* genes were PCR-amplified from Sp*FTRA*/pET23a and Sp*FTRB*/pET23d, respectively, using KOD DNA polymerase™ (TOYOBO) and the following oligonucleotides: for *FTRA*, 5′-cgcgaaattaatacgactcactat-3′ (T7 promoter primer) and 5′-cttgtctgaaggttccaccatggtctgtttccttatggtaccattagacttctactctgctatgatttcgaat-3′; for *FTRB*, 5′-attcgaaatcatagcagagtagaagtctaatggtaccataaggaaacagaccatggtggaaccttcagacaag-3′ and 5′-gctagttattgctcagcggtggca-3′ (T7 terminator primer). Two amplified DNA fragments were simultaneously incorporated into the *Nde*I and *Hin*dIII sites of pET23a (Merck Millipore) by seamless cloning using SLiCE from a laboratory *E. coli* JM109 strain [21,22]. The DNA sequence of the resulting Sp*FTRAB* single T7 expression vector was confirmed by DNA sequencing. The Sp*FTRAB* expression plasmid under the control of a single T7 promoter was named as Sp*FTRAB*-single/pET23a.

2.3. Expression and purification of the spinach FTR complex

E. coli BL21 (DE3) cells carrying Sp*FTRAB*-tandem/pET23d, the FTR complex expression plasmid under the control of tandem T7 promoters, were cultured at 37 °C in 12 L of 2 × YT medium supplemented with ampicillin (100 mg/L), and FTR protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final 0.5 mM) and incubation at 25 °C. At 3 h after induction, *E. coli* cells expressing the FTR-α and FTR-β proteins were harvested as wet cells (45.2 g). The recombinant spinach FTR complex was purified as follows: *E. coli* cells were suspended in 25 mM Tris–HCl (pH 7.5) and disrupted by sonication (Sonifier 250; Branson, Danbury, CT) at 4 °C. The disrupted cells were centrifuged at 100,000× *g* for 40 min, and the supernatant (crude extract) was applied to a Toyopearl DEAE-650M column (ϕ50 mm × 200 mm, Tosoh, Tokyo, Japan), which was previously equilibrated with 25 mM Tris–HCl (pH 7.5). Proteins were then eluted using a linear gradient of NaCl (0–300 mM) in 25 mM Tris–HCl (pH 7.5). The peak fractions (fraction No. 54–59) containing FTR-α and FTR-β were collected, and solid ammonium sulfate was added to obtain a final concentration of 1.7 M. The solution was then applied to a Toyopearl Butyl-650M column (ϕ25 mm × 200 mm, Tosoh) and eluted using an inverse gradient of ammonium sulfate (1.7–0 M) in 25 mM Tris–HCl (pH 7.5). The FTR-α and FTR-β peak fractions (fraction No. 25–28) were collected and dialyzed against 10 mM sodium phosphate buffer (pH 6.8). The dialyzed protein solution was applied to a hydroxyapatite column (HA-ultrogel; ϕ25 mm × 100 mm; Pall, New York, USA) and eluted with a linear gradient of sodium phosphate buffer (10–300 mM, pH 6.8). The resulting FTR peak fractions (fraction No. 25–30) were collected and stored at −80 °C in 16% (v/v) glycerol. In total, 40 mg of FTR complex protein was obtained from 12 L of 2 × YT medium.

2.4. Characterization of spinach FTR protein

Absorbance of the purified FTR complex protein was measured at 250–600 nm by a spectrophotometer, V-650 (JASCO, Tokyo, Japan). N-terminal amino acid sequences were determined as follows. Purified FTR complex was subjected to 15% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were blotted to a polyvinylidene difluoride membrane, ProBlott (Applied Biosystems, Foster City, CA). The blotted membrane was stained with Coomassie Brilliant Blue R-250, and the 16- and 14-kDa protein bands were excised. The pieces were analyzed by a peptide sequencer (Procise 491HT; Applied Biosystems) [18,23] by Nippi, Incorporated (Tokyo, Japan).

2.5. Protein preparation

Spinach Trx *f* [24] and Trx *m* [25,26] were purified in the absence of dithiothreitol (DTT) as described previously. *Arabidopsis* NADP-malate dehydrogenase (MDH; AT5G58330) was purified without an affinity tag as follows: *Arabidopsis* NADP-MDH-overexpressing *E. coli* cells were suspended in 25 mM Tris–HCl (pH 7.5) and disrupted by sonication (Sonifier 250; Branson) at 4 °C. The disrupted cells were centrifuged at 100,000 × *g* for 40 min, and the supernatant (crude extract) was applied to a Toyopearl DEAE-650M column (Tosoh) and eluted using a linear gradient of NaCl (0–300 mM) in 25 mM Tris–HCl (pH 7.5). Peak fractions containing NADP-MDH were collected, and solid ammonium sulfate was added to obtain a final concentration of 2.1 M. The solution was then applied to a Toyopearl Butyl-650M column (Tosoh) and eluted using an inverse gradient of ammonium sulfate (2.1–0 M) in 25 mM Tris–HCl (pH 7.5). Peak fractions containing NADP-MDH were collected and concentrated using Amicon Ultra-15 (10,000 NMWL membrane; Merck Millipore); the buffer in the protein solution was exchanged with 25 mM Tris–HCl (pH 7.5). Purified NADP-MDH was stored at −80 °C in 16% (v/v) glycerol.

2.6. Antibodies

Polyclonal FTR-β antibody was raised against a synthetic peptide, KSVEIMRKFSEQYAR, corresponding to residues 39–53 of the *Arabidopsis* FTR-β protein (Sigma–Aldrich, St. Louis, MO). Polyclonal Trx *f*, Trx *m*, and NADP-MDH antibodies were raised in