

Chapter 2

Quantification of Poly(ADP-Ribose) In Vitro: Determination of the ADP-Ribose Chain Length and Branching Pattern

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Abstract

The structural integrity of eukaryotic genomes, to a great extent, depends on highly regulated and coordinated enzymatic chromosomal poly(ADP-ribosyl)ation cycles that target chromatin proteins for specific covalent epigenetic poly(ADP-ribose) modification. As a result, the accurate determination of poly(ADP-ribosyl)ation amino acid specificity, as well as, a detailed characterization of the structural complexity of the protein-bound ADP-ribose polymers generated, e.g., linear versus branched ADP-ribose chains, need to be carefully sorted out. In this chapter, we describe well-established and reproducible laboratory methods and protocols typically used to determine: (1) the ADP-ribose chain length(s) and (2) the molecular stoichiometry of the protein–poly(ADP-ribosyl)ation reaction, e.g., number of ADP-ribose chains/polypeptide unit. While the methodology described here is exclusively for in vitro purified systems that can be used with high reliability, the reader is advised that application of these protocols to whole cell extracts and tissue systems must take into consideration the rapid turnover rate of protein-bound ADP-ribose polymers in vivo. Indeed, these extremely low-abundance chromatin-bound polymeric molecules are notoriously characterized for displaying a short half-life, typically from a few seconds to a few minutes. We also discuss potential methodological pitfalls, such as: (1) the chemical stability of protein–(ADP-ribose) n adducts and (2) the requirement for polymeric radiolabeling.

Key words: ADP-ribose, Polymer complexity, Chain length, Poly(ADP-ribosyl)ation stoichiometry, Linear homopolymers, Branched chains, High-resolution PAGE, SAX-HPLC

1. Introduction

The susceptibility of chromosomal histone and nonhistone proteins, including transcription factors and DNA-metabolizing enzymes (1), to covalent poly(ADP-ribose) polymerization by poly(ADP-ribose) polymerase-1 (PARP-1 [EC 2.4.2.30]), a ubiquitous chromatin-bound DNA-dependent enzyme that utilizes β NAD⁺ as a substrate (2–4), is a well-established epigenetic response to the

formation of single and double DNA strand breaks in the cell nucleus of higher eukaryotes. In fact, other less-prominent and abundant, but equally important, protein–poly(ADP-ribosyl)ation covalent modification pathways that may involve either PARP-2 (5), Tankyrase 1 (6), and/or V-PARP (7) as ADP-ribose-polymerizing enzymes and perhaps others (8) have also recently been reported in the literature. Interestingly, in spite of the increasing importance of protein–poly(ADP-ribosyl)ation cycles in human health and disease (9), a comprehensive analysis of the biochemical reaction stoichiometry (10) and a detailed analysis of the ADP-ribose polymer size distribution generated, when different individual protein acceptors (3–7, 11–13) are used as *in vitro* polypeptide targets, have seldom been reported.

Therefore, in this chapter, we aim to describe the standard sequence of laboratory steps needed to simultaneously determine the ADP-ribose chain length(s) of both radioisotopically labeled linear and branched ADP-ribose polymers following their amino acid-specific chemical release from polypeptide acceptor molecules by high-resolution (HR) polyacrylamide gel electrophoresis (PAGE) and strong anion-exchange (SAX)-high-pressure liquid chromatography (HPLC) below. The main drawback to the experimental approach described here is that it cannot be easily applied to the analysis of intracellular chromatin-bound ADP-ribose polymers because β NAD⁺, the substrate for protein–poly(ADP-ribosyl)ation, is not permeable to intact cells *in vivo* (2). Furthermore, even if β NAD⁺ could be made permeable to cultured cells, as it was previously described (14), the rapid turnover rate of ADP-ribose polymers synthesized *in vivo*, e.g., following genotoxic damage, would be a serious negative factor. Interestingly, following extensive DNA damage, the enzymatic activity of poly(ADP-ribose) glycohydrolase (PARG), the main poly(ADP-ribose) catabolic enzyme, is activated and needs to be taken into account (15). Thus, a different experimental approach to determine the structural complexity of the protein-bound ADP-ribose polymers, e.g., *in vivo* conditions under which PARG is inhibited (3), would have to be utilized.

