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Purification of Arp2/3 complex from Saccharomyces cerevisiae

Lynda K. Doolittle[1], **Michael K. Rosen**[1], and **Shae B. Padrick**[1]

[1]Department of Biophysics and Howard Hughes Medical Institute, UT Southwestern Medical Center, Dallas, TX 75390, USA

Summary

Much of cellular control over actin dynamics comes through regulation of actin filament initiation. At the molecular level, this is accomplished through a collection of cellular protein machines, called actin nucleation factors, which position actin monomers to initiate a new actin filament. The Arp2/3 complex is a principal actin nucleation factor used throughout the eukaryotic family tree. The budding yeast *Saccharomyces cerevisiae* has proven to be not only an excellent genetic platform for the study of the Arp2/3 complex, but also an excellent source for the purification of endogenous Arp2/3 complex. Here we describe a protocol for the preparation of endogenous Arp2/3 complex from wild type *Saccharomyces cerevisiae*. This protocol produces material suitable for biochemical study, and yields milligram quantities of purified Arp2/3 complex.

Keywords

Actin nucleation factor; Arp2/3 complex; biochemical purification; endogenous; source; Saccharomyces cerevisiae; detailed protocol

1. Introduction

Plant, animal and fungal cells all make use of dynamic rearrangements of actin filaments to move and reshape themselves (1). In yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, these rearrangements have been intensely studied in both vesicle trafficking (2,3) and in cell division (4). Force generation needed during yeast clathrin dependent endocytosis requires precise control of the initiation of actin filaments by the Arp2/3 complex (3,2).

The Arp2/3 complex is composed of one copy each of seven polypeptides (5–7), all of which are needed for function (8,9). Two of these polypeptides are the actin related proteins Arp2 and Arp3, from which the complex derives its name. There are five additional subunits, with no homology to actin, known as ArpC1, ArpC2, ArpC3, ArpC4 and ArpC5. The complex is basally inhibited, but can be activated by a collection of ligands known as nucleation promoting factors (10,7). Nucleation promoting factors are able to integrate a broad range of cellular signals to activate Arp2/3 complex at specific places and times (11). These ligands must both trigger an activating conformational change in the complex (12–

Correspondence to Shae B. Padrick, Shae.Padrick@utsouthwestern.edu, Telephone: 214-645-6357.

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14), and deliver the actin monomers that become the first subunits in the nucleated filament (15–17). The activity of the *Saccharomyces cerevisiae* Arp2/3 complex may be assayed *in vitro* through the increase in fluorescence of pyrene labeled actin upon polymerization (18) or through the microscopic observation of fluorescent actin filaments assembling on beads (19).

Given the complexity of this multi-protein complex, it is most typically purified from endogenous sources. In addition to purification schemes that use only standard chromatography (20–25), endogenous Arp2/3 complex can be purified through the use of an affinity column with an immobilized ligand. Immobilized ligand columns were used to originally identify the complex (5). More recently, the use of affinity beads bearing the VCA domain of the nucleation promotion factor N-WASP has proven of broad utility. This method was first described for the purification of Arp2/3 complex from bovine brain (26), but the general strategy has since been adapted to the purification of Arp2/3 complex from *Saccharomyces cerevisiae* (27), from *Acanthamoeba castellanii* (28), and from *Schizosaccharomyces pombe* (29).

We describe here our version of a protocol to purify Arp2/3 complex from commercially available bakers' yeast (*Saccharomyces cerevisiae*). The described protocol is derived from published work (27), but has several added purification steps that improve the final purity of the complex when isolated from commercial baker's yeast. We describe the Arp2/3 complex purification at a scale where milligram quantities may be prepared. We hope that others may use this protocol to purify Arp2/3 complex from *Saccharomyces cerevisiae*, and as a template to develop new protocols for the purification of the complex from additional sources. The purification protocol typically requires four days of work, after completion of the two additional protocols. We recommend that two people be involved during the first two days of the purification, and a single person complete the third and fourth days of work.

2. Materials

All buffer and salt stocks are prepared using ultrapure water (>18 MOhm, using Millipore brand Milli-Q water purification system). Except where noted, all solutions are filtered through a 0.22 μm cellulose acetate membrane. Working buffers are prepared by dilution of buffer stocks into prechilled ultrapure water. Where specific sources are recommended, the manufacturer and part numbers are indicated.

2.1. Stock Materials and Solutions

1. Baker’s yeast (*Saccharomyces cerevisiae*) (*see* Note 1).

[1]The wild type budding yeast (*Saccharomyces cerevisiae*) used in this protocol can be grown in the lab or acquired from commercial sources. We have had good success with both routes. This protocol can be performed using in house grown yeast from either shaker flasks (yeast grown to roughly OD_{600} of 1 – 2 in 18 L YPD is a good starting point for this protocol), or produced at high density using a fermentation system. Given the cost and time needed to produce this quantity of yeast, we recommend purchasing yeast from a commercial source. Dry yeast should not be used. 1 lb cakes of fresh yeast are commonly used in small commercial bakeries. The yeast has a shelf life of about one month, and thus most bakeries receive frequent shipments and are willing to part with one or two cakes at a price that is much less than the cost of media used to grow the yeast in house. Alternatively, one may be able to acquire a very fresh case of cakes directly from a distributor. This protocol has had good results with Red Star #05020 cake yeast, although washing, resuspending, freezing and storing at −80°C an entire 20 lb case is impractical.

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2. Leupeptin hydrochloride (Bachem #N-1000.0100): 1 mg/mL stock in ultrapure water, filtered and stored at −20°C in 250 μl aliquots.
3. Antipain dihydrochloride (Sigma #A6191): 1 mg/mL stock in ultrapure water, filtered and stored at −20°C in 250 μL aliquots.
4. Phenylmethanesulphonyl fluoride (PMSF; Sigma #P7626: 100 mM stock in isopropanol, filtered and stored in 1 mL aliquots at −20°C until needed (*see* Note 2).
5. Dithiothreitol (DTT): 1 mM stock in ultrapure water, filtered and stored at −20°C in 1 mL aliquots. When thawing, place tube in cool water until solution is liquid then transfer to ice until needed. Once added to buffers, assume DTT is no longer functional after 48 hours when stored at 4°C.
6. Adenosine 5' triphosphate disodium salt (ATP; Sigma #A7699): 100 mM stock in 100 mM Tris-HCl pH 8.0, and titrated to pH 7.4 to 7.5 with sodium hydroxide. The solution is filtered and stored at −20°C in 1 mL aliquots.
7. 1 M HEPES pH 7.5 and 1M HEPES pH 7.0: Prepared from HEPES acid, and titrated with sodium hydroxide to pH 7.5 or pH 7.0 at room temperature.
8. 0.5 M EGTA: Prepared using ethylene glycol tetraacetic acid powder and titrated to pH 8.0 with sodium hydroxide. Initially, EGTA is insoluble, but comes into solution as the pH is adjusted.
9. 1 M $MgCl_2$.
10. 2 M KCl
11. 5 M NaCl
12. 1 M Tris-Hcl pH 8.0: Prepared using tris base, and titrated to pH 8.0 at room temperature with concentrated hydrochloric acid.
13. 0.5 M EDTA: Preparedusing ethylenediaminetetraacetic acid powder and titrated to pH 8.0 with sodium hydroxide. Like EGTA, EDTA is initially insoluble, but comes into solution as the pH is adjusted to 8.0.
14. 1 M Imidazole pH 7.0: Titrated with hydrochloric acid to pH 7.0 at room temperature.
15. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution: 1 M in ultrapure water, filtered and stored at −20°C in 1 mL aliquots.
16. Construct for the expression of GST N-WASP VCA in *E. coli* (*see* Note 3).
17. Chemically competent BL21(DE3) $T1^R$ cells.

