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Purification of cytoplasmic actin by affinity chromatography using the C-terminal half of gelsolin

Takashi Ohki [a], Chikanori Ohno [a], Kotaro Oyama [a], Sergey V. Mikhailenko [b], Shin'ichi Ishiwata [a,*]

[a] Department of Physics, Faculty of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan
[b] Department of Physics, Faculty of Science, Gakushuin University, 1-5-1 Mejiro, Toshima-ku, Tokyo 171-8588, Japan

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ABSTRACT

A new rapid method of the cytoplasmic actin purification, not requiring the use of denaturants or high concentrations of salt, was developed, based on the affinity chromatography using the C-terminal half of gelsolin (G4–6), an actin filament severing and capping protein. When G4–6 expressed in *Escherichia coli* was added to the lysate of HeLa cells or insect cells infected with a baculovirus encoding the beta-actin gene, in the presence of Ca^{2+} and incubated overnight at 4 °C, actin and G4–6 were both detected in the supernatant. Following the addition of Ni–Sepharose beads to the mixture, only actin was eluted from the Ni–NTA column by a Ca^{2+}-chelating solution. The functionality of the cytoplasmic actins thus purified was confirmed by measuring the rate of actin polymerization, the gliding velocity of actin filaments in an *in vitro* motility assay on myosin V-HMM, and the ability to activate the ATPase activity of myosin V-S1.

Introduction

Actin filaments in living cells undergo continuous, dynamic turnover and remodeling. These processes involve polymerization, depolymerization, severing, capping, and branching of actin filaments through interaction with a vast array of actin-binding proteins. Gelsolin, a member of an actin scavenger system, rapidly severs actin filaments at substoichiometric concentrations, remaining as a cap on their barbed ends [1]. Gelsolin is composed of six repeating domains of sequence (G1–6) and contains three distinct actin binding sites, two that bind to G-actin (G1 and G4–6) and one that binds to filaments (G2) [2]. The C-terminal half (G4–6) provides the Ca^{2+}-sensitivity of gelsolin, namely, the presence of Ca^{2+} induces a significant structural rearrangement in the C-terminal half, which facilitates severing and capping of actin [3]. In the sedimentation assay [4], when F-actin was mixed for 8 h at 4 °C with G4–6 expressed in *Escherichia coli* and pelleted, the amount of actin in the supernatant increased compared with the absence of G4–6, suggesting that G4–6 induces the depolymerization of F-actin by increasing the critical concentration of actin which was capped by G4–6 in a Ca^{2+}-dependent manner.

Cytoplasmic actin has so far been purified by various methods that include cycles of polymerization/depolymerization, anion exchange, gel filtration, and DNase I or profilin affinity chromatography [5]. DNase I and profilin are the G-actin-binding proteins with the dissociation constant, K_d, of 50 pM [6] and 0.1–5 μM [7,8], respectively. The agarose column-immobilized DNase I was used to purify cytoplasmic actin from HeLa cells [9]. Due to the very high affinity for G-actin, the elution of actin from the column requires high concentration of formamide (10 M), urea (6 M), or guanidine hydrochloride (3 M), which are known to denature proteins [10,11]. Profilin is a small protein with a molecular weight of ~19 kDa, which promotes the nucleotide exchange in the actin monomers released from filaments [7]. When profilin is added to a plant-cell extract, it facilitates the depolymerization of actin filaments and forms a profilin–G-actin complex, which is then isolated by affinity chromatography on poly-L-proline-Sepharose [12]. Purified plant-actin was eluted with a high ionic strength solution (1 M KCl) followed by a G-buffer.

To eliminate the possibility of potential denaturation and structural instability of actin induced by denaturants and high concentrations of salts, we developed a rapid and gentle method of purification using the C-terminal half of gelsolin (G4–6), which was found to dissociate from actin at less than millimolar concentrations of Ca^{2+}, for isolating functional actin from the non-muscle cells.

Recombinant actins were expressed in several organisms for the mutational analysis [13,14]. Recently, Joel et al. succeeded in preparation of recombinant actins in the baculovirus/Sf9 expression system, which produces the foreign gene proteins with high levels in the insect cells [15–17]. Sf9 cells are known to be capable of post-translational modifications of eukaryotic proteins [18]. Here we expressed mouse beta-actin using baculovirus and purified it by G4–6

* Corresponding author. Fax: +81 3 5286 3437.
E-mail address: ishiwata@waseda.jp (S. Ishiwata).