Our method is constituted of essentially five different steps. First, the pure protein target to be tested is incubated with a homogeneous preparation of PARP and β NAD⁺ in the presence of nicked dsDNA for PARP activation, if required. Second, after the protein–poly(ADP-ribosyl)ation is completed, protein-bound polymers of ADP-ribose generated are detached from their polypeptide target by incubation in strong alkali. Third, upon neutralization, the protein-free ADP-ribose chains are purified by affinity chromatography on a boronate resin. Upon lyophilization, in the fourth and most important step, aliquots of the [³²P] radiolabeled homopolymers are simultaneously subjected to either high-resolution PAGE for the analytical determination of polymer size distribution (1/4 of the sample) or

the quantitative conversion (3/4 of the sample) to mononucleotide components (AMP, PRAMP, and PR₂AMP) by enzymatic digestion with snake venom phosphodiesterase (SVPD) following gel filtration chromatography by HPLC to separate linear from highly branched ADP-ribose polymers. Ultimately, in the fifth and final step of our protocol, separate samples of linear and highly branched polymers of ADP-ribose hydrolyzed to branched (PR₂AMP), internal (PRAMP), and terminal (AMP) polymeric units are separated by SAX-HPLC. Upon quantification of each chromatographic peak by scintillation counting, the size of the polymer and the frequency of branching are calculated with the following formulas:

$$\text{AMP} + \text{PRAMP} + \text{PR}_2\text{AMP} / \text{AMP} - \text{PR}_2\text{AMP} = \text{ADP-ribose chain length}$$

and

$$\text{PR}_2\text{AMP} / \text{AMP} - \text{PR}_2\text{AMP} = \text{Number of branches / polymer.}$$

2. Materials

1. Several Econo-columns (10 ml) and one Bio-Sil TSK-125 HPLC column from Bio-Rad (Richmond, CA). A Guard HPLC column with the same material.
2. One PARTISIL-10 SAX HPLC column from Whatman (Piscataway, NJ). A guard column with the same material.
3. Fifty milliliters of dihydroxyboronyl-Bio Rex 70 prepared, standardized, and stored at 5°C in the dark as detailed elsewhere (16).
4. Tris-HCl buffer, pH=7.8 (100 mM); Tris-phosphate buffer (pH=6.0); Tris-borate buffer, pH=8.3 (50 and 100 mM); ammonium formate buffer, pH=9.0 (100 mM); ammonium acetate buffer, pH=9.0 (100 mM); sodium phosphate buffer, pH=4.7 (125 mM); EDTA stock solutions (10 and 50 mM).

3. Methods

3.1. Poly(ADP-Ribose) Radiolabeling of Specific Protein Targets

1. Homogeneously pure poly(ADP-ribose) polymerase, e.g., PARP-1, is added to the reaction mixture at the optimal protein concentration to minimize automodification and enhance the poly(ADP-ribosyl)ation of the protein target. Typically, the concentration is around 20–50 nM.
2. Homogeneously pure target protein, e.g., DNA polymerase beta, is added at the optimal concentration for heterodimerization with the ADP-ribose polymerase, e.g., PARP-1. This protein

concentration varies from protein acceptor to protein acceptor. For DNA pol beta (13), we have observed 100–200 nM to be optimal.

3. Homogeneously pure ADP-ribosylation substrate, e.g., [^{32}P]- βNAD^+ , of high specific radioactivity ($\sim 1.0 \mu\text{Ci}/\text{pmol}$) is used at a final concentration between 1 and 10 μM . Important to note that below 1.0 μM is not typically of high efficiency and above 10 micromolar βNAD^+ , the poly (ADP-ribose) polymerase, e.g., PARP-1, becomes the main protein acceptor and is hyper-poly(ADP-ribosyl)ated.
4. Buffer requirements include 100 mM Tris-HCl buffer of pH 7.8 for optimal enzymatic conditions in the presence of 10 mM MgCl_2 and nicked dsDNA for activation.

3.2. LDS- Polyacrylamide Gel Electrophoresis

1. An 8–15% LDS-polyacrylamide gradient gel (see notes below [section 4]) containing 10 mM Tris-phosphate buffer of pH=6.0, 0.1% LDS, and 2 mM EDTA is used for the resolution of poly(ADP-ribosyl)ated protein.
2. Following LDS-PAGE, the band containing the modified target protein used for hetero-poly(ADP-ribosyl)ation is cut out from the gel, and the radiolabeled material is eluted before chemical release.