[2]PMSF is toxic. Care should be used when preparing this stock. In particular, use of gloves, lab coats, protective eye wear and dust masks will reduce inhalation and cross contamination. One effective strategy is to purchase <100 g bottles, and prepare the entire bottle at one time trusting the manufactures provided weight. The working concentrations are high enough that small errors due to variability in the manufacturer's weight will not have a significant effect. The stock should be stored as 1 mL aliquots in 1.5 mL microfuge tubes at −20°C. Once the stocks have been frozen PMSF will crystallize out of solution. Warming in a room temperature beaker of water for a few minutes is usually sufficient to bring the PMSF back into solution. Agitation by inversion may be needed. When adding PMSF to a buffer, avoid splashing the stock and buffers onto gloves, skin and eyes.

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18. Ampicillin stock, prepared as 100 mg/mL in water, filtered and stored at −20°C in 1 mL aliquots.
19. LB agar plates supplemented with 100 μg/mL of ampicillin.
20. Luria (LB) broth.
21. Ammonium sulfate.

2.2. Working Buffers

1. Buffer IBS: 150 mM NaCl, 10 mM imidazole pH 7.0.
2. UB: 50 mM HEPES pH 7.5, 100 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT.
3. UBpi: UB with 2 μg/mL antipain, 20 μg/mL leupeptin, and 1 mM PMSF. Add antipain and leupeptin directly to the buffer, add PMSF once buffer is added to cells, but prior to resuspending the cells.
4. Buffer QA1: 20 mM Tris-HCl pH 8, 2 mM EDTA, 1 mM DTT.
5. Buffer QB1: 20 mM Tris-HCl pH 8, 2 mM EDTA, 1 mM DTT, 1 M NaCl.
6. Buffer QLB: 20 mM Tris-HCl pH 8, 2 mM EDTA, 1 mM DTT, 70 mM NaCl.
7. Buffer GSH-WB1: 20 mM HEPES pH 7, 2 mM EDTA, 1 mM DTT, 100 mM NaCl.
8. Buffer GSH-WB2: 20 mM HEPES pH 7, 2 mM EDTA, 1 mM DTT, 700 mM NaCl.
9. Buffer A: 20 mM HEPES pH 7.5, 25 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 100 μM ATP.
10. Buffer B: 20 mM HEPES pH 7.5, 200 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 100 μM ATP.
11. Buffer C: 20 mM HEPES pH 7.5, 200 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 0.5 mM EGTA, 100 μM ATP.
12. Buffer QC: 20 mM Tris-HCl pH 8, 1 mM DTT, 10 mM EGTA, 2 mM $MgCl_2$.
13. Buffer QD: 20 mM Tris-HCl pH 8, 1 mM DTT, 10 mM EGTA, 2 mM $MgCl_2$, 1 M NaCl.
14. Buffer KMEHd-A: 100 mM KCl, 10 mM HEPES pH 7.5, 1 mM $MgCl_2$ 1 mM EGTA, 0.5 mM DTT, 100 μM ATP.

[3]For this protocol, we use N-WASP VCA to capture Arp2/3 complex because it was used in the first reported version of this protocol. N-WASP VCA binds Arp2/3 complex more tightly than most VCAs. Also of note, the GST fusion to N-WASP VCA means that, in addition to binding to the target affinity column, the VCA will be a dimer. VCA dimers are important here as they increase the affinity towards Arp2/3 complex appreciably, and the protocol may not work with lower affinity VCA monomers. It is not clear whether VCA constructs will perform better than CA constructs, but we have used a VCA construct composed of amino acid residues 393–504. This is expressed from a modified pGEX 2T (GE) vector such that VCA has an N-terminal GST fusion, with a short linker and a thrombin cleavage site separating them.

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15. Buffer F: 100 mM KCl, 10 mM HEPES pH 7.5, 1 mM $MgCl_2$ 1 mM EGTA, 0.5 mM DTT, 100 μM ATP and 60% sucrose. This is prepared by adding appropriate volumes of salt stocks for 10 mL of buffer to 6 g of dry sucrose, and adding water to ~9 mL. The solution is mixed until most of the sucrose has come into solution, and the volume is corrected to 10 mL. The solution is then allowed to come completely into solution. At that point it is passed through a 0.22 μm PES filter. Prior to adding to the protein solution it is chilled on ice for ~10 minutes. This is enough time to reduce the temperature, but will not result in sucrose crystallizing out of solution. Use buffer F the same day that it is prepared.

2.3. Chromatography Columns

Recommended flow rates and operating pressures are typical for a column in good condition.

1. DEAE Sepharose FF: 40 ml of DEAE Sepharose FF (GE #17-0709-01) is packed into a 26 mm wide medium pressure column such as an XK26/20 (GE #28-9889-48). The maximum flow rate and pressure for this column is 26.5 mL/min and 0.5 MPa, respectively. We run this column at a flow rate of 8–15 mL/min at less than 0.5 MPa.

2. GSH sepharose column: Bulk Glutathione Sepharose 4B (GE #17-0756-05) is either handled as a batch suspension, or drained through 2.5 cm low-pressure column (*e.g.*, Bio-Rad Econo-Column #737-2512). To achieve reasonable flow rates, the column has a 2-position stopcock and approximately 8 cm of 1.6 mm inner diameter tubing (Bio-Rad silicone tubing #731-8211) attached via a Luer fitting.

3. 50 mL G-25 desalting column: Dry G-25 Medium Sephadex resin (GE# 17-0033-01) was hydrated according to manufacturer's instructions and packed into an XK 26/20 hardware (GE #28-9889-48). The maximum pressure is 0.5 MPa. We typical run at flow rates of 8 – 18 mL/min at less than 0.3 MPa. Prepacked HiPrep Desalting column (GE# 17-5087-01) can also be used, but with reduced flow rates. The maximum pressure for prepacked columns is 0.15 mPa.

4. 4 mL SOURCE 15Q column: Bulk SOURCE 15Q (GE# 17-0947-05) was packed into an empty Tricorn 10/50 GL column (GE #8-1163-14). The maximum flow rate is 23.5 mL/min at less than 4 MPa. We use this column at flow rates of 4 mL/min operated at < 1.0 MPa.

5. Superdex 200 pg column: This is a prepacked HiLoad 26/600 Superdex 200 pg column, with 320 mL of resin in XK26/600 column hardware (GE# 28-9893-36). The maximum flow rate is 4.25 mL/min with a maximum pressure of 0.5 MPa. We run this column run at 1 – 2.5 mL/min at less than 0.4 MPa back pressure.

2.4. Hardware and Equipment

1. A liquid chromatography system capable of delivering linear gradients and operating columns at the described flow rates and pressures.

