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Application of preparative disk gel electrophoresis for antigen purification from inclusion bodies

Yuki Okegawa [1], Masanori Koshino [1], Teruya Okushima, 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

Specific antibodies are a reliable tool to examine protein expression patterns and to determine the protein localizations within cells. Generally, recombinant proteins are used as antigens for specific antibody production. However, recombinant proteins from mammals and plants are often overexpressed as insoluble inclusion bodies in *Escherichia coli*. Solubilization of these inclusion bodies is desirable because soluble antigens are more suitable for injection into animals to be immunized. Furthermore, highly purified proteins are also required for specific antibody production. Plastidic acetyl-CoA carboxylase (ACCase: EC 6.4.1.2) from *Arabidopsis thaliana*, which catalyzes the formation of malonyl-CoA from acetyl-CoA in chloroplasts, formed inclusion bodies when the recombinant protein was overexpressed in *E. coli*. To obtain the purified protein to use as an antigen, we applied preparative disk gel electrophoresis for protein purification from inclusion bodies. This method is suitable for antigen preparation from inclusion bodies because the purified protein is recovered as a soluble fraction in electrode running buffer containing 0.1% sodium dodecyl sulfate that can be directly injected into immune animals, and it can be used for large-scale antigen preparation (several tens of milligrams).

1. Introduction

Antibodies are commonly utilized to study the localization and conformational changes of proteins in cells [1], organelles [2,3], or tissues such as plant leaves and roots [4–6]. Antigen preparation is an important step for antibody production, and pure antigen facilitates the production of better antibodies [7]. Recombinant proteins from *Escherichia coli* are commonly used as a source of antigens for antibody production [3,8,9], but they are often overexpressed as inclusion bodies in *E. coli* [2,10–13]. To prepare antigen from inclusion bodies, several methods have been used including the detergent-washing of inclusion bodies [7,14], purification of the protein using immobilized-metal affinity chromatography (IMAC) under denaturing conditions [15,16], and the fragmentation of sodium dodecyl sulfate (SDS) polyacrylamide slab gel or nitrocellulose membrane strips containing the protein [1,7,17].

On the other hand, polyacrylamide gel electrophoresis (PAGE) with discontinuous buffer [18] has been applied for large-scale purification of denatured proteins [19–22]. Preparative disk gel electrophoresis using an electroosmotic medium pump is a simple electrophoresis method that does not require an electronic peristaltic pump or similar apparatus [21].

Acetyl-CoA carboxylase (ACCase) is an enzyme that catalyzes the formation of malonyl-CoA from acetyl-CoA, which is the first committed step of fatty acid synthesis [23]. Plastidic ACCase is a light-dependent redox-regulated protein [24], and its activity is regulated by thioredoxin (Trx) [25,26]. ACCase is composed of the α and β subunits, and is activated by reduction of an intermolecular disulfide bond between the α and β subunits [27]. To investigate changes in the redox state of ACCase *in vivo*, a specific antibody against ACCase is required.

Here, we report an alternative method for antigen preparation by purifying inclusion bodies using preparative disk gel electrophoresis. This method is especially suitable for purification of recombinant proteins without any affinity tag, which cannot be

Abbreviations: ACCase, acetyl-CoA carboxylase; IMAC, immobilized-metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Trx, thioredoxin.

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

[1] These authors contributed equally to this work.

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purified by affinity chromatography such as IMAC. In addition, protein purified by preparative disk gel electrophoresis can be recovered at a high concentration as soluble protein in SDS electrode running buffer. The purified protein solution can be directly used as antigen for antibody production without any subsequent protein concentration step.

2. Materials and methods

2.1. Cloning of Arabidopsis ACCase β subunit (AccD)

The AccD gene (ATCG00500) encoding the β subunit of *Arabidopsis* ACCase was obtained from an *Arabidopsis* cDNA library [28] by polymerase chain reaction (PCR) amplification using KOD -Plus- DNA polymerase™ (TOYOBO, Osaka, Japan), a derivative of DNA polymerase from *Thermococcus kodakaraensis* KOD1 [29]. The following oligonucleotides were used: 5′-aactgcagcatatggaaaaatcgtggttcaat-3′ (*Nde*I) and 5′- cggaattcttaatttgtgttcaaaggaaa-3′ (*Eco*RI). Restriction sites for the enzyme shown in parentheses are underlined. The amplified DNA fragments were cloned into the *Nde*I and *Eco*RI sites of pET23a (Merck Millipore, Billerica, Massachusetts). The length of cloned DNA inserts was confirmed by colony-PCR using Blend Taq DNA polymerase (TOYOBO, Osaka, Japan) [30,31]. Plasmids were prepared from colony-PCR positive clones and the inserted DNA sequence of plasmids was confirmed by DNA sequencing.

2.2. Insert-check by colony PCR in transformed E. coli

Each colony was picked with a sterile toothpick and placed into the bottom of a 0.2-mL 8-strip PCR tube. After the toothpicks were removed from the PCR tube, 10 μL of Blend Taq DNA polymerase (TOYOBO, Osaka, Japan) PCR mix was added to each sample; this mixture included primers corresponding to the T7 promoter and T7 terminator sequences of the pET vectors [30,32]. The sample solutions were reacted according to the Blend Taq DNA polymerase standard protocol.

