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Generating Protein-linked and Protein-free Mono-, Oligo-, and Poly(ADP-Ribose) In Vitro

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Abstract

ADP-ribosylation is a covalent post-translational modification of proteins that is catalyzed by various types of ADP-ribosyl transferase (ART) enzymes, including members of the poly(ADP-ribose) polymerase (PARP) family. ADP-ribose (ADPR) modifications can occur as mono(ADP-ribosyl)ation, oligo(ADP-ribosyl)ation, or poly(ADP-ribosyl)ation, depending on the particular ART enzyme catalyzing the reaction, as well as the specific reaction conditions. Understanding the biology of ADP-ribosylation requires facile and robust means of generating and detecting the modification in all of its forms. Here we describe how to generate protein-linked mono(ADP-ribose), oligo(ADP-ribose), and poly(ADP-ribose) (MAR, OAR, and PAR, respectively) in vitro as an automodification of PARPs 1 or 3. First, epitope-tagged PARP-1 (a PARP polyenzyme) and PARP-3 (a PARP monoenzyme) are expressed individually in insect cells using baculovirus expression vectors, and purified using immunoaffinity chromatography. Second, the purified recombinant PARPs are incubated individually in the presence of different concentrations of NAD⁺ (as a donor of ADPR groups) and sheared DNA (to activate their catalytic activities) resulting in various forms of auto-ADP-ribosylation. Third, the products are confirmed using ADPR detection reagent that can distinguish among MAR, OAR, and PAR. Finally, if desired, the OAR and PAR can be deproteinized. The protein-linked and free MAR, OAR, and PAR generated in these reactions can be used as standards, substrates, or binding partners in a variety of ADPR-related assays.

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Keywords

ADP-ribose (ADPR); ADPR binding domain (ARBD); ADP-ribosylation; ADP-ribosyl transferase (ART); Automodification; Mono(ADP-ribosyl)ation (MARylation); Nicotinamide adenosine dinucleotide (NAD⁺); Oligo(ADP-ribosyl)ation (OARylation); Poly(ADP-ribose) polymerase (PARP); Poly(ADP-ribosyl)ation (PARylation); Post-translational modification (PTM)

1. Introduction

ADP-ribosylation is a post-translational modification of proteins that results from the covalent addition of ADP-ribose (ADPR) moieties derived from donor beta-nicotinamide adenine dinucleotide (β NAD⁺) on glutamate, aspartate, serine, lysine, arginine, and possibly other amino acid residues [5, 8, 19, 27]. The modification is catalyzed by a diverse family of ADP-ribosyltransferase (ART) enzymes, including (1) bacterial toxins (e.g., Cholera and Diphtheria toxins) [6, 28], (2) ecto-ADP-ribosyltransferases (ectoARTs) [10], (3) members of the sirtuin family [13, 25, 31], and (4) members of the poly(ADP-ribose) polymerase (PARP) family [2, 8]. ARTs generally catalyze mono(ADP-ribosyl)ation (MARylation) of their substrate proteins, although some PARPs, such as PARP-1, catalyze oligo- and poly(ADP-ribosyl)ation (OARylation and PARylation, respectively) [6, 8, 10, 14, 32] (Figure 1). Many ARTs are capable of modifying themselves (i.e., automodification). ADP-ribosylation of proteins has emerged as a dynamic regulator of a wide variety of cellular and molecular processes, including transcription, RNA processing, DNA damage repair, unfolded protein responses, and cellular signaling [8, 11, 20, 26].

In addition to ARTs, which add ('write') ADPR, cells express glycohydrolases, which remove ('erase') ADPR, and proteins containing ADPR binding domains (ARBDs), which bind ('read') mono-, oligo-, and/or poly(ADP-ribose) (MAR, OAR, and PAR, respectively) [3, 7, 8, 11, 14, 29]. Interestingly, nature has devised protein modules that specifically recognize and bind to various forms of ADP-ribose [3, 29]. The latter (i.e., ARBDs 'readers'), which are found in a variety of proteins with diverse functions, including a number of PARP family members [3, 8, 29], include macrodomains and WWE domains [3, 29]. Macrodomains bind MAR, as well as the terminal ADPR moieties in OAR and PAR, allowing them to bind to all three forms of ADPR (i.e., MAR, OAR, and PAR) [16, 18, 30]. On the other hand, WWE domains bind the iso-ADPR linkages joining ADPR monomers, limiting their binding to OAR and PAR [15, 33, 34]. ARBDs can be used as research tools for the recognition of various forms of ADPR by fusing them to other protein domains or modules [1, 4, 9, 21, 23, 24, 30], including the Fc region of rabbit immunoglobulin to generate antibody-like ADPR binding proteins [9, 21].