2. Three centrifuges are needed: (1) A low speed refrigerated centrifuge with swinging bucket rotor, capable of spinning 1 L bottles at 1,600 × *g*. (2) An ultracentrifuge (Beckman Optima or equivalent) with Type 45 Ti rotor. (3) A refrigerated high-speed centrifuge (Beckman Coulter Avanti Centrifuge), equipped with JA-10 and JA-25.50 rotors. Centrifuges equipped with a Beckman JA-20 or Sorvall SLA-3000 and SS-34 are equivalent for this purpose. All centrifugation is performed at 4°C.
3. Three each: large stir plates, magnetic stir bars and 4 L beakers.
4. Cold room or cold box with space to plug in and run stir plates.
5. 50 kDa MWCO dialysis tubing (Spectra/Por 6 #132544) and clamps.
6. Cell extruder(s). A system capable of lysing bacteria is needed as is a system capable of lysing yeast cells. These may be the same system. We use an Avestin EmulsiFlex-C5 microfluidizer for lysing bacteria, and a Microfluidics M-110P microfluidizer with an H10Z 180μ Diamond homogenizing chamber for lysing yeast cells. The latter is capable of achieving the high pressures (> 22000 psi) needed to lyse yeast cells (*see* Note 7).
7. Liquid nitrogen and suitable vessels for freezing cell suspension.

3. Methods

This is a time intensive protocol and greatly benefits from having 2 people working together. Using commercially sourced yeast, one person can perform **Section 3.1** (Preparing Yeast Suspension) with two 1 lb/454 g blocks of yeast in less than a day. Typically, we repeat the procedure several times over 1 – 2 days, producing enough frozen prepared yeast suspension for several preparations. **Section 3.2** (Expression of GST N-WASP VCA in *E. coli*) can be performed by one person, and may be performed while the yeast suspension is being prepared. The described protocol prepares enough GST N-WASP VCA for multiple Arp2/3 complex preparations. Both **Sections 3.1** and **3.2** must be completed before the Arp2/3 complex can be purified. The protocol for Arp2/3 complex purification takes four full days, which we break into four Sections here (**3.3 – 3.6**). Two people working in a team are needed during the first two days (**Sections 3.3** and **3.4**). Typically, these two days require 12 – 14 hour workdays at the scale of prep described here. Working as a team keeps the process manageable. The third and fourth days (**Sections 3.5** and **3.6**) can be completed by a single person.

3.1. Preparing Yeast Suspension

The preparation described here removes any preservatives and stabilizers from the suspension, or any remaining media components if produced in house. **Sections 3.3 – 3.6**

[7]Lysis of yeast cells requires more aggressive physical methods than does lysis of bacteria. Common ways to lyse yeast cells include grinding in liquid nitrogen, agitation with glass beads, and extrusion at high pressure. The latter can be achieved using the same type of equipment used for bacterial lysis, but efficient lysis requires substantially higher pressures. We lyse yeast cells by passing the cell suspension through a Microfluidics M-110P microfluidizer operated at ~25,000 psi, then repeating for a total of three passes. To address the heat generated, particular care is used to cool the system and lysate. Cooling is achieved by packing much of the system in wet ice, and by stopping the flow each time 90 mL had been homogenized to allow several minutes for the system to cool.

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yield approximately 1 mg of purified Arp2/3 from 150 g of yeast cells. The described preparation begins with ~300 g of yeast cells, but we typically prepare more than one preparations worth of cell suspension at a time. Two 1 lb/454 g blocks can be washed at one time using a centrifuge equipped with a six position, 1 L swinging bucket rotor. This protocol generates roughly >2 L of resuspended cells; ensure that sufficient liquid nitrogen and freezer space are available to complete the protocol.

1. Obtain wild type *Saccharomyces cerevisiae* through fermentation or by purchase (*see* Note 1).
2. Resuspend in 5 mL of IBS per gram of wet cell weight in a large beaker. Commercial blocks can be crumbled by hand, and then stirred, and finally pipetted until homogeneous. Allow any dried out yeast to settle briefly before decanting homogenous suspension into 1 L centrifuge bottles. Any yeast that does not resuspend is discarded.
3. Centrifuge for twenty minutes at ~4,700 × *g* in 1L bottles (4,000 rpm in Sorvall H6000A rotor).
4. Decant supernatant, retain cell pellet.
5. Repeat steps (2) through (4) for a total of three washes.
6. Resuspend the final cell pellet with 2 mL of fresh UBpi per gram of wet cell weight. Appreciable loss of cell weight relative to starting weight is common.
7. Flash freeze the cell suspension by slowly dripping it directly into liquid nitrogen in a clean vessel, such that is freezes as individual pellets. Collect the cell suspension and store in a tightly sealed plastic container at −80°C.

3.2. Expression of GST N-WASP VCA in E. coli

1. Obtain a construct for the expression of GST N-WASP VCA in *E. coli* (*see* Note 3)
2. Transform vector into chemically competent BL21(DE3) T1^R cells. Plate on LB agar supplemented with 100 μg/mL of ampicillin. Incubate at 37°C overnight.
3. The following day prepare 1.5 L LB broth in 4 L flasks and autoclave. We typically prepare six of these flasks, but this is sufficient for many Arp2/3 complex purifications (*see* Note 4). In the evening, use the center of a single colony to inoculate a 7 mL LB culture (supplemented with 100 μg/mL ampicillin). Repeat

[4]The quantity of GST N-WASP VCA used to prepare the affinity column is not a critical element. As the binding of Arp2/3 complex from solution is fairly inefficient, there should be an excess of GST-VCA present. Using our bacterial expression system, we use cells from 0.5 – 1 L of LB culture for each 100 g of yeast cells lysed or 300 g of cell suspension lysed (as prepared in **Section 3.1**). The DEAE column will bind nearly all of the VCA dimers from solution, and the glutathione sepharose should be overloaded with VCA dimer. By overloading this column, we minimize contaminants at the cost of yield. This saves additional purification steps, and thus greatly shortens the VCA purification.

We have found that Arp2/3 complex is never completely captured when working with beads suspended in batch. Thus, we save the flow through following separating beads from lysate after a binding step. The flow through is then reapplied to the beads a second time, and the purification repeated. We have found that additional Arp2/3 complex can be purified by additional cycles of capture from the lysate, but that the purity and amount captured decreases with each cycle. Typically, the second pass has only 30 – 80% of the Arp2/3 complex captured in the first pass, but shows only an incremental increase in contaminants. The third pass typically shows a similar decrease in quantity captured, but a substantial increase in contaminants. Thus, two cycles seems the best compromise in terms of yield, purity and time involved.

such that two 7 mL cultures are prepared for each 1.5 L culture planned. Place tubes at an angle in a shaking incubator and grow with shaking (200 – 250 rpm for a 1 inch orbit) overnight at 37°C.