3.3. Chemical Release of Linear and Branched ADP-Ribose Chains from Protein

1. Radiolabeled ADP-ribose polymers [^{32}P] are first released from protein in 2.0 ml of 0.1 N NaOH at 60°C for 2 h in the presence of 20 mM EDTA. See Fig. 1 for a schematic representation of the three ADP-ribose isomeric polymer(s) released in alkali.
2. Upon chemical release, the alkaline solution containing protein-free [^{32}P] radiolabeled ADP-ribose polymers is neutralized with 0.1 N HCl and immediately subjected to purification from the contaminating protein(s) via affinity chromatography on a boronate resin (16).

3.4. Purification of Protein-Free ADP-Ribose Polymers by Affinity Chromatography on a Boronate Resin

1. The solution containing the mixture of protein and free ADP-ribose chains is diluted to 10 ml of 100 mM NH_4HCO buffer, pH=9.0, and immediately applied to a 1.0 ml bed of dihydroxyboronyl-Bio Rex 70 (16) previously equilibrated with 10 ml of the affinity chromatography loading buffer.
2. Immediately after loading the diluted sample, the 1.0 ml Econo-column from Bio-Rad (Richmond, CA) is washed twice with 10 ml of 100 mM NH_4HCO buffer, pH=9.0, prior to the elution of the retained protein-free ADP-ribose polymers on the column with 4 ml of water at 37°C.

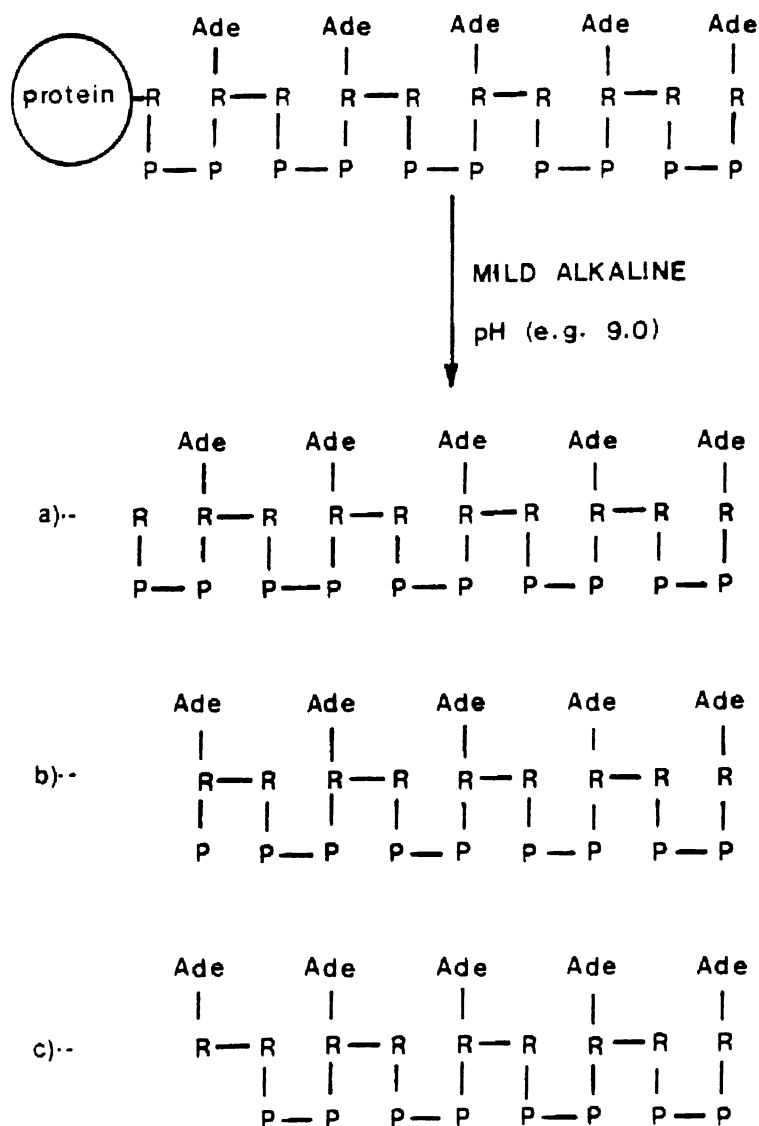


Fig. 1. Chemical release of ADP-ribose polymers from protein in mild alkali. Abbreviated structure of the three isoforms of ADP-ribose chains generated after polypeptide release in 0.2 N NaOH. A linear oligomer of five ADP-ribose units is displayed only for simplicity purposes: (a) pentameric ADP-ribose chain with an intact reducing end; (b) pentameric ADP-ribose chain with a 5'-terminal phosphate; and (c) pentameric ADP-ribose chain with a 5-hydroxyl group.