2.3. Expression of Arabidopsis ACCase β subunit (AccD)

Overexpression of the recombinant AccD protein was induced in *E. coli* BL21 (DE3) cells harboring AtAccD-pET23a plasmid at 37 °C, by adding isopropyl-β-D-thiogalactopyranoside (IPTG, final concentrations of 0.5 mM) at OD_{600} = 0.8 of *E. coli* cell density [33]. After induction, *E. coli* cells were grown for 3 h at 37 °C and then harvested by centrifugation at 5,000 × *g* for 10 min at 4 °C. The recombinant AccD protein was recovered as inclusion bodies.

2.4. Detergent-wash of Arabidopsis AccD inclusion bodies

AccD inclusion bodies were washed as described follows [2,13]. *E. coli* cells overexpressing AccD (1.8 g wet cells) were suspended in lysis buffer (40 mL) containing 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA, disrupted by sonication (Sonifier 250; Branson, Danbury, CT), and centrifuged at 11,000 × *g* for 15 min. The precipitate of inclusion bodies was suspended in inclusion body wash buffer (20 mL) containing 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 2% (w/v) Triton X-100 using a homogenizer, gently mixed using a rotator for 30 min at room temperature, and centrifuged at 11,000 × *g* for 10 min. This washing step was repeated 4 times. Finally, the inclusion bodies were washed twice with inclusion body wash buffer minus EDTA (20 mL). Inclusion bodies were washed total 7 times to remove detergent soluble *E. coli* contaminated proteins, thoroughly. The washed inclusion bodies were stored at −20 °C.

2.5. Purification of Arabidopsis AccD inclusion bodies by preparative disk gel electrophoresis

A half of the detergent-washed AccD inclusion bodies was further purified by preparative disk gel electrophoresis [21] (NA-1800; Nihon Eido, Tokyo, Japan). The washed AccD inclusion bodies (24 mg) were dissolved in 3 mL of a sample buffer containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 62.5 mM Tris–HCl (pH 6.8), 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue. The sample was loaded onto a preparative disk gel electrophoresis column (ϕ36 mm × 51 mm, 12.5% (w/v) gel), without heat-denaturation step. Sample separation by gel electrophoresis was performed at 100 V constant voltage at 4 °C. The SDS electrode running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) was deaerated to siphon the eluted sample from the gel to the fraction through a glass capillary. After elution of bromophenol blue from the disk gel, the eluted samples were fractionated every 20 min (700–900 μL) using a fraction collector (FC 203B; Gilson, Middleton, WI), and the fractions containing AccD protein were identified by SDS-PAGE. The purified AccD protein was stored at −80 °C. To confirm N-terminal amino acid sequence of purified AccD protein, the N-terminal sequence was determined as described follows. Purified AccD protein was loaded on 15% (w/v) 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 protein band was cut out. The cut piece was analyzed with a peptide sequencer (Procise 491HT; Applied Biosystems) [1]. The peptide sequence analysis was performed by Nippi, Incorporated (Tokyo, Japan).

2.6. Preparation of antibodies

Polyclonal anti-AccD antibodies were raised in rabbits against the detergent-washed AccD inclusion bodies or against AccD protein purified by preparative disk gel electrophoresis. The antibodies were prepared by Medical & Biological Laboratories (Nagoya, Japan).

2.7. Preparation of Arabidopsis chloroplast

Intact chloroplasts were purified as previously described [3] with the following modifications. One gram of leaves from 4- to 5-week-old plants was homogenized by Polytron (PT 10–35 GT) for a few seconds in a medium containing 400 mM sorbitol, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM EDTA, 10 mM $NaHCO_3$, 0.5% (w/v) bovine serum albumin (BSA), 5 mM ascorbate, and 20 mM Tricine-NaOH (pH 8.4) and filtered through Miracloth (Calbiochem, La Jolla, CA). After centrifugation for 5 min at 3,000 × *g*, the pellet was gently resuspended in 400 mM sorbitol, 5 mM $MgCl_2$, 2.5 mM EDTA, and 50 mM HEPES-KOH (pH 7.6). The concentration of chlorophyll was determined as previously described [34].

2.8. Estimation of antibodies against Arabidopsis AccD protein by western blotting

To estimate the reactive sensitivity of antibodies against the AccD protein, western blotting analysis was performed using serial dilutions of the purified AccD protein at a range of concentrations (1–100 ng) and chloroplast lysates from *Arabidopsis* (0.1–5 μg chlorophyll). All samples were separated on SDS-PAGE (10% (w/v)) and electroblotted to Immun-Blot polyvinylidene difluoride membrane (Bio-rad Laboratories, Hercules, CA) using a semidry blotting apparatus. After blocking with ECL-prime Blocking Reagent (GE healthcare, Little Chalfont, England), the membranes were probed by anti-AccD antiserum using enhanced chemiluminescence [35].