4. The following morning, spin down overnight cultures at ~2,000 × *g* (3,500 rpm in a Sorvall Legend centrifuge with swinging bucket rotor) for 10 minutes at 4°C. Decant the supernatant and resuspend all cultures in fresh LB with 100 μg/mL ampicillin, using a volume easily divided among the 1.5 L cultures (*e.g*., 12 mL for 6 flasks). Reserve a small volume of fresh media for blanking optical density measurements. Inoculate large-scale growths with the resuspended cultures.
5. Place the large cultures in a 37°C shaking incubator, shaking at 225 to 250 rpm for 1 inch orbit. Begin checking optical density after approximately 2 hours. When optical density at 600 nm reaches between 0.6 and 0.9 for a 1 cm path-length, induce the culture with 1 mL of a 1 M IPTG stock per L of culture volume. Save a small volume of the culture for pre-induction gel sample (*see* Note 5).
6. Return the induced culture to 37°C with shaking for 3 hours.
7. Collect post induction gel sample (*see* Note 5).
8. Harvest the culture by centrifugation at ~6,000 × *g* (4,500 rpm in a Sorvall H6000A rotor) for 25 minutes at 4°C.
9. Decant spent media and resuspend with 25 mL of QLB per L of culture volume. Add 250 μL of 1M PMSF stock per 25 mL of QLB near the end of resuspension. Cells should be completely resuspended prior to freezing. Check resuspension by watching as the suspension is pipetted against the bottom of the centrifuge bottle, there should be no visible cell clusters. Place resuspended cells into 50 mL plastic conical tubes and freeze at −80°C.

3.3. Arp2/3 Complex Purification, Day 1

On the first day of the protocol, yeast cell suspension is lysed using a cell extruder and clarified by high-speed centrifugation. These are the principle limiting steps in the entire protocol and available hardware will greatly influence the overall preparation scale. If the preparation changes in scale, use the given VCA column and SOURCE15Q column sizes to guide rescaling of the column sizes. Finally, if the prep is scaled up substantially, it may be impractical to increase the volume of dialysis buffer. In that case, additional buffer change steps may be used. As described, this protocol takes approximately 14 hours to complete, with a second person needed through the first 8 – 10 hours.

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[5]The induction of proteins can be best seen in SDS-PAGE analysis if the samples are well matched. We have good results by collecting ~1.5 mL of culture and measuring its optical density at 600 nm (OD_{600}). Calculate how much of this sample is needed to prepare a matched expression sample using the formula: volume = $0.15/OD_{600}$ * mL. Place that volume of culture in a 1.5 mL tube, and pellet the cells by spinning at ~10,000 × *g* for 2 minutes. Remove the medium, and resuspend the cells completely in 50 μL of water or neutral buffer by pipetting up and down. Add an additional 50 μL of 2× concentrated SDS sample buffer and mix. After induction, repeat the process, adjusting the volume for the increase in optical density. Heat the samples for five minutes immediately before loading 6 – 8 μL onto a denaturing SDS-PAGE gel. We routinely use continuous 15% acrylamide PAGE gels for the analysis of GST N-WASP VCA expression.

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1. Weigh out the desired quantity of frozen yeast cell suspension. The protocol is designed for roughly 900 g of cell suspension, equivalent to 300 g of wet cell weight (*see* **Section 3.1 Step 6**). Place frozen cell suspension into a glass beaker and thaw (*see* Note 6).
2. Lyse the yeast by extrusion at high pressure (*see* Note 7). Use three passes operated at 24,000 – 30,000 psi. Verify lysis by placing 250 μL of cell suspension before extrusion, and after each pass, into 1.5 mL centrifuge tubes, and spinning in a microfuge to clarify for 10 minutes at 16,000 × *g*. (This spin can be performed at room temperature.) Compare sample before extrusion and after each pass for changes in pellet size and supernatant color. The pellet should reduce in size and the supernatant should become more turbid and golden brown with each pass through the extruder. Lysis is deemed complete when the centrifuged samples from the final and penultimate passes through the extruder are roughly the same. Microscopic inspection or release of protein to the supernatant could also be used to follow lysis. If the cell disruptor is operated at the lower end of the pressure range, an additional 1 – 2 passes may be needed. In this case, lysis efficiency may be assessed after only a portion of the lysate has been processed.
3. Clarify lysed yeast suspension by centrifugation at 138,000 × *g* for one hour at 4°C (42,000 rpm using Type 45 Ti rotor and compatible 70 mL polycarbonate bottle assemblies). As the volume of lysate produced exceeds the capacity of the rotor, the lysate is split across multiple cycles of centrifugation. To save time, a portion of the lysate is centrifuged while additional cell suspension is lysed. The volume processed here, 900 g of cell suspension, will need to be divided across three centrifugation cycles.
4. Inspect the clarified lysate. Four layers should be visible: a solid tan pellet at the bottom of the tube, a jelly-like darker brown layer on top of the pellet, a golden brown liquid layer on top of the jelly layer and a thin stark white layer at the very top (which may not completely cover the surface). The golden brown liquid layer contains the soluble Arp2/3 complex. Decant clarified liquid supernatant through four layers of cheesecloth (to catch most of the white top layer and any of the jelly layer that comes loose as the supernatant is poured off) into a glass beaker packed into wet ice.
5. Measure the combined supernatant volume, while keeping it at 4°C (or on wet ice). Return the measured lysate to the beaker from **step 4** and add a clean magnetic stir bar.
6. Place the beaker on a magnetic stir plate and begin stirring. Stirring should be aggressive enough to create a vortex with a depression which reaches at least

[6]Thawing the yeast cell suspension can be performed more than one way. For any method, it is important to remember to keep the yeast cell suspension cool, to thaw it as evenly as possible and to use it soon after thawing. One suggestion is to place the weighed frozen cell suspension in a 1 L glass beaker with a clean stir bar. Cover the 1 L beaker with aluminum foil and place it in a plastic 4 L beaker with cool water. Hold the 1 L beaker down with a lead ring and place the nested beakers on a stir plate. Once the yeast begins to thaw begin stirring. As the water in the lower beaker is chilled by the thawing cell suspension, replace it with fresh cool (but not cold) water. Alternatively, once the cell suspension is sufficiently thawed, it can be stirred with a serological pipette.

halfway down the height of the liquid. The depression should not contact the stir bar, which would introduce air. Weigh out enough solid ammonium sulfate to bring the solution to 25% saturated (134 g/L dry ammonium sulfate powder). Add ammonium sulfate slowly while stirring in cold room (*see* Note 8).

7. Continue to stir for an additional 30 minutes.

8. Spin down pellet at ~9,000 × *g* (9,000 rpm in a JA-10 or equivalent) for 15 minutes. Use half full 500 mL centrifuge bottles, splitting into two spins if necessary. Pool, measure, and retain the supernatant.

9. While supernatant from **step 8** is held at 4°C in a cold room, weigh out enough ammonium sulfate to bring this 25% saturated solution to 55% saturated (an additional 179 grams of ammonium sulfate solid per L of supernatant). Slowly add the ammonium sulfate powder while stirring (*see* Note 8).

10. Continue to stir for an additional 30 minutes.

11. Spin down pellet at ~9,000 × *g* (9,000 rpm in a JA-10 or equivalent) for 15 minutes. Use half full 500 mL centrifuge bottles, splitting into two spins if necessary.

12. Decant and discard supernatant from **step 11** and place bottles on ice at an angle such that the pellet is away from the ice and excess liquid can collect at the lowest part of the bottle. Let sit for 3 minutes and remove any accumulated liquid with a pipette.