The field is faced with a number of fundamental questions about ADP-ribosylation. For example, (1) which proteins are substrates (i.e., targets) for ADP-ribosylation?, (2) which specific amino acid residues within those substrate proteins are modified? (3) what is the nature of the ADPR modification (mono-, oligo-, or poly)?, and (4) how does ADP-ribosylation modulate the activity or function of the substrate proteins? Answering these questions requires a facile and robust means of generating and detecting ADPR in all of its forms. Herein, we describe methods for (1) the expression and purification of recombinant

PARP-1 and PARP-3, (2) using these proteins to generate protein-linked MAR, OAR, and PAR in vitro as an automodification of PARPs 1 or 3, and (3) deproteinizing the automodified PARP-1 to generate free OAR or PAR (Figure 2). The protein-linked and free ADPR forms can be used as standards in a variety of ADPR-related assays.

2. Materials

All reagents should be the highest quality molecular biology and biochemistry grade.

2.1. Expression of Recombinant PARP-1 and PARP-3 Proteins

1. Sf9 insect cells.
2. Appropriate serum-free or serum-containing Sf9 cell growth medium (e.g., Sf-900™ II SFM medium).
3. Recombinant baculoviruses for expressing FLAG epitope-tagged wild-type PARP-1 or PARP-3.
4. Cell culture spinner flasks.
5. Low RPM magnetic cell culture stir plate.
6. 27°C incubator.
7. Phosphate-buffered saline (PBS), ice cold.

2.2. Purification of Recombinant PARP-1 and PARP-3 Proteins

1. >18 MΩ double distilled H₂O (ddH₂O).
2. Protease inhibitor mix (e.g., Roche cOmplete™ EDTA-free Tablets provided in glass vials, which can be dissolved to the appropriate concentration) (see Note 1).
3. FLAG Lysis Buffer: 20 mM HEPES (pH 7.9), 0.5 M NaCl, 4 mM MgCl₂, 0.4 mM EDTA, 20% glycerol, 250 mM nicotinamide, 2 mM β-mercaptoethanol, 2x protease inhibitor mix (see Notes 2 and 3).
4. Dounce homogenizer with a type B (tight) pestle.
5. High speed centrifuge with an SS-34 rotor (Sorvall) or a comparable centrifuge/rotor pair.
6. FLAG Dilution Buffer: 20 mM HEPES (pH 7.9), 10% glycerol, 0.02% NP-40 (see Note 3).
7. Probe-style sonicator.
8. Anti-FLAG M2 agarose resin, 50% slurry.

¹. Make fresh for each use.

². While this buffer can be made with the base components in advance and stored, the reducing agent (β-mercaptoethanol) and protease inhibitors (PMSF, aprotinin, leupeptin, or protease inhibitor mix) should be added immediately before use.

³. This buffer/solution should be ice cold for use.

9. FLAG Wash Buffer #1: 20 mM HEPES (pH 7.9), 150 mM NaCl, 2 mM MgCl₂, 0.2 mM EDTA, 15 % glycerol, 0.01% NP-40, 100 mM nicotinamide, 0.2 mM β-mercaptoethanol, 1 mM PMSF, 1 μM aprotinin, 100 μM leupeptin) (see Notes 2 and 3).
10. FLAG Wash Buffer #2/High Salt: 20 mM HEPES (pH 7.9), 1 M NaCl, 2 mM MgCl₂, 0.2 mM EDTA, 15% glycerol, 0.01% NP-40, 100 mM nicotinamide, 0.2 mM β-mercaptoethanol, 1 mM PMSF, 1 μM aprotinin, 100 μM leupeptin) (see Notes 2 and 3).
11. FLAG Wash Buffer #3: 20 mM HEPES (pH 7.9), 150 mM NaCl, 2 mM MgCl₂, 0.2 mM EDTA, 15% glycerol, 0.01% NP-40, 0.2 mM β-mercaptoethanol, 1 mM PMSF, 1 μM aprotinin, 100 μM leupeptin) (see Notes 2 and 3).
12. FLAG peptide (Sigma), 5 mg/mL dissolved in Tris buffered saline [TBS: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl] (see Notes 2 and 3).
13. FLAG Elution Buffer: Use FLAG Wash Buffer #3 containing 0.2 mg/mL FLAG peptide (see Notes 2 and 3).
14. Bradford protein assay solution.