3. The eluted [^{32}P] material is next lyophilized to dryness and stored at -20°C until ready for the analysis of the polymer size distribution by high-resolution PAGE on a 20% homogeneous polyacrylamide gel (*vide infra*).
4. The sample is dissolved in high-resolution electrophoresis loading solution containing 100 mM Tris-borate pH=8.3,

50% urea, 25 mM NaCl, 4 mM EDTA, and 0.02% xylene cyanol (XC) and 0.02% bromophenol blue (BPB) to be used as markers of electrophoresis.

3.5. High-Resolution Polyacrylamide Gel Electrophoresis

1. A small aliquot (no more than 25% of the total) and 20,000–40,000 cpm of [^{32}P] radiolabeled poly(ADP-ribose) are loaded on a homogeneous 20×20 cm 20% polyacrylamide gel containing 19:1 acrylamide:bis-acrylamide ratio and 50 mM Tris–borate buffer of pH=8.3, containing 2 mM EDTA and 50% urea, for 3–5 h at 400 V for the high resolution of protein-free ADP-ribose polymers by electrophoresis and until the bromophenol blue marker has migrated 9 cm from the origin in 20-cm long gels.
2. Following HR PAGE on the 20% gel, the gel is left on one of the glass plates and covered with saran wrap prior to exposure to a high-quality X-ray film at -80°C sandwiched between the frozen gel and an amplifying screen.
3. After 24–72 h, depending on the efficiency of poly(ADP-ribosylation), the X-ray film is developed and the xylene cyanol and BPB marks on the frozen gel are used as markers for the 20- and 8-mer of linear ADP-ribose chains displayed on the X-ray film. As shown in Fig. 2 below, the three distinct isomeric forms of every ADP-ribose oligomer produced [from (ADP-ribose) $_2$ to (ADP-ribose) $_8$], upon alkaline release from protein, and subsequent boronate purification and electrophoresis on a 40-cm long 20% polyacrylamide gel can be efficiently resolved into individual [^{32}P] radiolabeled bands.

3.6. Gel Filtration of Protein-Free ADP-Ribose Polymers by HPLC

1. A large aliquot (up to 75% of the total) of the protein-free [^{32}P] radiolabeled ADP-ribose polymers obtained above is subjected to molecular sieve chromatography on a Bio-Sil TSK-15 column (300 mm long × 7.5 mm ID) from Bio-Rad preceded by a guard column (75 mm long × 7.5 mm ID) containing the same material. A total of 1 ml of [^{32}P] radiolabeled sample is injected to separate highly branched ADP-ribose polymers from linear ADP-ribose chains as fully described elsewhere (2). The chromatographic efficiency works well with a turnaround time between sample injections of less than 60 min from sample to sample to allow for full reconstitution of the chromatographic conditions.
2. Briefly, the chromatogram is developed under isocratic conditions using 100 mM Na_2HPO_4 , pH = 6.8, at a flow rate of 1.0 ml/min. Fractions of 500 μl are collected and small aliquots of each fraction are moved for graphical localization of the highly branched polymers of ADP-ribose, that typically elute immediately following the void volume of the HPLC system used, by liquid scintillation counting.