13. Resuspend ammonium sulfate cut pellet in 40 mL of buffer A per 300 g of cell suspension (*see* Note 9).

14. Place resuspended pellet into 50 kDa MWCO dialysis tubing and dialyze against ~4 L of buffer A per 300 g of cell suspension (*see* Note 9) overnight at 4°C, with gentle stirring (*see* Note 10).

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[8]A common mistake during the ammonium sulfate cut is to add the solid ammonium sulfate too quickly or all at once. This results in locally high concentrations of ammonium sulfate, which can cause undesired proteins to precipitate in a non-equilibrium fashion. A few precautions can minimize this problem.
First, inspect the ammonium sulfate as it is weighed out. Often there are clumps of crystals present, some of which may be larger than 5 mm. If this is a problem, break them up by mashing them with a mortar and pestle. The goal is not to smash the crystals into powder (which does help, but not enough to warrant routinely doing) but just to break up any large clumps of crystals that may be present.
Second, for the amounts described here the addition of ammonium sulfate should occur over 20 to 25 minutes. Time can be scaled down somewhat for smaller volumes. The stirred solution should be checked periodically while the solid is added. Look at the bottom of the beaker, make sure solid ammonium sulfate is not accumulating. If it does, the ammonium sulfate is being added too quickly. Wait a few minutes for the accumulated solid to disperse, then continue to add, but more slowly. A practical way to add the solid is to put half to one third of it in a plastic weigh boat, and to tap it with a spatula. By varying the frequency of tapping a reasonably controllable and uniform addition rate may be found.

[9]Here, cell suspension weight refers to cells resuspended as in **Section 3.1 Step 6**, where one third of the suspension weight is wet cell mass. If cells are resuspended at a different cell density, the protocol should be scaled according to the wet cell weight lysed, not according to the cell suspension weight.

[10]Cut and rinse lengths of 50 kDa cut-off dialysis membrane (Spectra/Por 6, #132544) with ultrapure water. Enough dialysis tubing should be prepared to hold the resuspended volume, plus a 70% increase in volume due to the difference in osmotic strength between the resuspended pellets and buffer A. For the indicated dialysis tubing cut a total of 50 cm per 60 mL, split across at least two pieces. Affix dialysis clips onto one end of the tubing and transfer the resuspended pellets into the dialysis tubing, leaving at least 50% of the length as slack. Close off the other end with additional dialysis tubing clips. Dialyze the pellets against 12 L of buffer A overnight (greater than 6 hours) at 4°C, stirring slowly. It is usually necessary to use two medium or large dialysis clips at either end of the tubing to give the suspension/tubing sufficient buoyancy to prevent it from hitting the stir bar. Alternatively, hang the dialysis tubing from a rod arranged over the dialysis vessel.

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3.4. Arp2/3 Complex Purification, Day 2

The second day of the protocol begins with the dialyzing samples from the first day. This day prepares the GST-VCA column while dialysis continues, and then performs the affinity column step twice. If the prep has been scaled up substantially, it may be necessary to add an additional dialysis buffer change step. As described, this protocol takes approximately 14 hours to complete, with a second person needed through the first 8 – 10 hours.

1. Change the dialysis buffer once with an equal volume of buffer A. Continue to dialyze during the GST N-WASP VCA purification.

2. Thaw the cell pellets from 2–3 L of *E. coli* expressing GST N-WASP VCA (prepared in **Section 3.2**) in cool water. Lyse using 2–3 passes through an Avestin Emulsiflex C5 cell disruptor/homogenizer, cooling the lysate as it passes out of the aperture. The system should be operated at >8,000 psi (*see* Note 11).

3. Load lysate into 50 mL round-bottom centrifuge tubes and clarify by spinning for 40 minutes at 30,000 × *g* (19,000 rpm using a JA25.50 rotor).

4. Equilibrate a clean DEAE column (*see* Note 12) in 92% QA1 / 8% QB1.

5. Pool the supernatant from **step 3** and load onto the DEAE column at 5–10 mL/min. Wash with 2 column volumes of 92% QA1 / 8% QB1, then with 3 column volumes of 85% QA1 / 15% QB1. Elute GST N-WASP VCA with 3 column volumes of 50% QA1 / 50% QB1, collecting and saving the eluate. Save 20 μL samples from the supernatant, column flow through, column wash (combine 8% QB1 and 15% QB1 washes) and elution fractions for SDS-PAGE analysis.

6. Load 6 mL of Glutathione Sepharose 4B (*see* Note 13) into a 2.5 cm low-pressure column and equilibrate by passing 60 mL of buffer GSH-WB1 over the beads (*see* Note 14).

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[11]*E. coli* expressing GST N-WASP VCA can be lysed by a number of common methods. We have used sonication and extrusion using an Avestin EmulsiFlex-C5 cell disruptor. In either case, some care should be used to minimize heating of the sample. When using sonication, use a series of sonication pulses (about 10 seconds total sonication time) and place the solution on ice for 1–2 minutes between pulse series. For extrusion, the cell disruptor has a heat exchanger mounted immediately following the lysis aperture, and chilled water is flowed through this during lysis.

[12]The DEAE column should be sized according to how much culture is lysed. At least 10 mL of DEAE Sepharose FF should be used per L of LB VCA culture. When lysing volumes larger than 4 L it is usually more practical to repeat the DEAE step and pool the results than to use a larger column.

The DEAE Sepharose column can be reused many times. The principle reason the column should be repacked is accidental introduction of air. Between uses, the column should be thoroughly cleaned. The final high salt wash (in 100% QB1) does a reasonable job removing most protein contaminants, but leaves behind a significant amount of nucleic acid. This can be depleted with washes at different percentages of QB1 (with QA1 making up the balance). Wash the DEAE column with ~1 column volume at 5% QB1, followed by 35%, 65%, 100%, 5%, 35%, 65%, and 5% QB1. If there is still substantial absorbance at 254 nm eluted during the 35% QB1 step, add an additional 1 –2 cycles of 35%, 65% and 5% QB1.

[13]To save costs, we routinely regenerate Glutathione Sepharose 4B after use in purifying bacterially expressed proteins. The resin is regenerated with 3 column volumes of 6 M guanidine hydrochloride (98% purity), and then washed extensively with water. The resin can be further washed with 2% SDS, although extensive washes with water are necessary between the guanidine and SDS washes. After the initial use, capacity of the resin drops by roughly half, and then is more or less stable for subsequent uses. Regenerated resin can be used here, but adjust the total column volume for the two-fold decrease in capacity. Given the different possible contaminants, once used with yeast lysate glutathione resin is not regenerated but is instead discarded.

[14]A smaller column such as a 1.5 cm diameter disposable column (*e. g*., Bio-Rad Econ-Pac #732-1010) is needed when scaling down the prep (~100 *g* of cells) and less than 2.5 mL of Glutathione Sepharose 4B resin is used. These smaller columns can also be used in the prep described here as an alternative to the 2.5 cm glass Econo-Column. If using for this prep, split the 6 mL of resin into three 1.5 cm columns. In either case, a stopcock and a piece of tubing attached to the bottom of the column will help to keep the solution flowing (due to the increased height between the top of the liquid and the column outlet) and will allow stopping the flow to recover the resin.