2.3. In Vitro Auto(ADP-ribosyl)ation Reactions with PARP-1 and PARP-3

1. Purified recombinant full-length FLAG-tagged PARP-1 and PARP-3 (from Sections 3.1 and 3.2 below).
2. Sonicated salmon sperm DNA.
3. Bovine serum albumin (BSA)
4. Automodification Buffer: 20 mM HEPES (pH 8.0), 5 mM MgCl₂, 5 mM CaCl₂, 0.01% NP-40, 25 mM KCl, 1 mM DTT, 0.1 mg/mL sonicated salmon sperm DNA, 0.1 mg/mL BSA.
5. NAD⁺, 10 mM, dissolved in 10 mM Tris-HCl (pH 7.9) (see Note 4).

2.4. Confirmation of Protein-Linked ADPR Products by Western Blotting

1. 10% PAGE-SDS gel and an appropriate electrophoresis apparatus.
2. 4X SDS-PAGE Loading Buffer: 200 mM Tris-HCl (pH 6.8), 10% SDS, 40% glycerol, 0.04% bromophenol blue, and 400 mM DTT (see Note 5).
3. Nylon-backed nitrocellulose membrane.
4. Western transfer apparatus.
5. Anti-mono(ADP-ribose) binding reagent (EMD Millipore: MABE1076, RRID: AB_2665469).

⁴.NAD⁺ is labile. Distribute in small, single-use aliquots after dissolving. Store at -80°C.

⁵.DTT is labile. Make the 4X SDS-PAGE Loading Buffer without DTT and add the DTT immediately before use from a fresh stock solution. β-mercaptoethanol (0.7 M or 5% of the final sample) can be added immediately before use as an alternative to DTT as the reducing agent.

6. Anti-poly(ADP-ribose) binding reagent (EMD Millipore:MABE1031; RRID: AB_2665467).
7. Anti-pan(ADP-ribose) binding reagent (EMD Millipore: MABE1016, RRID: AB_2665466).

2.5 Generation and Analysis of Protein-Free OAR and PAR

1. Trichloroacetic acid, 100% (w/v) (see Note 3).
2. Trichloroacetic acid, 20% (w/v) (see Note 3).
3. Ethanol, 100%.
4. TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
5. DNase I, 10 units/pL.
6. SDS, 10% (w/v).
7. Proteinase K, 20 mg/mL.
8. 1 M KOH/50 mM EDTA solution.
9. Hydrochloric acid (HCl).
10. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
11. Chloroform.
12. Glycogen, 10 mg/mL dissolved in nuclease-free water.
13. 3 M sodium acetate (pH 5.2).
14. 70% ethanol.
15. Urea-PAGE Loading Solution: 6 M urea, 25 mM NaCl, 4 mM EDTA, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromophenol blue.
16. 5X TBE Electrophoresis Buffer: 0.45 M Tris base (pH 7.6), 0.45 M boric acid, and 10 mM EDTA.
17. Ammonium persulfate (APS), 10% (w/v) prepared in water (see Note 6).
18. *N,N,N',N'*-tetramethylethylenediamine (TEMED)
19. Polyacrylamide gel, 20% (w/v) with a 19:1 (w/v) acrylamide/bisacrylamide ratio: 100 mL of 30% 19:1 (w/v) acrylamide/bisacrylamide, 30 mL 5X TBE Electrophoresis Buffer, and 10 mL ddH₂O. Add 0.75 mL of 10% APS and 6 µL of TEMED immediately before use to initiate polymerization.
20. Silver stain kit.

⁶.APS is labile. The 10% stock solution should not be stored at 4°C for more than two weeks.