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affinity chromatography. Purified recombinant beta-actin was evaluated by the ability to polymerize and interact with myosin V.

Materials and methods

Plasmid construction. The expression vector for the G4–6 construct in pCold-I (Takara Bio, Otsu, Japan) was produced as follows. The cDNA encoding G4–6 was amplified by KOD+ DNA polymerase (Toyobo, Osaka, Japan) from the mouse spleen cDNA library with a pair of oligonucleotides: 5′-gctcgagatggccgctcagcacg-3′ and 5′-cgaattctcaggcagccagctcag-3′, containing the XhoI and the EcoRI sites, respectively. The amplified cDNA was digested with XhoI and EcoRI and ligated into the pCold-I vector in the polylinker region.

The baculovirus transfer vectors for beta-actin and myosin V-HMM (1–1091 aa) and myosin V-S1 (1–807 aa) constructs were produced as follows. The cDNA encoding beta-actin was amplified by PCR from the mouse brain cDNA library with a pair of oligonucleotides: 5′-agaattcaccatggatgacgatatcgctgcg-3′ and 5′-ataagcttctagaagcacttgcggtgc-3′, containing the Kozak sequence and the EcoRI and the HindIII sites, respectively. The amplified cDNA was digested with EcoRI and HindIII and ligated into pFastBac-1 vector (Invitrogen Japan, Tokyo) in the polylinker region. The cDNA encoding myosin V (HMM or S1) was amplified by PCR from the mouse brain cDNA library with a pair of oligonucleotides: 5′-gccatggccgcgtccgagctc-3′ and 5′-tcactagtgcgctcctccaggcgactg-3′ (HMM) or 5′-tcactagtctgcatacacaggtatctcttcctt-3′ (S1). The amplified cDNA was digested with NcoI and SpeI in both cases and ligated into pFastBac-HT in the polylinker region. A myc-tag was introduced at the C-terminal end of the construct by site-directed mutagenesis. The baculoviruses encoding beta-actin and myosin V (HMM or S1) were produced by using Bac-to-Bac system (Invitrogen).

Expression and purification of recombinant proteins. *Escherichia coli* strain Rosetta 2 (DE3; Novagen, Madison, WI) was transformed by plasmid pCold-G4–6 and grown in MMI broth medium containing 0.1 mg/mL ampicillin at 37 °C to the optical density (OD 600) of 1.9. Then, the foreign protein expression was induced by refrigerating at 15 °C without isopropylthio-D-galactoside (IPTG). After cultivation at 15 °C for 12 h, the harvested cells were lysed by sonication in a binding buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.0), 5 mM $CaCl_2$, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.01 mg/mL leupeptin, 1 mM ATP). Following centrifugation at 62,000*g* for 15 min, the supernatant was mixed with Ni–Sepharose 6 Fast Flow (GE Healthcare, Tokyo, Japan) beads in a conical tube on an end-to-end rotator for 60 min at 4 °C. The resin suspension was then loaded on a disposable column and washed with a 10-fold volume of a binding buffer. G4–6 was eluted with a binding buffer containing 0.2 M imidazole (pH 8.0) and dialyzed against a binding buffer.

To express recombinant myosin V-HMM, Sf9 cells were co-infected with two viruses, expressing heavy chain and light chain (calmodulin), respectively. Cells were cultured at 27 °C for 3 days, then lysed by pipetting in a lysis buffer (20 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM EGTA, 1 mM PMSF, 0.01 mg/mL leupeptin). Following centrifugation at 62,000*g* for 15 min, 0.3 M KCl and 5 mM ATP were added to the supernatant, and the solution was mixed with Ni–NTA agarose (Qiagen) in a conical tube on an end-to-end rotator for 60 min at 4 °C. The resin suspension was then loaded on a column and washed with a 10-fold volume of a lysis buffer containing 0.3 M KCl and 5 mM ATP. Myosin V-HMM was eluted with a lysis buffer containing 0.2 M imidazole–HCl (pH 8.0) and 40 mM KCl. Myosin V-S1 was expressed and purified similarly.