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7. Resuspend GSH Sepharose beads using 10 mL of GSH-WB1 and add to the eluate from **step 5**. Distribute eluate/bead suspension into 50 mL conical tubes, filling the tubes to >90% of capacity and topping off with additional buffer GSH-WB1 as necessary. Allow proteins to bind to the beads in batch mode for 30 minutes, with gentle rotation at 4°C.
8. Separate the beads from the liquid by passing the suspension through the low-pressure column used in **step 6**. Retain the beads and collect the flow through.
9. Wash the beads 5 times with 2.5 column volumes of buffer GSH-WB1, pooling and retaining the wash. Clean the beads further by washing 5 times with 2.5 column volumes of buffer GSH-WB2. Finally, wash the beads an additional 5 times with 2.5 column volumes of buffer GSH-WB1. Save 20 μL samples of flow-through, pooled wash and a small quantity of the washed beads for SDS-PAGE analysis (*see* Fig. 1).
10. Clarify the yeast dialysate from **step 1** by centrifugation at 30,000 × *g* for 30 minutes (19,000 rpm in JA-25.50 rotor, or equivalent). The volume will have increased and this may fill more than eight tubes. Decant the supernatant through four layers of cheesecloth, into a 600 mL glass beaker on ice. Supernatant will not appear completely clear following centrifugation.
11. Equilibrate the affinity column from **step 9** with 30 mL of buffer A. Once the column has drained, apply another 30 mL of buffer A to the column and allow it to flow through.
12. Stop liquid flow through the column and re-suspend the resin with a small amount of buffer A. Mix resuspended resin with the clarified dialysate from **step 10**. Distribute into 50 mL conical tubes, filling nearly full and topping off with buffer A as necessary. Incubate with gentle mixing for 25 minutes at 4°C.
13. Drain bead suspension through the 2.5 cm low-pressure column, collecting the flow through.
14. Wash the beads 5 times with 4 column volumes of buffer A, collecting the wash. Then wash the beads 10 times with 5 column volumes of buffer B, collecting this wash separately. Finally, pass 2.5 column volumes of buffer C over the resin collecting the eluted proteins. Repeat the buffer C wash a total of five times, collecting each elution as a separate fraction.
15. Re-equilibrate the resin in buffer A as in **step 11**. Repeat **steps 12–14** substituting the flow-through from **step 13** for the clarified dialysate in **step 12**. This second pass captures about half the Arp2/3 complex captured on the first pass over the beads (*see* Note 4).
16. Determine buffer C elution fractions to pool by SDS-PAGE analysis. Arp2/3 complex should be visible by coomassie staining at this step (*see* Fig. 1 and Note 15).
17. Once fractions are pooled, they can be held overnight at 4°C (*see* Note 16).

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3.5. Arp2/3 Complex Purification, Day 3

The third day of the protocol begins with the pooled GST-VCA elution fractions. The buffer is exchanged and Arp2/3 complex is further purified using SOURCE15Q ion exchange chromatography. A preparative gel filtration column is then run as an overnight step. A single person can perform desalting, ion exchange, SDS-PAGE analysis, and beginning the gel filtration column in 8 – 10 hours.

1. Exchange pooled buffer C elution into buffer QC using the 50 mL G-25 desalting column. Equilibrate the column in 1.5 column volumes of QC and inject 13–18 mL of sample onto the column. Continue to pass QC over the column and collect the peak containing the eluted protein.

2. Repeat **step 1** until the entire sample has been buffer exchanged. For the combined pool of two passes of the yeast cell lysate over the VCA column, roughly ten injections onto the desalting column will be needed. Pool all desalted eluate.

3. Equilibrate the 4 mL SOURCE 15Q column in 100% QC. Inject desalted pool onto the column. Bind and run at 1–2.5 mL/min. Wash out unbound sample with 2 column volumes of QC. Quickly shift to 96% QC / 4% QD, then run a linear gradient from 96% QC / 4% QD to 71% QC / 29% QD over 50 column volumes, collecting 2.5 mL fractions. Assess purity by SDS-PAGE, pool relevant samples (*see* Fig. 2).

4. Purify Arp2/3 complex using Superdex 200 gel filtration chromatography. Equilibrate a 320 mL Superdex 200pg column with at least 300 mL KMEHd–A. If necessary, concentrate the SOURCE 15Q pool to ~10 mL using an Amicon Ultra-15, 30,000 MWCO centrifugal concentrator unit. Inject the concentrated Arp2/3 complex pool onto the column at 1 mL/min. Elute the Arp2/3 complex by flowing an additional column volume of buffer over the column at 1.5 – 2.5 mL/min, collecting 5 mL fractions beginning after 0.3 column volumes of buffer. Arp2/3 complex typically elutes as the dominant peak, with its maxima occurring when ~0.6 column volumes of buffer have been applied to the column. This is typically begun as an automated overnight step and allowed to run unattended. If possible arrange to have the column run somewhat slower than usual such that the Arp2/3 complex containing fractions elute in the early morning.

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[15]The *Saccharomyces cerevisiae* Arp2/3 complex ArpC1 subunit (ARC40) has been reported to have an odd mobility on SDS-PAGE gels (30). We have routinely noted this as well. The protein may not appear on the gel at all when samples are prepared in a standard fashion (mixed with 2× reducing SDS loading buffer and heated at 95°C for 3–5 minutes). This seems to be specific to ARC40 at moderate to high concentrations, and is dependent on heating of the SDS-PAGE samples. If it is necessary that ARC40 completely enter the gel, dilute the sample to ~40 nM or less prior to the addition of SDS and reducing agent, and omit the heating of the sample. In many cases, this drops the concentration out of standard coomassie gel staining sensitivity, necessitating silver staining or Western blotting methods.

[16]Given the length of the second day of the preparation (**Section 3.4**), once Arp2/3 complex is eluted from the GST-VCA column it is held at 4°C overnight and then exchanged into buffer QC the following day. A desalting column is employed to save time (**Section 3.5, steps 1 and 2**). If necessary, this allows delaying of SDS-PAGE assessment and pooling until the morning of the third day. Alternatively, dialysis could be set up against 4 L of buffer QC at the end of the second day and **steps 1** and **2** of **Section 3.5** skipped.

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3.6. Arp2/3 Complex Purification, Day 4

The fourth day of the protocol begins by assessing the overnight gel filtration results by SDS-PAGE. If necessary a second pass over gel filtration is used to complete the purification. Otherwise, the complex is concentrated, quantified and frozen. A single person can complete the fourth day in about five hours if the additional gel filtration step is not needed, and in about 10 hours if the additional gel filtration step is needed. Concentrating and freezing of the complex can be delayed until a fifth day.

1. Assess purity using SDS-PAGE analysis, pool relevant fractions (*see* Fig. 3).
2. If contaminating proteins are observed, typically a doublet at ~100 kDa, inject the pool back onto the Superdex 200pg column as described in **Step 4** of **Section 3.5**, dividing it into two injections. Assess purity by SDS-PAGE, pool relevant fractions (*see* Fig. 4).
3. Measure concentration (*see* Note 17); concentrate using an Amicon Ultra-15 30,000 MWCO centrifugal concentrator if desired.
4. Pooled materials can be diluted to 1.5-fold the desired working concentration, mixed with buffer F, aliquoted and frozen (*see* Note 18).