3. Methods

3.1. Expression of PARP-1 and PARP-3 in Insect Cells

Insect cells are useful eukaryotic cells for the high level expression of recombinant proteins, which in many cases retain their natural post-translational modification state. The protocol below describes how to generate high titer baculoviruses, which can be used for expressing recombinant full-length FLAG-tagged PARP-1 and PARP-3 in insect cells. The expressed proteins can then be purified as described in section 3.2 below.

1. Grow Sf9 cells using your preferred growth conditions (see Note 7).
2. Amplify PARP-expressing recombinant baculoviruses at a low multiplicity of infection (MOI) to generate more viruses by infecting Sf9 cells (see note 7). Use multiple rounds of low MOI amplifications as needed to generate 75 to 100 mL of high titer virus. Collect and store the baculovirus stocks at 4°C protected from light.
3. In parallel to the baculovirus amplification, expand uninfected Sf9 cells in suspension culture so that 250 to 500 mL of cells in logarithmic growth are available for a large scale infection (see Note 8). The cells should be grown at 27°C in a spinner flask placed on a low RPM magnetic cell culture stir plate.
4. Infect the suspension cultures of Sf9 cells in logarithmic growth from Step 3 with 50 to 100 mL of freshly amplified high titer recombinant baculovirus expressing FLAG-tagged PARP-1 or PARP-3 from Step 2. You will need one 250 to 500 mL suspension culture for each protein preparation.
5. 48 hours after infection, collect the Sf9 cells by centrifuging the culture at 1,000 x *g* for 10 minutes in a large centrifuge bottle (see Note 9).
6. Gently resuspend the Sf9 cell pellet in 40 mL of ice-cold PBS and transfer the suspension to a 50 mL conical tube.
7. Collect the cells again by centrifuging at 1,000 x *g* for 10 minutes in the 50 mL conical tube. Remove the PBS.
8. Flash-freeze the cell pellets in liquid N₂ and store them at –80°C.

3.2. Purification of PARP-1 and PARP-3 from Insect Cells

Baculovirus vectors allow for the high level expression of recombinant affinity-tagged proteins in insect cells. The protocol below describes how to purify recombinant full-length FLAG-tagged PARP-1 and PARP-3 expressed in insect cells, which can be used in the biochemical assays described below, as well as a host of other assays not described here.

1. Thaw PARP-1 or PARP-3-containing Sf9 cell pellets on wet ice.

⁷Please refer to other sources for detailed descriptions of Sf9 cell culture, as well as baculovirus generation and use.

⁸Healthy Sf9 cells in suspension cultures at densities between 5×10^5 and 2×10^6 cells/mL should be in logarithmic growth phase.

⁹Expression of the recombinant PARP proteins is usually detectable after 15 hours of infection and increases until ~48 hours post-infection. We recommend determining the time of maximal protein expression in a time course experiment.

2. Resuspend the cells in 7 mL of FLAG Lysis Buffer per 100 mL of initial insect cell culture volume.
3. Lyse cells by dounce homogenization 10 times on ice with a tight pestle. Transfer to a 50 mL screw capped centrifuge tube.
4. Incubate on ice with intermittent gentle mixing to allow salt extraction to occur.
5. Clarify the lysate by centrifugation at 27,000 x *g* at 4°C for 20 minutes (see Note 10). Collect the supernatant and mix with an equal volume of ice-cold FLAG Dilution Buffer (see Note 11).
6. Sonicate the lysate briefly using a probe sonicator (15 seconds, 65% amplitude), while keeping the sample on ice.
7. Clarify the lysate again as in Step 5.
8. While the sample is being centrifuged, wash and pre-equilibrate anti-FLAG M2 agarose in FLAG wash buffer #1.
9. Mix the clarified lysate with equilibrated anti-FLAG M2 agarose resin (200 µL of a 50% slurry per 250 mL of initial insect cell culture volume) in a screw capped conical tube.
10. Incubate the lysate with the anti-FLAG M2 agarose resin for 3 hours at 4°C with gentle continuous mixing (e.g., using a nutator).
11. Centrifuge the mixture in the conical tube at 1000 x *g* for 10 minutes at 4°C in a benchtop centrifuge.
12. Wash the resin by gently resuspending it in 40 mL of FLAG Wash Buffer #1, incubating it for 5 minutes at 4°C with gentle mixing, and collecting it by centrifugation in the conical tube at 1000 x *g* for 10 minutes at 4°C in a benchtop centrifuge.
13. Repeat step 12.
14. Wash the resin with 25 mL of FLAG Wash Buffer #2 as described in Step 12.
15. Repeat Step 14.
16. Wash the resin with 25 mL of FLAG Wash Buffer #3 as described in Step 12.
17. Repeat Step 16, for a total of six washes with three different buffers (see Note 12).
18. Elute the FLAG-tagged PARP proteins from the anti-FLAG M2 agarose resin using 200 µL of FLAG Elution Buffer (see Note 13). Add the elution buffer to the washed resin and incubate on ice with gentle mixing for 10 minutes. Collect