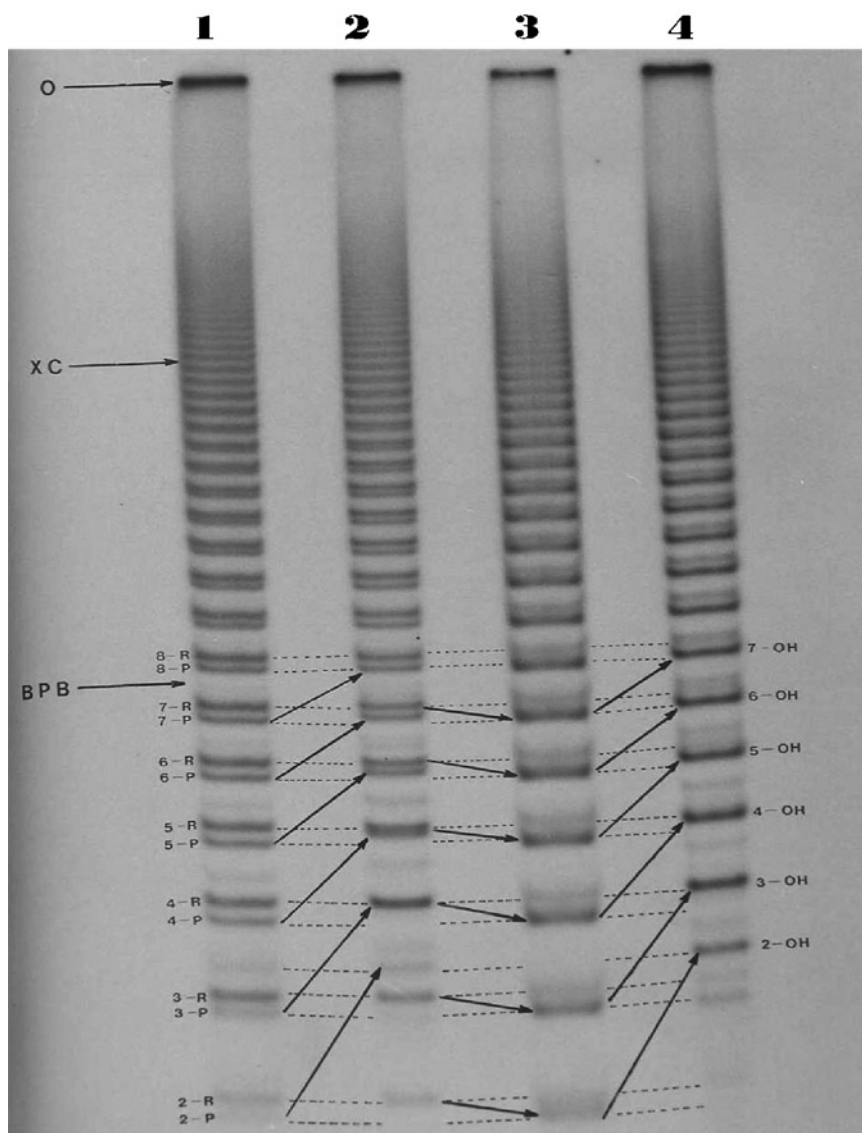


Fig. 2. Size distribution of protein-free ADP-ribose polymers by high-resolution polyacrylamide gel electrophoresis on a 40-cm long 20% polyacrylamide gel. *Lane 1* displays a typical ladder of ADP-ribose polymers obtained after mild alkaline release from protein (control). *Lane 2* shows the control sample treated with 10 units/ml of bacterial alkaline phosphatase (BAP) at 37°C for 60 min in 25 mM NH_4OAc , pH=9.0. *Lane 3* shows the control treated with 0.2 N NaOH and 20 mM EDTA at 60°C for 3 h. *Lane 4* displays the control sample exposed to 0.2 N NaOH and 20 mM EDTA at 60°C for 3 h followed by incubation with 10 units/ml of BAP at 37°C for 60 min in 25 mM NH_4OAc , pH=9.0. Abbreviations: **O** origin of application, **XC** xylene cyanol, **BPB** bromophenol blue. The ribosylated and 5' phosphorylated forms are shown with an R and a P, respectively, to the left of the electrophoretogram. The electrophoretic migration of the 5-hydroxylated forms of each oligomer is abbreviated by an OH to the right of *lane 4*. Oligomeric ADP-ribose chain length is indicated by Arabic numbers to the left or right side of the figure. The arrows indicate the electrophoretic migration changes for each band of each triplet containing the three different isomers for that oligomer (see legend to Fig. 1 for comparison) following exposure to either BAP or alkaline treatment or both. The dotted lines indicate that those polymeric species are equivalent units on different lanes of the electrophoretogram.

**3.7. Enzymatic
Digestion of Protein-
Free ADP-Ribose
Polymers into AMP,
PRAMP, and (PR)₂AMP
with Snake Venom
Phosphodiesterase**

- Typically, the first 2–4 fractions of 1.0 ml contain the highly branched polymers that must be purified one more time by boronate affinity chromatography (*vide supra*) prior to enzymatic digestion to mononucleotides with SVPD (*vide infra*).
- Commercially available SVPD from (Worthington, NJ) is purified on a blue sepharose column (0.7×7 cm) as indicated by Hayaishi and coworkers (17).
- Fractions of 500 µl containing pure ADP-ribose polymers as collected from the molecular sieve chromatography column (*vide supra* in step 3.6) are diluted to 1.0 ml with double-distilled water. The pH of the samples is adjusted to pH 8.0 for optimum enzymatic activity and MgCl₂ is added to a final concentration of 10 mM. The enzymatic reaction mixture is completed with the addition of 0.2 units of SVPD and the solution is incubated at 37°C for 2 h. Figure 3 schematically displays the phosphodiesterase digestion of a protein-bound ADP-ribose polymer. The same is true for a protein-free polymer after chemical release from the polypeptide acceptor.