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[17]Our method of quantifying Arp2/3 complex is based on tryptophan UV absorption. An extinction coefficient at 280 nm of 245240 M^{-1} cm^{-1} was estimated from the number of tryptophan and tyrosine residues (24 and 76, respectively) in a complex. As the complex may bring an ATP or ADP nucleotide along with it during purification, we routinely measure absorption at 290 nm, and correct for any scatter by using the absorption at 314 nm. By measuring the relative absorbance of a sample judged to be devoid of nucleotide at 280 nm and 290 nm, we found the A_{290}/A_{280} ratio to be 0.6. Thus, we routinely measure the concentration using an extinction coefficient at 290 nm of 147000 M^{-1} cm^{-1}. Our experience using laser interference to quantify the complex during analytical ultracentrifugation experiments shows this extinction coefficient to be within 10% of the correct value.

[18]Directly freezing small aliquots of Arp2/3 complex in liquid nitrogen results in a slight degree of aggregation, and measurable loss of activity. For routine assay at 10 nM of Arp2/3 complex in pyrene actin polymerization assays, we supplement 600 nM Arp2/3 complex in KMEHd-A with one half of a part buffer F (*i. e*., 5 mL of buffer F is added to 10 mL of 600 nM Arp2/3 complex solution). Small volumes (*e.g*., 80 μL) can then be placed into small tubes (200 μL thin wall PCR tubes are convenient for this) and snap frozen in liquid nitrogen. For additional commentary, see the bovine Arp2/3 complex purification notes (23).

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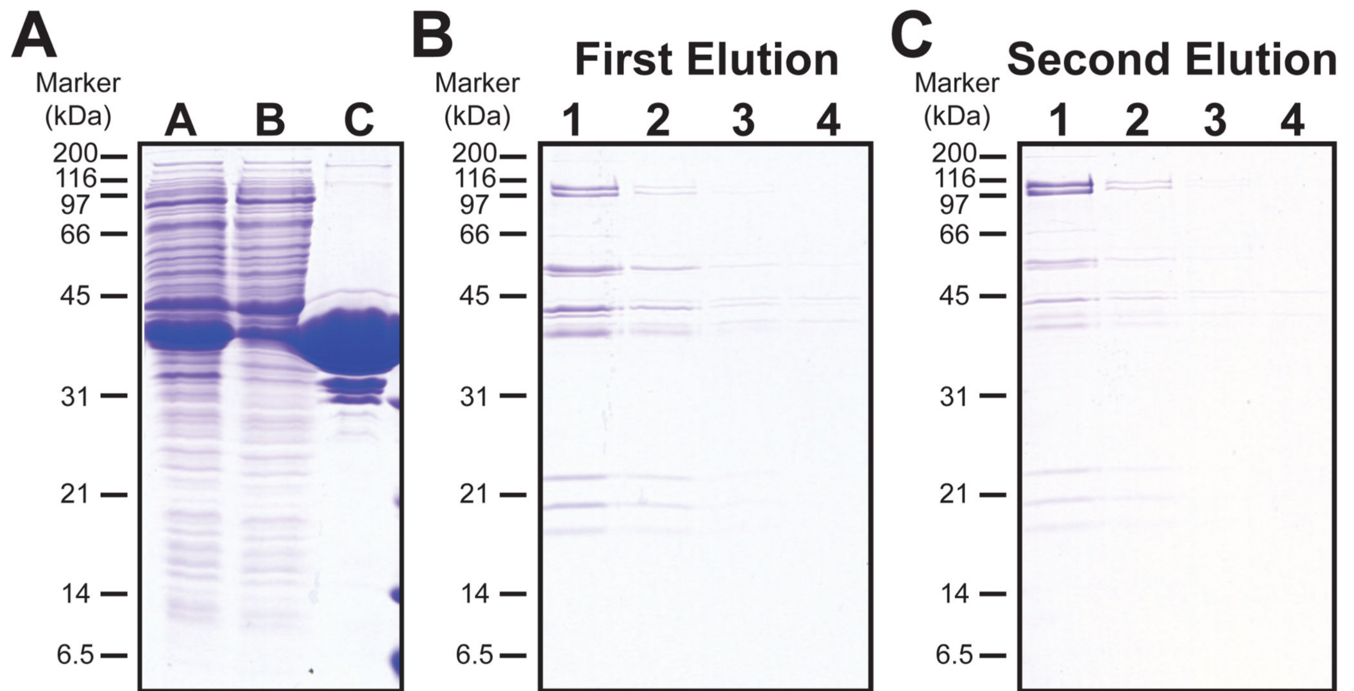

Fig. 1. Preparation and use of GST-VCA column

(**A**) Preparation of the GST-N-WASP VCA column using over-expressed protein. GST N-WASP VCA is bound and eluted from DEAE sepharose (elution shown in Lane A). The DEAE Sepharose elution is passed over glutathione sepharose beads, with the flow through (Lane B) and final washed beads (Lane C) shown. (**B**) Dialysate from **Section 3.4, step 1** is passed over the GST-VCA beads, washed extensively and eluted with Buffer C. Elution fractions #1 – #4 are shown. (**C**) Flow through from the first application onto GST-VCA beads is reapplied to the column, washed extensively and eluted with Buffer C. Elution fractions #1 – #4 are shown. Position and mass of molecular weight standards are indicated to the left of each panel. Elution fraction #1 – #3 were pooled for both the first and second binding passes.

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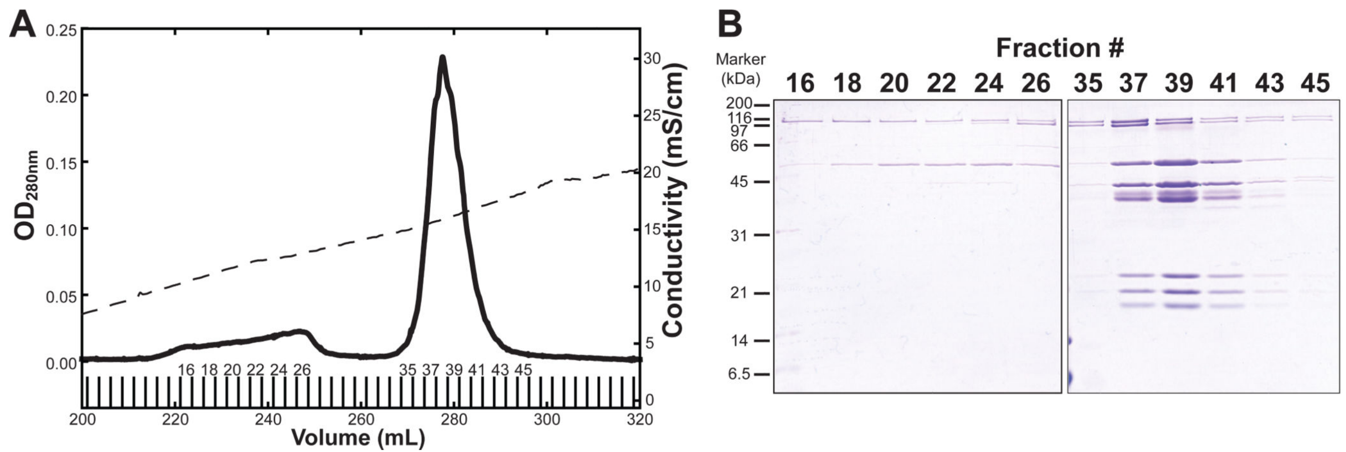