Actin purification. Skeletal actin was prepared from rabbit skeletal muscle [19]. All experimental procedures conformed to the "Guidelines for Proper Conduct of Animal Experiments" approved by the Science Council of Japan, and were approved by the Steering Committee for Animal Experimentation at Waseda University. Cytoplasmic actin used as a control of the present study was purified from HeLa cells by DNase I chromatography as described [9].

Purification of cytoplasmic and recombinant beta-actin by G4–6 affinity chromatography. HeLa cells and Sf9 cells infected with baculovirus expressing beta-actin were lysed by pipetting in a binding buffer without KCl (10 mL/2×10^7 cells), after which 50 mM KCl, 30 mM imidazole–HCl (pH 8.0), and purified G4–6 (0.4 mg/6.7×10^7 cells in case of confluent culture in 150 mm^2 culture dishes or 4 mg/8×10^7 cells in case of 40 mL suspension culture) were added and mixed at 4 °C overnight. After centrifugation at 200,000*g* for 20 min, the supernatant was mixed with Ni–Sepharose 6 Fast Flow and stirred for 60 min at 4 °C. The resin suspension was then loaded on a disposable column and washed with a 10-fold volume of a binding buffer containing 30 mM imidazole–HCl (pH 8.0). Actin was eluted with a Ca^{2+}-chelating buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.0), 0.1 mM EGTA, 7 mM β-mercaptoethanol) and, after the addition of 2 mM $MgCl_2$, were further purified by one cycle of polymerization and depolymerization.

Measurement of actin-activated ATPase activity. The actin-activated ATPase activity of myosin V-HMM (0.1 μM) was determined as described [20] at 25 °C using various concentrations of actin in the solution containing 0.08 mg/mL pyruvate kinase (Sigma–Aldrich, Tokyo, Japan), 2 mM phosphoenol pyruvate (Sigma–Aldrich), 1 mM ATP, 20 mM Tris–HCl (pH 7.2), 2 mM $MgCl_2$, 40 mM KCl, 1 mM EGTA, 1 mM dithiothreitol.

In vitro motility assay. An *in vitro* motility assay was performed as described [21]. Briefly, a glass coverslip was coated with anti-c-myc antibody (Invitrogen). The surface of the coverslip was then blocked with BSA (10 mg/mL), and myosin V-HMM (2 mg/mL) was applied. The ATP concentration in the assay buffer was 1 mM. The gliding velocity of actin filaments labeled with rhodamine phalloidin was calculated from the translocation distance and the elapsed time between the successive snapshots.

Actin polymerization assay. Actin polymerization rates were measured by change in pyrenyl fluorescence upon incorporation of pyrene into actin filaments [22]. Pyrene-labeled actin (2% of total actin concentration) in G-buffer (10 mM Tris–HCl (pH 8.0),

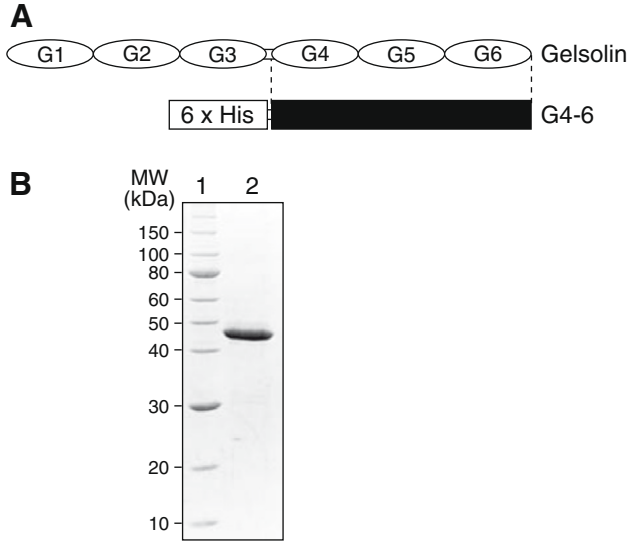

Fig. 1. Structure of gelsolin and G4–6. (A) Schematic representation of the sixfold repeating sequence of gelsolin and the deletion mutant, G4–6. (B) SDS–PAGE (12% polyacrylamide). Lane 1, molecular weight marker; lane 2, the purified G4–6. The yield was typically 150 mg/L of culture.