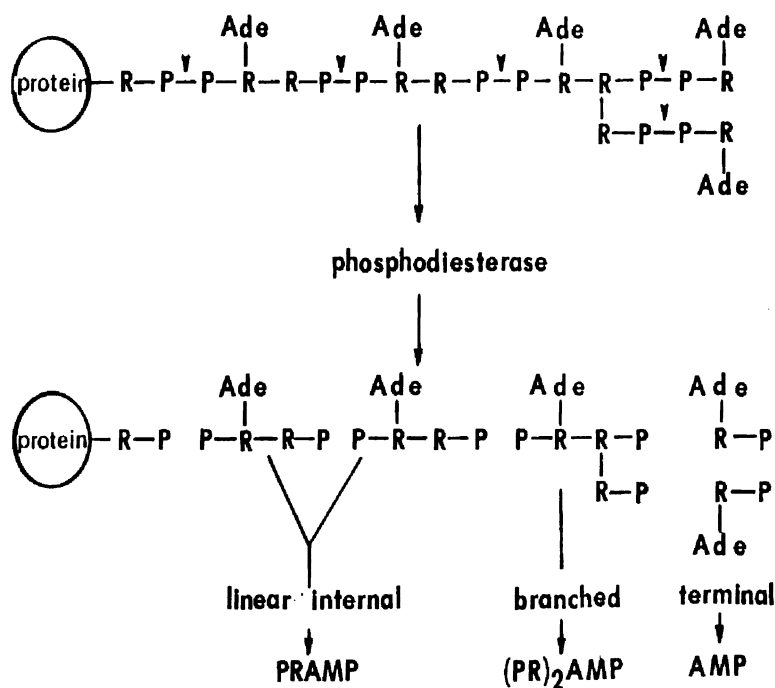


Fig. 3. Schematic representation of the enzymatic hydrolysis of protein-bound ADP-ribose polymers with snake venom phosphodiesterase. Abbreviations: *R* ribose, *P* phosphate, *Ade* adenine, *AMP*-5' adenosine monophosphate, *PRAMP*-5'' phosphoribosyl-adenosine 5'-monophosphate, *PR₂AMP*-5''' phosphoribosyl-5''-phosphoribosyl-adenosine 5'-monophosphate.

3.8. Separation of AMP, PRAMP, and (PR)₂ AMP by SAX-HPLC

1. The nucleotides obtained following phosphodiesterase enzymatic digestion (*vide supra* in step 3.7) are separated by HPLC using a Whatman PARTISIL-10 SAX column (250 mm long × 4.6 mm ID) preceded by a guard column (50 mm long × 1.5 mm ID) containing the same material.
2. Just before injection of each sample into the SAX column, KCl is added to a final concentration of 50 mM.
3. The column is next eluted isocratically with 125 mM KH₂PO₄, pH = 4.7, containing 0.5 M KCl at room temperature and a flow rate of 1.0 ml/min.
4. Finally, radioactivity is monitored by collecting 1.0 ml fractions for scintillation counting and the relative amounts of [³²P] radiolabel in each nucleotide is used to calculate both the average ADP-ribose chain length as well as the number of points of branching per molecule using the formulas described above in Subheading 1.
5. For the determination of ADP-ribose chain length(s) and number of ADP-ribose branching points per polymer, the amounts of AMP (polymeric tails), PRAMP (polymeric internal), and PR₂AMP (branching residues) must be determined with radioactivity amounts well above the background levels observed. Figure 4 displays the chemical structures of AMP, PRAMP, and PR₂AMP, respectively. It should also be noted that we previously published the SAX-HPLC chromatographic profile of the nucleotides generated following the SVPD digestion of [³²P] radiolabeled ADP-ribose polymers (2) in solution. However, up until now, we had not shown the formation of the same reaction products following HR PAGE on a 20% polyacrylamide gel. Figure 5 below displays the generated PRAMP and AMP nucleotides following the time-dependent enzymatic digestion of [³²P] radiolabeled ADP-ribose polymers.