¹⁰ 27,000 x *g* can be achieved at 15,000 RPM using an SS-34 rotor in a Sorvall centrifuge.

¹¹ This will bring the NaCl concentration to 250 mM, which is compatible with the FLAG affinity purification.

¹² The washes with Wash Buffer #1, a low salt buffer containing 150 mM NaCl, removes protein contaminants. The wash with 1 M NaCl (FLAG Wash Buffer #2) removes DNA and RNA contamination from PARP-1 and PARP-3, which can be significant for these nucleic acid binding proteins. Nicotinamide is included in Wash Buffer #1 and #2 to inhibit automodification of PARP-1 and PARP-3. Nicotinamide is then omitted in the Wash Buffer #3.

the resin by centrifugation as described above and carefully remove the supernatant to a new tube on ice, making sure not to bring any resin along with the eluate. Repeat at least three times (see Note 14).

19. Quantify the eluted protein by using a Bradford protein assay or by comparison to increasing amounts of a known protein (e.g., BSA) run side by side with the elutions on a Coomassie-stained SDS-PAGE gel (see Notes 15 and 16) (Figure 3).
20. Aliquot the purified protein, flash-freeze in liquid N₂, and store at −80°C.

3.3. In Vitro Auto(ADP-ribosyl)ation Reactions with PARP-1 and PARP-3 to Generate Protein-Linked Mono-, Oligo-, and Poly(ADP-ribose)

Protein-linked mono-, oligo-, and poly(ADP-ribose) can be generated by taking advantage of the fact that PARP-1 and PARP-3 undergo auto(ADP-ribosylation) when activated by free DNA ends - a process called automodification. At high NAD⁺ concentrations, PARP-1 produces poly(ADP-ribose) in its automodification reaction, whereas at lower, more limiting NAD⁺ concentrations, PARP-1 produces oligo(ADP-ribose) in its automodification reaction. PARP-3 is a mono(ADP-ribosyl)transferase that produces oligo(ADP-ribose) in its automodification reaction. In this section, we describe how to generate protein-linked mono-, oligo-, and poly(ADP-ribose) using purified recombinant PARP-1 and PARP-3 using automodification in vitro.

1. Incubate 500 ng of purified recombinant PARP-1 or PARP-3 at 25°C in a 100 µL reaction volume in Automodification Buffer under the following conditions (see Notes 17 and 18):
 - a. PARP-1 with 250 µM NAD⁺ for 5 minutes to generate poly(ADP-ribose)
 - b. PARP-1 with 3 µM NAD⁺ for 30 minutes to generate oligo(ADP-ribose)
 - c. PARP-3 with 250 µM NAD⁺ for 30 minutes to generate mono(ADP-ribose)
2. Stop the reactions by the addition of 4X SDS-PAGE Loading Buffer, followed by heating at 100°C for 5 min (see Note 19).

¹³Elute with the same volume of wash buffer as the packed bed volume of beads. For example, if you used 400 µL of a 50% slurry, this will contain a packed bed volume of 200 µL of beads.

¹⁴Multiple elutions should be performed to monitor for efficiency of elution. The peak of elution will usually occur in the first two elutions, but significant useable protein may be eluted out to elutions 4 or 5, depending on the expression and purification conditions.

¹⁵Running the purified proteins (~300 to 500 ng) on an Coomassie-stained SDS-PAGE gel will allow an assessment of the purity and quality of the purified proteins (Figure 3).