Fig. 2. Purification of Arp2/3 complex using SOURCE 15Q anion exchange chromatography

(**A**) Chromatogram for the resolving portion of the protocol, showing one dominant peak in the UV signal, occurring near a conductivity of 17 mS/cm. Spaces between long vertical lines beneath the UV trace represent the collected fractions, with relevant fraction numbers labeled. (**B**) SDS-PAGE analysis of fractions from the SOURCE 15Q column. Bands at 48, 42, 40, 35, 19, 18 and 15 kDa are Arp2/3 complex. Fractions containing substantial quantities of Arp2/3 complex and avoiding the contaminant at ~33 kDa (fractions #36 – #40) were pooled. Position and mass of molecular weight standards are indicated to the left of the panel.

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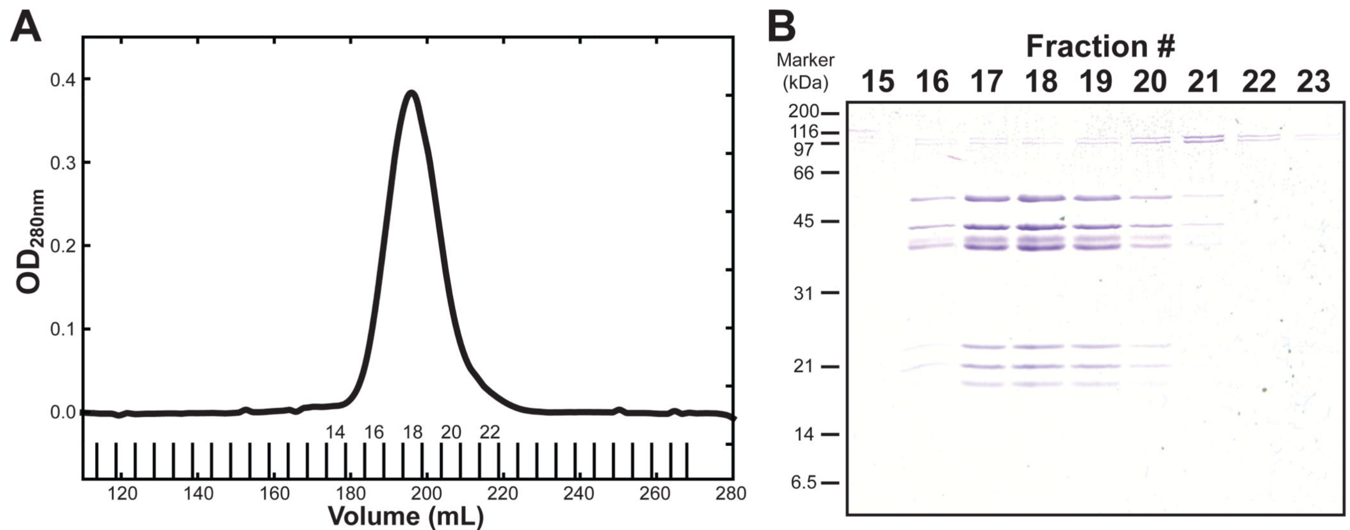

Fig. 3. Purification of Arp2/3 complex using Superdex 200pg gel filtration chromatography

(**A**) Chromatogram for the included volume portion of the protocol, showing one dominant, slightly trailing, peak in the UV signal, eluting with a maximum near 195 mL. Spaces between long vertical lines beneath the UV trace represent the collected fractions, with relevant fraction numbers labeled. (**B**) SDS-PAGE analysis of fractions from the Superdex 200pg gel filtration column. Bands at 48, 42, 40, 35, 19, 18 and 15 kDa are Arp2/3 complex. Fractions containing substantial quantities of Arp2/3 complex and avoiding the contaminating doublet near 100 kDa (fractions #16 – #19) were pooled. Position and mass of molecular weight standards are indicated to the left of the panel.

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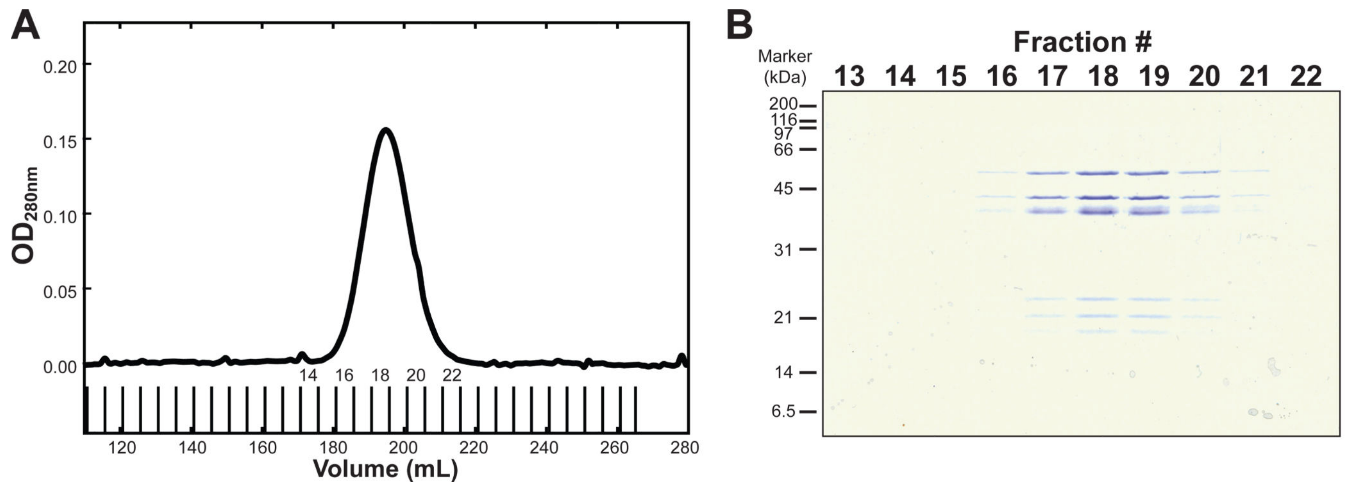

Fig. 4. Further purification of Arp2/3 complex using Superdex 200pg gel filtration chromatography

(**A**) Chromatogram for the included volume portion of the first injection of the protocol, showing one dominant peak in the UV signal, eluting with a maximum near 195 mL. The second injection is indistinguishable from the first. Spaces between long vertical lines beneath the UV trace represent the collected fractions, with relevant fraction numbers labeled. (**B**) SDS-PAGE analysis of fractions from the Superdex 200pg gel filtration column. Bands at 48, 42, 40, 35, 19, 18 and 15 kDa are Arp2/3 complex. Fractions containing substantial quantities of Arp2/3 complex and avoiding the contaminating doublet near 100 kDa (fractions #16 – #19) were pooled. Position and mass of molecular weight standards are indicated to the left of the panel.