4. Notes

1. Lithium dodecyl sulfate instead of the more typical sodium form, e.g., SDS, is highly recommended for step 3.2 to avoid the pre-electrophoresis artifactual chemical release from ADP-ribose polymers from protein that usually happens in 30–60 min at mild alkaline pH, e.g., above 8.0.
2. For the chemical release of ADP-ribose polymers from protein, if [³H] radiolabel is used to tag the adenine ring(s), the incubation in 0.2 N NaOH must be carried out at 37°C to avoid the loss of radiolabel by tritium exchange reactions.

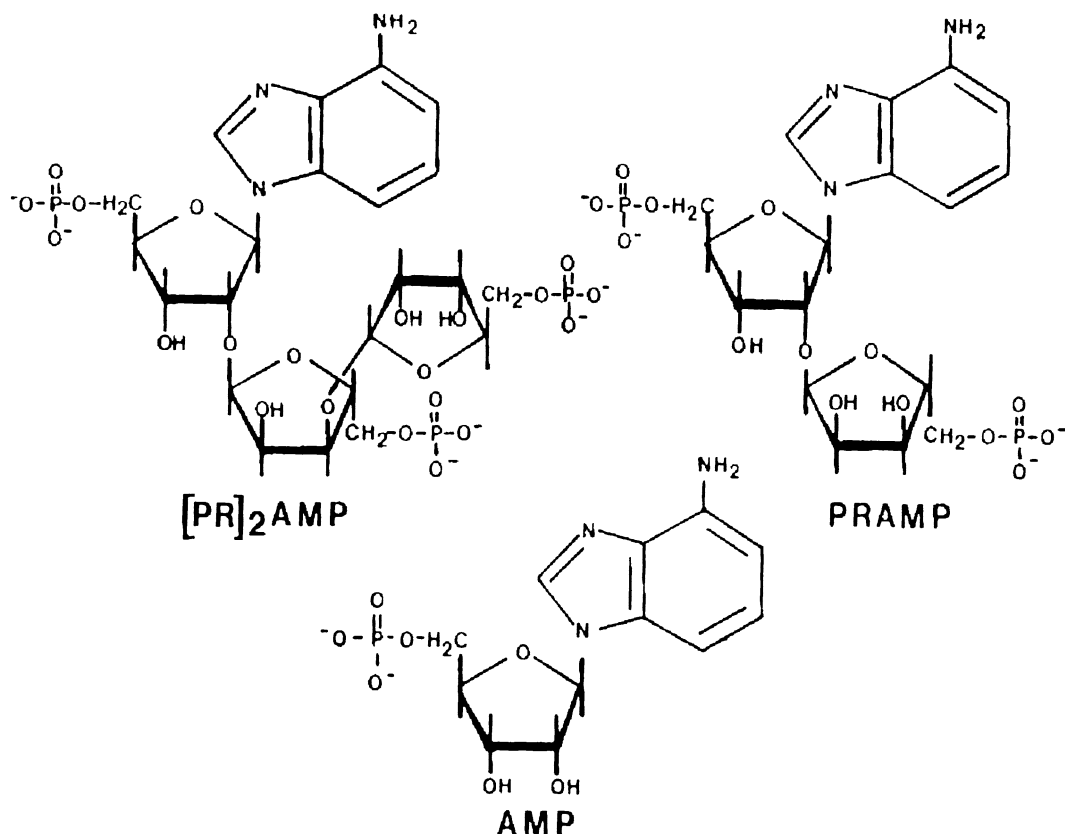


Fig. 4. Chemical structures of AMP-5'-adenosine monophosphate; PRAMP-5''-phosphoribosyl-adenosine 5'-monophosphate; and PR₂AMP-5''-phosphoribosyl-5''-phosphoribosyl-adenosine 5'-monophosphate.

- When running a 40-cm long 20% polyacrylamide gel instead of the standard 20-cm long gel, high-resolution electrophoresis must be carried out at 400 V maximum until the BPB marker has migrated 19 cm from the origin.
- Typically, the radiolabeled band observed at the origin of the gel following high-resolution PAGE of the protein-free polymers on 20% polyacrylamide corresponds to highly branched ADP-ribose polymers which must be purified by gel filtration chromatography and further subjected to SAX-HPLC analysis, as indicated above, following complete digestion to mononucleotide components with purified SVPD.
- When the phosphodiesterase digestion of highly complex polymers is not quantitatively efficient, the complete hydrolysis of ADP-ribose chains into individual mononucleotides, e.g., AMP (polymeric tails), PRAMP (polymeric internal), and PR₂AMP (branching residues), may be achieved by alkaline digestion in 0.2 N NaOH and 10 mM MgCl₂ for 3 h at 60°C in a final volume of 10 µl following another round of boronate affinity chromatography and lyophilization.