¹⁶The expected yield is ~0.4 – 0.5 mg/mL in elutions 1 and 2, and ~0.1 – 0.3 mg/mL in elutions 3 and 4, for a total combined yield of ~250 µg (i.e., 800 µL of a ~0.3 mg/mL solution if all elutions were combined).

¹⁷Sonicated salmon sperm DNA is added because PARP-1 and PARP-3 are DNA-dependent PARPs whose catalytic functions are activated by free DNA ends. The addition of sheared DNA may not necessary for other PARP family members.

¹⁸We do not routinely quantify the catalytic activity of our PARP-1 and PARP-3 preparations. The conditions described here for automodification have worked well across many different preparations of PARP-1 and PARP-3. If the expected automodification results are not achieved with a particular preparation of PARP-1 or PARP-3, try varying the amount of NAD⁺ in the reactions or the length of the incubation.

3. Assess the progress of the autoPARylation reaction with PARP-1 by running 25% of the reaction on a Coomassie-stained SDS-PAGE gel (see Notes 20 and 21) (Figure 4).
4. Analyze the protein-linked MAR, OAR, and PAR products by running 5% of the reaction products on Western or dot blots using antibody-like ADP-ribose binding reagents (see Note 22) (Figure 5).

3.4. Generation of Protein-Free Oligo(ADP-ribose) and Poly(ADP-ribose)

For some applications, it may be useful to separate the OAR or PAR from PARP-1 to use in a protein-free state for some assays [note that MAR (i.e., ADPR) can be purchased as in a protein-free state]. This section describes a method for deproteinizing and purifying protein-free OAR or PAR.

1. Perform automodification reactions using purified PARP-1 protein as described in Section 3.3, Step 1.
2. Stop the reaction by precipitation with ice-cold trichloroacetic acid (20%, v/v added from a 100% stock solution) on ice for 15 minutes.
3. Collect the precipitates by centrifugation at 15,000 x *g* for 15 minutes at 4 °C in a microcentrifuge.
4. Wash the pellets twice with ice-cold 20% trichloroacetic acid, followed by three times with 100% ethanol. After each wash, centrifuge briefly to make sure that the pellet does not become dislodged.
5. Remove any residual ethanol by air drying and then dissolve the pellets in 100 µL of TE.
6. Remove the DNA by adding 1 µL of DNase I (10 units/µL) and incubate for 15 minutes at 37°C.
7. Bring the reaction to 0.1% SDS from a 10% stock (see Note 23).
8. Add proteinase K to 0.2 mg/mL from a 20 mg/mL stock and incubate at 50°C for 2 to 3 hours or at 37°C overnight.
9. Add an equal volume of 1 M KOH/50 mM EDTA to detach the polymers from the digested proteins and incubate for 2 hours at 60°C.
10. Following the alkaline treatment, adjust the pH is to 8.0 using HCl.

¹⁹-If you plan to generate free OAR or PAR, skip this step and proceed directly to Section 3.4 (although, if this is your first time, you should confirm the reaction products as described Section 3.3 before proceeding).

²⁰-This step is optional, but recommended for the first time through the protocol.

²¹-As the reaction progresses, the PARP-1 protein band will smear into a slower-migrating automodified product, an outcome not observed with mono- or oligo(ADP-ribosylated) PARP-1 or mono(ADP-ribosylated) PARP-3 (Figure 3).

²²-The reactions conditions described here generate different levels of protein-linked ADP-ribose units. To achieve equal signals in Western or dot blots, you can standardize each reaction for the amount of terminal ADP-ribose units using anti-pan(ADP-ribose) binding reagent (EMD Millipore: MABE1016) (Figure 5).

²³-SDS helps the proteinase K digest proteins more efficiently.

11. Add an equal volume of phenol/chloroform/isoamyl alcohol and vortex for 30 seconds.
12. Centrifuge the mixture at 15,000 x *g* for 5 minutes at room temperature in a microcentrifuge to separate the aqueous and organic phases.
13. Transfer the upper aqueous phase to a new microcentrifuge tube.
14. Add an equal volume of chloroform and vortex for 30 seconds.
15. Repeat Steps 12 and 13.
16. Estimate the volume of the aqueous phase.
17. Add 1 μ L of 10 mg/mL glycogen, 1/10 volume of 3 M sodium acetate (pH 5.2), and 2.5 volumes of ice cold ethanol. Mix the solution well.
18. Incubate at -20°C for 1 hour (to overnight).
19. Centrifuge at 15,000 x *g* for 30 minutes at 4°C in a microcentrifuge.
20. Remove and discard the supernatant without disturbing the pellet.
21. Carefully wash the pellet with 1 mL of 70% ethanol and re-centrifuge at maximum speed for 5 minutes at room temperature in a microcentrifuge.
22. Repeat Steps 20 and 21.
23. Air dry the pellets and dissolve them in small volume of TE. Store the free polymers at -20°C in aliquots until needed. The protein-free products can be analyzed by dot blotting or urea-PAGE (see Notes 24 and 25).
24. For dot blotting, combine an aliquot of free OAR or PAR with Urea-PAGE Loading Solution, spot on a nylon-backed nitrocellulose membrane, and blot using ADPR detection reagents (Figure 6).
25. For PAGE analysis, prepare a 20 cm $\times$ 20 cm $\times$ 0.15 cm 20% polyacrylamide gel with a 19:1 (w/v) acrylamide/bisacrylamide ratio using the recipe described in Section 2.5, Step 19. Pre-run the gel for 1 hour in TBE buffer at 400 V. Replace the TBE buffer and run the free oligomers and polymers at a constant voltage of 400 V. Stain the gel using silver stain (Figure 7).

3.5 Using Protein-linked and Protein-free Mono-, Oligo-, and Poly(ADP-Ribose)

Protein-linked and protein-free MAR, OAR, and PAR can be used in a variety of assays as standards, substrates, or binding/interaction partners. The reaction conditions described herein generate OAR and PAR defined by lengths of 2 to 10 ADPR units and >10 ADPR units, respectively (see Notes 26 and 27).

²⁴Protein-free OAR can also be generated by controlled digestion of PAR with subsequent HPLC purification to isolate specific species of defined length.

²⁵Additional information about working with free polymers can be found in references [12, 22]

²⁶Free monoADPR can be purchased from Sigma (A0752). Producing free monoADPR by enzymatic means is inefficient. Free ADPR can be analyzed by thin layer chromatography, but is not suitable for analysis by SDS-PAGE or dot blotting.

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²⁷ Blotting with the ADPR detection reagents and the 10H monoclonal antibody can be used to confirm the length of the OAR and PAR chains generated under the reaction conditions described herein (Figures 5 and 6). The PAR chains generated under the reaction conditions described herein have an average length of ~25 to 35 units (Figure 7).

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Figure 1. Chemical structures of protein-linked ADP-ribose.

PARP family members and other ADP-ribosyl transferases (ARTs) catalyze the covalent attachment of ADP-ribose units derived from donor NAD⁺ molecules on substrate proteins.

Common residues of attachment include lysine, glutamate, and aspartate.

(A) Chemical structure of protein-linked mono(ADP-ribose) (MAR).

(B) Chemical structure of protein linked oligo- or poly(ADP-ribose) (OAR and PAR, respectively). Chain elongation can proceed in a linear or branched manner through different ribose-ribose linkages, as shown. For linear elongation, the adenine-proximal ribose (A-ribose) from a terminal ADP-ribose unit is joined through an $\alpha(1\rightarrow2)$ O-glycosidic bond to the nicotinamide-proximal ribose (N-ribose; relative to the nicotinamide moiety in NAD⁺) of the next ADP-ribose unit added to the growing chain. For branched elongation, the glycosidic bond occurs between the N-ribose of a terminal ADP-ribose unit and the N-ribose of the next ADP-ribose unit added to the growing chain. PAR polymers can grow to over

200 units in size (indicated by “Etc.”), with branching reactions occurring approximately once every 20 to 50 linear elongation additions [17].

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Figure 2. An approach for generating various forms of protein-linked ADP-ribose.

Flow diagram of the steps required to generate and test various forms of protein-linked ADP-ribose using biochemical approaches.

(A) Expression of recombinant PARPs in insect cells using a baculovirus-based expression system.