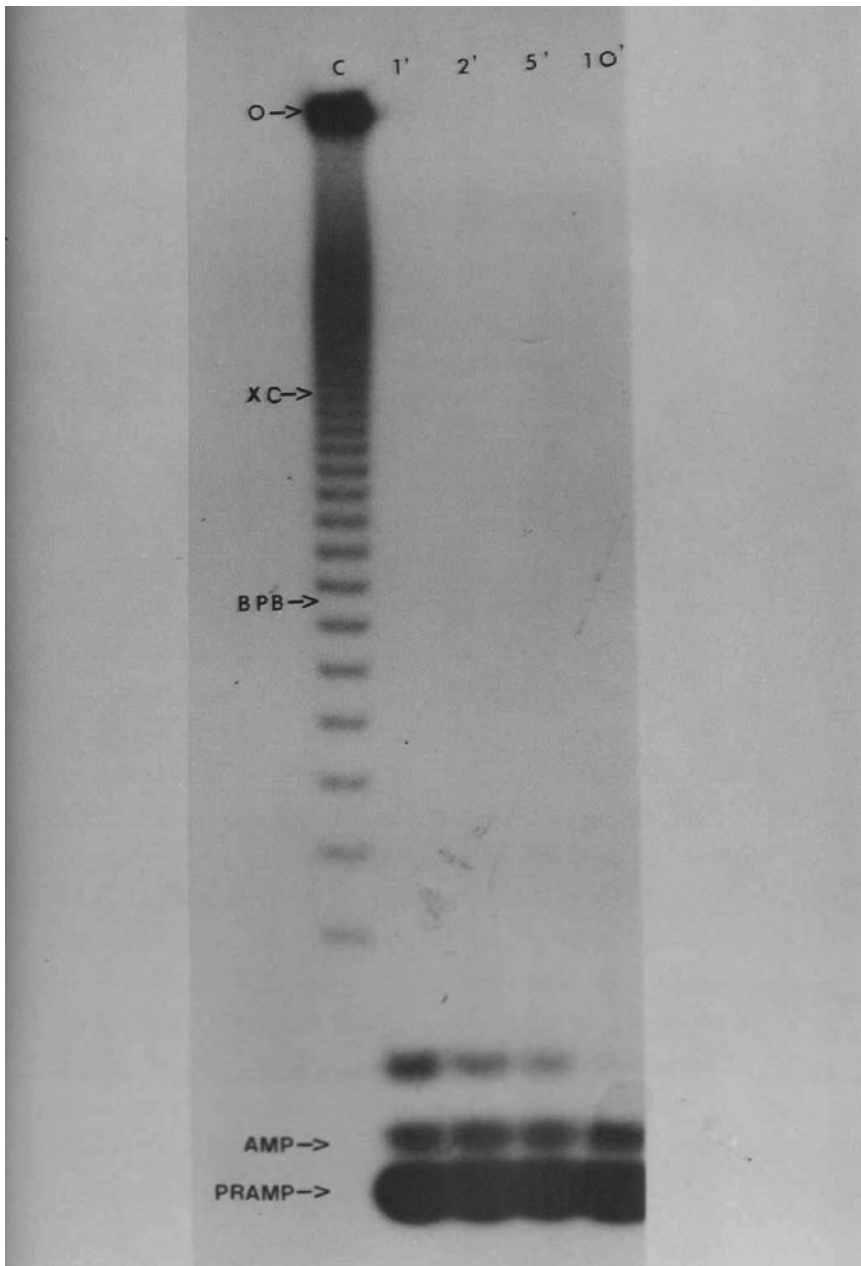


Fig. 5. High-resolution PAGE of [32 P] radiolabeled ADP-ribose polymers following SVPD digestion. C, untreated poly(ADP-ribose) control. 1', 2', 5', and 10' indicate the minutes of incubation with 2 units/ml of SVPD at 37°C in the presence of 25 mM NH_4HCO_3 buffer, pH=9.0, and 10 mM MgCl_2 . Abbreviations: O origin of application, XC xylene cyanol, and BPB bromophenol blue.

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