(B) Purification of catalytically active PARPs from insect cell lysates using affinity chromatography.

(C) Automodification of purified PARPs using controlled reactions conditions.

(D) Confirmation of the ADPR products by Western blotting using ADPR detection reagents.

Figure 3. SDS-PAGE analysis of purified recombinant PARP-1 and PARP-3.

Human PARP-1 and PARP-3 were expressed in insect cells using a baculovirus-based expression system and purified from cell lysates using affinity chromatography. Aliquots of the purified proteins were run on a 10% acrylamide-SDS gel, which was stained using Coomassie Brilliant Blue. Molecular weight markers (in kiloDaltons, kDa) are shown for reference. Black arrows indicate the locations of the purified proteins.

Figure 4. SDS-PAGE analysis of purified recombinant PARP-1 and PARP-3 subjected to in vitro automodification.

Purified recombinant human PARP-1 or PARP-3 (each at 500 ng/μL) were incubated with NAD⁺ (concentrations indicated) and sheared salmon sperm DNA (0.1 mg/mL) for the times indicated to promote automodification. Aliquots of the reactions were run on a 10% acrylamide-SDS gel, which was stained using Coomassie Brilliant Blue. Molecular weight markers (in kiloDaltons, kDa) are shown for reference. Incubation of PARP-1 with 250 μM NAD⁺ for 5 minutes produces poly(ADP-ribose), whereas incubation of PARP-1 with 3 μM NAD⁺ for 30 minutes produces oligo(ADP-ribose). Incubation of PARP-3 with 250 μM NAD⁺ for 30 minutes produces mono(ADP-ribose). Poly(ADP-ribosyl)ated PARP-1 exhibits a slower, heterogeneous migration (indicated as smearing, with a subsequent reduction of the primary band), which is not observed with oligo(ADP-ribosyl)ated PARP-1.

Figure 5. Western blot analysis of PARylated PARP-3, OARylated PARP-1, and PARylated PARP-1 using antibody-like ADPR detection reagents.

Purified recombinant human PARP-1 or PARP-3 was incubated with NAD⁺ and sheared salmon sperm DNA under the conditions specified in the legend to Figure 4. Aliquots of the reactions were run on a 10% acrylamide-SDS gel and subjected to Western blotting using the following antibody-like ADPR detection reagents: (1) anti-mono(ADP-ribose) binding reagent (EMD Millipore: MABE1076), (2) anti-poly(ADP-ribose) binding reagent (EMD Millipore: MABE1031), and (3) anti-pan(ADP-ribose) binding reagent (EMD Millipore: MABE1016). Molecular weight markers (in kiloDaltons, kDa) are shown for reference. The loading in each lane were standardized for the amount of terminal ADP-ribose units using anti-pan(ADP-ribose) binding reagent (EMD Millipore: MABE1016).

Figure 6. Dot blot analysis of protein-free OAR and PAR.

Purified recombinant human PARP-1 was incubated with NAD^+ and sheared salmon sperm DNA under the conditions specified in the legend to Figure 4 to generate OAR and PAR. The OAR and PAR products were deproteinized, precipitated, pelleted, dissolved, spotted on a nitrocellulose membrane in increasing amounts, and subjected to dot blotting using the following: (1) anti-PAR monoclonal antibody 10H ('PolyADPR Only'), (2) anti-poly(ADP-ribose) binding reagent (EMD Millipore: MABE1031) ('Mono + Poly'), or (3) anti-mono(ADP-ribose) binding reagent (EMD Millipore: MABE1076) ('MonoADPR Only'). For the latter, automodified PARP-3 was spotted as a positive control. The loading in each dot was standardized for the amount of terminal ADP-ribose units using anti-pan(ADP-ribose) binding reagent (EMD Millipore: MABE1016).

Figure 7. PAGE analysis of protein-free PAR.

Purified recombinant human PARP-1 was incubated with NAD^+ and sheared salmon sperm DNA under the conditions specified in the legend to Figure 4 to generate PAR. The PAR product was deproteinized, precipitated, pelleted, dissolved, and subjected PAGE analysis with subsequent silver staining. The migration of Bromophenol Blue, which runs at the location of chains of ~12 ADPR units, was used as a marker.